Extraction and Purification of *Salmonella spp*. enterotoxin isolated from Bovine in Basrah province

A. M. Al – Rodhan M. H. AL.Mayahi*
Coll. of Vet. Med./ Unive. of Basrah.

Abstract

One Hundred eighty fecal samples and (50) bile samples were collected from cattle of different ages and both sexes present in Basrah farms and Slaughterhouse. The results of the bacteriological and serological methods carried out on fecal and bile samples of cows detect *Salmonella spp* in the fecal samples of 3 cows (%1.66) and these bacteria were not detected (0%) in bile samples. Concerning the effect of months of study on the rate of *Salmonella spp*. isolation . The higher rate of isolation was encountered in march (6.66%) followed by February (2.38%), while in other months no Salmonella isolates were observed. Depending on the sex of animals the higher rate of Salmonella isolation was observed in males (%2.06) and it was in females (1.204%) . According to age group the higher rate of Salmonella isolation (%5.9)was observed in the third age group (3 < −9) followed by the second age group (1<-3) in which the rate was (%2.09) . There was statistical significance difference ($p< 0.05$) among age groups concerning the Salmonella isolation rate. Suckling mice and permeability of rabbit skin were show good result for detection of enterotoxin which were extracted from the more virulent isolate No. (161). The enterotoxin then were purified and fractionated by gel flirtation on sephadex (G-100). Results of gel flirtation showed that the toxin had two peaks , one of them were highly toxic. The chemical studying of enterotoxin characteristics revealed that it contained sugar moiety and it was a glycoprotein.

Introduction

Salmonella infection in farm animals and its health effects have been brought to great interest in view of their impact on human health. It has been observed that there was an increment in the rates of infection by Salmonella in humans and animals due to several reasons, including lack of caution required by the manufacturers and producers of food, which led to the emergence of medical conditions in various countries around the world on the consumption of animal products [(1)] Salmonellae food poisoning occurs after eating food or fluids contaminated with the Salmonella in sufficient numbers to cause poisoning. Of the most famous types of Salmonella that cause food poisoning is S .enteritidis *S.typhimurium* (2). These bacteria concentrated in the lymph nodes and Payer's patches and begin secreting enterotoxin witch was working with prostaglandin secreted from endothelial cells to increase the rate of Adenosine Monophosphate (CAMP) and thereby increase the absorption of water and fluids from the blood and collects in the cavity of the intestine. Enterotoxin is protein installed in the bacterial cell wall or in one of the components of the outer membrane of the bacterium (3), with specifications similar to the thermally stable (Heat stable) and to thermally un stable (Heat labile) enterotoxin of coliform bacteria also it has specifications similar to the heat-stable enterotoxin of *Vibrio choler* .This study aimed to : Isolate and Identify *Salmonella spp* from carrier and infected animals by using biochemical and serological tests, diagnose the enterotoxity of *Salmonella spp* by the extraction ,purification and detection of the toxicity of enterotoxin by biological tests and finaly the enterotoxin was chemically characterize.

Materials and Methods

Collection of samples

Fecal samples were collected directly from rectum of (180)cows . Bile samples were collected by sterilize syringe from gall bladder of slaughtered cows. This study was conducted through a period extended from October 2006 to March 2007

Isolation of *Salmonella Spp*

The presence of Salmonella in fecal samples were detected by selective enrichment media as tetrathionat and incubation at 37c^o for 24 hr followed by streaking on Salmonella Shighella Agar (SSA), MacConkey Agar and Brilliant Green Agar (BGA) with incubation at 37c^o for 24 hr. The presence of Salmonella in bile was determined by using SSA, MacConky Agar and BGA with incubation at 37c^o for 24 hr(2) .

Identification of *Salmonella spp*

Cultural characteristics

The growing colonies on SSA, MacConkey Agar and BGA were examined by naked eye concerning their color ,shape and size.

Specific biochemical tests

The biochemical characters of non lactose fermenting Salmonella spp, were determined by using Triple Sugar Iron (TSI), urea hydrolysis, Indol and citrate utilization test according to method of (4).

