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RESEARCH ARTICLE
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ERIC PCR AND BOX PCR MARKERS FOR GENETIC TYPING OF ENTERITIS SALMONELLA SPP. ISOLATED FROM CHILDREN WITH DIARRHEA.

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ABSTRACT

Background: Acute diarrheal diseases are a major cause of mortality and morbidity, with *Salmonella* being one of the leading causes of diarrhea. In this study, the objective was to determine the genetic distance between *Salmonella spp*. using *ERIC PCR and BOX PCR*.

Methods: The study involved collecting 250 samples from children suffering from diarrhea, and *Salmonella* strains were identified using the *VITEK2* device. The genetic distance was measured using *ERIC PCR and BOX PCR*.

Results: Out of the 250 diarrhea samples, 9 strains of *Salmonella bacteria* were identified using the VITEK2 device. The results of the genetic tree analysis showed that the study samples were divided into two subgroups based on the ERIC PCR, with a complete match between isolates No. 1 and 6. On the other hand, the *BOX PCR* showed three sub-groups with a correspondence between isolates 3 and 4.**In conclusion**, the study demonstrated the effectiveness of *ERIC PCR and BOX PCR* in determining the genetic relationship between *Salmonella* isolates.

Keywords: *Enteritis Salmonella spp*, *diarrhea, ERIC PCR and BOX PCR*

INTRODUCTION

Acute diarrheal illnesses cause significant mortality and morbidity. According to *the World Health Organization (WHO),* more than 2 billion people worldwide suffer from diarrheal diseases annually. One-third of the cases are caused by food (1). Diarrhea remains the main cause of morbidity and mortality in children worldwide, responsible for around 149 million deaths among children under five in 2019(2,3). Increased stool frequency and watery consistency, frequently coupled with vomiting, are the hallmarks of pediatric diarrhea. Weak immunity, dirty diets, and infections are all part of its complicated etiology, affecting children's growth, development, and general health(4,5). In addition to the causes that lead to diarrhea and are related to immunity and food quality, there are other main causes related to the imbalance of intestinal bacteria, bacterial infection, and viral infection. These harmful elements greatly affect children's growth, development, and physical and mental health(6,7) . *Salmonella* is a genus of gram-negative, oxidase-negative, catalase-positive, and non-spore-forming rods in the family Enterobacteriaceae. They are facultative anaerobes, and almost all species of *Salmonella* are motile via peritrichous flagella, except for *Salmonella enterica* ser. Gallinarium (8,9). The bacterium was first discovered by D. E. Salmon, an American bacteriologist, in 1884, who isolated it from the porcine intestine (10).

Up to 80% of salmonellosis cases are not recognized as part of a known outbreak and are considered sporadic cases. Moreover, some cases are not diagnosed at all (11). *Salmonella* is currently classified into two species, *Salmonella bongori* which is rarely associated with human infection, and *Salmonella enterica*. S. enterica is a diverse species that infects and colonizes many animals, including humans (12).The first classification was based on biochemical characteristics(13). There are more than 2500 species in the *Salmonella genus*, based on the one serotype-one species principle proposed by Kauffman-White according to O and H antigens (14). However, this classification is outdated since 2007, and there is no agreement on a replacement. Genomic approaches are promising, but standardization is needed. An issue is identifying isolates with unclear links between serovar and *DNA* (12).Molecular typing of *Salmonella spp*. is performed to examine genetic relatedness, discriminate closely related *Salmonella* isolates and reveal source-to-person strain transmission with sufficient precision to identify the specific source responsible for foodborne outbreaks (15). Enterobacterial Repetitive Intergenic Consensus *(ERIC)* sequences, which are repetitive imperfect palindromes, 127 bp in size, and occur in multiple copies on bacterial genomes, are used for genotyping. *ERIC-PCR* analysis is a PCR-only genotyping system that differentiates bacterial strains based on variations in the location of the ERIC sequences present in the bacteria genome (16). *BOX* sequences is another set of repetitive elements used for typing purposes is the repetitive extragenic palindromic (*REP*) sequence. This tool amplifies the repeated sequences of the bacterial genome using the *BOX* primer for the repetitive element sequence-based *PCR*. In earlier research, this primer was discovered in numerous bacterial genomes. The amplified repetitive areas' band profiles differ from one species to another or even from one species to another. As a result, band patterns can be used to identify various species (7,17). The aim of our study is to identify the genetic relation among the same *Salmonella ssp*. from diarrhea children's origins using *ERIC PCR and BOX PCR* markers.

