Molecular studies of virulence genes of *Salmonella Typhimurium* causing Clinical mastitis in dairy cattle.

[1]Ashraf A. Abd El- Tawab. and [2]Ashraf M. Nabih, [3]Mohsen A. Agag, [4]Marwah H. Abd Ali.

[1]*Bacteriology, Immunology and Mycology Department, Faculty of Veterinary Medicine, Benha University.*
[2] *Animal Reproduction Research Institute (ARRI), Haram / Giza*
[3] *Theriogenology Department, Faculty of Veterinary Medicine, Benha University.*
[4]*Veterinary Collage- Banha University*

ABSTRACT

A total of 213 milk samples from clinically mastitic cattle cows were collected from different Governorates of Egypt and transferred in ice box as soon as possible to Bacteriological lab. in Animal Reproduction Research Institute (ARRI) in Giza Governorate (AL-Haram) for bacteriological examination of most important pathogens causing clinical mastitis with special references for isolation and strict identification of *Salmonella species*. All samples were collected during the period from December 2016 till July 2017 from governorates of Egypt. The bacteriological investigations revealed that 8 (3.7%) of Salmonella isolates were identified biochemically from all examined samples. Serological study showed that a total of 5 (2.3%) of Salmonella isolates were typed as *Salmonella Typhimurium*. Two strains of *Salmonella Typhimurium* were isolated singly in the rate of (0.93%) from all examined samples, also another two strains of *Salmonella Typhimurium* were isolated mixed with *Staph aureus* in the rate of (0.93%), meanwhile only one strain of same species was isolated mixed with *E.coli* in the rate of (0.47%). Cefiquinom and Enrofloxacin were sensitively in the rate of (100%), Ampicillin, Chloraphincol, Cloxacillin were resistance in the rate of (100 %) to Streptomycin and Amoxicillin. The molecular examination confirmed that all 5 examined serotyped strains were *Salmonella Typhimurium*. Virulence genes *inv*A, *hil*A, *avr*A, were detected in examined sample by 100%, meanwhile sopE, ssaQ, and fimH genes were not detected by zero%. The objectives of the present study was to investigate the occurrence of Salmonella serotypes as well as to determine the frequency distribution of Six virulence genes (*inv*A, *hil*A, *avr*A, *ssa*Q, *sop*E and *fim*H) in salmonella isolates from cattle clinical mastitic milk, in addition to determine the drugs of choice for treatment of most *Salmonella* strains causing cattle clinical mastitis.

Key words: Cattle diseases- Clinical mastitis–Salmonella Infection-Molecular study- Virulence genes.

(http://www.bvmj.bu.edu.eg) (BVMJ-33(2): 27-37, DECEMBER, 2017)

1. INTRODUCTION

Mastitis means inflammation of the mammary gland and characterized by physical, chemical, microbiological and cellular changes in the milk as well as pathological changes in the udder (Merck Veterinary Manual., 2006), which have many adverse economic implications worldwide represented by decrease in quantity and quality of milk components and shorten the reproductive life of affected animals (Akopyan et al., 2007). Salmonella infection continue to be a major problem worldwide, it's a leading cause of foodborne illness in many countries (threlfall et al., 2014), but Salmonella are an infrequent cause of mastitis in dairy cows but several species of Salmonella have been documented to colonize udders and shed at high levels in milk (Fontaine et al., 1980). Several studies have identified milk borne pathogens including *Salmonella spp.*, which have been recovered with various prevalence rates from dairy farms (Fox et al., 2011). *Salmonella* are Gram negative, straight rods not exceeding 1.5 micrometers in width. They are facultative anaerobes usually motile by peritrichous flagella (Pawsey, 2002). The genus Salmonella compvises more than 2579 serotypes (Griment and Weill, 2007). An environmental reservoir is frequently the source of Salmonella, a gram negative bacterium that has the capacity not only to survive but multiply in the environment (peek et al., 2004), Unhygienic measures, contaminated equipments, mammary gland infected with Bacteria and hands of milkers during handling and processing of raw milk are considered the main cause of milk contamination (Scherrer et al., 2004).

Salmonella isolates have traditionally been classified by serotyping, the serologic identification of two surface antigens, O-polysaccharide and flagellin protein. Serotyping has been of great value in understanding the epidemiology of *Salmonella* and investigating disease outbreaks, production and quality control of the hundreds of antisera (McQuiston *et al.,* 2004). (Malorny *et al.* 2003) stated that as part of a major international project for the validation and standardization of PCR for detection of five major food-borne pathogens, four primer sets specific for *Salmonella species* were evaluated in-house for their analytical accuracy (selectivity and detection limit) in identifying *Salmonella spp*., The most selective primer set was the *inv*A gene. Antimicrobial resistance is a global public health problem. Although all countries are affected, the extent of the problem in the developing nations is unknown (Ang et al., 2004). The monitoring of drug resistance patterns among the Salmonella isolates not only gives vital clues to the clinician on the best therapeutic regime in each individual case, but is also an important tool in devising a comprehensive chemoprophylactic and chemotherapeutic drug schedule within a geographical area (Murugkar et al., 2005).

