Journal of University of Duhok, Vol. 22, No.1 (Pure and Eng. Sciences), Pp 148-154, 2019
https://doi.org/10.26682/sjuod.2019.22.1.16

MOLECULAR IDENTIFICATION OF *Salmonella Enterica* FROM PATIENTS WITH DIARRHEA IN DUHOK GOVERNORATE KURDISTAN REGION / IRAQ.

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(Received: May 16, 2019; Accepted for Publication: May 30, 2019)

ABSTRACT

Background and objectives: *Salmonella enterica* are responsible for causing the highest number of bacterial foodborne infections in the world. Methods: The Present study was carried out for detection and molecular identification of *Salmonella enterica* isolated from stool specimens of patients suffering from diarrhoea using traditional method and identification by using of RT-Polymerase Chain Reaction (PCR) techniques. Results: Out of the 121 patients with diarrhoea attended Azadi and Heevi Teaching Hospitals, 72 cases (59.504%) gave positive results for culturing. While by PCR method, 68 cases (56.198%) gave positive results for *Salmonella enterica*. In this study, (49) cases (40.495%) of non-*Salmonella spp*. gave negative results by culture and (53) cases (43.8%) of non-*Salmonella spp*. gave negative results by PCR method. Conclusions: The current study showed that RT-PCR technique is more accurate and sensitive compared to traditional methods.

***KEYWORD**: Salmonella enterica*, Food borne infections and RT-PCR.

INTRODUCTION

Salmonella are Gram-negative bacilli and non-spore forming, facultative anaerobic, non-lactose fermenting bacteria within the family of enterobacteriaceae. Infection with Salmonella is the most frequent food-borne gastrointestinal disease transmitted from animal products to human by food mainly through water, meat, eggs and poultry (Riyaz-Ul-Hassan, *et al*., 2004). Salmonella infection remains as a serious problem to public health which in turn is regarded as a major public health concern and responsibility for the significant cost in all around the world which leads to the thousands of deaths (Lynch, *et al*., 2006).

WHO estimated that the annual median number of non-typhoidal salmonellosis was 78.7 million foodborne illnesses with over 59 000 deaths, (Havelaar AH *et al.*, 2010). Non-typhoidal Salmonella species are a common cause of foodborne disease in the Middle East and North Africa (MENA). Moreover, *Salmonella* has been detected in an array of food products presented to consumers in the region. The number and quality of the studies differ substantially by country. To the best of our knowledge, there has been no published study that systematically reviewed, synthetized and assessed the available data on non-typhoidal enteric *Salmonella* in human and food in the MENA region (Havelaar AH *et al*., 2010).

There are many tools for diagnosis of salmonella infection: traditional bacteriological methods offered standardized procedures for microbial detection. However, they are time consuming that take approximately 4–7 days (Ferre et al., 2002) and not always compatible with short-time-to result demand. Therefore, Microbiology aims for supplementation of classical methods with molecular techniques based on detection of the microbial nucleic acids, which shorten the analysis time and lower the limit of detection. An important aspect in the characterization of bacteria is the molecular detection in determining the clonal and strain distributions among various environments. Traditional microbial methods, albeit generally considered to be variable, labor-intensive, and

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time-consuming, are of practical value in epidemiological investigations. Molecular detection methods are mainly based on the analysis of the genetic material of microbial agents (Ranjbar R, *et al*., 2014, Lin T, *et al*., 2014). This study was undertaken to determine the presence, of salmonella bacteria from patients with diarrhea using traditional, biochemical and Molecular tools in Duhok governorate, Kurdistan Region of Iraq.

PATIENTS AND METHODS

Salmonella isolates were collected from Azadi Teaching Hospital at emergency department and Heevi Pediatric Hospital in Duhok City. These isolates were obtained from stools samples taken from 121 patients with salmonellosis or suspicious salmonellosis were diagnosed in outpatient clinics at Azadi Teaching Hospital and in Duhok Medical Research Center (DMRC) during April to October 2018.

Stools samples were pre-enriched in peptone water in sterile conditions. Depending on reproduction abilities of colonies and incubated at 37˚c for 16-18 Hrs., enriched in Rappaport Vassiliadis(VRS) at 42˚c for overnight incubation and cultured on XLD agar according to International Organization for Standardization (ISO 6579-1:2017) then suspicious colonies taken for biochemical tests. DNA was extracted from 1 ml of the pre-enrichment broths using QIAamp DNA Extraction Mini Kit (Qiagen, Germany) following the manufacturers' instructions.

