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A COMPARATIVE STUDY OF CULTURE METHODS, API SYSTEM AND PCR ASSAY FOR *Salmonella* DETECTION ISOLATED FROM HUMAN, COWS AND POULTRY IN IRAQ

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ABSTRACT

The genus Salmonella is one of the most important enteric pathogens, over the world Salmonella Entritidis and Salmonella Typhimurium are the two most widespread serotypes that lead to salmonellosis in human and animals and are often transferred to humans by infected animals and their products. The present study was conducted to compare the culture methods, API 20E test and PCR assays for detection of *Salmonella spp.* isolated from 300 different samples collected from various sources included healthy and infected human, cows ,chickens, chickens eggs and other sources. To achieve this goal three selective media were used included (XLD, S.S. and KIA agar) in addition to using the chromogenic salmonella media and the result of culturing was compared with the results of API 20E test and PCR assays . We find that the using of chromogenic media and PCR assay for detection of Salmonella spp. is more current and efficient than using selective media and/or API 20E.

INTRODUCTION

Salmonellosis is an important infection disease in human and animals (1,2). Salmonella are inhabitant in the gastrointestinal lumen of a broad range of vertebrates (3, 4). Over 2610 variant serotypes (serovars) have been recognized by Kaufmann-White-Le minor scheme (5). Over the world Salmonella Entritidis and Salmonella Typhimurium are the two most commonly serotypes that led to salmonellosis in human and animals and are often transferred by infected animals and their products to humans (6, 7).Culture methods for detecting *Salmonella* are well established and

are routinely used for food testing, clinical diagnosis, and surveillance (8). In spite of the upgrading in the culture of *Salmonella* as a result of many years of productive research work, the isolation procedures currently in use are not ideal: false-positive and false-negative colonies are familiar. Both *Citrobacter* and *Proteus* spp. are commonly misidentified as *Salmonella* because of similar colonial features on selective xylose lysine desoxycholate (9). because the increased diffusion of *Salmonella* serovar *Enteritidis*, and its complicated life cycle, a lot of researchers emphasize the requirement and importance of finding a more quickly and effective detection technique as a basis of control (10,11). Now, *Salmonella* is find out by standard bacteriological, biochemical and serological method. These method are generally time-consuming, boring and expensive as they require hundreds of antisera as well as well-trained technicians (12,13). Many rapid and sensitive methods have been developed for conformity of *Salmonella* serotypes from clinical samples (14). These methods, however, still loss the required sensitivity and specificity. In recent times the amplification of DNA by the polymerase chain reaction (PCR) technique is consider a strong equipment in microbiological diagnostics. Bacterial PCRs target evolutionarily highly preserved genetic elements, e.g., bacterial 16S rRNA genes , Such PCRs with subsequent sequence analysis are very good confirmed techniques for the identification of bacterial pathogens (15), permitting for the find of a wide range of strains (16). Several protocols of 16S rRNA gene-based wide range PCRs for the diagnosis of infections have been suggested (17). PCR of short fragments give a higher sensitivity than PCR of longer fragments of the 16S rRNA gene, while longer fragments supply better differentiation in sequence analysis (18). Genotypic identification methods are appear as an alternative or complement to confirmed phenotypic identification process. For bacteria, 16S rRNA gene sequence analysis is a very accepted factor for molecular identification (19). The present study was aimed to find the most rapid and accurate method for detection of salmonella from different sources.

MATERIALS AND METHODS

Samples collection and preparations:

The period of the study was extending 10 months started from 2017 and involve both Basra and Baghdad province. The total collected samples was (300) distributed as following: The human samples were collected from two sources the first source was the healthy workers in the field