The aim of this study was to detect the spreading rate of Salmonella microorganism affecting lactating cattle suffering from clinical mastitis in some Governorate in Egypt and in addition to reporting the pathogenicity and severity of isolated Salmonella *Spp.* by molecular study of most important virulence genes. In addition to put a high light on most important drugs of choice for treatment and control the salmonella causing clinical mastitis in dairy cattle cows.

2. MATERIAL AND METHODES

2.1. Clinical examination:

The studied animals were subjected to clinical examination by visual inspection; palpation of the udder for swelling, redness and pain; beside the physical changes in the milk secreted from such udders.

2.2. Samples: According to ISO 6579: 2002 method.

A total of 213 milk samples from clinically mastitic cattle cows were collected from different Governorates of Egypt and transferred in ice box as soon as possible to bacteriological lab. in Animal Reproduction Research Institute (ARRI) in Giza Governorate (AL-Haram) for bacteriological examination of most important pathogens causing clinical mastitis with special references for isolation and strict identification of *Salmonella species*. All samples were collected during the period from December 2016 till July 2017 from Giza, Monofia, Fayoum, Ismailia, and Bani Suif Governorates.

2.3. Bacteriological examination (Quinn 2002)

2.3.1. Isolation of Salmonella and most important bacteria causing mastitis:

One ml of each collected milk sample was added on test tube contain selenite f. broth for activation of salmonella growth and on nutrient broth for propagation of other most common bacteria causing mastitis. A loop-full of material from selenite F. broth was transferred and streaked separately on the surface of xylose lysine deoxycholate agar (XLD agar), salmonella – sheigella agar (S.S), Brilliant Green (B.G. agar), meanwhile nutrient broth material was transferred and streaked separately on MacConkeys agar, Blood agar, Eosin methylene blue media (EMB), Mannitol salt agar; Baird Parker agar and Modified Edward's media. The plates were incubated in an inverted position at 37C for another 24 hours, then chacked for growth of typical salmonella and others microorganism's colonies. The pure colonies were kept in Semi-solid media for more identification.

2.3.2. Identification of suspected isolates: According to Quinn et al., 2002

Typical colonies of Salmonella and others microorganisms were identified by morphology, Gram staining -biochemical identification (traditional and API20 E test), serological methods and molecular study.

2.3.3. Diagnostic Salmonella antisera according to Kauffmann-White scheme (Kauffmann, 1974):

The isolates were identified serologically using different diagnostic monovalent salmonella antisera (Mast Company, London, England). Serological identification was carried out at Animal Health Research Institute, Dokki, Giza.

2.3.4. Antibiogram assay:

The disc diffusion method was used as described by (Nccl. 2002).

3. RESULTS

Table (1): Number of examined clinically mastitic milk from cattle cows in different governorates of Egypt.

Governorates	Monofia	Giza	Fayoum	Ismailia	Banisuif	Total
NO	73	32	29	38	41	213

Table (2): Oligonucleotide primers sequences. Source: Metabion (Germany).

Target MO	Target gene	Primers sequences	Amplified segment(bp)	Reference
Salmonella	*invA*	*GTGAAATTATCGCCACGTTCGGGCAA* *TCATCGCACCGTCAAAGGAACC*	284	Oliveira *et al*., 2003
	AvrA	*cct gta ttg ttg agc gtc tgg* *aga aga gct tcg ttg aat gtc c*	422	Huehn *et al*., 2010
	ssaQ	*gaa tag cga atg aag agc gtc gtc c* *cat cgt gtt atc ctc tgt cag c*	455	
	sopE1	*act cct tgcaca acc aaa* *tgc gga tgt cttctg cat ttc gcc acc*	422	
	hilA	*CATGGCTGGTCAGTTGGAG* *CGTAATTCATCGCCTAAACG*	150	Yang *et al*., 2014
	fimH	*GTGCCAATTCCTCTTACCGTT* *TGGAATAATCGTACCGTTGCG*	164	Hojati *et al*., 2013

Table (3): The different antimicrobial discs used in the agar diffusion method and interpretation of their sensitivity

Antimicrobial agents	Code	Concentration Of disks	Zone of inhibition		
			Resistant	Intermediate	Sensitive
Amoxicillin +Clavulinic acid	AMC	30 ug	13	14-17	≥ 18
Gentamycin	GM	10 ug	12	13-14	≥ 15
Neomycin	N	10 ug	11	12-13	≥ 14
Cefiquinom	CFQ	30 ug	13	14-17	≥ 18
Ampicillin	AMP	10 ug	11	12-13	≥ 14
Chloraphincol	C	30 ug	12	13-17	18
Enrofloxacin	ENR	10 ug	≤15	16-20	21
Oxytetracyclin	OX	30 ug	14	15-18	≥ 19
Streptomycin	S	10 ug	11	12-14	≥ 15
Penicillin g	P	20 ug	20-27	≥ 29	20
Amoxicillin	AM	25 ug	11	12-13	≥ 14

Table (4): Incidence of S*almonella* isolates from 213 Samples of clinically mastitic milk of cattle cows by using culture, different biochemical test.

Culture		By traditional methods		By API 20 E.	
NO	%	NO	%	NO	%
11	5.2	8	3.7	8	3.7

Table (5): Incidence of isolated single and mixed S*almonella Typhimurium* from different Governorates of Egypt.

