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STUDIES ON THE ROLE OF VIRULENCE FACTORS OF *S. ENTERICA* AND ITS PATHOGENICITY IN DAIRY FARMS

By

Gamil M.[*]; Amin M.M.[*]; Ebtsam E.Z.Kotb[**] and El-Ashmawy W.R.[*]

[*] Department of Internal Medicine and Infectious Diseases, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt. [**] Animal Reproduction Research Institute, Giza, Egypt.

ABSTRACT

Bovine Salmonellosis is a worldwide bacterial disease causing great zoonotic impact, animal losses and economic problems. The present study was conducted on a total number of 313 samples divided into188 milk samples (124 from bulk tank milk and 64 from individual mastitic milk) and 125 fecal samples (100 diarrheic fecal samples and 25 from apparently normal calves in contact with the diseased ones) which collected from different localities of governorates in Egypt. Samples were examined bacteriologically and serologically, revealed that in young diarrheic calves and apparent normal calves showed 11% and 4% were positive results, while mastitic caws exhibited 3.1% positive results and from bulk tank milk 3.2%. Serological 18 of *Salmonella* isolates were positive; fifteen of them were typed as *S. Typhimurium*, two as *S. sekondi* and only one as *S. chester* in percentage of 83.3%, 11.1% and 5.6% respectively. Epidemiological studies showed that some risk factors as (age, sex, housing, and hygiene) have significant effect on *Salmonella* prevalence. Statistical analysis showed that there were significant results .The highest rate of salmonellosis was recorded in small animals and in bad hygienic farms specially containing rodents and steer animals. Conventional PCR was performed on 18 *Salmonella* isolates for the presence of seven virulence genes (invA, bcfC, stn, pefA, mgtC, csgD, and fimH). These genes were detected in 18 (100%), 18 (100%), 16 (88.9%), 14(77.7%), 3(16.7%), 1 (5.5%) and 0 (0%) respectively. In conclusion, *Salmonella* is one of the most important causes of diarrhea in calves and in adult cattle causing mastitis. The application of PCR assay is important for rapid detection of virulence profile of *Salmonella*.

Key words:

Salmonella enterica, cattle, virulence genes, risk factors and PCR.

Corresponding author Ebtsam Kotb: dekotb@yahoo.com.

INTRODUCTION

Bovine Salmonellosis is a worldwide bacteria as have many impacts on animal causing economic losses in calves and has zoonotic importance in human health that are considered a major wide problem **(Youssef and El-Hiag 2012).** *Salmonella enterica* is a wide bacterial species that involved in field problems. Ninety-nine percent of human and animal infections are caused by the subspecies *enterica*. Within this subspecies more than 2600 serotypes have been classified, based on serological reactions to cell wall lipopolysaccharide (O), flagellar (H) and capsular (VI) antigens **(Sanderson and Nair, 2012, Agren (2017)**. Clinical signs of bovine salmonellosis including diarrhea, fever, anorexia, dehydration, abortion and evidence of endotoxaemia are the most excited although many infections remain asymptomatic **(Nielsen *et al.*, 2012b and OIE 2016)**. Detection and identification of *Salmonella* required not only isolation but also biochemical, serological and molecular assay that detect *Salmonella species* or even strain level for accurate epidemiological survey **(Azooz 2017).** DNA based method is becoming increasingly important in epidemiological studies and differentiation of *Salmonella species* and could be used as a rapid and reliable tool for epidemiological survey. PCR provides a specific method and superior ability to detect *S. enterica* serovars in the presence of other bacteria simultaneously **(Moussa *et al*., 2012)**. Virulence of *Salmonella* is linked to a combination of chromosomal and plasmid factors. *Salmonella* pathogenicity islands (SPIs) are large gene cassettes within the *Salmonella* chromosome that encode determinants responsible for establishing specific interaction with the host, and are required for bacterial virulence in a given animal. There are more than 20 SPIs have been described **(Sabbagh *et al.*, 2010 and Aleslamboly 2011).**

The most common detected virulence genes (invA and bcfC) using specific primer for each. InvA and bcfC gene were expressed in all examined *salmonella* serotypes **(Mohammed 2014).** Diarrhea induced by *Salmonella* infection is a complex phenomenon involving several pathogenic mechanisms including production of enterotoxin which mediated by *Salmonella* enterotoxin virulence gene stn **(Murugkar *et al*., 2003).** The objective of the present work is to investigate the risk factors associated with isolation of *Salmonella enterica* microorganisms and some of its virulence genes (invA, stn, pefA, csgD, bcfC, fimH and mgtC) by PCR.

