ejpmr, 2018,5(3), 11-17

SJIF Impact Factor 4.897

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

DIAGNOSIS OF SALMONELLA ASSOCIATED WITH DIARRHEA IN BOVINE CALVES AND ROLE OF LACTOFERRIN IN TREATMENT

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Article Received on 25/12/2017 **Article Revised on 15/01/2018** **Article Accepted on 05/02/2018**

ABSTRACT

Clinically, bacteriologically, serologically and by polymerase chain reaction (PCR); two hundred and fifty calves, with an age of 1 day to 2 months old were examined to diagnosed and identify salmonella and its virulence factors. Our study showed that the clinical signs ranged from yellowish watery to greenish watery diarrhea with blood clots and/or mucus. The mucous membranes ranged from normal, congested or pale. The eyes of affected animals were sunken in most cases due to dehydration while the bacteriological isolation revealed that 35(14%) calves were positive to salmonella including 57.14% cow calves and 42.85% buffalo calves. Serotyping of salmonella isolates revealed that *S. enteritidis* was 17.14%, *S. typhimurium* was 45.7%, *S. infantis* was 14.28%, *S. tsevie* was 14.28 and *S. magherafelt was* 8.57%. PCR carried out on 15 isolates; *inv*A gene was positive in 13 cases, *stn* gene was positive in 6 cases and *pef* gene was only in 2 cases. The antimicrobial sensitivity indicated that the most effective antibiotics were observed for Cefquinome (85.7%), Cefepime (77.1%), Ceftriaxone (60%) and Ciprofloxacin (57.1%). After using of Lactoferrin the total anti- oxidant capacity increased while H2O2 decreased and the cure rate of salmonella infected animals increased as the number of dead animals decreased from 6 (17.14%) to one (2.8%), so we concluded that the diarrhea of salmonella affected animals was ranged from yellowish watery to greenish watery diarrhea with blood clots and mucus, the most prevalent serotypes of salmonella were *S. Typhimurium* and *S. Enteritidis* and the treatment with antibiotics in addition to lactoferrin increased the cure rate and decrease the recurrency of the disease.

KEYWORDS: Salmonellosis in bovine calves, serological identification of Salmonella, PCR, lactoferrin.

INTRODUCTION

Bovine salmonellosis is a worldwide bacterial disease, it is the most serious infection usually attacks calves during the first ten weeks of life (Hoiseth and Stocker, 1981). It can be a costly disease for dairy producers because of treatment expenses, mortality and weight loss/decreased weight gain within the herd (Huston et al., 2002).

Salmonella is a zoonotic enteric pathogen that can cause significant disease in both calves and adult cattle. Clinical signs of bovine salmonellosis may include diarrhea, fever, anorexia, dehydration, decreased milk production, abortion and evidence of endotoxemia, although many infections remain subclinical (Divers and Peek, 2008).

The *invA* gene is essential for full virulence in salmonella and thought to trigger the internalization required for invasion of deep tissues; serotypes of salmonella that do not have the *invA* gene are not capable of expressing inv ABC genes, making them unable to invade mammalian cells (Khan et al., 1999).

Certain Salmonella serovars belonging to subspecies I carry a large, low-copy-number plasmid that contains virulence genes. Virulence plasmids are required to cause systemic disease; their involvement in the enteric stage of infection is unclear. Salmonella virulence plasmids *spv* are required for bacterial multiplication in the reticulo-endothelial system. Other loci of the plasmid, such as the fimbrial operon *pef*, the conjugal transfer gene *traT* and the enigmatic *rck* and *rsk* loci may play role in other stages of the infection process (Rotger and casadesus, 1999).

The standard method for detection of Salmonella has been developed and evaluated and an ISO-method (ISO6579:2002 Annex D) has now been adopted. The core of the standard method is pre-enrichment in buffered peptone water, enrichment on modified semi-solid Rappaport–Vassiliadis (MSRV) and isolation on xylose-lysine-deoxycholate (XLD) and an additional plate medium of choice (ISO, 2002).

Serotyping of Salmonella by Kauffman- White scheme (KW) is based on the antigenic structure of Salmonella serotypes. The cross absorption of antisera is used to reveal the antigenic structure of Salmonella (Helmuth, 2000).

Detection of Salmonella serovars by PCR is more rapid than conventional culture techniques. The sensitivity and specificity of PCR was 100% compared with culture techniques. PCR could be applied for rapid routine diagnosis (Stone et al., 1994).