Isolated Microorganisms	Governorates										Total	
	Monofia (n:73)		Giza (n:32)		Fayom (n:29)		Ismailia (n:38)		Bani suif (n:41)			
	No	%	NO	%	NO	%	NO	%	NO	%	NO	%
Salmonella Typhimurium	1	50.0	0	0.00	0	0.00	1	50.0	0	0.00	2	0.93
Salmonella Typhimurium +Staph. aureus	1	50.0	0	0.00	0	0.00	0	0.00	1	50.0	2	0.93
Salmonella Typhimurium + E.coli	0	0.00	0	0.00	1	100	0	0.00	0	0.00	1	0.47
Total	2	27.4	0	0.00	1	3.4	1	2.6	1	2.4	5	!.35%

Table (6): Incidence of S*almonella Typhimurium* isolates from clinically mastitic milk Samples of cattle cows.

N: (8)	By serology methods				y PCR methods :(5)	
	Serotype		An typing			
	NO	%	NO	%	NO	%
	5	62.5	3	37.5	5	100%

Table (7): serotyping of isolated salmonella serotypes from clinical mastitis cow milk.

Serology Strains	O antigen	H antigen	
		Phase I	Phase II
S.Typhimurium	1, 4, [5] ,12	I	1,2

Table (8): percentage of *Salmonella typhimurium* virulence genes from detected samples.

Salmonella Typhimurium	Virulence genes											
	*inv*A		*hil*A		*Ssa*Q		*Fim*H		*Avr*A		*Sop*E	
	NO	%	NO	%	NO	%	NO	%	NO	%	NO	%
NO: 5	1	100	1	100	0	.00	0	0.0	1	100	0	0.0

Table (9): -Antibacterial sensitivity tests of single and mixed isolated *salmonella typhimurim* from clinically mastitic cattle milk samples.

Antibacterial Disks		Single *salmonella* isolates (n:2)				mixed *salmonella* with other bacterial isolates (n:3)			
Name	Concentration Of disks	Sesitive		Risistant		Sensitive		Resistant	
		NO	%	NO	%	NO	%	NO	%
Amoxicillin +Clavulinic acid	30 ug	1	50	1	50	2	66.7	1	33.3
Gentamycin	10 ug	1	50	1	50	1	33.3	2	66.7
Neomycin	10 ug	1	50	1	50	0	0.0	3	100
Cefiquinom	30 ug	2	100	0	0.0	3	100	0	0.0
Ampicillin	10 ug	0	0.0	2	100	1	33.3	2	66.7
Chloraphincol	30 ug	0	0.0	2	100	2	66.7	1	33.3
Enrofloxacin	10 ug	2	100	0	0.0	0	0.0	3	100
Oxytetracyclin	30 ug	1	50	1	50	0	0.0	3	100
Cloxacillin		0	0.0	2	100	0	0.0	3	100
Streptomycin	10 ug	0	0.0	2	100	0	0.0	3	100
Penicillin g	10 ug	1	50	1	50	2	66.7	1	33.3
Amoxicillin		0	0.0	2	100	1	33.3	2	66.7

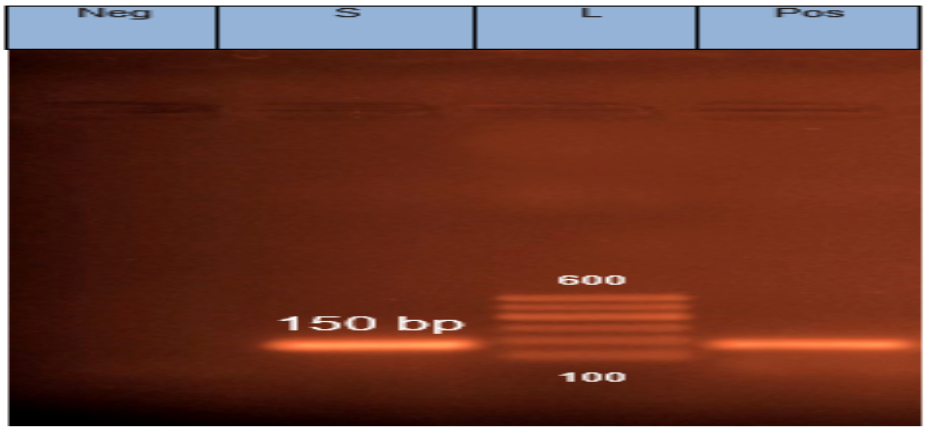

Figure (1): Electrophoresis gel show results of PCR amplification of hilA virulence gene of Salmonella Typhimurium analyzed on 2% agarose gel, Lane L: 100 bp DNA ladder; Lane negative control; PCR amplified 150 bp product of Salmonella typhimurium Positive sample Lane(S)