Serological testing

According to(5), all isolates were examined with polyvalent O and H antisera by slide agglutination test.

Extraction of enterotoxin

Cell – free culture supernatants (CFCS) of *Salmonella spp*. were prepared according to the procedure of (6) Briefly each Salmonella isolate was grown in brain heart infusion (BHI) broth on a shaker incubator at 37C$^\circ$ for 18h and then the culture was centrifuged (1000 rpm, 45 mint at 4C$^\circ$). The supernatant was collected after filteration by membrane Millipore filtere (0.45µm) and its' Protein concentration was estimated by method of (7).

Purification of enterotoxin

The CFCS of *Salmonella spp*. was precipitated with ammonium sulphate at 60% and 80% saturation level. After adding ammonium sulphate to CFCS, the contents were stirred for 20 minutes and kept at 4C$^\circ$ overnight. The precipitate was collected by centrifugation (10000 rpm for 30 minutes at 4C$^\circ$) and was redissolved in minimum quantity of distilled water (DW). Thereafter, the preparation was dialyzed in cellophane dialysis tubing (sigma) against DW at 4C$^\circ$ until it became completely free from ammonium sulphate ions (8).

Gel filtration

According to (8) the precipitated dialyzed preparation (PDP) was gel filtered through sephadex G-100. Two ml of PDP (25mg protein) was placed on column (80 × 1.5cm) of sephadex G- 100 equilibrated with 0.2 M phosphate buffer (pH 6.8). The material was eluted from the gel with same buffer at a flow rate of 15ml/h. Fraction, each of 2.5ml were collected separately. The contents of each peak pooled. The contents of each peak was tested for enterotoxicity by skin permeability tests (Delayed permeability factor).

Biological Detection of Salmonella Enterotoxin

Prepared supernatant was tested for presence of rapid (RPF) and Delayed(DPF)acting skin permeability factors on the back of rabbits by the method described by(9). The diameter of the reaction was measured and the area was calculated. A preparation giving reaction of $\geq$ 78.5 mm^2 was considered positive for PF. Suckling mice were used for the assay of entertoxicity. This test was performed as described by(10). Two sucking mice were used for this testing. A preparation yielding dilatation and increase in the intestinal weight percentage of 0.08 was considered as enterotoxic. Intestinal weight percentage was determined by dividing the average of intestinal weight of two mice by the body weight of these of two mice.

Detection of carbohydrate.

To determine the presence of carbohydrate in the enterotoxin, Mulish reagent was used. To 1ml of active fraction of PDP 1 ml of Mulish reagent were mixed and allowed to react. Appearance of purple ring after addition of (10) drops of H_2SO_4 to the mixture considered positive (Presence of carbohydrate). This test was performed as described by (11).

Statistical Analysis : In order to determine the statistical significance among different variables. Chi-square was applied to test the obtained results.

Results

Prevalence of Salmonella isolates according to diagnostic tests

The results of the bacteriological and serological methods carried out on fecal and bile samples of cows showed that all tests were able to detect Salmonella spp in the fecal samples of 3 cows (%1.66) and they were in able to detect them 0 (0%) in bile samples after 24 hr incubation of the Pre enrichment broth(Table 1) .The colonies of Salmonella on SSA,BGA and MacConkey agar were circular, smooth, convex and their color was pale with black center on SSA,on MacConkey agar was pale and was pink on BGA. All tested isolates of cows fecal samples 3(%1.66)revealed the inability of Salmonella to hydrolys urea and to split tryptophan to indol and its ability to use citrate as sole carbon source and to ferment the glucose and produce hydrogen sulfide gas on TSI medium. The positive results of polyvalent(O) and (H)antisera slide agglutination test appeared as cloudy, granular, dark milky mixture in 3 (%1.66) fecal Salmonella isolates(Table 1).

Table (1)Distribution of *Salmonella spp*.in the tested samples according to the bacteriological and Serological methods.

