Detection of Enterotoxigenic *Staphylococcus aureus* in Patients with Gastroenteritis in Erbil/Iraq

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Abstract

Gastroenteritis is one of the words serious public health problem, and enterotoxigenic *Staphylococcus aureus* is one of the causes of gastroenteritis. The aim of this study was the molecular detection of enterotoxigenic genes in *Staphylococcus aureus*, then detection the association of resistance patterns with the toxigenicity of strains. Microbiological analysis of fecal samples from patients with gastroenteritis was performed for detection of *S. aureus*. The samples were cultured and identified by routine bacteriological methods and VITEK 2 system. Subsequently PCR amplification of enterotoxin genes (*sea and seb*) was carried out on all *S. aureus* strains. The results indicated that in total of 417 fecal samples taken from patients with acute gastroenteritis, 30 grew *S. aureus* (7.19%), and of 417 fecal samples 96 yielded bacterial pathogens (23.02%), *S. aureus* was the most common bacterial pathogen found in 30/96 cases (31.25%). In 30 isolated of *S. aureus*, penicillin sensitive *S. aureus* were 2 (6.67%), methicillin sensitive *S. aureus* were 19 (63.33%), multidrug resistant *S. aureus* were 12 (40%), methicillin resistant *S. aureus* were 11 (36.67%), Extensive drug resistance were 3 (10%), and none of them was pan drug resistant,and all isolates were sensitive to vancomycin. The PCR results showed that 7/30 (23.33%) of the *S. aureus* isolates possessed the *sea* gene, and 3/30 (10 %) had the *seb* gene. In total of 30 isolates Staphylococcal enterotoxin genes *sea* and *seb* were more prevalent among methicillin resistant *S. aureus* were 6/11 (54.54%), 3/11 (27.27%) respectively, then in multidrug resistant *S. aureus* which were 6/12 (50%), and 3/12 (25%) respectively, and in Extensively drug resistance *S. aureus sea* was1/3 (33.33%) and there is no *seb genes*, while in methicillin sensitive *S. aureus sea* was 1/19 (5.26%), and there is no *seb genes*, and there was no Staphylococcal enterotoxin genes *sea* and *seb* in penicillin sensitive *S. aureus*. The result confirmed the involvement of enterotoxigenic *S. aureus* in the occurrence of gastroenteritis, and isolates with enterotoxin genes have a higher drug resistance rate.

Keywords: *Gastroenteritis, Staphylococcus aureus, sea and seb Enterotoxin genes.*

Introduction

Gastroenteritis is a common clinical syndrome. It poses one of the words major clinical and public health problems. The principal clinical manifestation of gastroenteritis is diarrhea. The Word Health Organization Syndromic definition of gastroenteritis is "three or more abnormally loose or fluid stools over 24 hours" [1]. Diarrhea is the second disease that causes death in children in the world. Every year, around there are 1.7 million cases of diarrhea and cause around 525,000 deaths in children under the age of five in the world [2,3]. Overall, 1.7 billion global cases of diarrheal disease are reported annually and are associated with an estimated 2.2 million deaths. The burden of diarrheal disease is most critical in developing countries, facilitated by unsafe water supplies, poor sanitation, and nutritional deficiencies. While less common in high-income countries, diarrheal diseases remain a significant health concern. There are an estimated 211 to 375 million episodes of diarrheal illnesses each year in the United States, with 1.8 million

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hospitalizations and 3,100 deaths [4,5].

A wide variety of viruses, bacteria, and protozoa may cause gastroenteritis [1]. Acute bacterial gastroenteritis (ABG) can cause more severe symptoms than acute viral gastroenteritis in children[6], and the list of bacteria that cause gastroenteritis is continuously growing, include *Campylobacter jejuni, Staphylococcus aureus, Bacillus cereus, Escherichia coli, Vibrio cholera, Shigella dysenteries, Salmonella enteriditis, Yersinia enterocolitica, Clostridium perfringens, and Clostridium difficile.* Micro-organisms cause gastroenteritis by a number of mechanisms. They may release performed toxins prior to ingestion, multiply and produce toxins within the gastrointestinal lumen, directly invade the bowel wall, or use a combination of toxins and invasion[1].

