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PREPARATION OF POLYVALENT DIAGNOSTIC ANTISERA FOR SALMONELLA SEROVARES TYPHI AND TYPHIMURIUM

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ABSTRACT

Polyvalent diagnostic antisera for *S.Typhi* and *S. Typhimurium* have been prepared during this study according to protocol being suggested by Florida State University. *Salmonella* isolates were received from laboratory of veterinary College of AL-Qadissya University. Preparation of heat killed s antigen for each isolates were prepared and injected in three groups of laboratory animals (rabbits). The first and second groupwere injected with different antigens in four successive doses with an interval time of three to four days, while the third group was injected with normal saline and used as control. After the last fourth injection, 10 ml of blood was collected from animals by heart puncture then serum has been separated by centrifugation. The separated antisera was tested with live and killed Salmonella antigens using the agglutination test. The results were highly positive and specific. To determine the purity of prepared antisera, it cross reaction with live antigens of some species belong Entrobactrecea represented by *E.coli, Klebsiella Spp.* and *Protus Spp*. The results indicated that there is no considerable reactions.

KEYWORDS: S. Typhi, S. Typhimurium, E.coli, Klebsiella Spp. and Protus Spp.

INTRODUCTION

Salmonellosis, is a disease conditions either enteric fever or gastroenteritis which caused by a large group of bacteria of genus *Salmonella* that can effect throughout the world. Each year, millions of persons become ill from food-borne especially Salmonellosis diseases, though many cases are not reported, so it is one of the health problems for humans and animals as

well (Erdem *et al*., 2005). There are many transmission routes of *Salmonella*, but the most common of humans infections are derived from consumption of contaminated water and food specially those of animal origin (Hernandez *et al*., 2005). This requires reducing the spread of the bacteria through rapid diagnosis and then treatment so this study aimed to prepare diagnostic antisera locally. Salmonella is belonging to the family Enterobacteriaceae, and it is Gram negative bacilli, motile, non-lactose fermenting, non capsulated and non sporing bacteria (Cheesbrough, 2000; Perilla, 2003).

Salmonella classified according to Kauffmann-White classification in to three major antigenic determinants consist of somatic O antigens, flagellar H antigens, and virulence (Vi) capsular antigens (Scherer and Miller, 2001). The somatic (O) antigen which found on the surface of outer membrane of bacteria and are determined by specific sequences on the cell surface, the O antigens are heat stable and upon which grouping of the organisms is based (Kondoh and Hotam, 1994).

MATERIALS AND METHODS

Culture media

Table (1) Culture media and their manufacture company

Culture media	Company
Api E20	BioMerieux ,France
Kligler iron agar	Himedia / India
MacConkey agar	Himedia / India
MR-VP medium	Himedia / India
Nutrient agar	Himedia / India
Selenite F broth	Himedia / India
Simmons citrate medium	Himedia / India
Urea agar base	Himedia / India
XLD agar	Himedia / India

LABORATORY ANIMALS

Six New Zealand albino healthy animals (white female rabbits) were obtained from the animal house of the faculty of science / university of kufa. They were kept under standard conditions for 10 days for adaptation. The animals weight ranged from 2.0 to 2.25Kg with an age at 16 weeks, the animals were divided into three groups by tow animals per each group . The first group was injected intravenously (using a marginal vein) with heat–killed *Salmonella* serotype *Typhimurium*, the second group was injected with heat–killed *Salmonella* serotype *Typhimurium* and the third group used as control be injected with sterile normal saline. These injections were doses upward. (Al- Hamadany, 2009; Al-Taae, 2013).

Table (2): Protocol Of Injection For The Laboratory Animals With Somatic Antigen Of Salmonella Typhi And Salmonella Typhimurium.

Antigen injection	Date	Group (A)	Group (B)	Group (C)
		Dose	Dose	Dose
Initial antigen injection	Day 0	0.5ml	0.5ml	0.5ml
First antigen booster	Day 3	1.0ml	1.0ml	1.0ml
Second antigen booster	Day 6	1.5ml	1.5ml	1.5ml
Third antigen booster	Day 9	2.0ml	2.0ml	2.0ml
Blood collection	Day 16		Day 16	Day 16

METHODS

Isolation and identification of bacteria using culture media

The stool samples were cultured in selenite-F medium which considered as enrichment media for *Salmonella*, then bacteria cultured on MacConkey agar an XLD agar for determined culture characteristics of *Salmonella*.