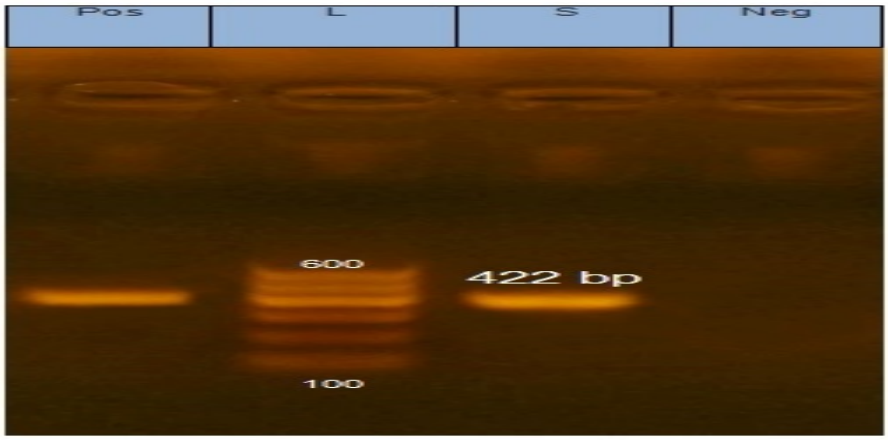

Figure (2): Electrophoresis gel show results of PCR amplification of avrA virulence gene of Salmonella Typhimurium analyzed on 2% agarose gel, Lane L: 100 bp DNA ladder; Lane negative control; PCR amplified 422 bp product of Salmonella typhimurium Positive sample Lane (S).

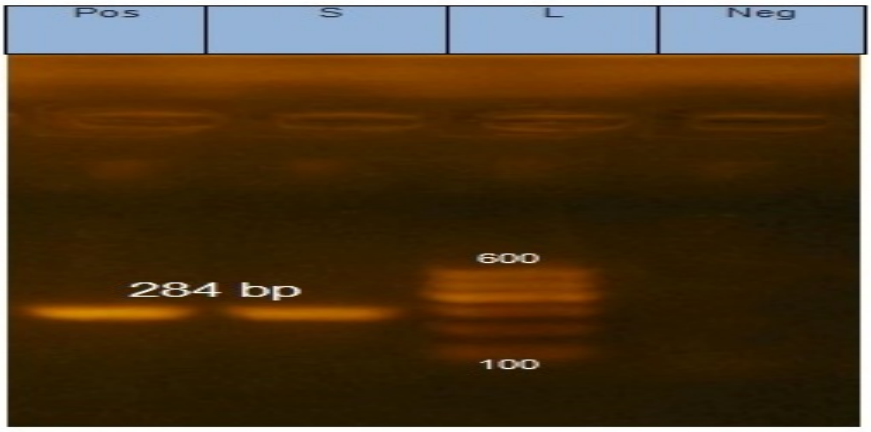

Figure (3): Electrophoresis gel show results of PCR amplification of invA virulence gene of Salmonella Typhimurium analyzed on 2% agarose gel, Lane L: 100 bp DNA ladder; Lane negative control; PCR amplified 284 bp product of Salmonella typhimurium Positive sample Lane(S).

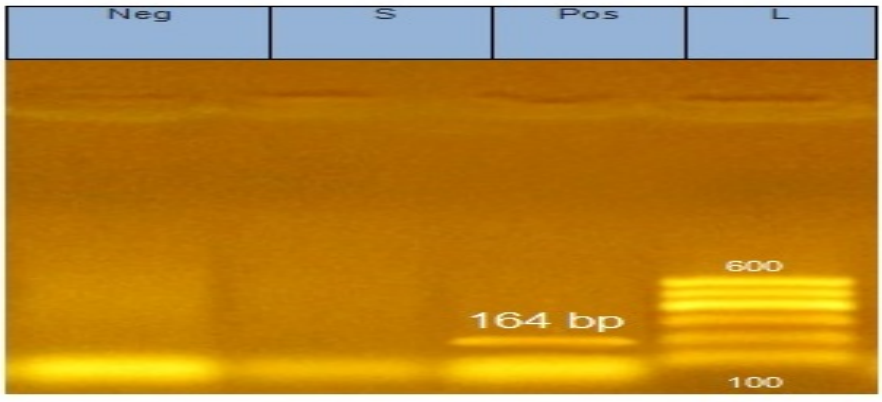

Figure (4): Electrophoresis gel show results of PCR amplification of fimH virulance gene of Salmonella Typhimurium analyzed on 2% agarose gel, Lane L: 100 bp DNA ladder; Lane negative control; PCR amplified 164 bp product of Salmonella typhimurium negative sample Lane(S).

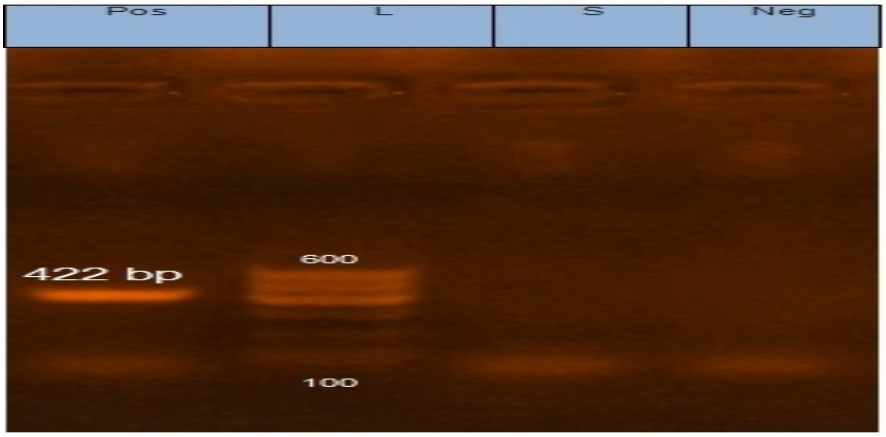

Figure (5): Electrophoresis gel show results of PCR amplification of sopE virulance gene of Salmonella Typhimurium analyzed on 2% agarose gel, Lane L: 100 bp DNA ladder; Lane negative control; PCR amplified 422 bp product of Salmonella typhimurium negative sample Lane(S) .