On 21 September 2006, the Austrian media reported the occurrence of a cluster of gastroenteritis cases in a boarding school in Eisenstadt, capital of the province Burgenland in eastern Austria. *S. aureus* was identified as the causative agent. From 45 stool samples, none of them contained *Salmonella*, *Campylobacter*, enterohaemorrhagic *Escherichia coli* and *Yersinia*. Of those 45 stool samples, 44 were positive for *S. aureus*[7]. *S. aureus* may cause diarrhea, gastrointestinal carriage of MRSA has been associated with antibiotic associated diarrhea [8.9].

The existence of several virulence factors such as staphylococcal exotoxins have an important role in *S. aureus* pathogenicity. One of these factors is enterotoxins which proved to be more tolerance to high temperature[10]. Staphylococcal enterotoxins are low molecular weight proteins (MW 26.900-29.600D)[11]. All enterotoxins are superantigens which are encoded by mobile genetic elements including phages, plasmids and pathogenicity islands[12,13]. Also *S. aureus* is one of the most important causes of food poisoning due to its ability to produce more than 22 different enterotoxins[14,15].

The *S. aureus* enterotoxins (SEs) can be separated into two groups; the 5 serological classical types (SEA-SEE)), and the newer (SEG-SE*l*Y and counting) enterotoxin groups. Many members from both these groups contribute to the pathogenesis of several serious human diseases. Only enterotoxins with demonstrated emetic potential in monkeys were designated “SE,” whereas enterotoxins that failed to do so or have not been evaluated in non-human primate models of emesis are designated enterotoxin like (SE*l*-) toxins [14,16]. Among these Staphylococcus enterotoxins, five of them (A, B, C, D and E) are known to be responsible for 95% of Staphylococcal food poisoning cases [17,15]. S. aureus strains can produce one or several of the five major SEs (SEA, SEB, SEC, SED, and SEE)[18]. Staphylococcal enterotoxin SEA and SEB are two of the most important gastroenteritis causing agents. SEA and SEB are the most food poisoning agents (> 60%) in USA and England. For the above-mentioned reason, these toxins can cause epidemic gastroenteritis[19,11]. While the majority of MRSA isolated from food products in Denmark was negative for major virulence factors such as enterotoxins[20].

Staphylococcal enterotoxins (SE) A and B are two of the most important gastroenteritis causing agents in USA and England. There are several methods for detection of enterotoxigenic bacteria. The phenotypical methods are not reliable in specificity, because staphylococcal enterotoxins serotypes are antigenically similar. Therefore, molecular techniques such as PCR are recommended for detection of *S. aureus* enterotoxins genes[13]. Despite the potential role of *S. aureus* enterotoxins in causing diarrhea a limited number of studies have focused on multidrug resistant *S. aureus* colonized diarrhea. Herein we explored the prevalence of *S. aureus* in the fecal of patients with gastroenteritis.

Materials and Methods

The study was carried out for six months from June to November 2020. Fecal samples were taken from patients admitted to Erbil Hospitals suffering from acute diarrhea and gastroenteritis infection.

Bacterial Identification:

Fecal samples under aseptic conditions were inoculated into a tube containing 10ml Tryptic soy broth.

The broth was incubated at 37℃ for 24h then streaked from the enriched broth onto a selective mannitol salt agar medium, and incubated at 37℃ for 24h. Suspected *S. aureus* colonies from each sample were evaluated based on morphology. All isolates were further identified for their species assignment by the automated VITEK2 compact system (bioMérieux, France). After the screening, the presumptive *S. aureus* isolates were stored at -20℃ in Tryptic soy broth (TSB) plus 20% v/v glycerol [21,22].

Criteria for finding multidrug resistance, methicillin - resistant *S. aureus,* extensively drug resistance, and pan drug resistance in *S. aureus*

Colonies of *S. aureus* were inoculated in 5ml of 0.85% saline, and the turbidity was adjusted to match 0.5McFarland standard. Then sterile cotton swabs were dipped into the inoculums and spread evenly on to Mueller Hinton agar, MHA[23]. The following antimicrobial discs were applied aseptically to the MHA plates and incubated overnight at 37°c: penicillin(P,10U), amoxicillin (AX,25µg), gentamycin (CN, 10µg) ,vancomycin (VA,30µg), erythromycin (E, 15µg), tetracycline (TE,30µg), methicillin (MET,10µg), cefoxitin (30µg), ciprofloxacin (CIP,5µg), amoxicillin-clavulanic acid (AMC,20µg/10µg), oxacillin (OXA, 1µg), fusidic acid (FA, 10µg), rifampicin (RA, 5µg), clindamycin, (DA, 2µg), and trimethoprim-sulfamethoxazole (SXT,1.25µg/23.75µg).