MATERIAL AND METHODES

Collecting Samples:

According to **Anon 2002 and Quinn *et al.*, 2002.**The present study was conducted on a total number of 313 different collecting samples divided into, 124 bulk tank milk samples, 64 individual milk samples and 125 fecal samples were collected from different locality of some Governorates in Egypt and full history for each case was taken from owners including age, sex and health status.Fecal samples should be fresh, preferably collected from the animal during the act of defecation or from the rectum using a fecal loop during the physical examination transferred in ice box as soon as possible to bacteriological lab. of Animal Reproduction Research Institute (ARRI) for bacteriological examination.

Bacteriological examination and biochemical tests:

Accordingto**(Anon,2002andQuinn*et al.*,2002).**Typicalcoloniesof*Salmonella* microorganisms were identified by morphology, Gram staining - biochemical identification (traditional tests), serological methods and molecular study.

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, and Giza.

Detection of Virulence genes in *Salmonella* isolates by PCR:

DNA extraction: DNA was extracted from bacterial colonies using QIAamp DNA mini kit (Qiagen, Germany, GmbH) instructions.

Oligonucleotide primers: primers used were supplied from (Metabion company, Germany) are listed in (Table 1)

PCR amplification: primers were utilized in a25 reaction containing 5µl of 5 taq PCR master Mix (jena Bioscience, Germany),1µlof each primer of 20pmol concentration,13µl of PCR grade water (jena,Bioscience,Germany) and 5 of DNA template . The reaction was performed in aBiometra thermal cycler.

Analysis of the PCR products:

The products of PCR were separated by electrophoresis on 1.5% agarose gel **(**Applichem, Germany, GmbH) in 1x TAE buffer at room temperature .for gel analysis, 6 µl of the products were loaded in each gel well. A 100bp DNA ladder (Jena bioscience, Germany) was used to

determine the fragment size. the gel was photographed by agel documentation system (Alpha innotech Biometra) and the data was analyzed through Bioedit software version 7.0.0.

Table (1): Primers sequences, target gene, amplicon size and cycling condition.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
csgD	TTACCGCCTGAGATTATCGT ATGTTTAATGAAGTCCATAG	651	94°C 5 min.	94°C 30 sec.	50°C 45 sec.	72°C 45 sec.	72°C 10 min.	Bhowmick *et al.*, 2011
mgtC	TGA CTA TCA ATG CTC CAG TGA AT ATT TAC TGG CCG CTA TGC TGT TG	677	94°C 5 min.	94°C 45 sec.	54°C 45 sec.	72°C 45 sec.	72°C 10 min.	Huehn *et al.* 2010
bcfC	ACC AGA GAC ATT GCC TTC C TTC TGC TCG CCG CTA TTC G	467	94°C 5 min.	94°C 30 sec.	52°C 30 sec.	72°C 30 sec.	72°C 7 min.	
pefA	TGT TTC CGG GCT TGT GCT CAG GGC ATT TGC TGA TTC TTC C	700	94°C 5 min.	94°C 45 sec.	55°C 45 sec.	72°C 45 sec.	72°C 10 min.	Murugkar *et al.*, 2003
Stn	TTG TGT CGC TAT CAC TGG CAA CC ATT CGT AAC CCG CTC TCG TCC	617	94°C 5 min.	94°C 45 sec.	59°C 45 sec.	72°C 45 sec.	72°C 10 min.	
fimH	GTGCCAATTCCTCTTACCGTT TGGAATAATCGTACCGTTGCG	164	94°C 5 min.	94°C 30 sec.	59°C 30 sec.	72°C 30 sec.	72°C 7 min.	Hojati *et al.*, 2013
invA	GTGAAATTATCGCCACGTTCGGGCAA TCATCGCACCGTCAAAGGAACC	284	94°C 5 min.	94°C 30 sec.	59°C 45 sec.	72°C 45 sec.	72°C 10 min.	Olivera *et al.* (2003)

Statistical analysis:

Frequencies were subjected to chi-Square test analysis to assess the significance between different variables (**SAS, 2004**).

RESULTS

Table (2): The prevalence rate of isolation of *Salmonella* spp. in examined animals and bulk tanks milk samples.