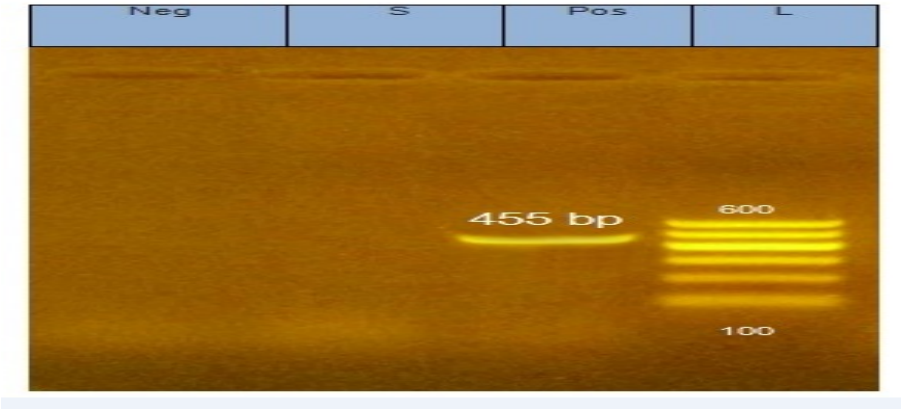

Figure (6): Electrophoresis gel show results of PCR amplification of *ssa*Q virulence gene of *Salmonella Typhimurium* analyzed on 2% agarose gel, Lane L: 100 bp DNA. ladder; Lane negative control; PCR amplified 455 bp product of *Salmonella typhimurium* negative sample Lane (S).

4. DISCUSION

Mastitis caused by Salmonella infection is very rare, the microbial contamination of milk is multifactorial originating from sources like the air, feed, soil, feces, grasses and the milking cow itself. Other possible sources of milk contamination include; utensils and water used in the collection and processing of milk (Coorevit et al., 2008). Salmonella contamination of milk and milk products has been reported in several parts of world confirming its grouping as a rare zoonotic pathogen (Van Kessel et al., 2004). Salmonella organism is one of the most important pathogenic bacteria in the world (Gast and Holt, 1997). In this study 213 clinical mastitis cattle milk sample were examined for presence of Salmonella, clinical examination of all studied Cows infected with clinical mastitis appeared that affected udder was warm, swollen, doughy to firm and painful. The milk was watery, purulent or with thick clots and samples were tinged with blood. Most cases showed only one or two quarters affected. The bacteriological results of our study showed isolation of 8 strains of *Salmonella Typhimurium* by (3.76%) from all examined milk samples Table (4), two single isolates of salmonella and 3 strains of *Salmonella Typhimurium* were mixed with other bacteria (one case was mixed with *E.coli and two cases were mixed with staph aureus.*) Table (5). Also the results of traditional biochemical methods and API20E methods table (4), confirmed that 8 isolates (3.76%) of *Salmonella Typhimurium* were detected. five isolates (2.3%) of *Salmonella Typhimurium* were identified by serotype method. These results were closely in agreement to that recorded by (Salihu, M.D., et al 2011) who carried out a total of 100 milk samples to determine the prevalence of clinical mastitis in lactating cows in some selected commercial dairy farms in Sokoto

