Evaluation of Molecular and Culture Method for Detection of *Campylobacter*, *Salmonella*, *E. coli* and *Shigella* in children infected with Diarrhea

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Abstract

Acute diarrhea is the second most common cause of children deaths worldwide. Bacterial pathogens have been confirmed as the major cause of acute diarrhea among children. In this study, 100 stool samples were investigated from pediatric children with acute diarrheal illness aged from 1 month to 5 years old. Bacterial investigated was performed for the common enteric pathogens: *Campylobacter*, *Salmonella*, *Shigella*, and Diarrheagenic *Escherichia coli* included Microscopy, culture and confirmatory identification by biochemical reactions included API 20E system. Finally, the real time PCR investigated for these enteric bacteria. The overall prevalence of the pathogens for *Campylobacter, Salmonella, Shigella* and *Diarrheagenic E. coli* were 9%, 8%, 3% and 14%, respectively. The results showed significant association of the clinical symptoms with *Shigella* and *Salmonella* infection. The RT-PCR markedly improves the detection rates of bacterial stool pathogens and offers culture methods.

Keywords*: Campylobacter, Salmonella, Shigella, RT-PCR*

Introduction

Diarrhea, as a global public health problem, causes a large number of infections and deaths every year. It is the second most common cause of childhood deaths worldwide and kills around 500,000 children less than 5 years every year. Diarrheal diseases are still a major medical conundrum in developing countries, causing considerable morbidity and mortality. It is the second leading cause of death in children under the age of five years in the world. On the basis of clinical criteria alone, it is not always possible to differentiate between viral, parasitic, and bacterial diarrhea. The identification of the etiological agents of acute bacterial diarrhea is critical for patient care and public health interventions [1-3].

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Campylobacter, Salmonella, Shigella, and *E. coli* are the common bacterial pathogens for diarrhea. *Campylobacter* is a foodborne zoonotic disease pathogen that is regarded as one of the most important causes of both developed and developing countries of the bacterial gastroenteritis. The laboratory diagnosis of bacterial infection is mainly based on traditional culture methods and confirmatory identification were done by the pattern of biochemical reactions using a standard bacterial identification system included API20E [4,5]. Simultaneously, the isolation for other four pathogenic organisms, *Campylobacter, Salmonella, Shigella, and E. coli* which including the enteropathogenic *Escherichia coli (EPEC), enteroinvasive E. coli (EIEC)* and enteroaggregative *E. coli (EAEC)* were also investigated for the samples in this study. Although *Escherichia coli* (*E. coli*) are one of the normal flora microorganisms in the human intestinal tract, it has five pathogenic bacteria types that can cause human diarrhea, known as diarrheagenic *E. coli*.

When people are infected, highly accurate and provide important diagnostic information for early identification of gastroenteritis, along with timely treatment [6,7]. Here, this is a modern way of identifying and analyzing a wide number of pathogenic strains using multiplexes real time PCR for enteric bacteria. This method has strong specificity and high sensitivity and detects multiple target sequences in one experiment, in compares with other methods, such as culture methods.

The aim of this study was to determine the frequency of *Salmonella* spp., *Campylobacter* spp., and *Shigella* spp., *E. coli* in pediatric patients with acute diarrheal illness using both conventional culture methods and Compared with multiplex real-time PCR.

Materials and Methods

Sample Collection

Pediatric patients who were admitted to the Children's Protection Teaching Hospital in Baghdad Medical City between 1 October 2020 and 31 December 2020. Conventional culture methods were used for *Salmonella, Shigella,* and DEC. One mL stool sample was inoculated into 5 mL selenite brilliant green broth and enriched at 36 ± 1°C for 18–24 hr [4]. After selective enrichment, a loop of the culture was streaked on xylose lysine desoxycholate (XLD) medium and MacConkey (MAC) plates and incubated at 36 ± 1°C for 18–24 hr. More than one presumptive *Salmonella* colony (usually 3–5 colonies) on the selective agar plate were inoculated on to triple sugar iron slant and incubated at 36 ± 1°C for 24 hr. Isolates with typical colony phenotypes were confirmed by systemic chemical tests and the pure culture for each suspected colony was identified using EPI 20E [7,8]. Isolation of *Campylobacter* spp. was carried out by filtration method (0.45 µm filter) according to [8]. Suspension for 1 mL stool was placed in a 4 mL buffer enrichment. In micro aerophilic atmosphere with 5 percent O2, 10 percent CO2, and 85 per cent N2, the enriched suspension was incubated for 24 hours at 42°C. Three hundred micronutrient cultivated enrichment suspension was then detected on the filter surface pasted onto two medium plates containing, respectively, the Karmali and Columbia agars. The plate was incubated at 42°C for 48 hour in a microaerophile environment. The suspected colonies were picked and identified by Gram stain and biochemical tests.