Polymerase Chain Reaction (PCR) was performed depending on amplification of the *invA* gene. The Final volume of amplification was 20µl, which described in Table (A-1). The PCR reaction consisted of 2X master mix and probed primer (10µl of Master Mix, 10µl of Extracted DNA).

The cycling parameters consisted of an initial PCR activation step, activation of HotStarTaq Plus DNA Polymerase (5 min, 95 °C), followed by 40×3step-cycles consisting of denaturation (15 sec. at 95°C), annealing for 15 sec. at 60 °C, and extension for 10 sec. at 72 °C, as in Table (A-2). Fluorescence detection was performed at the end of the annealing stage of each cycle. The amplification done by Rotor-gene Q instrument in Central Laboratory –Duhok.

Table A.1: Components of PCR reaction.

Component	Sample	Positive PCR control	Negative PCR control
Reconstituted *mericon* Assay	10 µl	10 µl	10 µl
Sample DNA	10 µl	–	–
Dissolved Positive Control DNA	–	10 µl	–
QuantiTect Nucleic Acid Dilution Buffer or RNase-free water	–	–	10 µl
Total volume	**20 µl**	**20 µl**	**20 µl**

Table A-2: PCR program of Cycling parameters.

Step	Time	Temperature	Comments
Initial PCR activation step	5 min	95°C	Activation of HotStarTaq *Plus* DNA Polymerase
3 step cycling			
Denaturation	15 s	95°C	Data collection at 60°C
Annealing	15 s	60°C	
Extension	10 s	72°C	
Number of cycles	40		
Detection	**Reporter**	**Excitation/emission**	**Channel**
Target	FAM	495/520 nm	Green
Internal control	MAX	524/557 nm	Yellow

ResultsThe isolation and identification of *Salmonella* were conducted according to International Organization for Standardization (ISO) 6579-1 (2017). Depending to the traditional culture methods of stool samples the percentage of *Salmonella spp.* isolation was 72 (59.504%), (Table A-3). There was a significant difference ($P<0.01$) between the positive and negative results. The colonies of *Salmonella spp.* on XLD agar were small, circular, smooth, convex and red in color with black center (Fig. B-1).

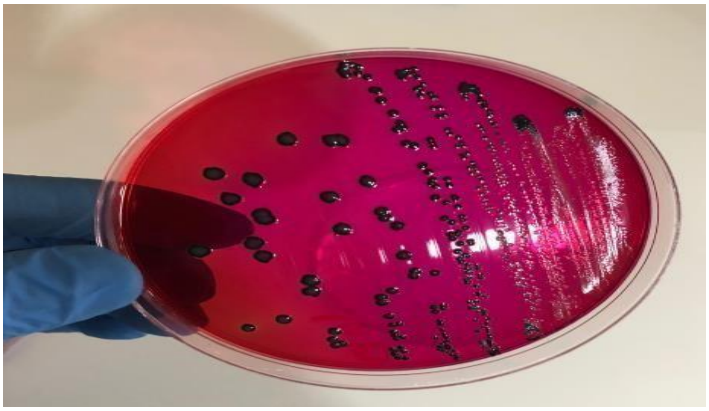

Fig (B-1): Salmonella Colonies on XLD agar

Subsequently colonies on XLD agar was confirmed with biochemical tests revealed the inability of Salmonella to urea hydrolysis (Fig B-2), ability of Salmonella to ferment the glucose and produce the hydrogen sulfide gas when tested on Triple Sugar Iron TSI media (Fig B-3) and Lysine Decarboxylation medium (LDC) To assist in the identification of Salmonellae (positive) Bacteria that are form Lysine Decarboxylase (LDC) in (Fig B-4).

