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Molecular pathological detection of *S. typhimurium* by fluorescence in situ hybridization (FISH) in lambs tissue sections

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Abstract

Despite many different methods used to diagnose *Salmonella typhimurium*, especially that depended on isolation and biochemical identification, but use technique, *Fluorescence in Situ Hybridization* (*FISH*), as a one method from molecular fashion probe, has been developed and applied for the direct diagnosis of *S. typhimurium* in bacterial cell smears of pure cultures, or in formalin-fixed sections, and in paraffin embedded tissue. Therefore, this study was designed to determine the single bacterial cells in the infected intestine, liver and gallbladder by used (Sal-3 prop), through orally administered for lambs via stomach tube, with a volume of 0.5 ml contain ($1x10^8$ cfu/ml *S.typhimurium*), after bacterial identification by cultures media mainly Salmonella chromogenic agar, and biochemical diagnosis by [Iraq-CDC/central public health laboratory (CPHL) in the Baghdad province]. So results was concluded that Sal3-prop of FISH technique, was a good instrument for fortuity *S. typhimurium* in the in histopathological tissue sections.

Keywords: *Fluorescence in situ hybridization, lambs, FISH, S. typhimurium,.*

Introduction:-

Salmonellosis is a one of most important zoonotic diseases, it have a direct occupational with public health and related with food-borne illness, *Salmonella* enterica subspecies *typhimurium* are one of enteropathogenic bacteria, non typhoid *Salmonellosis*, cause diseases that range from diarrhea to systemic infections in human and animals (1 ; 2). *S. typhimurium*, is a facultative intracellular pathogen, that multiplication in intestinal submucosa, and infected the mesenteric lymph node, which spread into liver and gallbladder (3).

This pathogen has been widely prevalence, that infected animals may carry these bacteria without any clinical symptoms, so they isolated from farm animal products like meat, milk, excretion of carrier animals, also from contaminated equipment, floors at slaughterhouse, and personnel worker in an abattoir (4 ; 5). *S. typhimurium* had a many diagnostic methods in the microbiological laboratory for recognition, by use cultivation on

culture media, also with biochemical tests i.e. manually like *Analytical profile index* (API 20E/NE), or automatically test systems such as (*MicroScan-WalkAway*, *VITEK* and *BD-phoenix*), but all these procedures usually require pure cultures (6- 9) that helped pathologist to give perfect diagnosis report.

Fluorescence in situ hybridization techniques(*FISH*), was considered as one of molecular pathological methods, help to study the single bacterial strains in complex ecological systems, through use specifically probes targeting ribosomes in *S. typhimurium*(10).

The aim of this study is using FISH technique via specific probe (Sal3) generate from oligonucleotide to targeting *S.typhimurium* which infected lambs tissue organs after embedding in paraffin wax, that main in formalin-fixed section.

Material and methods

***Animals used*:** Eight lambs, in 3-4 months old, (5.00 - 5.50 Kg) were obtained from (animal market at Al-Mahmudiya District) in Baghdad-Iraq, and it divided into:

I- Control group, included two animals

II- Infected group included six animals, infected by 0.5 ml contain ($1x10^8$ cfu/ml *S. typhimurium*), given as single dose, through orally administered lambs by stomach tube.

Then kept in animal house, range in dimensions (6x8) meter, air of the room was changed by air vacuum, under conditions were maintained at 20± 2C°, animals were fed on animal hay , grass and water. Experimental study was taken in department of pathology, college of veterinary medicine; Baghdad University, for one month. Sacrifice was occur via slaughter animals, at (week, 15 day and month) of experiment, and pieces of tissue was taken from animal, to cultivation on Salmonella chromogenic agar, whereas other pieces fixed in 10% formalin saline (11), remain at less for 72 hours, tissue processing, and embedded in paraffin, was occur according to (12), slides stained with (Hematoxylin and Eosin).