who was in contact with the domestic animals, the second human samples were collected from the diarrheic patient who attended the hospital. The number of samples for each type were 50 sample, a 5 g of the stool was taken and it was added to 10 ml of selenite broth to be mixed. And then was transported to the laboratory of microbiology in icebox to be incubated at 37^{o} C for 18-24 h. Cows samples: A fresh fecal samples from 50 cows were taken from the bowel as soon as possible after the animals were butchered. A 5g of feces were mixed with 10 ml of selenite broth and was transported to the laboratory of microbiology in icebox to be incubated at 37 ^{o}C for18 -24 h. The chicken samples: The 50 chicken swabs samples were done directly from the rectum by soft insertion of the swab stick after wetting it with selenite broth, then it was inoculated in 5 ml of selenite broth, and then it was transported immediately to the laboratory by icebox to be incubated at 37^{o} C for 18-24 h. The eggs samples: A 50 eggs were collected from irregular local house chicken and put immediately in sterile jar and transported to the laboratory for more processing. The eggs shells were cleaned thoroughly and wiped by cotton with 70% ethanol. Then two type of samples were collected first one included swab samples collected directly after remove the shell membrane of the eggs. While the second type of samples were included the taken whole eggs content and put directly in sterile container with 25 ml of peptone water and mixed well, then 3 ml was taken from the mixture and was added to 7 ml of selenite broth. The last Samples from other sources: included 50 swab samples were collected from drainage water and the ground of the field of domestic animals and slaughter tools, the swab samples were transported by a test tube containing selenite broth to the laboratory. All samples were transported immediately to the laboratory by icebox and incubated with selenite broth at 37^{o} C for 18-24 h., except the eggs samples were incubated for two days the first day with peptone water and the second day was incubated with selenite broth.

Isolation and identification:

Identification of *Salmonella spp.* was carried out according to the method of (20,21). After incubation of sample in selenite broth for 24h., a loopful of the selenite broth streaked on XLD, S.S. and KIA agar and were incubated at 37^{o}C for 24 to 48h and the dishes were examined for the morphology of Salmonella colonies. Positive samples were subsequently culture on Brian heart infusion broth for other biochemical tests and for streaking on chromogenic agar plates and

incubated at 37oC for the 24 to 48h. the plates were examined for the colour of colonies. Salmonella (including *S.typhi*, *S.paratyphi* and lactose positive salmonella) was appeared mauve colour. *E.coli* and Proteus, etc. was colorless or inhibited and Coliforms, etc. was blue colour. Another Conventional tests were done like Gram's stain, Motility test, Oxidase test and Urease test. Moreover the result was confirmed by inoculation the API 20 E system (BioMérieux, Inc., France).

API 20E

API 20E test is a plastic strips holding twenty mini-test tubes were inoculated with the distilled water suspensions of the cultures from nutrient agar that was compared with the McFarland standard solution for the density. Then the suspension was distributed in each tube. Some of tubes were completely filled (CIT,VP and GEL), and other tubes were overlaid with mineral oil for isolation from air reactions (ADH, LDC, ODC, H2S, URE). After incubation in a wet chamber for 24 hours at 37°C, the color reactions were read (some with the aid of added reagents as supplied by the kit). The data were analyzed by using the indicator book.

Bacterial DNA Extraction and PCR Analysis

DNA Extraction

Because Salmonella is Gram negative bacteria the boiling extraction method was used in DNA extraction. The procedure of this extraction was done by picked a five colonies from the XLD agar plate of the suspected bacteria, and then it was transferred into Eppendorf tubes(1.5 or 2 ml) containing 200 µl of distilled water. Before incubated at 100oC for 15 min in water bath the tubes was vortexed. Then 800 µl of distilled water was added to get 1ml and remixed well by vortex until the solution was homogeneous. Then the solution for 10 min was centrifuged at 12000 rpm in cool centrifuge. The last step was the taken the supernatant which contain the genomic DNA and transferred into an new Eppendorf tubes to be ready for PCR technique.

PCR technique:

For detection of Salmonella species a specific set of oligonucleotide primer were used in polymerase chain reaction (F:5′TGTTGTGG TTAATAACCGCA-3′, R:5′-

CACAAATCCATCTCTGGA-3′) which amplify 572bp specific region of 16rRNA (21). The PCR reactions were conducted in a total volume of 25µl,consisted of 5µl of master mix (Bioneer /Korea), 10µl of genomic DNA , 1µl of each primer and 8µl of nucleus free water. Amplification condition was obtained with an initial denaturation step at 95C° for 5 min followed by 35 cycle each at 95C° for 5min , 55C° for 30sec and 72C° for 1 min , with final extension period of 10 min at 72C°(21). The products amplified sizes were identified using 100 base pair DNA ladder (Bioneer/Korea). Five µl of PCR products were directly loaded in a 1.5% agarose gel electrophoresis and visualized by UV trans illuminator.