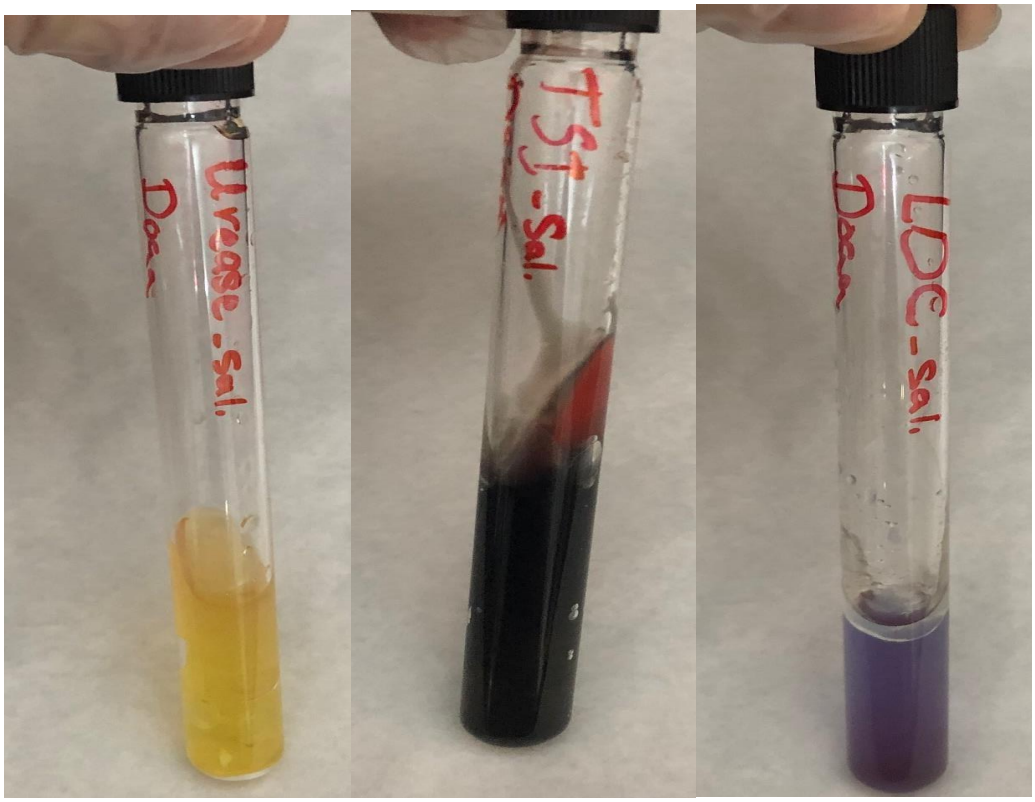

Fig (B-2): Urease test (-ve), Figure (B-3): TSI test (+ve) and Figure (B-4): LDC test (+ve)

To determine the presence or absence of salmonella pathogen based on amplification of *invA* gene using real time PCR, the sample considered to be positive if the corresponding fluorescence(FAM) curve lies clearly above the threshold line. The comparison between detection results of *Salmonella spp.* by traditional cultural methods and RT-PCR technique is described in Table (A-3). Out of 72 cases (59.504%) that gave positive results as *Salmonella spp*. on culture media, out of 49 cases (40.495%) that gave negative results in culture and biochemical tests. While 68 cases (56.198%) were positive for PCR techniques and 53 cases (43.801 %) were negative for PCR test.

Table (A-3): Percentages of *Salmonella spp.* isolation

Isolation Results	**by culture**		**by PCR**		**X^2 value**
	Number	**Percentage**	**Number**	**Percentage**	
Positive	**72**	**59.504%**	**68**	**56.198%**	**X^2 = 0.271 Prob.= 0.602**
Negative	**49**	**40.495%**	**53**	**43.801%**	
Total	**121**	**100%**	**121**	**100%|**	

DISCUSSION

According to phenotypic characteristic, which is the main method for detection of bacteria. However, in some cases, PCR technique required for specific detection of *Salmonella spp*., particularly when atypical culture characteristics appear (Cohen *et al.*, 1996, Daum *et al.*, 2002). As well as these methods are not reliable for species designation due to morphological similarity among these organisms and the need of an expert person. Low sensitivity and specificity of these methods leads to controversy in interpretation of result. In vitro amplification of DNA by PCR method is a powerful tool in microbiological diagnostics (Malorny *et al.*, 2003). Therefore, RT-PCR analysis with cultural methods were applied in this study to increase the detection specificity of Salmonella in Duhok city of Kurdistan Region and Iraq.

Salmonellosis has remained one of the most common causes diarrhoeal diseases in human, and gastroenteritis is the typical disorder caused by non-typhoidal Salmonella infection as recorded by (Oliveiria *et al*., 2003). This high occurrence may be due to increase in the number of supermarkets and restaurants in Duhok province. These supermarkets are selling meat in parallel with other different products such as poultry, fish and meat. The result of present study is coincided with a study done by OWAIED, D.Y.H., (2016). They found that type *S. typhimurium* is more sovereignty in the studied species.