Dose preparation:- Infected dose and concentration, was prepared in (Unit of Zoonotic disease, Department of Microbiology/College of Veterinary Medicine /Baghdad University). According to " McFarland standards

Preparation of Salmonella chromogenic agar:- According to manufacturer instruction,(37.1g.) of dehydrated media was dissolved in (1000 ml D.W.) at 80ºC. The mixture shacked and boiled for one minute to dissolve the medium completely then cooled, to about 50°C and poured into sterile petri dishes.

Selection of probe:- The systematic name of the Sal3-probe was [L-S-Sal- 1713-a-A-18], and sequences of Salmonella oligonucleotide probe obtained from (13 and 14), and (Sal3)specificity, is tested by hybridization with whole bacterial cells, and the slides prepare with prop was occur according to (15), that explain in (Table:1).

Probe	Type	Sequence (5'→3')	No. of Salmonella strains detected	No. of Non-Salmonella strains detected	Specificity (%)	Sensitivity (%)	reference
Sal-3	DNA	5'-AATCACTTCACCTACGTG	96	0	100	95.5	Nordentoft, *et al.*(1997).

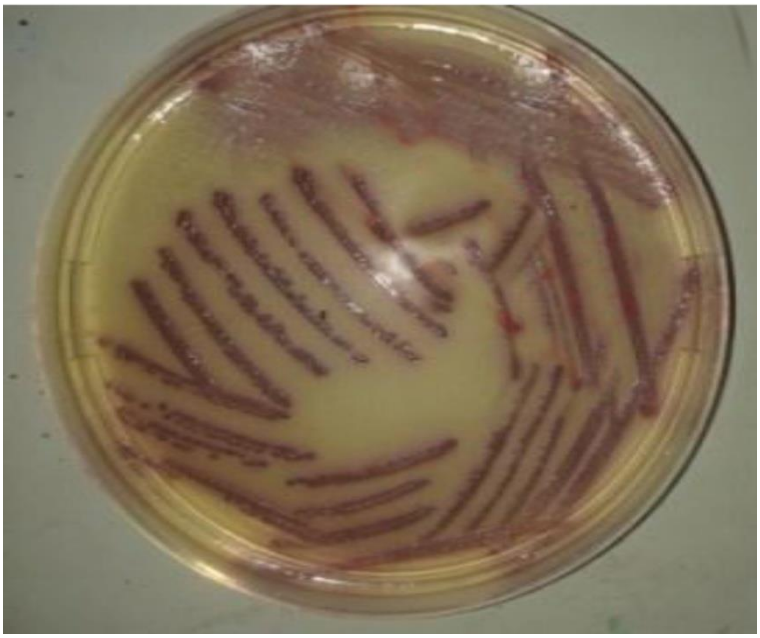
(Table:1) sequencing of FISH probes for detection of S. typhimurium in embedding tissue.

Results and Discussion:-

Bacterial isolation:- The results of isolated bacterial infection, from tissue organs, is illustrated in (figure:1) that shows the *S. typhimurium* growing on the culture media (*Salmonella* chromogenic agar), that appear as a mauve - magenta colonies in color, during vision. This result installed by diagnosis report of "Iraq-CDC/central public health

laboratory (CPHL) in the Baghdad province", that appear in (figure:2).

The magenta colonies appear due to chromogen, is a target for esterase activity present in *Salmonella*, which utilization of this chromogen results in mauve or magenta colonies in color. These result are agree with (16), which act on Isolation and Identification of several type of Salmonella spp. from human and animal sources in the Baghdad city. Also agreement with (17), that used the molecular and conventional detection of *S. typhimurium* , Isolation from different food sources.



(Figure:1) show magenta-mauve colonies of S. typhimurium colonies on chromogen agar.

جمهورية العراق
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التاريخ : ٤/ ٢٠١٧/

مختبر الصحة العامة المركزي
Central Public Health Lab.

يرجى الإشارة الى رقم الاصبارة عند الإجابة

إلى / وزارة التعليم العالي والبحث العلمي / جامعة بغداد / كلية الطب البيطري

م/ تشخيص عزلة

تحية طيبة . . .