Statistical analysis:

Statistical analysis was performed by ANOVA test by Minitab 17, using the Fisher LSD method. P-value less than 0.05 was considered as statistically significant and P-value less than 0.01 considered as highly significant .

Ethical Responsibilities

Protection of human and animal subjects: The authors declare that the procedures followed were in accordance with the Animal Welfare Regulations and Ethics.

RESULTS

Table and figure (1) show the difference among the results of using three selective media for isolate Salmonella sp. and use of conventional PCR as indicator for specific detection of salmonella species. Alternatively table and figure (2) reveal the differences among the results of using the Kligler iron, API20E, chromogenic media and PCR for diagnosis of salmonella spp. The result of PCR amplification that performed on the extracted DNA was confirmed by electrophoresis as the strands of the DNA which are resulted from successful binding between primers and the extracted DNA. These successful binding appear as a single band under U.V illuminator using ethidium bromide as a specific DNA stain. Bands with expected size (572bp) were observed figure (3).

Table 1 : Comparative between the XLD, S.S, chromogenic and PCR positive results according to the source of samples.

Test / Source	XLD agar %	S.S agar%	Chromogenic agar%	PCR %
Human	37/100(37%)	35/100(35%)	8/100(8%)	8/100(8%)
Cows	11/50(22%)	12/50(24%)	3/50(6%)	3/50(6%)
Egg	25/50(50%)	28/50(56%)	13/50(26%)	13/50(26%)
Chicken	15/50(30%)	15/50(30%)	4/50(8%)	4/50(8%)
Other	23/50(46%)	24/50(48%)	11/50(22%)	11/50(22%)
P value	0.005			

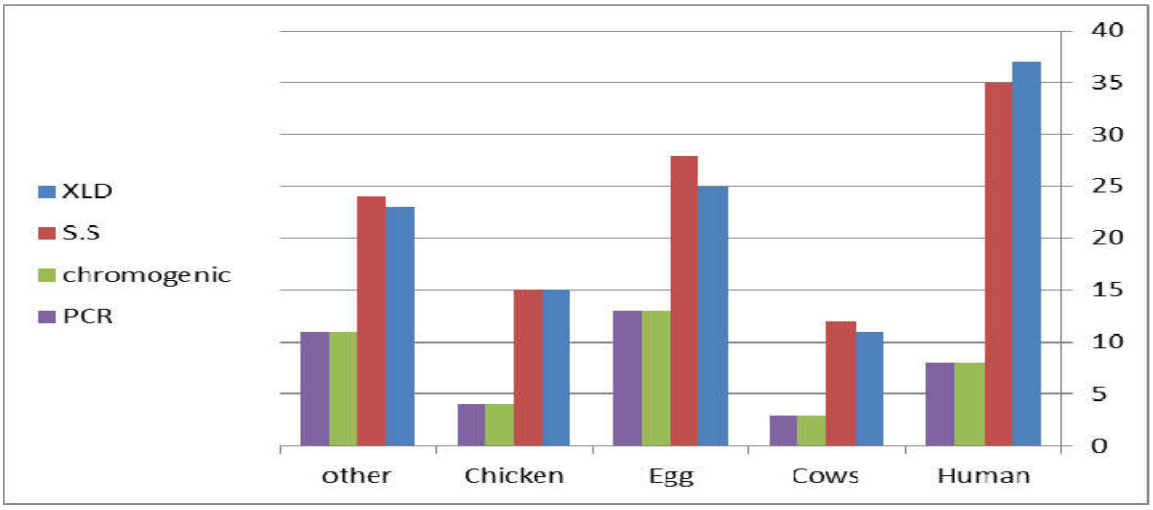

Figure 1 : Show the variation between the XLD, S.S, chromogenic and PCR results according to the source of samples

Table 2: Comparative between the KIA, API 20, Chromogenic and PCR positive results according to the source of samples

Test / Source	Kligler iron agar%	API20E%	Chromogenic media %	PCR%
Human	20/100(20%)	16/100(16%)	8/100(8%)	8/100(8%)
Cows	10/50(20%)	9/50(18%)	3/50(6%)	3/50(6%)
Egg	22/50(44%)	17/50(34%)	13/50(26%)	13/50(26%)
Chicken	13/50(26%)	9/50(18%)	4/50(8%)	4/50(8%)
Other	19/50(38%)	16/50(32%)	11/50(22%)	11/50(22%)
P value	0.013			