Molecular detection of *Campylobacter, Salmonella, E. coli*, and *Shigella* by real-time PCR

The *Campylobacter, Salmonella, E. coli* and *Shigella* were submitted for detection by real-time PCR using Acute Intestinal Infections (A.I.I) screen real-TM kit. The assay is based on two major processes: isolation of DNA for bacteria from specimens and Real-Time amplification of DNA. The experiment has an internal control (IC) it functions as an amplification control in place of every independently administered sampling and to identify potential procedure inhibition.

Statistical Analysis

A chi-squared test was used. A value of $p<0.05$ was considered to be significant.

Results and Discussions

Epidemiological Information and Pathogens Spectrum

A total of 100 diarrheal patients children less than five years (53 male and 47 female) were enrolled from the over the course from 1 October 2020 to 31 December 2020. Patients ranged in age from 1 month to 5 years old. 100 stool samples were collected from 100 diarrheal cases. There were 34 (34 %) cases that were positive for the tested enteric pathogens. The prevalence for *Campylobacter*, *Salmonella*, *Shigella*, and DEC were 9% (9/100), 8% (8/100), 3% (3/100), and 14% (14/100), respectively in chart (1). The age distribution show no significant difference according to the statistical analysis for the infection ratio (P = 0.74). Similar to another study, Bacterial etiology have been confirmed as the leading cause of acute diarrhea amongst patients [9,10].

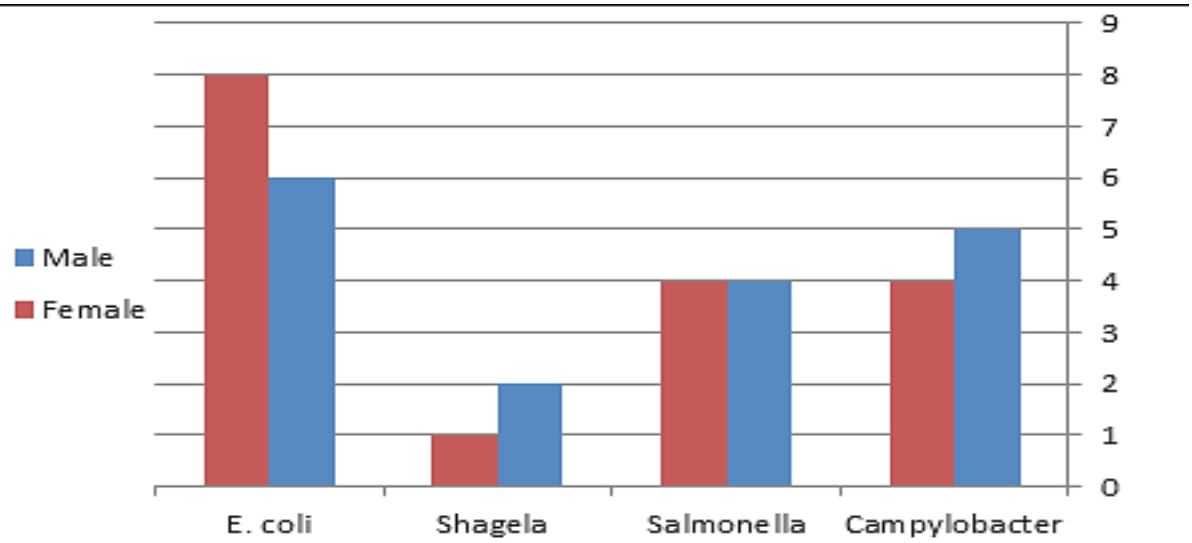

Chart (1): The distribution of enteric bacterial infection between the gender groups.