Samples	Examined No.	Positive No.	%
Feces	180	3	1.66
Bile	50	0	0

According to type of samples Statistically there was significant difference (P< 0.05) between feces and bile samples concerning the positivity of Salmonella isolation.

The effect of some epidemiological factors on Salmonella distribution:

The months of study

Higher percentage of *Salmonella spp*. isolation were encountered in march 6.66% followed by February 2.38% were as no Salmonella were isolated at other months. There was statistical significance difference (p< 0.05) among the months concerning the Salmonella isolation rate (Table-2).

Table (2) . Salmonella distribution according to months of study.

Months	Examined No. of fecal samples	Positive fecal sample No.(%)	Examined No. of bile samples	Positive bile sample No.(%)
October	24	0	0	0
November	25	0	16	0
December	23	0	13	0
January	36	0	15	0
February	42	1(% 2.38)	0	0
March	30	*2 (% 6.66)	6	0
Total	180	3(%1.66)	50	0

X^2= 35.58 P< 0.05

Sex of infected cows

According to table (3) the non significant higher rate of Salmonella isolation was observed in males (%2.06) in comparison to females (% 1.204).

The age of cows

According to age of animals in our study the higher rate of Salmonella isolation (%5.9)was observed in the third age group followed by the second age group (%2.09) . There was statistical significante difference ($p< 0.05$) among age groups concerning the Salmonella isolation rate (Table4).

Table (3) . The effect of sex on Salmonella distribution.

Sex	Examined No.	Positive No.(%)
Males	97	2(2.06)
Females	83	1(1.204)
Total	180	3(1.66)

X^2= 0.89 P>0.05

Table (4). The effect of age on Salmonella distribution

Age group (year)	Examined No.	Positive No.	%
1>	21	0	0
1<-3	73	1	2.09
3<-9	86	2	5.9
Total	180	3	1.66

X^2= 9.23 P<0.05

The biological detection of enterotoxicity of crude CFCS

Suckling mice and Rabbit skin permeability tests were used in the biological detection of enterotoxicity. The results of these tests were displayed in table(5) .These results revealed that the isolate No.(161) greatly affect the intestinal weight rate (0.087) followed by Isolate No.172 (0.083).Depending on Rabbit skin permeability the Isolate No.161 show larger zone of bluish coloration (14 mm) followed by the Isolate No.172 (8 mm).

Gel filtration:

The enterotoxic moiety was precipitated with ammonium sulphate and the precipitated dialyzed preparation (PDP) of *Salmonella spp*. which contain 8mg/ml was fractionated through sephadex G-100, into two peaks (Fig. 1). The first peak (A) which eluted close to the void volumes exhibited delayed and rapid Permeability activity (Fig-2), induced fluid accumulation in intestine of suckling mice (Table-6). None of these activates was detected in the second peak (B) contents.

Table (5) . The biological detection of enterotoxicity of crude CFCS

Enterotoxin of isolates (crude CFCS)	Suckling mice intestine weight %	Rapid skin permeability of Rabbit (mm)
Isolate No.134	0.054	6
Isolate No.161	0.087*	14*
Isolate No.172	0.083*	8*
Brain Heart Infusion broth	0.033	0
Phosphate Buffer		