Isolated M.O			No	%
Rate of distributed salmonella according to	Age	Young	12/125	9.6
		Adult	2/64	3.1
	Symptoms	Mastitis	2/64	3.1
		Diarrhea	11/100	11
		Apparent normal	1/25	4
Bulk tank milk			4/124	3.2
No. of isolates / Total examined samples			18/313	5.8

It was found that, the highest rate of salmonellosis was recorded in young diarrheic calves.

Table (3): Factors affecting the rate of isolation of *Salmonella* spp.

Category	Risk factor	Effects
Animals management	Grouping and avoid overcrowding	Decrease the infection rate
	Quarantine for new purchase and diseased cattle	
Hygienic measures	Sanitary condition in milk parlour	
	Manual milking	Increase the infection rate
Biosecurity measures	Birds, rodents and wild animals	
	Ration	Act as sources of infection
	Human	

From this study many management aspect such as grouping animals, herd size, manure management, equipment sanitation, feed composition, presence of rodents and wild animals have been associated with increase prevalence of *Salmonella* in cattle herds and may prolong persistence of these microorganisms on the farm.

Table (4): Serotyping of isolated *Salmonella*.

	Total No. of isolate	*S. typhimurium*			*S. sekondi*			*S. chester*		
		Somatic (O) antigen	Flagellar (H) antigen		Somatic (O) antigen	Flagellar (H) antigen		Somatic (O) antigen	Flagellar (H) antigen	
			Phase 1	Phase 2		Phase 1	Phase 2		Phase 1	Phase 2
		1 , 4 , [5],12	I	1,2	3,10	e,h	Z_6	1 , 4 , [5] 12	e,h	e,n,x
BTM	4	4			0			0		
Mastitic cow	2	2			0			0		
Diarrheic calves	11	8			2			1		
Apparently normal	1	1			0			0		
Total	18	15 (83.3%)			2 (11.1%)			1 (5.6%)		

The high percent of isolated M.O. in calves followed by BTM then mastitic cows and finally apparently normal.

Table (5): Detection of Some Virulence Genes in *Salmonella* isolates by PCR.

Samples No.	invA	bcfC	stn	pefA	mgtC	csgD	fimH
1	+	+	+	-	-	-	-
2	+	+	+	+	-	-	-
3	+	+	+	+	-	-	-
4	+	+	-	+	-	-	-
5	+	+	+	+	+	-	-
6	+	+	+	+	-	-	-
7	+	+	+	+	-	-	-
8	+	+	+	+	-	-	-
9	+	+	-	+	-	-	-
10	+	+	+	-	-	-	-
11	+	+	+	+	-	-	-
12	+	+	+	+	+	-	-
13	+	+	+	+	-	-	-
14	+	+	+	-	-	-	-
15	+	+	+	+	-	-	-
16	+	+	+	+	+	-	-
17	+	+	+	+	-	-	-
18	+	+	+	-	-	+	-
Total %	**100**	**100**	**88.9**	**77.8**	**16.7**	**5.5**	**0**

Conventional PCR performed on 18 *Salmonella* isolates for the presence of seven virulence genes: invA, bcfC, stn, pefA, mgtC, csgD, and fimH. These genes were detected in 18 (100%), 18 (100%), 16 (88.9%), 14 (77.7%), 3 (16.7%), 1 (5.5%) and 0(0%) isolates respectively.

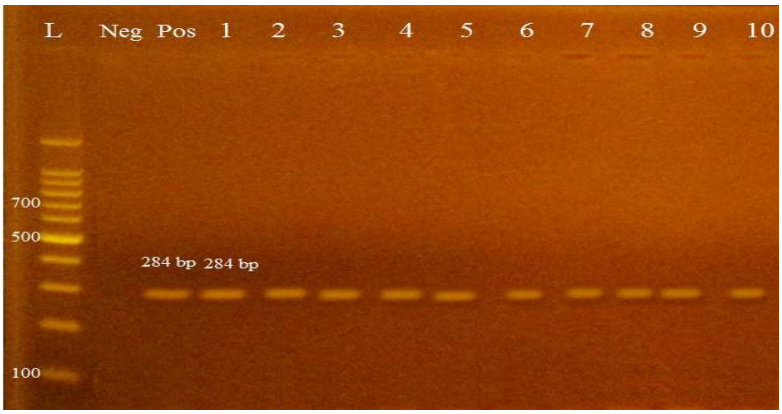

Fig.(1): 1.5% agarose electrophoresis gel show results of PCR amplification of invA 284 bp) virulence gene of *Salmonella* analyzed on,Lane L: 100 bp DNA ladder;Lane Neg: negative control; Lane Pos; positive control; Lane (1 - 10) positive samples.