أشارة الى كتابكم ذي العدد ٢٤٥ في ٢٠١٧/٣/٢٨

تم أجراء الفحوص التوكيدية على العزلة المرسلة من قبل طالب الدكتوراه (باسم محمد جواد) جامعة بغداد / كلية الطب البيطري لغرض اكمال متطلبات بحثه الموسوم (Molecular pathological changes induced by Salmonella typhimurium in lambs) ، وكانت النتيجة كما مبين في ادناه .

ID#	Specimen	Diagnosis
1	Sheep	Salmonella typhi-murium

للتفضل بالإطلاع مع الاحترام

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جبار هاشم ناصر
مدير مختبر الصحة العامة المركزي
٢٠١٧ /٤/٢

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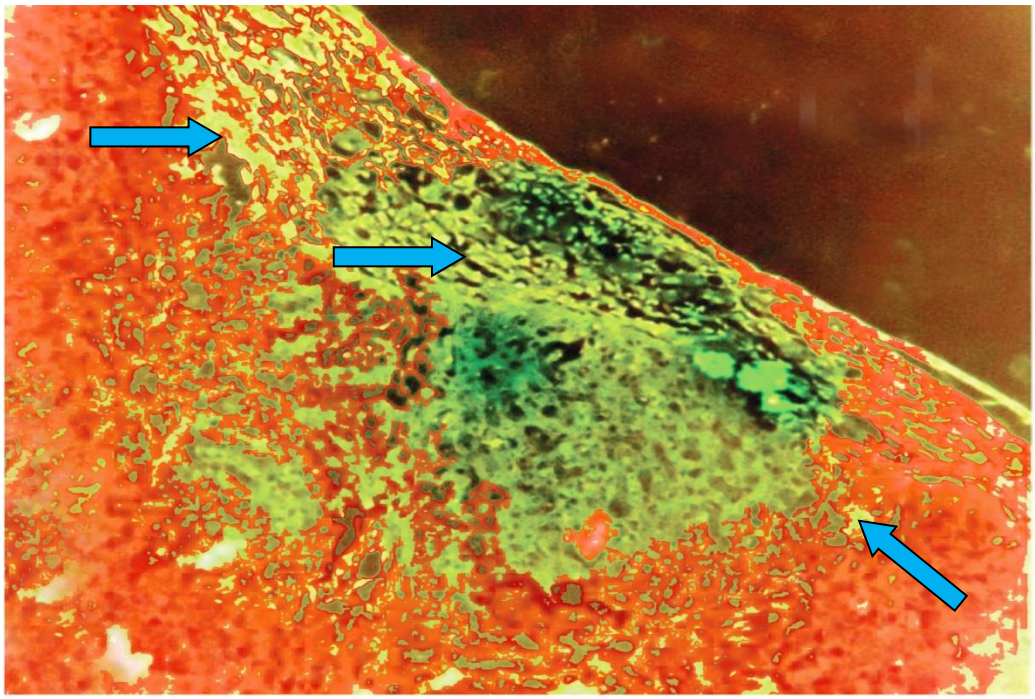
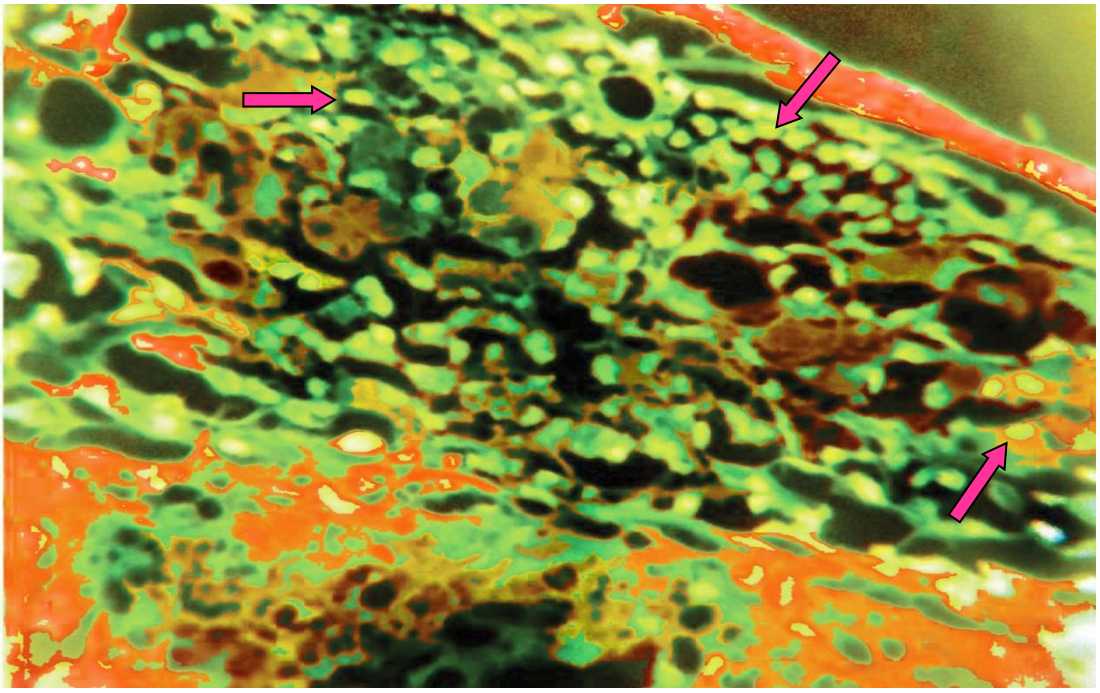
(Figure:2) show Baghdad-CPHL,certification for serological test.