MATERIALS AND METHODS

Two hundred and fifty calves (130 farm calves including 60 cow calves and 70 buffalo calves and 120 individually raised calves including 50 buffalo calves and 70 cow calves). Their age ranged from one day to two months. The animals were suffering from diarrhea and fever in most cases.

The animals examined clinically by measuring body temperature, noticing mucous membranes, sunken eyes and fecal characters for each case. The animals were clinically examined for health status, with special attention for diarrhea according to Kelly (1984).

Fecal Samples and swabs were collected aseptically from the rectum of animals by sterile swabs in tubes containing Rappaport–Vassiliadis broth or fresh feces and sent as soon as possible in an ice box to the laboratory for bacteriological examination.

Blood samples collected without anticoagulant for H_2O_2 and total anti-oxidant capacity; and the samples allow clotting for 30 min. at 25°C then put in the refrigerator for 2 hrs then centrifuge at 3000 rpm for 15 min. at 4°C, pipette off the top yellow supernatant, put in a centrifuge tube then centrifuge again then take the clear serum layer. Store serum on ice and if not assaying at the same day, freeze at -80°C.

The fecal samples and swabs were examined bacteriologically as following, the rectal swabs were enriched in 10 ml of Rappaport–Vassiliadis broth then incubated aerobically at 37Cº for 18 hours then cultured onto MacConkey's agar, Xylose lysine deoxycholate (X.L.D.) and Salmonella- Sigella agar media then inocubated at 37 Cº / 24 -48 hours. Suspected growing colonies were picked up and sub-cultured then pure culture was inoculated onto slant and semisolid agar tube and all Gram-negative bacilli were identified biochemically according to (Quinn et al., 2002).

Salmonella isolates were subjected to serological identification according to Kauffman-white scheme (Kauffmann 1974) as following**:** A loopfull of the colony was suspended in a drop of physiological saline solution on a slide, so as to make a homogeneous suspension. A drop of Salmonella antiserum was added to the suspension with a standard loop and thoroughly mixed, Positive agglutination occurred within a minute**.**

DNA was extracted from pure cultures by phenol-chloroform method according to Sambrook et al. (1989). Preparation of PCR Master Mix according to Emerald Amp GT PCR master mix (Takara) Code No. RR310A kit *(tab.1).*

Table 1: PCR analysis of Salmonella serovars achieved by the oligonucleotide primers.

Primer	Sequence	Amplified product	Reference
Stn	TTG TGT CGC TAT CAC TGG CAA CC	617 bp	**Murugkar *et al.*, 2003**
	ATT CGT AAC CCG CTC TCG TCC		
pefA	TGT TTC CGG GCT TGT GCT	700 bp	
	CAG GGC ATT TGC TGA TTC TTC C		
invA	GTGAAATTATCGCCACGTTCGGGCAA	284 bp	**Oliveira *et al.*, 2003**
	TCATCGCACCGTCAAAGGAACC		

Treatment based on the sensitivity test by antimicrobials as Cefquinome (30µg), cefepime (30µg), ceftriaxone (30 µg) and ciprofloxacin (5 µg) used**,** oral preparations as Sacrolyte used for rehydration in moderately dehydrated calves, while Saline 0.9% or Ringer lactate with isotonic sodium bicarbonate 1.3% intravenously in severe cases Smith and Berchtold (2014). Probiotics as *Lactobacillus rhamnosus* used for regeneration of the intestinal microflora Vanderpool et al. (2008). Flunixin Meglumin and Diclofenac sodium used in cases of fever and toxemia. Lactoferrin 100 mg (Pravotin sachets) used in 17 cases to show its effect on cure rate and as an anti-oxidant agent.

RESULTS

The clinical features of diarrhea in animals with salmonellosis ranged from yellowish watery to greenish watery diarrhea with blood clots and/or mucus. The mucous membranes ranged from normal, congested or pale. The eyes of affected animals were sunken in most cases due to dehydration.