We categorized a specimen as penicillin-sensitive *S. aureus* (PSSA) if it was susceptible to penicillin and methicillin, methicillin - sensitive *S. aureus* (MSSA) if it was resistant to penicillin and susceptible to methicillin, and methicillin - resistant *S. aureus* (MRSA) if it was resistant to both penicillin and methicillin. Resistance to methicillin was inferred by testing for oxacillin and cefoxitin resistance[24,25]. Multidrug resistance (MDR) one or more of these have to apply: (i) an MRSA is always considered MDR by virtue of being an MRSA (ii) non susceptible to at least one agent in three or more antimicrobial categories. Extensively drug resistance (XDR): non-susceptible to at least one agent in all but two or fewer antimicrobial categories. Thus, bacteria isolate that is characterized as XDR will also be characterized as MDR. Pan drug resistance (PDR) non-susceptible to all antimicrobial agents in all antimicrobial categories[26,27].

DNA extraction

Bacterial DNA was extracted from *S. aureus* using a DNA extraction kit (geneaid/Korea), according to the manufacture's instruction. Nanodrop was used for measuring the concentration and the purity of nucleic acid. The DNA extracted samples were stored at -20□ for further use as template for PCR.

Detection of Staphylococcal enterotoxin

Amplification of Staphylococcal enterotoxin genes was done by specific primers and polymerase chain reaction method. Two PCR primers sets were used to detect the staphylococcal enterotoxin *sea* and *seb* encode SEA and SEB respectively[28], as described in Table 1.

Table1: Primers for amplification of enterotoxin-encoding genes in *Staphylococcus aureus*

Gene	Primer	Oligonucleotide sequence (5'-3')	Amplicon size
sea	SEA1 SEA2	5' TTG CGA AAA AAG TCT GAA TTG C 3' 5' ATT AAC CGA AGG TTC TGT AGA AGT A 3'	552 bp
seb	SEB1 SEB2	5' TCG CAT CAA ACT GAC AAA CG 3' 5'AGG TAC TCT ATA AGT GCC TGC CT 3'	477 bp

The total of 25µl PCR master mix reaction volume was performed containing 1µl of genomic DNA, 12.5µl of 2x Go Tag green master mix (Promega, USA), and 1µl was added from each of the forward and reverse primer and completed with nuclease free water to 25 µl.

The PCR condition were as follows: a total of 32 cycles was performed with the first denaturation at 94°C for 5min, then consisted of 30 cycles, for denaturation at 94°C for one-minute, specific annealing temperature 55°C for one minute, extension at 72°C for one-minute, and the final extension at 72°C for 5min[29.28]. The PCR products were separated in a 1% agarose gel and stained with Safe dye (GenetBio, Korea). The gel was visualized under UV trans-illuminator (Synegene, UK).

Results

In total of 417 fecal samples taken from patients with acute gastroenteritis, 30 grew *S. aureus* (7.19%), and of 417 fecal samples 96 yielded bacterial pathogens (23.02%), *S. aureus* was the most common bacterial pathogen found in 30/96 (31.25%) cases.

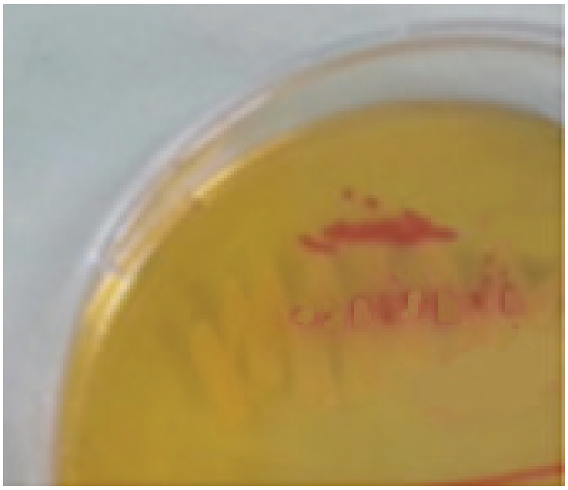

Figure (1): ***Staphylococcus aureus*** **on selective mannitol salt agar medium**

These isolates showed different susceptibility towards antibiotics. All isolates of *S. aureus* were sensitive to vancomycin. However, the effect of other antibiotics was variable among the isolates, the heigh effective antibiotics were clindamycin, fusidic acid, ciprofloxacin, gentamycin, and trimethoprim-sulfamethoxazole. Adversely, the less effective antibiotics were penicillin, and amoxicillin, as depicted in Figure 2.