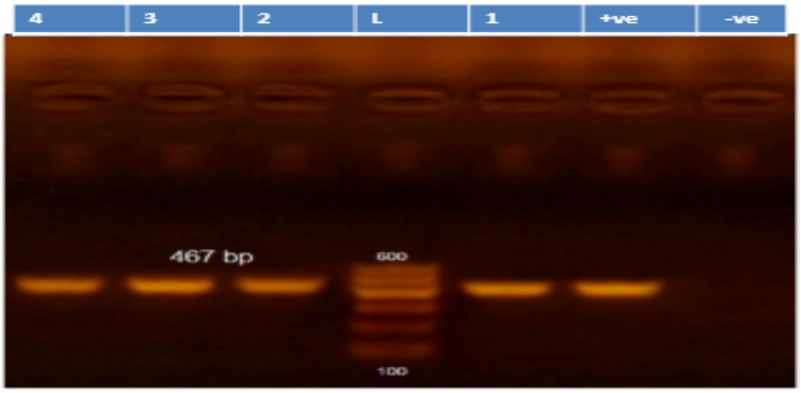

Fig. (2): 1.5% agarose electrophoresis gel show results of PCR amplification of bcfC (467 bp) virulence gene of *Salmonella* analyzed on, Lane L: 100 bp DNA ladder; Lane-ve: negative lane +ve positive control; Lane (1 - 4) samples.

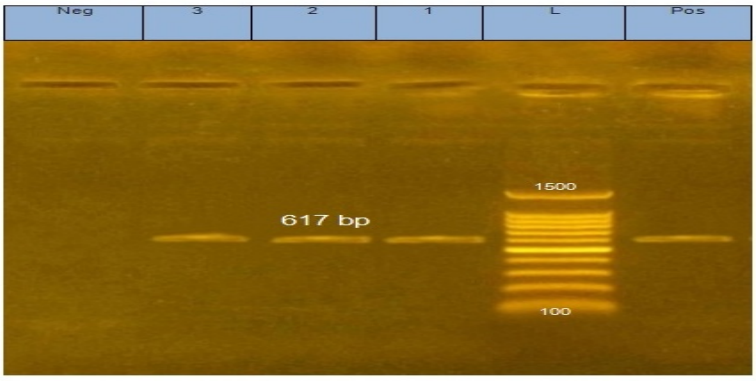

Fig. (3): 1.5% agarose electrophoresis gel show results of PCR amplification of stn (617 bp) virulence gene of *Salmonella* analyzed on, Lane L: 100 bp DNA ladder; Lane Neg: negative control; lane Pos: positive control; Lane (1-3) positive samples.

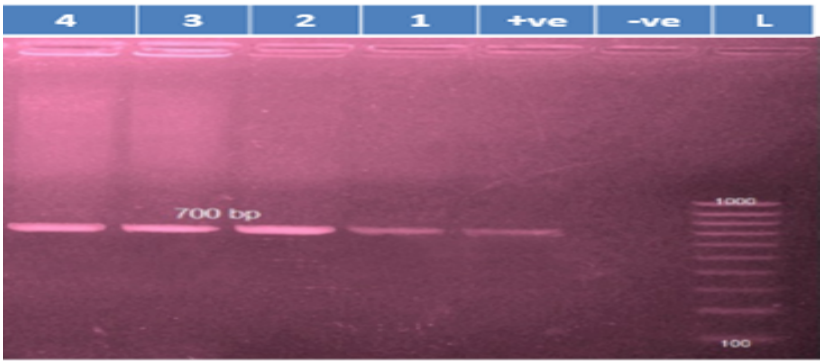

Fig. (4): 1.5%agrose electrophoresis gel show results of PCR amplification of pefA (700 bp) virulance gene of *Salmonella* analyzed on, Lane L: 100 bp DNA ladder; Lane (-ve) negative *8-control; lane Pos: positive control; lane (1 - 4) positive samples.