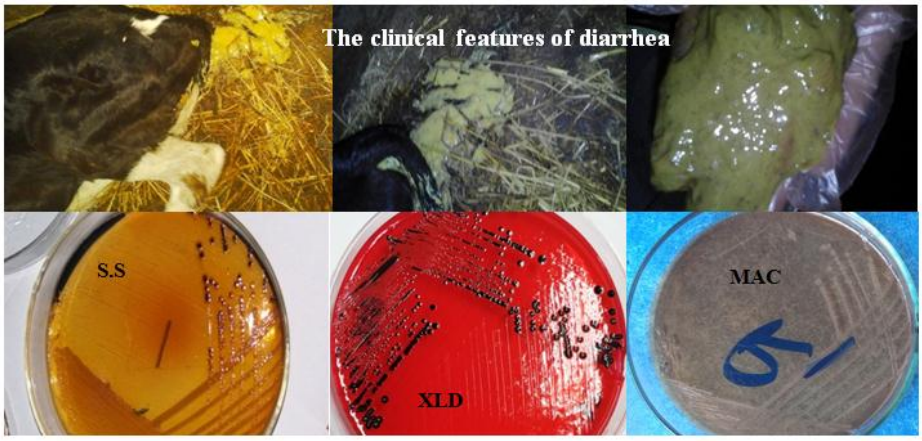

Figure 1: Shows the colonial characters on different media.

Salmonella isolates were tested biochemically and were negative to Indole, Urease and Voges proskauer while were positive to Cimon's Citrate, Methyl red and Triple sugar iron.

Table 2: Serotypes of the isolated Salmonella.

Calves	Salmonella isolates		
	Serotypes	No.	%
	S. Enteritidis	6	17.14
	S. Typhimurium	16	45.7
	S. Infantis	5	14.28
	S. Tsevie	5	14.28
	S. Magherafelt	3	8.57
Total	5	35	19.99

Table 3: Percentage of salmonella among diarrheic calves.

	Farm raised animals			Individually raised Animals		
	No.	+ve	%	No.	+ve	%
Cow calves	60	5	8.3	70	15	21.4
Buffalo calves	70	8	11.4	50	7	14
Total	130	13	10	120	22	18.33
Total of calves	250	35 salmonella infected calves				14%

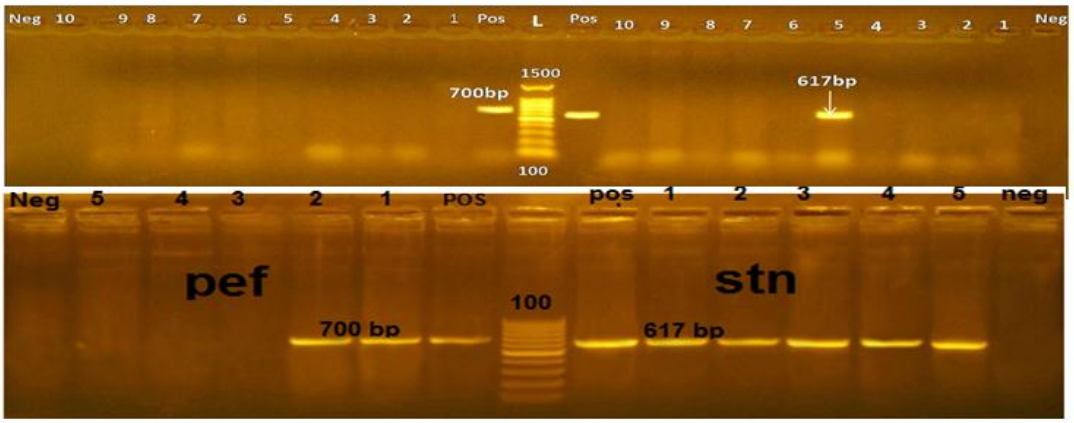

Figure 2: Lane L: DNA ladder. P: positive control; N: negative control. The others are positive isolates. PCR was carried out using primers specific for *pef*A (700bp) and *stn* (617bp) virulence genes. *stn* gene found in 6 cases, while *pef* gene found only in 2 cases