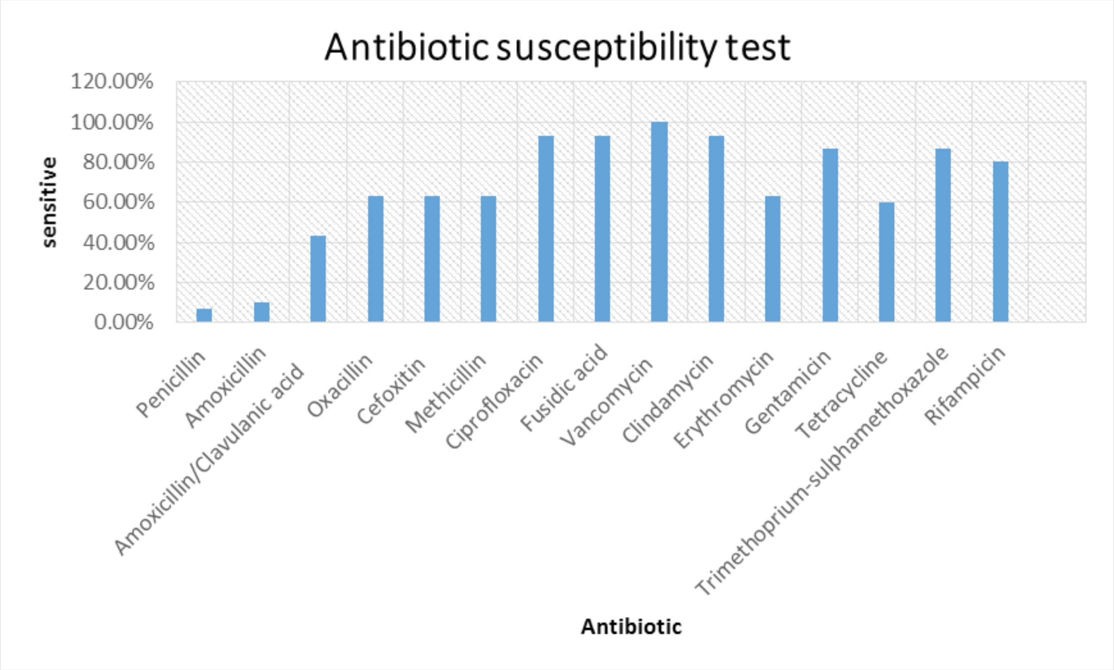

Figure (2): Antibiotic susceptibility test

Of 30 isolated *S. aureus*, penicillin sensitive *S. aureus* were 2 (6.67%)*,* methicillin sensitive *S. aureus* were 19 (63.33%)*,* multidrug resistant *S. aureus* were 12 (40%)*,* methicillin resistant *S. aureus* were 11 (36.67%)*,* extensive drug resistance (XDR) *S. aureus* were 3 (10%), Bacteria isolates that is characterized as MRSA and XDR will also be characterized as MDR*,* and none of them was pan drug resistant *S. aureus* because all isolates of *S. aureus* were sensitive to vancomycin, as depicted in Table 2.

Table 2: Percentage of penicillin-sensitive *S. aureus* (PSSA), methicillin- sensitive *S. aureus* (MSSA), multidrug-resistance (MDR), methicillin resistance S. aureus (MRSA), extensive drug-resistance (XDR), and Pan drug-resistance *S. aureu* (PDR)*s.* Bacteria isolates that is characterized as MRSA and XDR will also be characterized as MDR.

	Type of antimicrobial resistance	**S. aureus (30)**
1-	Penicillin- sensitive S. aureus (PSSA)	2 (6.67%)
2-	Methicillin- sensitive S. aureus (MSSA)	19 (63.33%)
3-	Multidrug-resistance (MDR)	12 (40%)
4-	Methicillin resistance S. aureus (MRSA)	11 (36.67%)
5-	Extensive drug-resistance (XDR)	3 (10%)
6-	Pan drug-resistance (PDR)	0%

The PCR results showed that 7/30 (23.33%) of the *S. aureus* isolates possessed the *sea* gene, and 3/30 (10 %) had the *seb* gene. In total of 30 isolates Staphylococcal enterotoxin genes *sea* and *seb* were more prevalent among methicillin resistant *S. aureus* (MRSA) were 6/11 (54.54%), 3/11 (27.27%) respectively, then in multidrug resistant *S. aureus* (MDR) which were 6/12 (50%), and 3/12 (25%) respectively, and in Extensively drug resistance (XDR) *S. aureus sea* was1/3 (33.33%) and there is no *seb genes*, while in methicillin sensitive *S. aureus* (MSSA) *sea* was 1/19 (5.26%), and there is no *seb genes*, and there was no Staphylococcal enterotoxin genes *sea* and *seb* in penicillin sensitive *S. aureus* (PSSA), as depicted in Table3, and Figures 3, 4.