Detection of ***Campylobacter*** by real-time PCR

Campylobacter was detected on the FAM (Red) channel with the tube that contained PCR-mix-1campylobacter (Figure 1). The sample is considered positive for *Campylobacter* where the value of Ct was lower than the boundary value. The sample is considered negative for *Campylobacter* when the result is positive only on the channel FAM with PCR-mix-1 IC (Green curve) and the Ct value is lower than the boundary value.

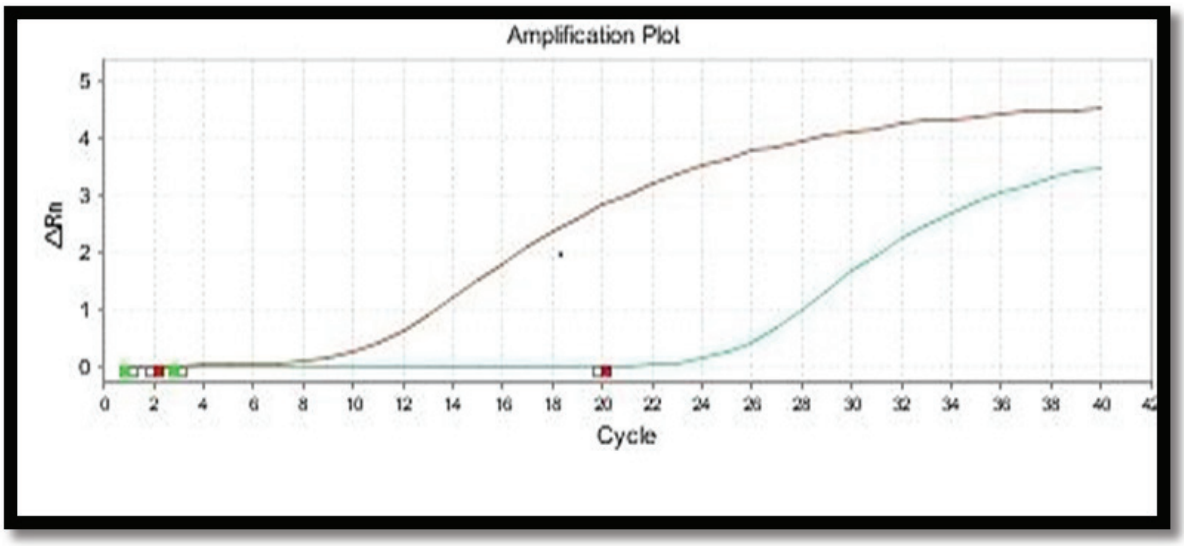

Figure 1. The RT-PCR multiplex amplification of *Campylobacter* red curve indicates the expression of *Campylobacter* (8.4 Ct), the green curve indicates the expression of IC (22.7 Ct).

The result of RT-PCR detection of *Campylobacter* showed that the *Campylobacter* was detected in 9(9%) out of 100(100%) stool specimens that collected from children with acute diarrhea. These results were similar to the results of the study carried out in Belem, Brazil and match with findings referee that when in prior studies state the prevalence of *Campylobacter* has been variable, ranging from 0.7% to > 30. Also, study in Iran, which showed a close percentage of *Campylobacter* 7.8% among children in stool samples [11, 12].

Detection of ***Salmonella*** by real-time PCR

Salmonella was detected on the JOE (blue curve) channel with the tube that contained PCR-mix-1 *Shigella* spp. / *Salmonella* spp (Figure 2). The sample is considered positive for *Salmonella* where the value of Ct was lower than the boundary value. The sample is considered negative for *Salmonella* when the result is positive only on the channel FAM with PCR-mix-1 IC (Green curve) and the Ct value is lower than the boundary value.