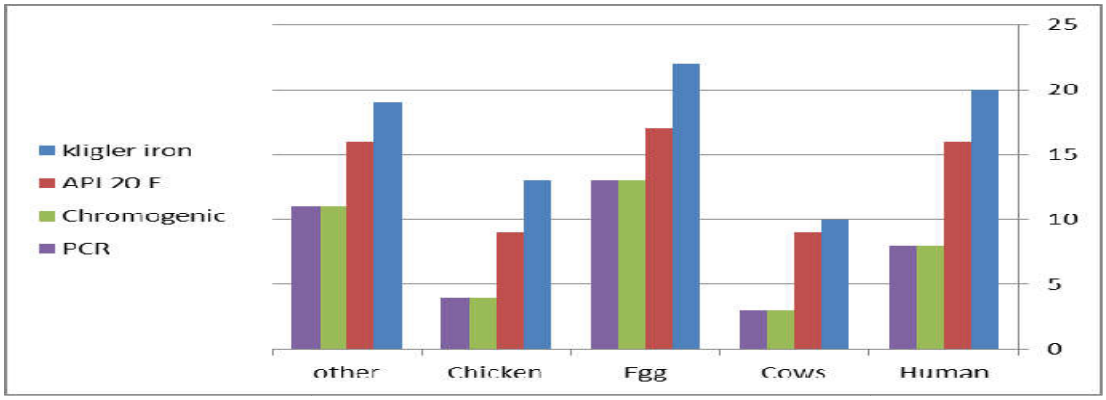

Figure 2 : Show the variation between the KIA, API 20, chromogenic and PCR results according to the source of samples.

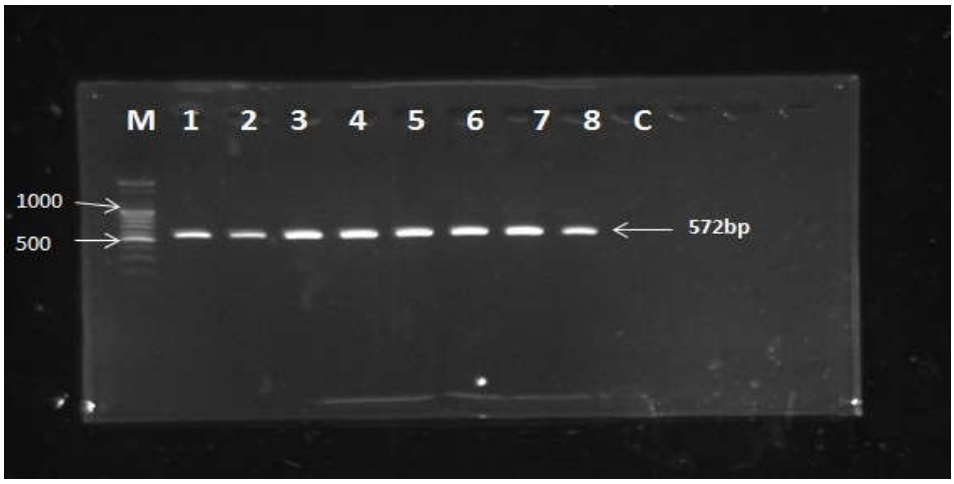

Figure. 3. Agarose gel electrophoresis reveals the PCR products of : Lane M: 100 bp DNA Ladder. Lanes 1- 8: PCR Products of 572bp region of salmonella spp. 16S rRNA gene and Lane c: control negative.