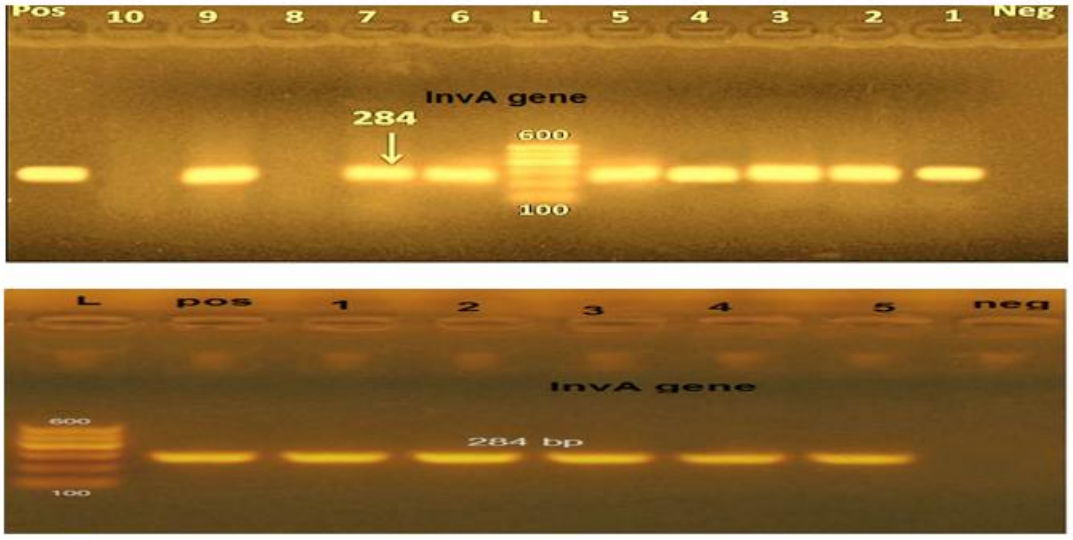

Figure 3: Lane L: DNA ladder. P: positive control; N: negative control the others are positive isolates. PCR was carried out using primers specific for *invA* (284bp) virulence gene. *invA* gene were positive in 13 cases

Table 4: Antimicrobial sensitivity of the isolated Salmonella.

Antimicrobial agent	cont.	S.		I.		R.	
		No.	%	No.	%	No.	%
Cefquinome (CEQ)	30 µg	30	85.7%	5	14.2%	0	0 %
Sulpha – trimethoprim (SXT)	25µg	9	25.7%	0	0 %	26	74.3 %
Ceftriaxone (CRO)	30 µg	21	60%	12	34.3%	2	5.7 %
Gentamycin (GN)	10 µg	3	8.6%	11	31.4%	21	60%
Amoxicillin & clavulonate	30 µg	8	22.9%	5	14.3%	22	62.9 %
Spectinomycin SH	100 µg	3	8.6%	1	2.9 %	32	91.4 %
Cefradine CE	30 µg	0	0%	0	0%	35	100 %
Streptomycin S	10 µg	2	5.7%	3	8.6 %	30	85.7 %
Ciprofloxacin CIP	5 µg	20	57.1%	14	40 %	1	2.9 %
Cefepime CMP	30 µg	27	77.1%	5	14.3%	3	8.6 %
Doxycycline	30 µg	0	0%	0	0 %	35	100 %
Chloramphenicol (C)	30µg	4	11.4%	1	2.9 %	30	85.7 %
Tetracycline (TE)	30µg	0	0%	0	0 %	35	100 %

Cont.: disc content; S. sensitive; I.: intermediate; R.: resistant.

Table 5: Effect of Lactoferrin on the cure rate.

Animals	Number of infected	Number of recovered	Cure rate
No. of animals treated without Lactoferrin	18	12	66.6
No. of animals treated with Lactoferrin	17	16	94.11
Total	35	28	80

Table 6: Bacteriological examination of Salmonella recovered animals.

	Farm raised animals (13)			Individually raised animals (22)		
	No. of infected	Re - infected animals	%	No. of infected	Re-infected animals	%
Cows calves	5	-	0	15	3	20
Buffalo calves	8	1	12.5	7	2	28.6
Total	13	1	7.69	22	5	22.7

Table 7: Total anti-oxidant capacity and hydrogen peroxide in the serum before and after the use of lactoferrin (400mg/day).

	Total anti-oxidant capacity(TAC) mmol/L		Hydrogen peroxide (H_2O_2) mmol/L	
	Before	After	Before	After
1	1.01	2.261	33.92	27.57
2	1.77	2.307	33.8	25.87
3	1.86	2.007	33.39	22.89
Mean	1.55	2.192	33.703	25.443

DISCUSSION

The clinical features of diarrhea showed that the feces were watery with variable amounts of mucus, fragments of the intestinal mucosa and blood clots with pyrexia in accordance with showed by Quinn et al. (2011) who mentioned that acute salmonellosis generally induces diarrhea, mucous at first, later becoming bloody and fibrinous, often containing epithelial casts.

