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

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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 a 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 filtration on sephadex (G-100). Results of gel filtration showed that the toxin had two peaks, one of them was 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 which 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 cholera*. 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 finally 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 sterile 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 37°C for 24 hr followed by streaking on Salmonella Shigella Agar (SSA), MacConkey Agar and Brilliant Green Agar (BGA) with incubation at 37°C for 24 hr (2). The presence of Salmonella in bile was determined by using SSA, MacConkty Agar and BGA with incubation at 37°C 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 37°C for 18h and then the culture was centrifuged (1000 rpm, 45 mint at 4°C). The supernatant was collected after filtration by membrane Millipore filter (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 4°C overnight. The precipitate was collected by centrifugation (10000 rpm for 30 minutes at 4°C) and was redissolved in minimum quantity of distilled water (DW). Thereafter, the preparation was dialyzed in cellophane dialysis tubing (sigma) against DW at 4°C 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 ≥ 78.5 mm² was considered positive for PF. Suckling mice were used for the assay of enterotoxicity. 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$_2$SO$_4$ 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 (66.66%) 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 (66.66) fecal Salmonella isolates( Table 1).

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

<table>
<thead>
<tr>
<th>Samples</th>
<th>Examined No.</th>
<th>Positive No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feces</td>
<td>180</td>
<td>3</td>
<td>1.66</td>
</tr>
<tr>
<td>Bile</td>
<td>50</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Months</th>
<th>Examined No. of fecal samples</th>
<th>Positive fecal sample No.( %)</th>
<th>Examined No. of bile samples</th>
<th>Positive bile sample No.( %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>October</td>
<td>24</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>November</td>
<td>25</td>
<td>0</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>December</td>
<td>23</td>
<td>0</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>January</td>
<td>36</td>
<td>0</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>February</td>
<td>42</td>
<td>1(2.38)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>March</td>
<td>30</td>
<td>2(6.66)</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>180</td>
<td>3(1.66)</td>
<td>50</td>
<td>0</td>
</tr>
</tbody>
</table>

$X^2= 35.58$  $P< 0.05$
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 significant difference (p< 0.05) among age groups concerning the Salmonella isolation rate (Table4).

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

<table>
<thead>
<tr>
<th>Sex</th>
<th>Examined No.</th>
<th>Positive No.(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>97</td>
<td>2 ( 2.06)</td>
</tr>
<tr>
<td>Females</td>
<td>83</td>
<td>1 ( 1.204)</td>
</tr>
<tr>
<td>Total</td>
<td>180</td>
<td>3 ( 1.66)</td>
</tr>
</tbody>
</table>

X² = 0.89  P>0.05

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

<table>
<thead>
<tr>
<th>Age group (year)</th>
<th>Examined No.</th>
<th>Positive No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1&gt;</td>
<td>21</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1&lt;3</td>
<td>73</td>
<td>1</td>
<td>2.09</td>
</tr>
<tr>
<td>3&lt;9</td>
<td>86</td>
<td>2</td>
<td>5.9</td>
</tr>
<tr>
<td>Total</td>
<td>180</td>
<td>3</td>
<td>1.66</td>
</tr>
</tbody>
</table>

X² = 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

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

*= positive result

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**Figure (1):** Elusion profile of *Salmonella* enterotoxin

- A
- B

**Absorption (280 nm)** vs **(Elusion volume) ml**
Table (6) The biological detection of enterotoxicity of purified CFCS

<table>
<thead>
<tr>
<th>Tested material</th>
<th>prepartion</th>
<th>Protein mg</th>
<th>Suckling mice intestine weight %</th>
<th>Rabbit skin permeability (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>RPF</td>
</tr>
<tr>
<td>Salmonella enterotoxin</td>
<td>SG–</td>
<td>PA</td>
<td>0.085</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>PB</td>
<td>0.052</td>
<td>-</td>
</tr>
<tr>
<td>Controls</td>
<td>BHI Broth</td>
<td></td>
<td>0.38</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td></td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>


Figure (2) Rapid permeability of purified enterotoxin in rabbit skin
(A) - represent peak A  (B) - represent peak B
Carbohydrate detection:
The test of carbohydrate detection by using Mulish reagent revealed presence of carbohydrate binned to protein. The enterotoxin composed of glycoprotein (Figure 4).

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 moiety 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.

**Reference**


bacteria, Williams and Wilkins , U.S.A.
استخلاص وتقنيه الذئفي المعوي لجرثومة السالامونيلا المعزولة من أبقار مدينة البصرة

عنوان مؤدي حنون المياحي
كلية الطب البيطري/جامعة البصرة

الخلاصة
شملت الدراسة 180 عينة براز و50 عينة عصارة الصفراء جمعت من ابقار في أعمال واحجام مختلفة من مزارع تربية الابقار ومزرعة البشرة. أظهرت نتائج الفحص الجرثومي والمصلي التي أجريت على عينات البراز وعصارة الصفراء أنه تم الكشف عن جنس السالامونيلا في براز 3 ابقار (1.66%) ولم يتم الكشف عن هذه الجرثومي في عينات الصفراء (0%). فيما يتعلق بتأثير إشهر الدراسة على نسبة عزل جنس السالامونيلا فقد لوحظ أن أعلى نسبة عزل للسالامونيلا كانت في شهر آذار (6.66%) وليلها شهر شباط (2.38%) ولم تظهر السالامونيلا في شهر الدراسات الأخرى. واعتمادًا على جنس الحيوانات المفحوصة كانت أعلى نسبة عزل السالامونيلا في الذكر (2.06%) في حين كانت في الإناث (3.20%). أما بالنسبة للفيتامن الغذائي فقد لوحظت أعلى نسبة عزل السالامونيلا في الفيتامن المغذي (3-9) تليها الفيتامن المغذي الثاني (9-12) ولم تظهر السالامونيلا في الفيتامن في الفيتامن المغذي النقي. واعترفت قدرة جراحات السالامونيلا على انتاج الذئفي المعوي باستخدام طريقتين هما طريقة الفتران الرساعية وطريقة الفرعية لجلد الأرنب وقد أظهرت مطال للفيتامن تأثير موجبة للكشف عن الذئفي المعوي الذي هي أحد عوامل الضرب لجرثومة هذه الحالات. وقد تم استخلاص الذئفي لجرثومة السالامونيلا من العينة (161) وكانت هذه العينة من اسث الذئفي المواقع. استخدمت تقنية التشخيص الهلامي (Sephadex G-100) كتقنية وتجربة الذئفي المعوي وتبين أنه يتكون من فئتين بروتينيتين احدهما أظهرت سببية عالية. درست مواصفات الذئفي المعوي كيمياء وظهر أنه يحتوى في تركيبه على وحدات سكري وان بروتيني سكري.