DISCUSSION

Understanding the limits of presently used selective and differential media, it is request to improve the specificity and the sensitivity of the medium while keeping cost-effectiveness. Particularly, it is favorite to differentiate *Salmonella* spp. from *Proteus* spp., as well as from *Citrobacter* spp. (22). Many quick methods have been developed to detect pathogens (23). However, traditional selective and differential media are still remarkable, due to many advantages, including cost-effectiveness, ease of use, and knowing among users (24). In the present study table and figure (1) show the difference among the results of using three selective media for isolate Salmonella sp. and use of conventional PCR as indicator for specific detection of salmonella species, we find that the chromogenic media result was similar to the result of (PCR) while the XLD and S.S. agar culturing result was show highly significant different(p-value < 0.008 and 0.006 respectively) if compared with chromogenic, this due to the growth of some type of bacteria that have similar characters of salmonella the led to false diagnosis. And this finding agree with the previous study that say, both Citrobacter and Proteus spp. are commonly misidentified as Salmonella because of similar colonial features on selective xylose lysine desoxycholate (9). Moreover XLD agar are the most widespread media for finding of *Salmonella* spp., and their recognition abilities depend on characteristics of *Salmonella*, such as hydrogen sulfide generating and the nonfermentation of lactose (22). However, these features are shared with other bacteria, such as *Proteus* and

Citrobacter (25). Although XLD has a high sensitivity and specificity, *Proteus* and *Citrobacter* give colonies indistinguishable from those of *Salmonella* on this agar (22). On the other hand table (2) and figure (2) reveals that chromogenic media culturing and (PCR) show the same results in compare with two other test KIA and API 20E, we find that the KIA test (p-value <0.006) is highly significant different in compare with chromogenic agar this was due to salmonella and Proteus cause glucose fermented with H_2S this may led to false result, and some type of salmonella may not give black or weak black result, while the API 20 was less different in the result (p-value 0.064), in spied of the interpretation of the test is fixed on two option positive or negative sometime it is difficult to recognize between colorless and weak pale color this may led to mistake in the result, it was more fixed and specific than KIA test. Additionally the acid formation as outcome of carbohydrate fermentation may affect hydrogen sulfide production (22). Under the acid conditions of carbohydrate metabolism, H_2S-positive *Enterobacteriaceae* were unable to produce the black precipitate of iron sulfide. *S.* Gallinarum and *S. Pullorum* rarely generate hydrogen sulfide, and the reaction occurred slowly (22). Also the hydrogen sulfide producing *S. Typhi* is weak or negative (26). Although the chromogenic media culturing result was similar to PCR result (100%) this was due to inhibit other type of bacteria or appeared in another color that make the detection of salmonella more easy and faster. Over the last 30 years, a range of chromogenic media has been developed that are designed to target patho-genes with high specificity. Such media take advantage of enzyme substrates that release colored dyes at hydrolysis, thus resulting in pathogens forming colored colonies that can easily be recognize from other bacteria. Ideally, other bacteria should either be inhibited completely by selective agents or give colorless colonies to allowing pathogens to emerge against background .This make easy differentiation of microbes have the enzyme from those that do not. This is very important when trying to detect specific pathogens within polymicrobial cultures. The substrate and products of hydrolysis should not restrained microbial growth (27).We concluded that using of chromogenic media and PCR assay for detection of Salmonella spp. is more current and efficient than using selective media and API 20E test.

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دراسة مقارنة لطرق الزرع و،نظام API و اختبار PCR للكشف عن السالمونيلا المعزولة من الانسان ،الابقار والدواجن.

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الخلاصة

جنس السالمونيلا واحد من أهم مسببات الأمراض المعوية، وعالميا تعتبر كل من Salmonella Entritidis و Salmonella Typhimuriumمن الانواع المصلية الأكثر انتشارا والمسببان للسالمونيلا في الإنسان والحيوان، وكثيراً ما تنتقل إلى البشر من قبل خلال الحيوانات المصابة بها ومنتجاتها. أجريت الدراسة الحالية للمقارنة بين استخدام الاوساط الزرعية واختبار API 20E وال PCR في الكشف عن أنواع السالمونيلا المعزولة من ٣٠٠ عينة جمعت من مصادر مختلفة شملت عينات من اشخاص اصحاء ومصابين من الأبقار المصابة، الدجاج ، بيض الدجاج، ومصادر أخرى. ولتحقيق هذا الهدف استخدمت ثلاث اوساط انتقائية شملت (XLD, S.S. and KIA agar) بالإضافة إلى استخدام وسط chromogenic salmonella agar وقورنت نتائج التشخيص بالزرع مع نتائج اختبار API 20E و فحص PCR. وقد وجدنا من خلال النتائج بان استخدام chromogenic salmonella agar وفحص PCR للكشف عن السالمونيلا هو أكثر دقة وكفاءة من استخدام الاوساط الانتقائية واختبار API 20E.

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