BIOCHEMICAL TESTS

The biocamicals tests were used identification of *Salmonella Typhi* and *Salmonella Typhimurium* after inoculated these bacterial isolates on MR-VP medium, Simmons citrate medium, Urea agar medium and Kliglar iron agar.

API -20 E TECHNIQUE

API 20E system consists of twenty micro tubes containing dehydrated substrates. According to manufacturing company (BioMerieux) Microtubes were inoculated with a bacterial suspension equivalent to **(**MacFarland tube No 0.5). Metabolism produces color changes either spontaneous or by addition of appropriate reagents includes: oxidase, Voges-Proskaure reagents [VP1: 40% potassium hydroxide and VP2: 6% alpha-naphthol], 10% Ferric chloride and Kovac's reagents). Test was performed separately and reactions were read after 24 hrs. according to the standard results as shown in table (3) the identification was obtained by referring to the analytic profile.

A. Preparation of galleries: 5ml of tap water were dispensed into the incubation tray to provide a humid atmosphere during incubation.

B. Preparation of bacterial suspension: Single colony from plating medium was taken and suspended in 5 ml sterile distilled water by rubbing against the side of the tube and mixed thoroughly.

C. Inoculation of galleries: With a sterile Pasteur pipette, the 21 microtubes were inoculated with the bacterial suspension under test. According to the manufacture instructions, both the tubes and couple section of CIT, VP and GEL microtubes were filled with bacterial suspension. After inoculation, couple section of the ADH, LDC, ODC, H2S and URE microtubes were completely filled with mineral oil.

D. Incubation of the Galleries: After inoculation, the plastic lid was placed on the tray and the galleries were incubated for 18-24 hours at 37°C.

E. Reading the Galleries: All reactions that not requiring reagents were recorded first, and then the following reagents were added to the corresponding microtubes:

• 1 drop of 3.4% ferric chloride was added to the TDA microtube.
• 1 drop of Kavoc's reagent was added to the IND microtube.
• 1 drop of Voges-Proskauer reagent was added to VP microtube.
• 1 drop of oxidase reagent was added to each of H2S or ONPG microtube.

Table (3) the test and their standard results for Salmonella Spp.

Microtube test	Positive	Negative
ONPG	Yellow	Color less
ADH	Red/ Orange	Yellow
LDC	Red/ Orange	Yellow
ODC	Red/ Orange	Yellow
CIT	Blue-Green / blue	Pale green / yellow
H2S	Black deposit-thin line	Color less / greyish
URE	Red/Orange	Yellow
TDA (TDA – immediate)	Reddish brown	Yellow
IND (James-immediate)	Pink	Color less / pale green/yellow
VP (VP1 + VP2 / 10 min)	Pink-Red	Color less/pale pink
GEL	Diffusion of black pigments	No diffusion
GLU	Yellow / greyish yellow	Blue /blue green
MAN	Yellow	Blue /blue green
INO	Yellow	Blue /blue green
SOR	Yellow	Blue /blue green
RHA	Yellow	Blue /blue green
SAC	Yellow	Blue /blue green
MEL	Yellow	Blue /blue green
AMY	Yellow	Blue /blue green
ARA	Yellow	Blue /blue green

PREPARATION OF IMMUNOGEN

For this experiment single S.typhi and S.typhmurium isolates were used for preparation of specific immunogens. Somatic S.typhi and S.typhmurium antigens were isolated and purified from bacterial cell wall that was killed by heating at 100 C°.

Nutrient agar was used for inoculated and growth of bacteria, after 18-20 hrs., the growth of bacteria was harvested by sterile loop in sterile screw-cap contains 10 ml sterile normal saline. After that bacterial suspension was placed in boiling water bath for 2½ hrs. At the end of heating period, the sterility of this suspension was checked by inoculating this suspension on nutrient agar and incubated for 24 hrs at 37 C°, and examined for growth. Suspension was stored in refrigerator then it was used for injection into laboratory animals as recommended by (Garvy al., 1981; Al-Hamadany, 2009; Al-Taae, 2013).