metropolis, the prevalence of *Salmonella* were (2.17%), also Inter J Agri Biosci, (2012) examened 200 clinically mastitic milk samples collected from 50 dairy cows, and they resulted (4%) of Salmonella infected cases. These results were nearly similler to our results in this study. Exactelly like our study, but from another source of infection Fadel and Ismail (2009) isolated *Salmonella spp*. from 3.7% of dairy workers' hands swabs. On the other hand our results were differed from reported by Zeinhom and Abdel-Latef (2014) who examined 25 milker's hand swabs,as acausitive manner for salmonella infection leads to clinical mastitis, collected from different farms in Beni-Suef Governorate.The results revealed that *Salmonella spp*. failed to be detected in any of the examined samples. This result may be attributed to another souces of infections causes clinical mastitis .In our investigations *Salmonella Typhimurium* isolates were serotyped using poly and monovalent O and H antisera and the results of this study revealed that 5 strains which were isolated from clinical mastitic cattle milk from different Governorates in Egypt, were all from *Salmonella Typhimurium* (62.5 %) , and 3 (37.5 %) isolates were untyped strains. Thesee results confirmed that *S. Typhimurium* participated with the mainly isolated strain than other serotypes of *Salmonella*, 100% (all the 5 isolates were *Salmonella Typhimurium*) tables (6) and (7). Mituniewicz et al., (2007) who analyzed *Salmonella bacillus* infections causing clinical mastitis of dairy cows found that *Salmonella Typhimurium* was the mainly isolated strains. Finally we concluded that 5 cases of clinical mastitis of lactatig cows (2.3%) were infected by *Salmonells Typhimurium* from a total of 213 examined clinical mastitic cattle milk samples. Other studies reported the isolation of *Salmonella spp.* with the prevalence of 6.1% in USA (Jayarao and Henning, 2001), 6% in Pennsylvania (Jayarao et al., 2006) and 2.6% in USA (Van Kessel et al., 2004). Higher isolation rates of 28.1% (Van Kessel et al., 2011) and 28.6% (Addis et al., 2011) were documented in USA and Ethiopia, respectively. This gape of different results of other studies may be returned to many factors like, different management, sanitation, ways of rearing, methods of milking, grazing methods, collection and transportation of milk production, cleaning of milk tanks or milk pipes of production lines or even due to workers clothes and hands. According to the results concerning antimicrobial susceptibility tests in table (8) two single *salmonella* isolates from a total of 213 clinical mastitis cattle milk samples showed the highest percentage of resistance (100 %) to Ampicillin, Chloraphincol, Cloxacillin, Streptomycin and Amoxicillin , and give resistant in the rate of (50 %) to Amoxicillin + Clavulinic acid , Gentamycin ,Neomycin , Oxy tetracyclin and Penicillin g. Khan et al., (2010) stated that all *Salmonella* isolates exhibit (100 %) resistant to cephalexin and rifampicin while about 90% and 88% of the isolates were resistant to ampicillin and tetracycline. Pan et al., (2009) who reported that *Salmonella* displayed a high level of resistance to ampicillin, streptomycin and tetracycline, (Alali et al., 2010) who showed that *Salmonella* was resistant to streptomycin and ampicillin. On other hand also the two single salmonella isolates from examined species were sensitive to Cefiquinom and Enrofloxacin in the rate of (100 %), and these single isolates were sensitive in the rate of (50%) to Amoxicillin + Clavulinic acid,and Gentamycin . The obtained results were agree with Jodas and Hafez (2003) who reported that all of the examined *Salmonella* isolates were sensitive to enrofloxacin (98%). Our reported results were disagreement with (Habrun et al., (2012) who reported that all *Salmonella* isolates were sensitive to chloramphenicol and streptomycin (100 %) while (58 %) were sensitive to Nalidixic acid and (41.7%) were sensitive to all antimicrobials. Also were differed with Cardoso et al., (2006) who reported that *Salmonella* showed sensitivity to doxycycline hydrochloride with 100%. The different results of another studies were mainly attributed to individual physiological differencs, importent role of misuse of antibiotics treatement wihout bacteriological investigations, using of different antibiotics groups or even use of the same antibiotics names in the treatement without applying new trade of drug production, Shivhare et al., (2000) found that highest sensitivity of *Salmonella* isolates to Norfloxacin was 92 % and the lowest percentage was to Cloxacillin 8%. All isolates were resistant to Sulfonamides and trimethoprim.

PCR was perfect tool for accurate detection of Salmonella virulence genes and the PCR technique is capable pf identifying the pathogenic *Salmonella Typhimurium*, these isolates can be used as the basis for the production of a powerful vaccine to be used against infections. Based on the fact that virulence varies not only among different species but also among strains of the same species. Thus, numerous studies have been conducted to identify virulence factors of isolated *S.Typhimurium* strains (Akiba *et al.* 2011). So the present study was directed mainly to genotypic detection of *Salmonella typhimurium* and virulence genes that may play a role in virulence of *Salmonella* by using one of the recent developments molecular

biological techniques (PCR) These genes were *inv*A , *hil*A , *avr*A *gene* , *sop*E , *fim*H *and ssa*Q genes. The PCR results for Salmonella typhimurium table (9) fig (3) revealed that in the studied strain that invA gene was detected in the rate of 100%. These result agreement with Malorny et al., (2003). Also *avrA*, and *hilA* genes were detected in the rate of 100 % in the studied strain table (9) fig (1) and (2). Meanwhile the results of PCR showed that *fim*H , *Sop*E and *ssa*Q genes were negative result in the studied strain in the rate of (0.0 %) table (9) fig (4), (5), (6).

Finally, from results of the present work it could be concluded that, clinical mastitis is a serious disease of cattle cows with economic and public health importance at Egypt Governorate. *Salmonella Typhimurium*, rare causes of clinical mastitis. Also, PCR could indicate that, *inv*A , *avr*A and *hil*A genes were detected in the studied strain in the rate of (100 %). *Sop*E, *ssa*Q and *fim*H genes were not detected in the studied strain (0.0 %).

5. REFERENCES

Addis, Z.; Kebede, N.; Sisay, Z.; Alemayehu, H.; Yirsaw, A. and Kassa, T. 2011. Prevalence and antimicrobial resistance of *Salmonella* isolated from lactating cows and in contact humans in dairy farms of Addis Ababa: a cross sectional study. BMC Infectious Diseases 11: 222-228.

Akopyan, A.S., Haruthyunyan, H.A., Gasparyan, V.K., Haruthyunyan, H.D., Khocanyan, V.V. and Kamalyan R.G. 2007. The indices of nitrogen metabolism mastitis in caw s blood.National Academy of Sciences of RA. Electronic J. Natural Sciences, 2 (9).