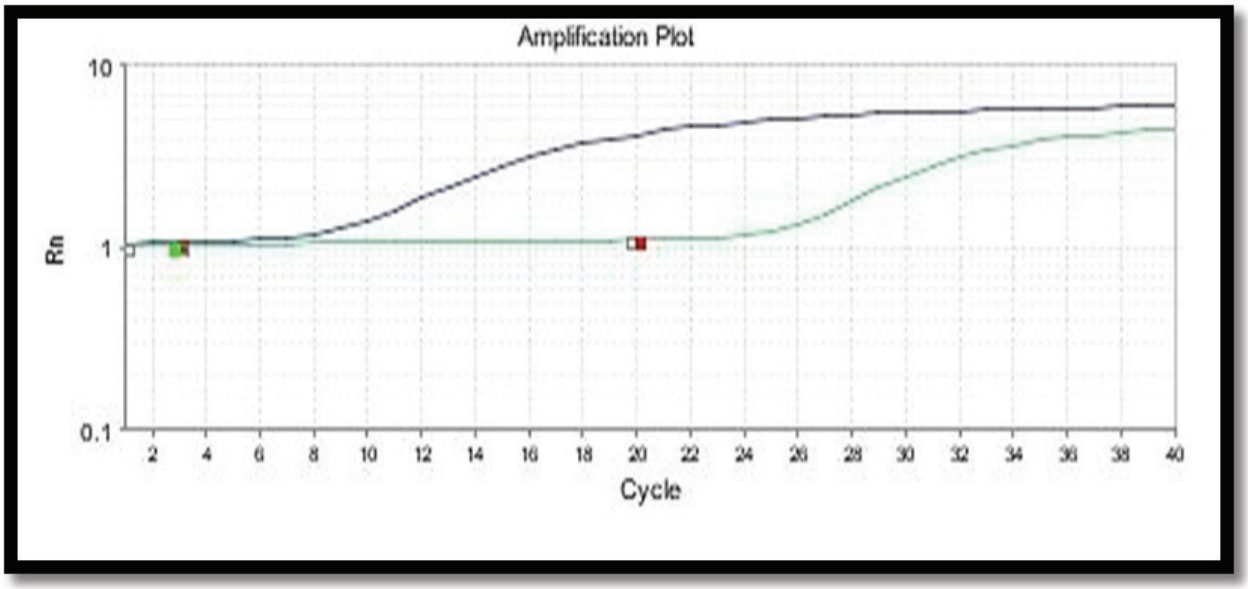

Figure 2. The RT-PCR multiplex amplification of *Salmonella*, blue curve indicates the expression of *Salmonella* (8.2 Ct), the green curve indicates the expression of IC (24.3 Ct).

The result of RT-PCR detection of salmonella showed that the salmonella was detected in 8 (8%) out of 100(100%) stool specimens that collected from patient with acute diarrhea. this result matched to findings of Kylla *et al*. [13] that show A total of 8.31% *Salmonella* were identified with higher prevalence. Also, another study showed that the prevalence of *Salmonella* isolates was 6.9% Children aged between 1 to 3 years were significantly associated with *Salmonella* infection, Studies conducted in Ethiopia also revealed an increasing trend in the prevalence of *Salmonella* isolates and *Shigella* spp [14].

Detection of ***Shigella and E. coli*** by real-time PCR

Shigella and E. coli were detected on the same channel, FAM (Red) channel with the tube that contained PCR-mix-1 *Shigella* spp. / *Salmonella* spp (Figure 3). The sample is considered positive for *Shigella and E. coli* where the value of Ct was lower than the boundary value. The sample is considered negative for *Shigella and E. coli* when the result is positive only on the channel FAM with PCR-mix-1 IC (Green curve) and the Ct value is lower than the boundary value.