*= positive result

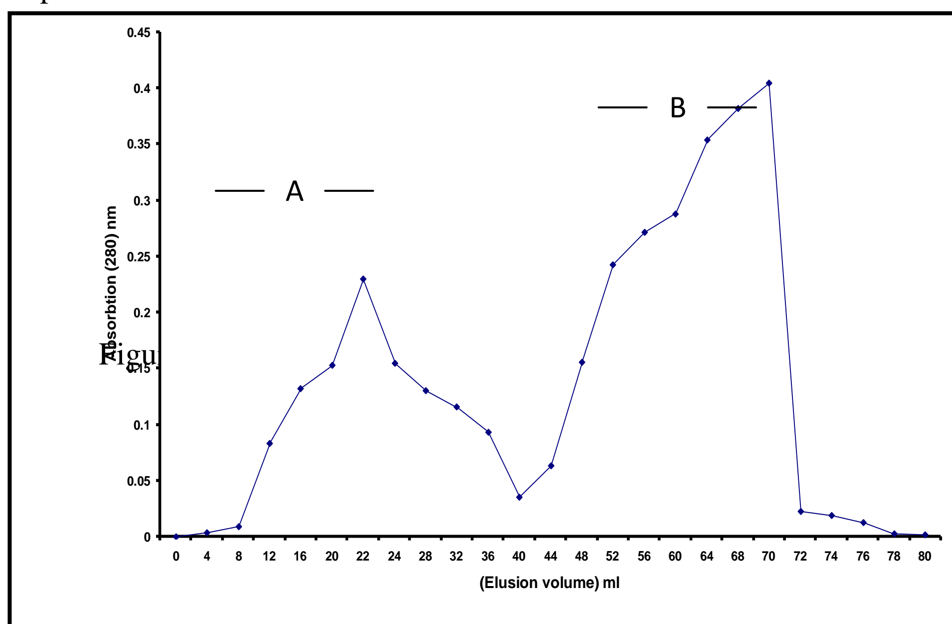

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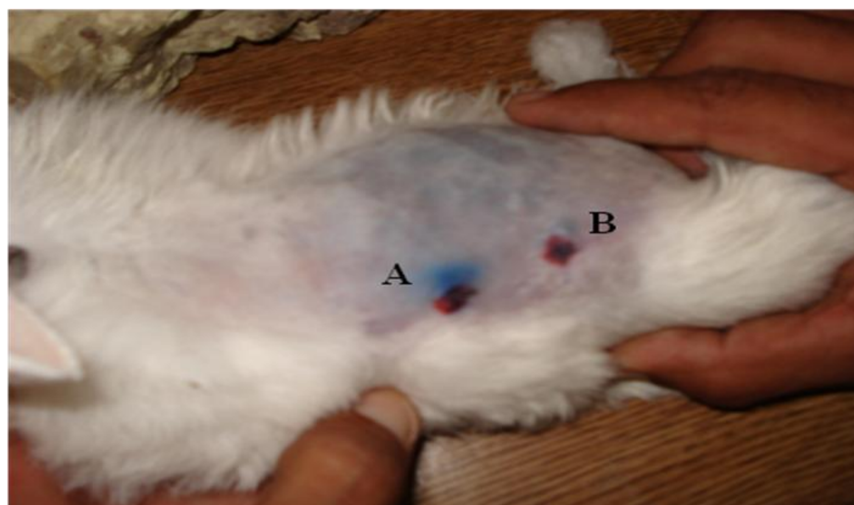
Table (6) The biological detection of enterotoxicity of purified CFCS

Tested material	prepartion		Protein mg Suckling mice intestine weight %	Rabbit skin permeability (mm)	
				RPF	DPF
Salmonella enterotoxin	SG–	PA	0.085	15	17
	100	PB	0.052	-	-
Controls	BHI Broth		0.38	-	-
	PBS		-	-	-

SG: Sephadex G, P. A : Peak A , P. B: Peak B. RPF: Rapid permeability Factor., DPF: Delayed permeability Factor, BHI Broth: brain heart infusion Broth, PBS: Phosphate Buffer



Figure(2)Rapid permeability of purified enterotoxin in rabbit skin
(A)- represent peak A (B) - represent peak B