METHODS

Sample Collection

250 stool samples were collected from visitors to Salah al-Din General Hospital in Tikrit for children who suffered from diarrhea, during the period from December 2022 to May 2023, by taking about 1-2 grams of stool, then putting them into clean, sterilized containers with a tight lid. Completely write the patients' names, age, and history in the boxes. Completed the transplantation immediately after collection.

Identification

Isolates were diagnosed at the genus level and then subspecies level through a rapid phenotypic study for its colonies on a combination of media SS agar, HE agar, MacConkey agar, and Nutrient agar This was completed, and a set of biochemical tests necessary for diagnosis were performed. The diagnosis was also confirmed by Using Vitek 2 system.

DNA Extraction And PCR Amplification

DNA was extracted from nine clinical samples that tested positive for Enteritis *Salmonella spp*. The extracted *DNA* was estimated using a 1% agarose gel, and the amount and purity were determined using a Nanodrop 2000 spectrophotometer from Thermo Scientific in Waltham, MA, USA. Specific primers for *ERIC* and *Box-PCR* assays (Table 1) were used for *PCR amplification*. The *ERIC-PCR* reaction involved an initial denaturation at 95°C for 2 minutes, followed by 35 cycles of denaturation at 92°C for 30 seconds, annealing at 50°C for 1 minute, and extension at 65°C for 8 minutes. The final extension step was performed at 65°C for 8 minutes, and the samples were stored at 4°C. The Box PCR protocol was different, starting with denaturation for 2 minutes at 95°C, then 30 cycles of denaturation for 3 seconds at 94°C, and 30 seconds at 92°C. Annealing was performed for 1 minute at 50°C, elongation for 8 minutes at 65°C, and final elongation for 8 minutes at 65°C. The fragments obtained were examined through electrophoresis on a 1.5% agarose gel.

Table 1: Sequencing for each primer used in the current study.

Locus	Sequencing
ERIC	5'-ATG TAA GCT CCT GGG GAT TCA C-3
	5'-AAG TAA GTG ACT GGG GTG AGC G-3'
Box	5′-CTACGGCAAGGCGACGCTGACG-3′

Phylogenetic Analysis

Differences in the sizes of the amplified fragments indicate the variability of genomic *DNA* sequences, and computer-assisted techniques can be used to analyze the various *DNA* fragment profiles to cluster distinct patterns and create phylogenetic trees (18). Estimates of Evolutionary Divergence were conducted in *MEGA X* (28), while the Heat map and phylogenetic tree variants among 9 *Salmonella spp* were done by heatmapper.ca (19).

Both the *BOX-PCR , ERIC-PCR* discriminating indices (D) were computed using Simpson's Diversity Index. Several 0 (zero) denotes an identical pattern between isolates, while a value of 1 denotes a total dissimilarity. The typing tool's discriminatory power increases with a greater Simpson's Diversity Index (20).

RESULTS AND DISCUSSION

The results of the current study showed that the number of male children with diarrhea was 165 (66%), while the number of female children was 85 (34%). This study is consistent with other studies that showed a higher infection rate in males (21,22, 23). After recording the results of the culture media, the results of the *VITEK2* device were relied upon, as it showed that the percentage of *Salmonella* isolates was 9 diagnosed isolates, which recorded a high probability ranging between 96 to 98%. Table 2. Representative distribution of patients based on sex and age.

Patient sex	General patients	Percentage	No. of Salmonella spp.	Percentage
Male	165	66 %	6	66.67 %
Female	58	34 %	3	33.33%
Total	250	100 %	9	100%

Table 2: the distribution of patients categorized by their sex and age.