Alali,W.Q.;Thakur S.; Berghaus R.D.; Martin,M.P. and Genreyes,W.A. 2010. Prevalence and distribution of *Salmonella* in organic and conventional broiler poultry farms . Foodborne pathog. Dis.7(11):1363-1371.

Ang , J.Y.; Ezike, E.and Asmar , B.I. 2004. Antibacterial resistance. indian Journal of Pediatrics. 71, 299-239.

Cardoso, M.O., Ribeiro, A.R., Santos, L.R., Pilotto, F., Moraes, H.S., Salle, C.P.,Rocha, S.S., Nascimento, V.P. 2006. Antibiotic resistance of *SalmonellaEnteritidis* isolated from broilercarcasses. Braz. J. Microbiol., 37:368-371.

Coorevits A., De Jonghe V., Vandroemme J., Reekmans R., Heyrman J., Messens W., De Vos P., Heyndrickx M. 2008. Comparative analysis of the diversity of aerobicspore-forming bacteria in raw milk from organic and conventional dairy farms System. Appl. Microbiology

Fadel, H. M. and Ismail, J. 2009. Prevalence and significance of *Staphylococcus aureus* and *Enterobacteriaceae species* in selected dairy products and handlers. International Journal of Dairy Science 4 (3): 100-108.

Fontaine, R. E., Cohen, M. L., Martin, W. T., Vernon, T.M. 1980. Epidemic salmonellosis from cheddar cheese—surveillance and prevention. Am J Epidem 111:247.

Fox EM, Leonard N, Jordan K. 2011. Molecular diversity of Listeria monocytogenes isolated from Irish dairy farms. Foodborne Pathogene 8: 635–641.

Gast , R.K ., and Holt , P.S. 1997. Asseessing the sensitivity of egg yolk antibody testing for detection *Salmonella* Entertidis infection in laying hens. Poult. Sci., 67: 798-801.

Ghanbarpour, Salehi. 2010. Determination of Adhesin Encoding Genes in *Escherichia coli* Isolates from Omphalitis of Chicks. American Journal of Animal and Veterinary Sciences 5 (2): 91-96.

Grimont PAD., Weill FX. 2007. Antigenic formulae of the *Salmonella* serovars, 9th revision World Health Organization Collaborating Center for Reference and Research on *Salmonella,* Pasteur Institute, Paris, France.

Habrum , B.; Simpraga , B.; Kompes , G. and Krstulovic , F. 2012. Antimicrobial resistance and serotyping of *Salmonella enterica subsp*. enterica isolated from poultry in Croatia. Veterinarski Arhiv;. 82 (4):371-381.

Hojati, Z.; Zamanzad, B.; Hashemzadeh, M.; Molaie, R.; Gholipour, A. 2013. Detection of FimH Gene in Uropathogenic *Escherichia coli* Strains Isolated From Patients With Urinary Tract Infection. Jundishapur J Microbiol. ; 8(2): e17520.

Hu, Q.; Tu, J.; Han, X.; Zhu, Y.; Ding, C. and Yu, S. 2011. Development of multiplex PCR assay for rapid detection of *Riemerella anatipestifer, Escherichia coli,* and *Salmonella enterica* simultaneously from ducks. Journal of Microbiological Methods 87 (1): 64–69.

Huehn S., La Ragione RM., Anjum M., Saunders M , Woodward MJ., Bunge C., Helmuth R, Hauser E., Guerra B., Beutlich J., Brisabois A., Peters T., Svensson L., Madajczak G.,

Litrup E., Imre A., Herrera-Leon S., Mevius D., Newell DG., Malorny B. 2010. Virulotyping and antimicrobial resistance typing of *Salmonella enterica* serovars relevant to human health in Europe. Foodborne Pathogens Dis 2010; 7:523-35.

Inter J Agri Biosci, 2012, 1(1): 16-19. Shrestha S and YR Bindari, Prevalence of sub-clinical mastitis among dairy cattle in Bhaktapur District, Nepal. www.ijagbio.com Sulochana Shrestha and Yugal Raj Bindari Himalayan College of Agricultural Sciences and Technology, Bhaktapur, Nepal.

International Standard Organization (ISO 6579/2002).ISO standard 6579:2002 (E). General guidance on methods for detection of *Salmonella.*

Jayarao, B.M. and Henning. D.R. 2001. Prevalence of foodborne pathogens in bulk tank milk. J. Dairy Sci. 84:2157-2162.

Jayarao, B.M.; Donaldson, S.C.; Straley, B.A.; Sawant, A.A.; Hegde, N.V. and Brown, J.L. 2006. A survey of food borne pathogens in bulk tank milk and raw milk consumption among farm families in Pennsylvania. J. Dairy Sci., 89:2451-2458.

Jodas, S., and Hafez , H. M . 2003. Surveillance on antimicrobial resistance in *Escherichia coli* and *Salmonella* isolates from poultry between 1993 and 1999. Archiv fur Geflugelkunde ;67(1); 16-21.

Kauffmann, F. (1974):"Kauffmann white scheme WHO, AO/72,1."Rev.I. Acta Pat. Microbiol. Scand., 61 : 375-387.

Khan ,M .; suryanarayan ,P.;Ahmed ,M.; Vaswani ,R.and Faheem,S. 2010. Antimicrobial susceptibility of *Salmonella* isolates from chicken meat samples in Dubai ,UNited Arab Emirates .Iternational journal of food ,Nutrition and puplic Health ,3(2):149-159.