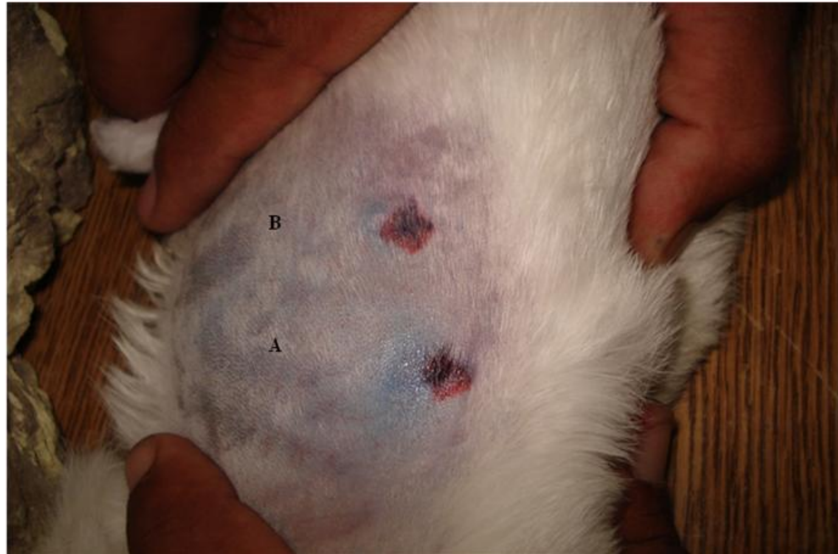


Figure (3) Delayed permeability of purified entertoxin in rabbit skin
(A) - represent peak A (B)-represent peak B

Carbohydrate detection:
The test of carbohydrate detection by using Mulish reagent revealed presence ofcarbohydrate binned to protein .The enterotoxin composed of glycoprotein(Figure4).

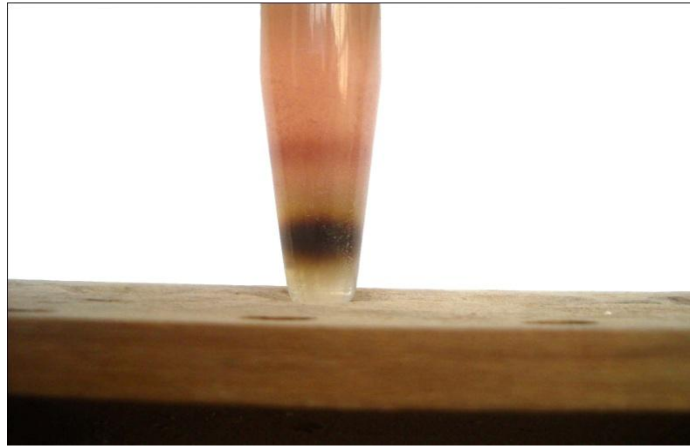


Figure (4). Carbohydrate detection test

Discussion

In consideration to Salmonella importance as one of the causative agent of human and animal food. poisoning. So the present study aimed to isolate and identify *Salmonella spp*. In cows. Only (3) *Salmonella spp*. isolates(%1.66) were identified in fecal samples by the biochemical and serological testing of all fecal and bile samples. The present identification rate was lower than the reported rates of other studies (12,13) who reported % 2.1 and %3 respectively. Other study (14) reported higher rate (%4.6) than the present rate. The variation in results of present study and other studies may be related to one or more of these factors including differences in methods of sustenance, strains , methods of identification and geographical factors.These two testing failed to identify *Salmonella spp*

in bile samples, the explanation of this result could be due to the presence of Salmonella in other organs as liver, spleen and mesenteric lymph nodes. One Iraqi study previously conducted in Basrah province by (12) sport the present result. Concerning the effect of some epidemiological factors on the rate of Salmonella isolation, the present results revealed that There was statistical significance difference ($p< 0.05$) among the months concerning the Salmonella isolation rate and high rate of *Salmonella spp*. isolation were encountered in march 6.66% followed by February 2.38%. These results in constant with other Iraqi studies(15, 6)which indicate that there was an increment in Salmonella isolation rate associated temperature elevation in studied months. On the other hand sex of cows showed statistically non significant effect and higher rate of Salmonella isolation was observed in males (%2.06) in comparison to females (% 1.204). According to age group there was statistical significance difference ($p< 0.05$) among age groups concerning the Salmonella isolation rate the higher rate (%5.9)was observed in the third age group. These results in line with(17) who reported that calves and cows equally infected with Salmonella and the severity of the infection depend on the dose of bacteria and immune status of animals