Result of (FISH) in paraffin embedding tissues:-

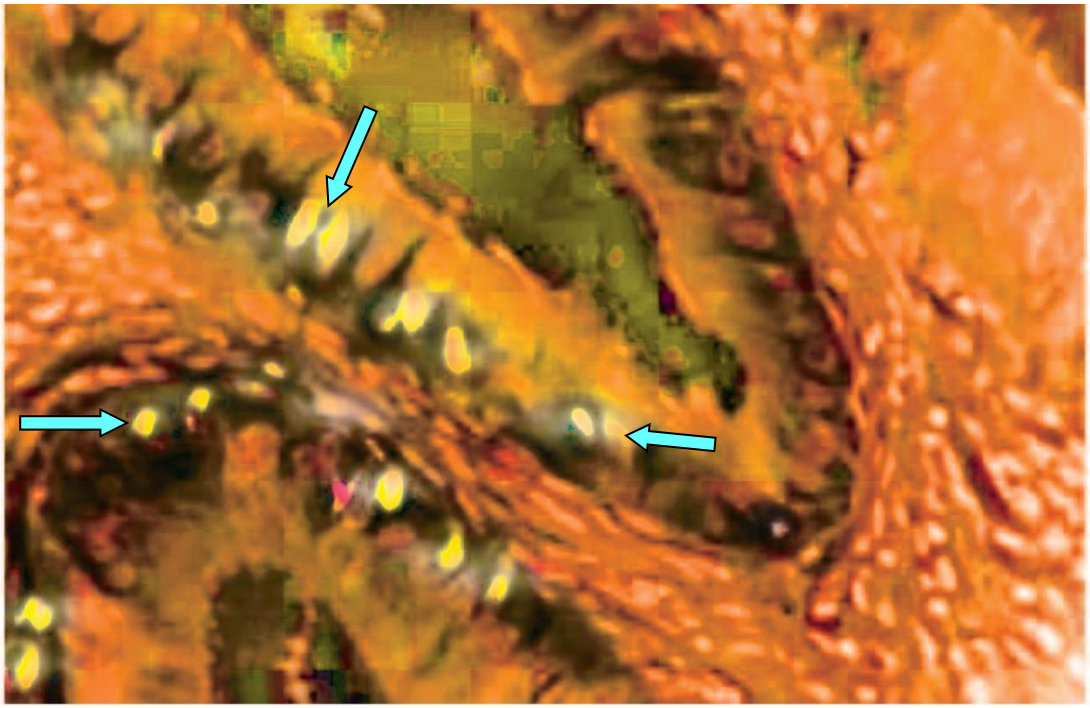
Results of microscopic detection about presence of *S. typhimurium* by using DNA-probe(Sal3), applied by fluorescent in situ hybridization on paraffin embedding tissues of liver and intestine of lambs after oral infected 0.5 ml contain ($1x10^8$ cfu/ml *S. typhimurium*), were indicating the presence *S. typhimurium* inside the epithelial cell of liver, that demonstrate in (figure:3) and (figure:4). Also within intestinal villi in the lamina propria show the infected cells that exhibition in (figure: 5) and (figure: 6), an equally important, all examined tissue, which illustrated as green shiny color, from signal for tissue injury through presence of bacteria in tissue epithelial cells of intestine and gallbladder within liver, during the period of experiment (7, 15 and 30 days) with paraffin embedded tissue section of lambs (liver, gallbladder and GIT).