INTRAVENOUS INJECTIONS (I.V) FOR RABBIT

Rabbit was placed in restraining position; topical anesthesia was used at site of injection using 2% lidocaine. Intravenous injection was performed in the marginal ear vein. The marginal vein is on the inner edge of the dorsal surface of the ear. The base of ear pressed lightly and the blood that returns to the body was restricted in the vein which appears clearly. The injection area was cleaned with 70% alcohol then needle (26-gauge) was inserted into the vein. When the needle appears to be in the vein, if blood appears in the needle hub slowly the immunogenic content of the syringe were injected. Sterile gauze was placed on the injection site as the needle is removed (Halliday et al., 2004; Florida State University, 2007). The values of dosses indicated in table **(**3**)** were considered (Al-Hamadany, 2009; Al-Taae, 2013).

INTRACARDIAC BLOOD COLLECTION

Intracardiac puncture for large volume blood collection (10-15ml) was performed under general inhalation anesthesia using Diethyl ether/ 98%. The injection area was cleaned with 70% alcohol.

After that needle (22-gauge) was inserted into the lateral thoracic region toward the area of maximal heart beat between the ribs of the rabbit's left side midway between its sternum and back under the left elbow A volume of 10 ml of blood was collected slowly then sterile cotton was placed over the injection site as the needle is removed. The blood was decanted immediately in sterile test tube (Florida State University, 2007).

SEPARATION AND PRESERVATION OF ANTISERA

Freshly blood that was collected from Rabbits allowed standing after collected for 2 hrs. at room temperature for clot formation and centrifuged at (2500 rpm with a rotating radius of 14 cm for 10 minutes). Then the serum was removed carefully by sterile pasture pipette and decanted into sterile Eppendrof test tube. Repeated centrifugation was performed by using Eppendrof centrifuge to obtain a clear serum, part of the serum was collected and stored in frozen at -18°C for long periods and the other part of serum was stored at (4°C) by addition of sodium azide at final concentration 0.10 % (Garvey et al., 1981).

THE CAPABILITY OF SEPARATED ANTISERA

The capability at separated antisera was checked by reaction of these antisera with killed and live antigen to both Salmonella Typhi and Salmonella Typhimurium Agglutination of the reacted mixture indicates for positive result.

RESULTS AND DISCUSSION

Morphological And Culture Characterization

This study based on colonial morphology and biochemical tests for identification of Salmonella Spp. The Salmonella colonies that grown on culture media represented by: XLD agar and MacConkey agar have the optimum growth at 37 ºC and pH 7.0 revealed the typical characteristics being described by referential studies (Quinn et al., 2004; Patrick et al., 2005; Pakzad et al., 2007). The Colony of Salmonella Typhi and Salmonella Typhimurium have the same characteristics on culture media with little differences in some biochemical tests. The selient- F broth medium that use in the study as enrichment medium contains additive factors such as (Sodium Selenite) that selectively permits the salmonella to grow and increase its number in stool sample, due to the sodium selenite which inhibits the growth of Gram-positive bacteria and many Gram- negative bacteria (Kelly et al., 2003). On McConkey agar the Salmonella colonies are colorless and smooth (Murray et al., 1999). The inability of salmonella to ferment lactose leading to no acid production and, no change in pH leading to no changes the neutral red which is considered as indicator for change the color of media. On XLD medium the Salmonella colony is red with black center (Anderson et al., 2005; Murray et al., 2011). The black center of Salmonella colonies on XLD medium is due to inability of Salmonella to metabolize thiosulfate in media to produce hydrogen sulfide and allows them to be differentiated from the similarly colored Shigella colonies on this medium (Nye et al., 2002).

BIOCAMICAL TESTS

The biochemical tests indicated in table (4) were achieved to differentiation between the isolates that showed growth and morphological characteristics, which are closely related to Salmonella Spp. The results showed that the isolates (Salmonella Typhi and Salmonella Typhimurium) revealed negative reactions for Oxidase, urease, and Voges-prosckaur where its revealed positive results for Catalase and Methyl red but S.Typhi exhibit negative result for utilization of citrate when inoculated on Simmons citrate medium while S. Typhimurium exhibited positive result and S.Typhi produced little amount of H2S with no gas when inoculated on Kliglar iron medium while S.Typhimurium produce high amount of H2S with gas production. These results are conformity with those results being reported for Salmonella Spp. by other studies (Woo et al., 2001; Brenner et al., 2005; Tindall et al., 2005).