The biological detection of enterotoxicity

The results of the present study indicated that *Salmonella spp*. isolated from cows produced and released enterotoxin into the culture supernatants as their CFCS induced fluid accumulation in the suckling mice intestine and increased permeability of the rabbit skin. Enterotoxic activity in the CFCS of Salmonella has also been reported (6), while others failed to detect activity in the extracellular medium (18).The present study revealed that suckling mice was authenticated, cheep method able to detect the enterotoxicity of CFCS. Other study(19) sport this finding, while (20) indicate the inability of this test in detection of the enterotoxicity of CFCS.Enterotoxicity of the precipitated dialyzed preparation revealed that the enterotoxic moity was precipitated with ammonium sulphate and was non-dialyzable. The presence of two peaks on gel filtration (Sephadex G-100), indicated that the purification of enterotoxic moiety was achieved to apparent homogeneity through salt precipitation and gel filtration. Other have reported the presence of two peaks on gel filtration (Sephadex G-1000 , only the first one contained toxic moiety also the presence of carbohydrate moiety was detected in the CFCS (6).The presence of rapid and delayed PF in the gel filtrated CFCS are in accordance with the observation made by earlier worker(21) . In conclusion the presence of enterotoxic activity of CFCS which is detected by sucking mice test and presence of rapid and delayed in the same peak indicted that entrotoxic activity was due to the single moiety.

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أستخلاص وتنقية الذيفان المعوي لجرثومة السالمونيلا المعزولة من أبقار مدينة البصرة

عدنان موسى الروضان   مؤيد حنون المياحي
كلية الطب البيطري /جامعة البصرة

الخلاصه

شملت الدراسه 180 عينة براز و50 عينة عصارة الصفراء جمعت من ابقارفي اعمار و اجناس مختلفه من مزارع تربية الابقار ومجزرة البصره . اظهرت نتائج الفحص الجرثومي والمصلي التي اجريت على عينات البراز وعصارة الصفراء انه تم الكشف عن جنس السالمونيلا في براز 3ابقار(1.66%) ولم يتم الكشف عن هذه الجراثيم في عينات الصفراء (0%) . فيما يتعلق بتاثير اشهر الدراسه على نسبة عزل جنس السالمونيلا فقد لوحظ ان اعلى نسبة عزل للسالمونيلا كانت في شهر اذار (66. 6 %) يليها شهر شباط (2.38%) ولم تعزل السالمونيلا في اشهر الدراسه الاخرى .واعتمادا على جنس الحيوانات المفحوصه كانت اعلى نسبة عزل السالمونيلا في الذكور(2.06%) في حين كانت في الاناث (1.204%).اما بالنسبه الى للفئات العمريه فقد لوحظت اعلى نسبه لعزل السالمونيلا فى الفئه العمريه الثلثه(< 3-9) تليها الفئه العمريه الثانيه (< 1-3) ولم تعزل السالمونيلا في الفئات العمريه الاخرى.واختبرت قدرة جراثيم السالمونيلا على انتاج الذيفان المعوى باستخدام طريقتين هما طريقة الفئران الرضيعه وطريقة النفوذية لجلد الارنب وقد اعطت هاتان الطريفتان نتائج موجبه للكشف عن الذيفانات المعويه التي هي احد عوامل الضراوه لهذه الجرثومه .وقد تم اسخلاص الذيفان لجرثومة السالمونيلا من العزله(161) وكانت هذه العزله من اشد العزلات ضراوه.استخدمت تقنية الترشيح الهلامي (Sephadex G-100)لتنقية وتجزئة الذيفان المعوى وتبين انه يتكون من قمتين بروتينيتين احدهما اظهرت سميه عاليه .درست مواصفات الذيفان المعوى كيمياويا وظهر انه يحتوى في تركيبه على وحدات سكريه وانه بروتيني سكرى.