The results of genotyping when using the *ERIC-PCR* markers showed several general and different bands, as in Figure 1, the difference in the number and locations of the bands is due to the difference in the targeted regions in the bacterial genome. In general, the total locus was 72, of which 45 loci showed amplification bands. The separation was coded in binary, with a standard score of 1 for group presence and 0 for group absence. Each band position functioned as a locus.

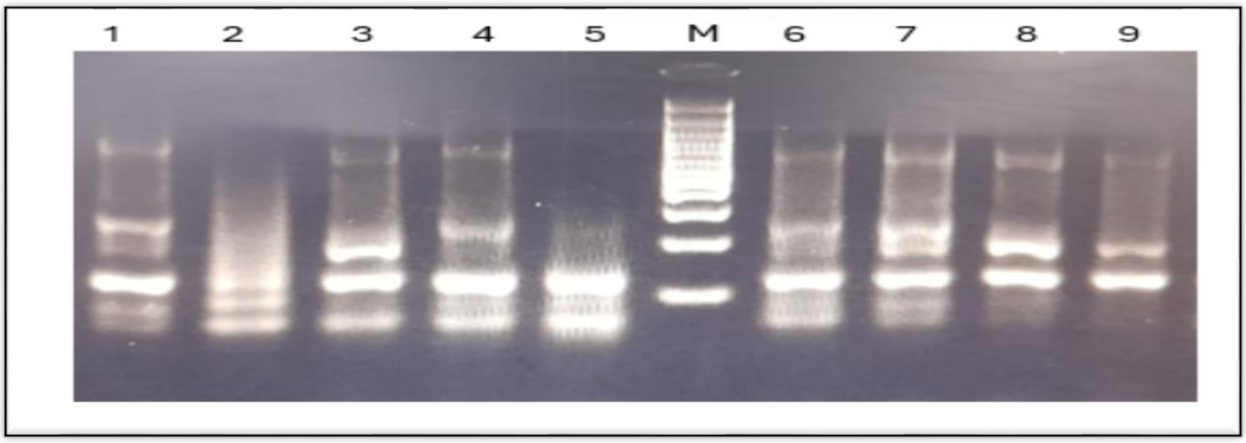

Figure 1: PCR product of *ERIC PCR* markers electrophoresis on 2% agarose, 1 to 9 represent isolates and M= DNA marker.

The following findings are shown in Figure 2, which represents the phylogenetic tree based on the *ERIC PCR* marker results. The study samples were divided into several subgroups based on their distance and genetic proximity. For instance, we observed that isolates No. 1 and No. 6 matched, while sample No. 3 was genetically distinct from the rest of the isolates. In general, the study isolates can be categorized into two groups: the first group includes isolates 1, 2, 4, 5, 6, and 7, and the second group includes isolates 3, 8, and 9.