Malorny, B.; Hoorfar, J.; Bunge, C.and Helmuth, R. 2003. Multicenter validation of the analytic accuracy of *Salmonella* PCR: toward an international standard. Appl. Environ Micronial. 69: 290-296

McQuiston JR, Parrenas R, Ortiz-Rivera M, Gheesling L, Brenner F and Fields PI. (2004): Sequencing and comparative analysis of flagellin genes *fliC*, *fljB*, and *flpA* from *Salmonella*. J Clin Microbiol. May;42 (5):1923-32.

Merck Veterinary Manual, 2006. http://www.merckvetmanual.com.htm

Mituniewicz, T.; Sowinska, J.; Iwanczuk-Czernik , K.; Witkowska, D.; Wojcik A.; kleinszmidt K. and Sobczak , J. 2007. *Salmonella* bacteria occurrence on poultry farms in warmia and Mazury province between 2001-2005. Medycyna weterynaryjna. 63(9):1081-1083.

Murugkar , H.V.; Rahman , H.; Kumar , A. and Bhattacharya , D . (2005): Isolation , phage typing and antibiogram of Salmonella from man and animals in northern Indian . Indian J. Med . Res ., 122 (3): 237-242.

Nccls.2002*; Diagnostic Microbiology and Infectious Disease .Volume 42, Issue 2, February 2002, Pages 137-139.*

Olivera, S.D.; Rodenbusch, C.R.; Ce, M.C.; Rocha, S.L.S. and Canal, C.W. 2003. Evaluation of selective and non selective en-richment PCR procedures for *Salmonella* de-tection. Lett. Appl. Microbiol., 36: 217-221.

Pan,Z.;Wang,X.;zhang,x.;Geng,S.;Chen,X.;pan, W.;cong,Q.;Liu,X.;Jiao,X.andLiu,X. 2009. Changes in antimicrobial resistance aming *Salmonella enterica* subspecies enterica serovar pullorum isolates in china from 1962 to 2007. Vet Microbial.136(3-4):387-392.

Pawsey, R.K. 2002. Case study in food microniology for food safety andquality. Cornwell. TJ International Ltd. pp. 179-190

Peek, SF., Hartmann FA., Thomas CB., Nordlund KV. 2004. Isolation of *Salmonella spp* from the environment of dairies without any history of clinical salmonellosis. J Am Vet Med Assoc 2004; 225:574-577.

Quinn, P, J., Markey, B.K.; Carter, M.E., Donnell , W,J,C., and Leonard, F,C. 2002. "Veterinary Microbiology and Microbial Diseases " Blackwell Science , London ,UK.Rocz.Panstu .Zaki .Hig.,41(5-6):263-268.

Salihu M.D. , Junaidu A.U , Tambuwala F.M. , Magaji A.A. and JaafaruAdv S. 2011. Prevalence of Mastitis in Lactating Cows in some selected Commercial Dairy Farms in Sokoto Metropolis Appl. Sci. Res., , 2 (2):290-294.

Scherrer, D.; Corti, S.; Muehlherr, J.E.; Zweifel, C. and Stephan, R. 2004. Phenotypic and genotypic characteristics of *Staphylococcus aureus* isolates from raw bulk-tank milk samples of goats and sheep. Vet Microbiol 101: 101-107.

Shivhare S, Sharda R, Sharma V and Reddy AG (2000). Antibiogram and drug resistance pattern of Salmonella isolates of avian origin. Ind. J. of Comp. Microbiol. Immuno and Infect. Dis. 21(1):76–78.

Threlfall, E.j.j., wain, T., peters C., lane, E., De pinna, Cl., Little, A.D. Wales , R.H., Davies. 2014. Egg-borne infections of humans with

salmonella: not only an S. enteritidis problem. Worlds poultry Sciences Journal 70(01): 15-26.

Van Kessel JS., Karns JS., Gorski L., McCluskey BJ., Perdue ML. 2004. Prevalence of *Salmonella, Listeria monocytogenes*, and fecal *coliforms* in bulk tank milk on US dairies. J. Dairy Sci., 87(9): 2822-2830.

Van Kessel, J.A.; Karns, J.S.; LOMBARD, J.E. and KOPRAL, C.A. 2011. Prevalence of *Salmonella enterica*, *Listeria monocytogenes*, and *Escherichia coli* Virulence Factors in Bulk Tank Milk and In-Line Filters from U.S. Dairies. J. Food Protect. 74: 5

Yang, X., Brisbin2, J., Yu, H., Wang, Q., Yin, F., Zhang, Y., Sabour, P., Sharif, S. and Gong, J. 2014. Selected Lactic Acid-Producing Bacterial Isolates with the Capacity to Reduce *Salmonella* Translocation and Virulence Gene Expression in Chickens. PLOS ONE | www.plosone.org 1 Volume 9 (4): e93022.

Zeinhom, M.A. and Abdel-Latef, G.K. 2014. Public health risk of some milk borne pathogens; beni-suef university journal of basic and applied sciences xxx (2014); 1(7):209-215.available at: http://dx.doi.org/10.1016/j.bjbas.2014.10.006.