Table (4) results of biocamical tests for S.Typhi and S.Typhimurium

Tests	S. Typhi	S.Typhimurum
Oxidase test	-	-
Catalase test	+	+
Urease test	-	-
Kliglar	Red/Yellow with little H2S production	Red/Yellow with high H2S production
simmon citrate test	-	+
Methyl red	+	+
Voges prosckaur	-	-

*** + positive / - Negative**

API 20 E system

Api 20E is a standardized colorimetric identification system for enteric bacteria and it is considered as an suitable method for identification of the more commonly-occurring members of the family Enterobactrecea (Jawetz et al., 2007). In this study API 20E system was used as complementary method for identification of S.Typhi and S.Typhimurium included in this study. By using the analytical profile index of this system the identification percentage was (id%= 99.9) for S.Typhi and (id%= 99.5) for S.Typhimurium which was in correspondent with trust worthy references Holt et al. (1994) and Quinn et al., (2004).

Table (5) showed the results of API 20 tests.

Testes	Reactions / Enzymes	S.Typhi	S.Typhimurium
ONPG	β-galacosidase Ortho NitroPhenyl - β D-Galactopyranosidase	–	–
ADH	Arginine DiHydrolase	_	+
LDC	Lysine DeCarboxylase	+	+
ODC	Ornithine DeCarboxylase	_	+
CIT	Citrate utilization	_	+
H_2S	H_2S production	+	+
URE	Urease	_	_
TDA	Tryptophane DeAminase	_	_
IND	Indole production	_	_
VP	Acetoin production (VogesProskauer)	_	_
GEL	Gelatinase	_	_
GLU	Fermentation/oxidation (Glucose)	+	+
MAN	Fermentation/oxidation(Mannitol)	+	+
INO	Fermentation/oxidation (Inositol)	_	+
SOR	Fermentation/oxidation (Sorbitol)	+	+
RHA	Fermentation/oxidation (Rhamnose)	_	+
SAC	Fermentation/oxidation (Saccharose)	_	_
MEL	Fermentation/oxidation (Melibiose)	+	+
AMY	Fermentation/oxidation (Amygdalin)	_	_
ARA	Fermentation/oxidation (Arabinose)	_	+

The first isolate revealed positive results for ornithine decarboxylase, citrate utilization and was fermenter for arabinose, rhamnose and inositol. these results, together with other results mentioned above are in agreement with those results be described by Brenner et al. (2005); indicating, that this isolate belongs to the genus Salmonella and it is S. Typhimurium. The second isolate on other hand, revealed negative results for ornithine decarboxylase, citrate utilization, while it exhibited positive results to ferment arabinose, rhamnose and inositol. These comparison of my results with other closely related results Nucera et al. (2006); indicates that they are in accordance indicating that this isolate belongs to the genus Salmonella and it is S.Typhi.

PREPARATION OF ANTISERA

In this study, diagnostic antisera were prepared by using heat killed somatic antigen of Salmonella Typhi and Salmonella Typhimurium isolates, since antisera were widely used to rapidly confirm the diagnosis of Salmonella Spp. and to differentiate the serotypes of these organisms (Aliexo et al., 1984; Kimmi et al., 2008). Heat killed antigen is somatic (O) antigen in fact, which regarded as specific antigen. Other non specific e.g. flagellar and capsular Ags. Which are fully distorted by heat (boiling temperature for at least 2hrs.).

Somatic antigen prepared by this study was used to induce antibodies in Lab. Animals. NewZeeland albino female rabbits were used to prepare antisera because these animals are easily of handling, cost-benefit ratio and hyper immune which respond rapidly when injected with antigens as stated by (Florida State University, 2007; Al-Hamadany, 2009; Al-Taae, 2013). Equal amount of prepared antisera was mixed with killed antigen or (live bacteria) (S.Typhi and S.Typhimurium) on clean slide, the results revealed strong agglutination with, both types of antigen indicating for the high immunogenic property of O-Ag.

In the current study, inoculation of rabbits with O Ag of Salmonella Typhi and Salmonella Typhimurium resulted in stimulation of significant high titers of antibody in the experimental groups compared with control. These results were agreement with George et al., (1985) who reported that immunization of rabbits with O Salmonella antigen resulted in high titer of antisera. Mittrucker and Kaufmann, (2000) recorded that the rabbits injected with live bacterial cells or isolated somatic antigen of Salmonella strains produced higher cellular and humoral immune response, which are important for control of primary infection and protection against secondary infection attack. According to Kimmi et al., (2008) the increased manifestation of Salmonella infections in latest years have accentuated the requirement for the proration of Salmonella antisera, which are successfully employed to confirm diagnosis of microorganism or/and for serotyping of Salmonella as a base for proper diagnosis, identification of infection and contaminated organism.

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