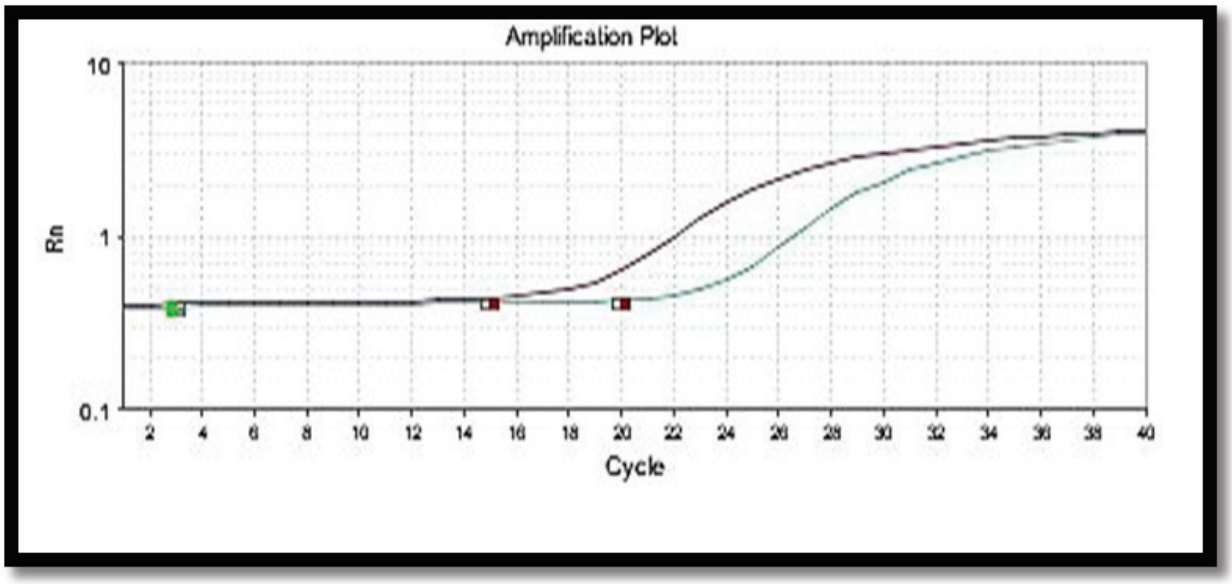

Figure 3. The RT-PCR multiplex amplification of *Shigella and E. coli*, blue curve indicates the expression of *Shigella and E. coli*, (16.8 Ct), the green curve indicates the expression of IC (21.9 Ct).

The result of RT-PCR detection of *Shigella and E. coli* showed that the *Shigella and E. coli* was detected in 3 (3%) and 14(14%), respectively, out of 100(100%) stool specimens that collected from children with acute diarrhea. Although the same target channel is used for the detection of *Shigella* and *E. coli* in RT-PCR assay, they are distinguished by culture and confirmed by EPI 20E test for all positive RT-PCR stool samples. This results similar to result state that the prevalence of *Shigella* and *E. coli* isolates was 4.3%, 24%, respectively [15,16].

Performance of RT-PCR vs conventional culture methods

The Sensitivity of real time PCR was 100% for all four enteric bacterial detection in stool samples, while the Sensitivity of culture methods of *campylobacter, salmonella, E. coli and shigella* detection in stool samples was 81%, 77%, 100%, and 75%, respectively. The result of *P*. value was statistically significant (0.03). The specificity of real time PCR was 100% for all four enteric bacterial detection in stool samples by both methods. The differences of Sensitivity between the four types due to growth requirements of culture of each bacteria type.

The result matched with study that states the positive laboratory result for enteric bacteria in 109 (14.7%) patients. PCR was positive in all 109 (100%) patients, but only 32 (29.4%) of them were culture positive. *Salmonella* spp. was the most common bacteria detected by culture while *Campylobacter* spp. was mostly detected by PCR. *Campylobacter* spp., which are fastidious organisms, are probably the most difficult for clinical laboratories to detect because of specimen transport and specific culture requirements. Also, study demonstrated that culture-based methods miss a substantial proportion of *Campylobacter* infections. One of the benefits of nucleic acid amplification tests (NAATs) is the ability to detect low levels of fastidious organisms despite poor growth [17].

Studies shown that the multiplex RT-PCR is a sensitive and specific assay for the diagnosis of enteric pathogens. RT-PCR testing detects bacterial DNA, not viable organisms, and positive RT-PCR results must therefore be interpreted in conjunction with clinical presentation. When RT-PCR is used for diagnosis, cultures are required for antimicrobial susceptibility testing for positive RT-PCR cases [18]. Diagnostic testing for enteric pathogens has rapidly evolved in the past

decade. Although culture independent diagnostic tests are still most commonly being used for *Campylobacter* and STEC, the Foodborne Diseases Active Surveillance Network found the highest percentage increase in use for *Shigella* and *Salmonella* compared with the previous 3-year average. They concluded that the most likely reason for this change was the implementation of newly available DNA-based syndrome panels in participating laboratories [19,20].

Conclusions

In conclusion, Reel time-PCR markedly improves the detection rates of bacterial stool pathogens and offers rapid identification. Therefore, we recommend using Real time-PCR with culture to achieve optimal results. In our study, *Salmonella* and *E. coli* were most commonly detected by culture while *Campylobacter* and *Shigella* were most commonly identified by real time-PCR.

Conflict of Interest: None

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Ethical Clearance: Not required

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