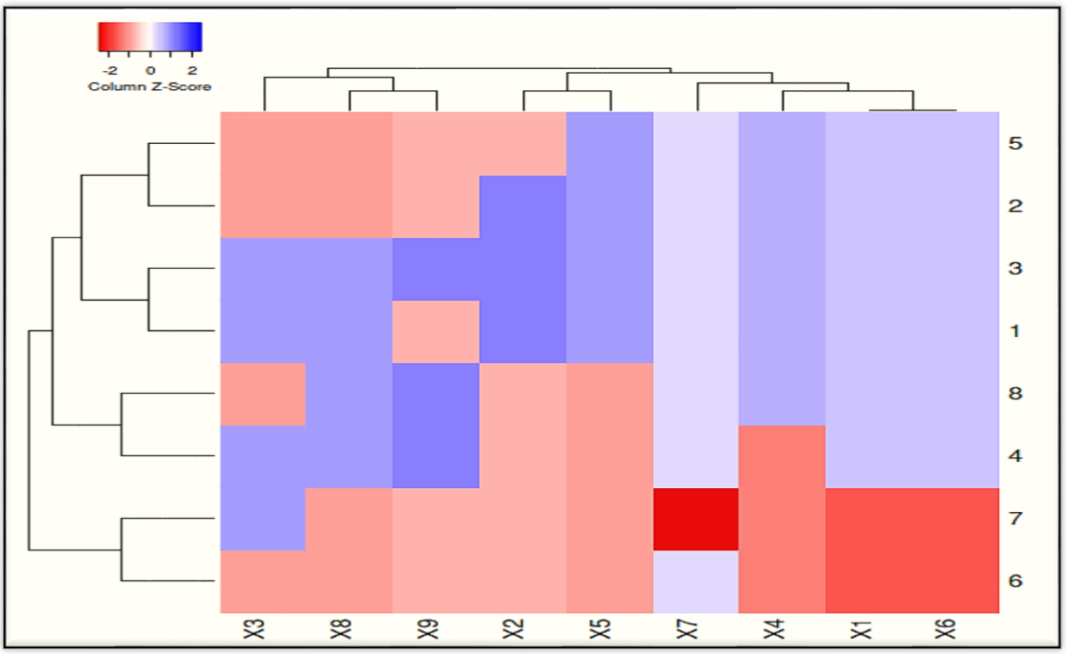

Figure 2: phylogenetic heat map based on product of *ERIC PCR* markers among Salmonella spp.

The *BOX-PCR* markers used for genotyping produced diverse and varied bands, as shown in Figure 3. The difference in band number and position is due to the variation in targeted regions in the bacterial genome. In total, there were 99 loci, out of which 48 showed amplification bands. The separation was coded using binary, with a standard score of 1 for group presence and 0 for group absence. Each band position functioned as a locus.

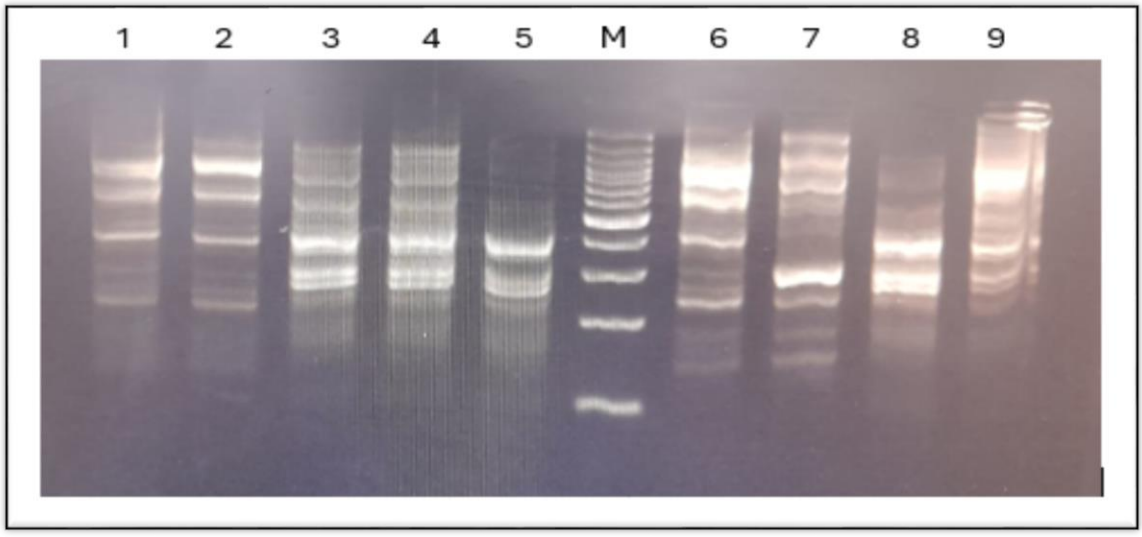

Figure 3: *PCR* product of Box PCR markers electrophoresis on 2% agarose, 1 to 9 represent isolates and M= *DNA* marker.

In Figure 4, we present the findings of our study, which is based on the results of the *BOX- PCR* marker. We have divided the samples into several subgroups based on their distance and genetic proximity. For instance, we noted that isolates No. 3 and No. 4 were identical. In general, we have categorized the study isolates into three groups: the first group consists of isolates 1 and 2, the second group consists of isolates 3, 4, 5, 8, and 9, and the third group consists of 6 and 7 isolates.