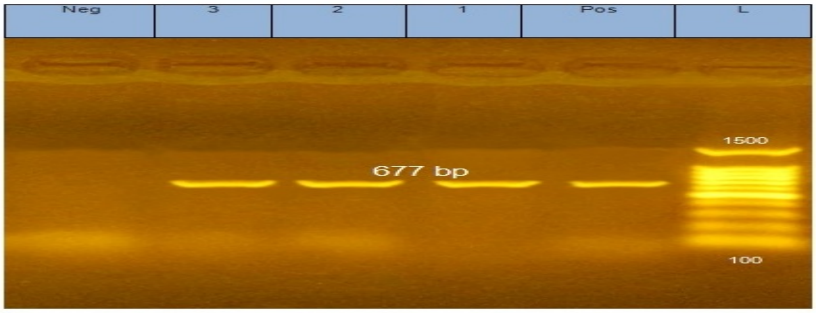

Fig. (5): 1.5 % agarose electrophoresis gel show results of PCR amplification of mgtC (677 bp) virulence gene of *Salmonella* analyzed on, Lane L: 100 bp DNA ladder; Lane negative control; lane post :positive control and samples Lane (1-3).

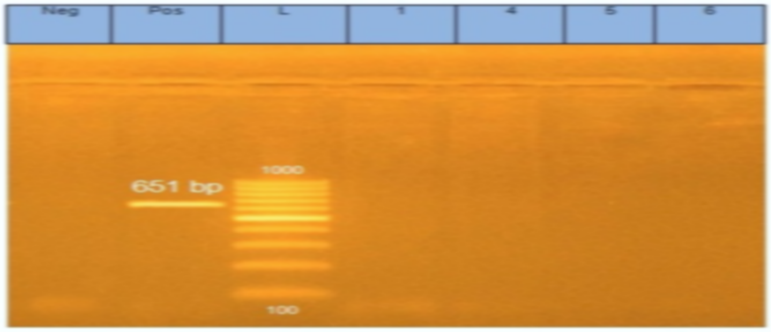

Fig. (6):1.5% agarose electrophoresis gel show results of PCR amplification of csgD (651 bp) virulence gene of *Salmonella* analyzed on, Lane L: 100 bp DNA ladder; Lane Neg: negative control; lane Pos: positive control; Lane (1-4) negative samples.

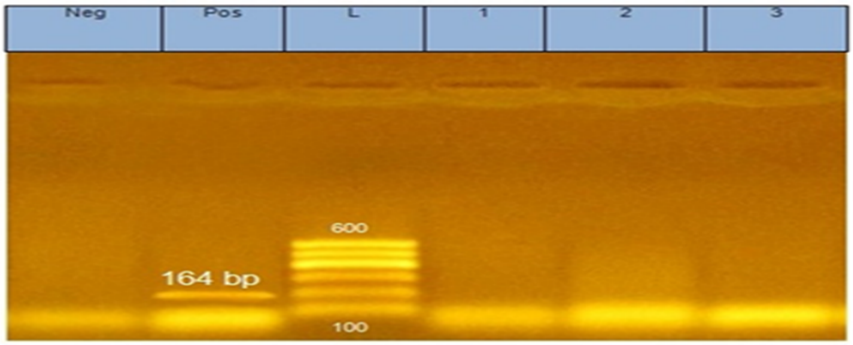

Fig. (7):1.5% agarose electrophoresis gel show results of PCR amplification of fimH (164 bp) virulence gene of *Salmonella* analyzed on, Lane L: 100 bp DNA ladder; Lane Neg: negative control; lane Pos: positive control; Lane (1-3) negative samples.

DISCUSSION

Salmonella infection among the examined cattle as the control program is mostly based on passive surveillance and bacterial culture positive results, as shown in (Table 2); we isolated *salmonella* spp. from 5.8% of total collected samples. As 12 (9.6%) feacal swabs included 11 diarrheic calves and one apparent normal in percentage of 11% and 4% respectively.

This result was slight lower than that of **Seleim *et al*., (2004)** who collected fecal samples from calves suffering from diarrhea revealed the isolation of *Salmonella was* 17.5% of the cases, while in the contact apparently healthy calves was 3.4% **Yousef and El-Hiag (2012) and El-Seedy *et al.,*(2016)** reported that salmonellosis prevalence was 18.6% and 18.1% among diaherric calves respectively in Egypt and the result was higher than that reported by **Hagag and khaliel (2002),Moussa *et al.,* (2013), Selim (2015),Azooz (2017) and Rizk (2018)** which were 4% ,4.4%, 1.2% ,1.28% and 1% among diahrric calves respectively in Egypt. Calves affected with acute clinical salmonellosis may shed 10^8-10^{10} *Salmonella*/gram feces and when recovered from infection may persist shedding of microorganisms for 2-12 weeks post recovery without clinical signs being chronic infected carrier as mentioned by **Ola *et al.,* (2014)**, This explained the results of isolation rate of Salmonella from apparently normal animal which is 4% as shown in (Table 2).