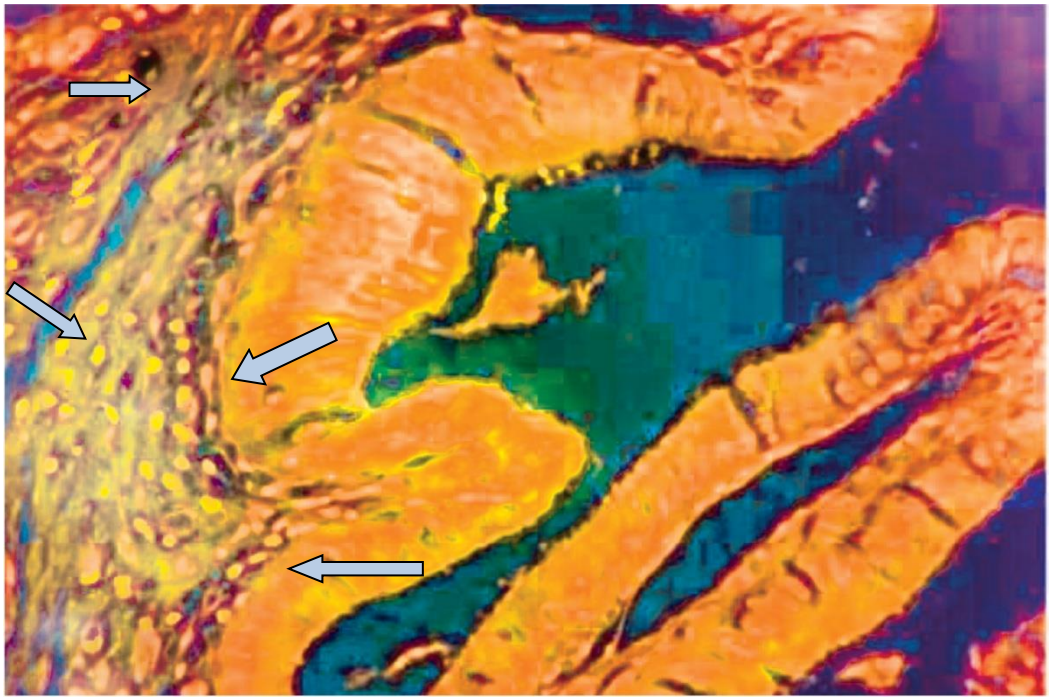
(Figure:3) Microscopic picture of lambs liver, which patronized with (Sal3), after 7 day from infected by, show infected cells appear as green in color ⇨ (40X)



(Figure:4) Microscopic picture of lambs liver, which patronized with (Sal3), after 7 day from infected by, show green shiny appearance inside infected cells, ⇨ are seen indicating the presence of both components of the probe (400X).



(Figure:5) Microscopic picture of lambs intestin, which patronized with (Sal3), after 15 day from infected by, show green shiny appearance inside infected cells, ⇨ are seen indicating the presence of both components of the probe (400X).