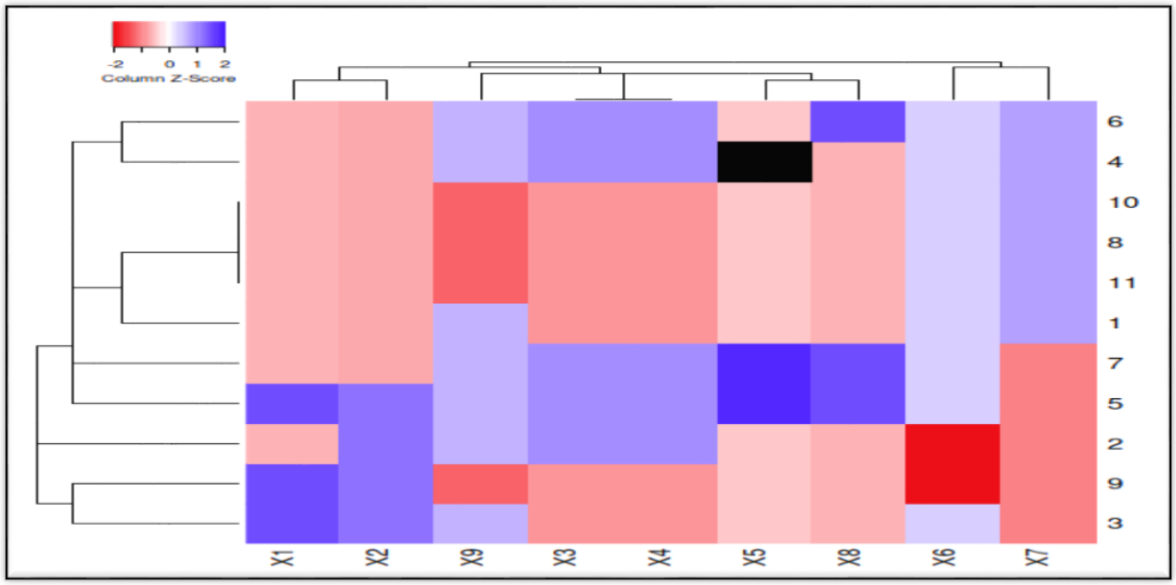

Figure 4: phylogenetic heat map based on product of *BOX PCR* markers among Salmonella spp.

Although *ERIC PCR* and *BOX PCR* techniques have been used for genotyping to differentiate strains of a specific species, their application for identification has been limited but suggested.
The *ERIC PCR* method has proven to be highly effective and user-friendly in epidemiological research. It is particularly useful for studying genetically diverse pathogenic members of the Enterobacteriaceae family, such as Salmonella. Through the use of a single primer pair complementary to short repeat sequences, a genomic *DNA* fragment is amplified in this manner, producing an intricate and accurate fingerprint (24). This study clearly shows that ERIC-PCR is also able to discriminate between different isolates. Using *ERIC-PCR*, only 8 of the 9 isolates (88.88%) could be typed, resulting in the generation of 8 different *ERIC-PCR* genotypes. The results of the current study are consistent with the Ahem study in 2022, which found the ERIC-PCR could be typing 86.7% of bacterial isolates (25).
This study provides evidence that *BOX-PCR* can differentiate between different isolates. While ERIC-PCR could only type 8 of the 9 isolates (88.88%), resulting in 8 different *ERIC-PCR* genotypes, *BOX-PCR* provided more accurate results. A study conducted by Lozanoal. et al in 2019 also showed that *BOX-PCR* is effective in differentiating the genetic relationship between *Salmonella* isolates (26). Molecular approaches for genetic typing of *Salmonella* Enteritidis include *ERIC-PCR , BOX-PCR; BOX-PCR* has greater discriminatory power than *ERIC-PCR* (92.91%). By successfully differentiating strains, these methods support epidemiological studies and the comprehension of genetic variation (27).

CONCLUSION

The recent study has demonstrated that the *ERIC PCR and BOX PCR* markers play a significant and influential role in identifying the genetic diversity among *Salmonella* isolates. The results of both markers are highly effective in determining the genetic dimension. However, one of the drawbacks of the study is that it did not employ the DNA sequencing method to determine genetic variation and compare it with the study's findings. In the future, the two markers could be utilized with bacterial strains that demonstrate unique characteristics related to antibiotic resistance.

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