Mastitis is a significant problem for dairy cows which causing severe economic losses and impact on production as it increased treatment costs, discarded milk, lost quarters, premature culling, death rate and decreased milk yield. *Salmonella* were isolated from mastitic caws (3.1%) as reported in (Table 2), that near to the results recorded by **Abd El-tawab *et al.,***

(2017) who isolated *Salmonella* from mastitic milk samples (3.78 %) and **Salihu *et al.*, (2011)** who determined the prevalence of *Salmonella* in clinical mastitic milk samples as 2.17%**.** On the other hand *salmonella* isolated from BMT in 3.2%in rate lower than detected by **Omar *et al*., (2018)** that in bulk farm milk was 14% and **Singh *et al.*, (2018**) showed that prevalence of *salmonella* spp. in raw milk was 11.9 %. This percent of isolation may be contributes to the calving pens harbor number of microorganisms, contamination during milking due to inadequate milking sanitation.

From this study many epidemiological investigations were compared and identified as route means of spread e.g. management aspect such as animals grouping, herd size, manure management, equipment sanitation, feed composition, presence of rodents and wild animals generally poor hygienic conditions which have been associated with increase prevalence of salmonella in cattle herds and may prolong persistence of these microorganisms on the farm as shown in (Table 3).These results were agree with that recorded by **Kirk *et al.*, (2002),** suggesting that birds are a potential source of external contamination that deserve further scrutiny. This is because birds can transport and disseminate pathogens over long distances.

Also **El-Seedy *et al.*,(2016),Azooz (2017**) who recorded that, the risk factors that were found to be associated with the presence of this pathogen included purchasing calves from dealers,herds size, group housing ,dirty muddy, calf floor area ,inadequate cleaning of feeding and watering utensils,sign of rodents in feed storage area ,presence of dogs and cats, lack of barn disinfection, age of calves, breeds, gender and season.

S. typhimurium were the most frequently isolated serovars as shown in (Table 4) in rate 88.3% as mentioned by **Ahmed *et al.*, (2009) El-Seedy *et al.*,(2016) Eman Khalifa *et al*., (2017)** followed by *S. sekondi* 11.1% then *S. chester* 5.6% which rare to be isolated but we could isolated and identified them serologically.

The *Salmonella* infection can determine by the host status and microorganisms conditions. As the age, nutrition and environmental hygienic factors can determine host status, while the status of microorganisms is detected by virulence factors which powerful marker for microorganisms based on the fact that virulence varies not only among different spp. but also among strains of the same spp. thus numerous studies have been conducted to identified the virulence genes **Ola *et al.*, (2014)**. The results recommended that, the identification of *Salmonella* isolates virulence genes by PCR using two sets of primers targeting the invA and bcfC sequences from *Salmonella* spp. As the chromosomally located invasion A (invA) and

(bcfC) gene which triggers the pathogen to invade the host cell, has been considered a universal genetic marker identified from mostly all the *Salmonella* serovars. Also, the enterotoxin (stn) gene and *pefA* gene sequences which are another virulence gene, encodes a protein causing severe diarrhea,regarded as a unique PCR marker for *Salmonella* identification regardless of their serovars. As the results appeared invA, bcfC, stn and pefA genes present in 100%, 100% 88.9% and 77.8% in all serovars respectively; while mgtC gene, csgD gene and fimH gene were detected in 16.7%, 5.5% and 0% respectively.

These results were similar to those recorded by **Ola *et al.*, (2014), Mohamed (2017), Singh *et al*., (2018)** that showed that invA gene was present in all *salmonella* positive samples and can be used for rapid and accurate diagnosis of bovine *salmonellosis*.

It could be concluded that, the performance of hygienic measures during milking and raring of calves are essential to minimize the risk of infection with *Salmonella*. Moreover, the application of PCR targeting in vA and bcfC genes provides a valuable tool for the rapid identification of *Salmonella* from milk and fecal swabs.

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