In most parts of the world, surveys have reported that *S. Enteritidis* and *S. Typhimurium* are the major serovars found in human (Saba *et., al*., 2013 and Antoine *et., al*. 2008). In Ghana and most other African countries, these types were also the most frequently isolated species from bloodstream infections (Evans *et al.*, 2004 and Wilkens *et al.*, 1997) and from diarrhoeal diseases (Bonkoungou *et al.*, 2013), which is confirmed also by the findings of the present study**.**

ACKNOWLEDGEMENTS

We would like to acknowledge the staff including the directorate of medical college at university of Duhok, the staff of Azadi Teaching Hospital including lab members and Central public health Lab.

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پوختە

پاشینە و ئارمانج :

سالمونیلا گەدەیی بەرپرسە ژ تووشبوونێ دپتریا هەودانێن بەکتریا دهێتە ڤەگوهاستن ب خوارنێ ل جیهانێ .

ڕێك :

کارێ ڤەکولینا نوکە هاتەکرن بو ڤەدیتن و نیاسینەکا بەشەکی یا سالمونیلا گەدەیی و ڤەدەرکری ژ نموونەیێن پیساتیا وان نەخۆشان ئەوێن ئامادەبووین ل نەخۆشخانا ئازادی و هیڤی کو تووشی زك چوونێ بووینە. ئەو ڤەدیتن و نیاسین هاتەکرن بکارئینانا ڕێکەکا ئاسایی و ڤەدیتنێ بکارئینانا تەکنیکا کارلێکا بەلمەڕێنێ .

ئەنجام :

دناڤبەرا 121 نەخۆشان کو تووشی زك چوونێ بووینە 72 حالەت (59,504 %) ژوان ئەنجامێن ئەڕێنی یا سالمونیلا گەدەیی دان ئەوژی ب ڕێکا چاندن ب چینێن چاندنێ. لێ ب ڕێکا تاقیکرنێن کارلێکا بەلمەڕێنا ڕێزبەند 68 حالەت (56,198 %) ژوان ئەنجامێن ئەرینی یا سالمونیلا گەدەی دان. ڤێ ڤەکولینێ 49 حالەت (40,495 %) ژبلی سالمونیلا شوینگر ئەنجامێن نەڕێنی دان ئەوژی ب ڕێکا چاندنێ و (53) حالەت (43,8 %) ژبلی سالمونیلا شوینگر. ئەنجامێن نەڕێنی دان ب ڕێکا تاقیکرنان ب تەکنیکا کارلێکا بەلمەڕێنا ڕێزبەند. PCR

دەرئەنجام :

ڤەکولینا مە دیارکر کو تەکنیکا RT – PCR پتر یا هویر و هەستیارە ب بەراوردی دگەل ڕێکێن ئاسایی.

Journal of University of Duhok, Vol. 22, No.1 (Pure and Eng. Sciences), Pp 148-154, 2019
https://doi.org/10.26682/sjuod.2019.22.1.16

الخلاصة

الخلفية والأهداف: السالمونيلا المعوية هي المسؤولة عن التسبب في أكبر عدد من الالتهابات البكتيرية المنقولة بالغذاء في العالم. الطريقة: تم إجراء العمل البحثي الحالي للكشف والتعرف الجزيئي للسالمونيلا المعوية المعزولة من عينات البراز من المرضى الذين حضروا مستشفيي آزادي وهيفي يعانون من الإسهال باستخدام طريقة التقليديه والكشف باستخدام تقنيات تفاعل البلمرة .

النتائج: من بين 121 مريضاً أصيبوا بالإسهال ، 72 حالة (59.504٪) أعطت نتائج إيجابية للسالمونيلا المعوية بطريقة الزرع بالاطباق الزرعية، بينما من خلال اختبارات تفاعل البلمرة المتسلسل أعطت 68 حالة (56.198 ٪) نتائج إيجابية للسالمونيلا المعوية. هذه الدراسة ، (49) حالة (40.495 ٪) من غير السالمونيلا النيابة أعطت نتائج سلبية عن طريق الزرع و (53) حالة (43.8 ٪) من غير السالمونيلا النيابة. أعطى نتائج سلبية عن طريق اختبارات بتقنية تفاعل البلمرة المتسلسل

PCR

الاستنتاجات: أظهر بحثنا أن تقنية

RT-PCR

.أكثر دقة وحساسية مقارنة بالطرق التقليدية