Serotyping of salmonella isolates revealed that six isolates were *S.*enteritidis (17.14%), sixteen isolates were *S.*typhimurium (45.7%), five isolates were *S.*infantis (14.28%), five isolates were *S.* tsevie (14.28%) and three isolates were *S.* magherafelt (8.57%) with agreement with Moussa et al. (2010, 2012) who found that the most prevalence of Salmonella infections in calves was *S.*typhimurium and *S.* enteritidis serovars and Rasha and El-Behiry (2014) who published that the isolated Salmonella strains from cow-calves and buffalo-calves were belonging to 6 serovars namely: *S.* typhimurium, *S.* entritidis, *S.* infantis, *S.* anatum, *S.* dublin, and *S.* meleagridis *(tab. 2).*

The percentage of Salmonella among diarrheic calves was 14% and this in agreement with Rasha et al. (2015) who found that the percentage of Salmonella was 16.25% and disagree with Eid (2010) who found the percentage of salmonella was 46% and Younis et al. (2009) who reported that Salmonella spp. was found in 4.09% *(tab. 3).*

The conventional PCR performed on 15 Salmonella isolates for detection of three virulence genes: *inv*A, *stn* and *pef*A. The *inv*A gene was detected by a percentage of (86.66%) and this higher than the results obtained by Amr (2015) who found that the percentage of *(invA)* gene was 70% and lower than that obtained by Amini et al. (2010) who found that the percentage of it was (100%), while the *stn* gene percentage was (40%) and this was higher than the results of Rasha et al. (2015) and Amr (2015) who found that the percentage of it was (15.38% and 20% respectively) and lower than the results of Muthu et al. (2014) who found that the percentage of it was100%. Also our results showed that *pef* gene percentage was 13.33% and this disagree with the result obtained by Muthu et al. (2014) who stated that *pef* gene was not found in any of the tested strains of Salmonella and lower than Murugkar et al. (2003) who reported that *pef* gene was found in 89% of isolates *(fig. 2,3).*

The antibiotic sensitivity showed that all the isolates were highly sensitivite to Cefquinome (85.7%), Cefepime (77.1%), Ceftriaxone (60%) and Ciprofloxacin (57.1%) while all the isolates were resistant to doxycycline, Tetracycline and Cefradine (100%), spectinomycin (91.4%), sulfa-trimethoprim (74.3%), chloramphenicol (85.7%), Amoxicillin & Clavulonate (62.9%) and gentamycin (60%) and this in agreement with Aida et al. (2012) who found that the highest sensitivity was to Ceftriaxone and Enrofloxacin and Frye et al. (2008) who found that salmonella was more sensitive to the 4th generation cephalosporines as Cefquinome and Cefepime *(tab. 4).*

The cure rate of salmonella infected animals increased after using of Lactoferrin as the number of dead animals decreased from 6 (17.14%) to one (2.8%) and this come in agreement with Robblee et al. (2003) who explained that Lactoferrin has a good effect in the treatment of salmonella infected calves as it has a great affinity to bind to the iron which is one of the elements essential for the growth of bacteria, is responsible for the bacteriostatic effect of lactoferrin. A lack of iron inhibits the growth of iron-dependent bacteria such as *E. coli* and Salmonella *(tab. 5).*

After recovery of *salmonella* positive cases by 20 days; samples from these cases were taken and the results showed that one (12.5%) calve from farm animals affected with salmonella while in individually raised calves were 5 (22.7%) with a total percent 17.14% and this disagree with Amr (2015) who found no Salmonella were isolated from examined apparently healthy calves and higher than the results obtained by Rasha and El-Behiry (2014) who found that Salmonella was isolated from 5% of apparently healthy cow calves *(tab. 6).*

Our results explained that after using of lactoferrin the total anti-oxidant capacity increased from 1.55 mmol /L to 2.192 mmol/ L (70.71%) and this come in agreement with Leila et al. (2015) who found that LF at concentrations of 6.25–100 µg/ml significantly increased the FRAP (ferric reducing antioxidant capacity) levels in intra-cellular fluid and at the concentrations of 12.5–100 µg/ml in extra-cellular fluid concentration-dependently, while the H2O2 decreased in blood from 33.703 mmol/ L to 25.443 mmol / L (75.49%) and this is nearly similar to Sophie et al. (2000) who found that 20 and 50 µg/ml Lf caused 36.7 ±7.2% and 55.4 0.5% inhibitions of the intracellular hydrogen peroxide production, respectively

(tab. 7). So we concluded that the diarrhea of salmonella affected animals was ranged from yellowish watery to greenish watery diarrhea with blood clots and mucus, the most prevalent serotypes of salmonella were *S. Typhimurium* and *S. Enteritidis*, lactoferrin acts as anti-oxidant, antibacterial and cause denaturation of the lipopolysaccharides and the treatment with antibiotics in addition to lactoferrin increased the cure rate and decrease the recurrency of the disease.

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