(Figure:6) Microscopic picture of lambs intestin, which patronized with (Sal3), after 30 day from infected by, show green shiny appearance inside infected cells, ⇨ are seen indicating the presence of both components of the probe (400X).

From other hands, The scores of green signals appear in (table:2), were indicating the presence of Salmonella typhimurium in the organ of the animals during the period of experiment (7, 15 and 30 days) with paraffin embedded tissue section of lambs (liver and GIT). Whereas reduced autofluorescence from color of the tissue, that appears red in background, easily for detection of single infected bacteria.

Scores during (7 days), was highest after infected by *Salmonella typhimurium*, so the scores with paraffin embedded tissue was recorded in all infected animals (100%), so it has a sign that *S. typhimurium* infection with tissue organ was more severe in the period of infection during the first seven days of infection. However, notes that the two groups were equalized by the severity of the infection and spread the bacteria in the body within the period of 15 and 30 days after liver infection, where from (table:2) in the intestine, we note that the proportions of the score were compared among them whether the infection with presence in liver or in intestine, so at end of the 15 days the green signals were found in all score that main the spread within animal from each observation, was recorded (100%) that recorded after 30 day.

Table(2) :The scores of the (Sal3) DNA probe

Time	Green signal score of paraffin embedded tissue section of intestine lambs infected by 10^8 *S. typhimurium*			Green signal score of paraffin embedded tissue section of liver lambs infected by 10^8 *S. typhimurium*		
	Score 1	Score 2	Score 3	Score 1	Score 2	Score 3
7 days	0	1 B c	1	1	1 A c	1
15 days	3	0 A b	0	3	0 A b	0
30 days	0	1 B a	2	3	0 A a	0

Small letters significant differences between group at ($p \leq 0.01$)
Capital letters significant differences with group at ($p \leq 0.01$).

The results that show a shiny green color appear in the infected epithelial cell, occur due to (Sal3) prop within FISH technique, was used to identification of S. typhimurium through alignment of mRNA gene sequences of representative Salmonella sequences, which characterized by percentage of specificity to Salmonella strains detected was (100%) and the sensitivity was (95.5%), and it did not hybridize with any non-Salmonella sequence.

Furthermore, probe reaction with two regions from sequence variability in bacteria, which have previously been shown to exhibit high degrees of conservation in the probe target region, that making detection and spatial localization of single bacterial cells in tissue samples.

Also used the paraffin embedding tissue with these probes, because the fixative tissue in paraffin wax, help to opening up the bacterial wall for probe penetration, also it protecting the ribosomes from degradation by endogenous RNase activity, and enhance the level of discrimination of the signal from single bacteria in tissue sections, and simultaneous excitation in two separate bands of the DNA, therefore enabling it to reduce autofluorescence from tissue considerably by turning the color of the tissue red and thereby allowing for the detection of single bacteria.

This finding was supported by results which reported by (18) in dog ; (19 - 21) they explained that "the development of a [fluorescence-labelled specific oligonucleotide probe] makes the (FISH) technique a promising tool for the rapid identification of S. enterica in bacterial smears, as well as for the detection of S. enterica in histological tissue sections" .

We have verified in present study investigated that used DNA-probe(Sal3), applied by fluorescent in situ hybridization on paraffin embedding tissue of liver and intestine of lambs after oral infected 0.5 ml contain ($1x10^8$ cfu/ml *S. typhimurium*), give a stability of the ribosome target allowed "*mRNA-DNA-S.typhimurium*", for the detection of single cells in clinical infection tissue, therefore we suggest that the FISH technique can be used for the detection of *S. typhimurium*, inside infective tissue after fixation and embedding in paraffin wax.

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Doctor of Philosophy in the department of Pathology.

Animal Rights Statement:- *The experiments and procedures involving local lambs, were approved by scientific committee of department of pathology; in the College of Veterinary Collages of Veterinary Medicine, University of Baghdad, Iraq, and were conducted according to the guidelines of the committee.*

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