Table 3: Prevalence of Staphylococcal enterotoxin genes among penicillin- sensitive *S. aureus* (PSSA), methicillin- sensitive *S. aureus* (MSSA), multidrug-resistance (MDR), methicillin resistance *S. aureus* (MRSA), extensive drug-resistance (XDR), and Pan drug-resistance (PDR) *S. aureus*. Bacteria isolates that is characterized as MRSA and XDR will also be characterized as MDR.

Type of antimicrobial resistance		No.	Staphylococcal enterotoxin genes sea	Staphylococcal enterotoxin genes seb
1-	Penicillin- sensitive S. aureus (PSSA)	2	0	0
2-	Methicillin- sensitive S. aureus (MSSA)	19	1/19 (5.26%)	0/19
3-	Multidrug-resistance (MDR)	12	6/12 (50%)	3/12 (25%)
4-	Methicillin resistance S. aureus (MRSA)	11	6/11 (54.54%)	3/11 (27.27%)
5-	Extensive drug-resistance (XDR)	3	1/3 (33.33%)	0
6-	Pan drug-resistance (PDR)	0	0	0

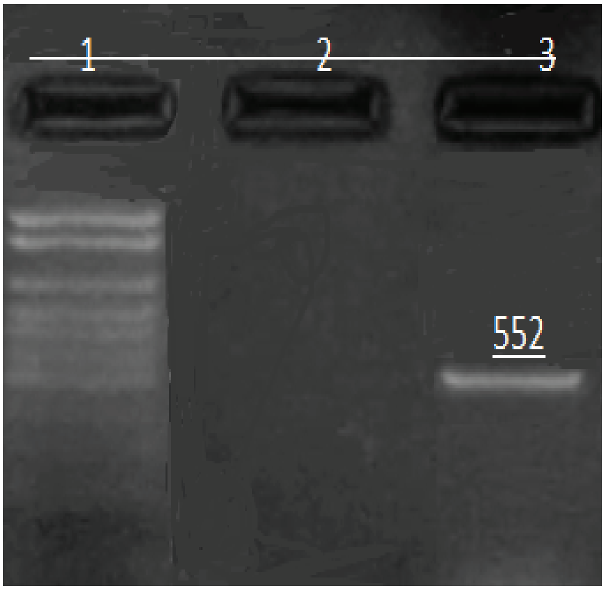

Figure (3): Electrophoresis of the PCR products in 1% agarose gel. Lane 1: 100bp DNA ladder, Lane 2: Negative control, Lane 3 *sea* gene (552bp).

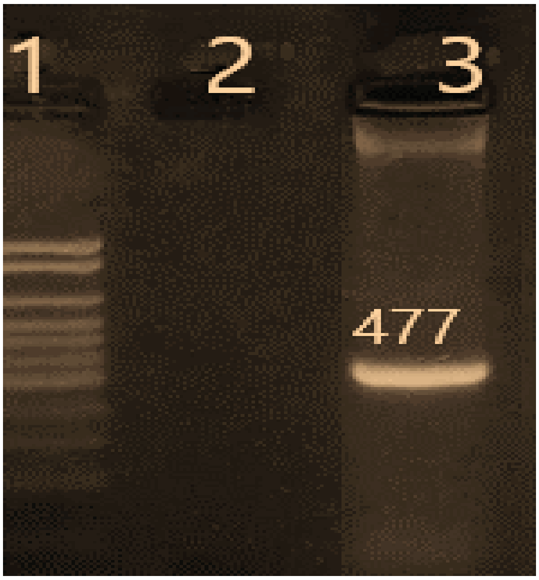

Figure (4): Electrophoresis of the PCR products in 1% agarose gel. Lane 1: 100bp DNA ladder, Lane 2: Negative control, Lane 3 of *seb* gene (477bp).

Discussion

S. aureus is one of the major pathogens able to infect every organ, damaging tissues and causing severe, chronic, and/or recurrent infections, which require prolonged antimicrobial treatment, with a dramatic impact on the patient's quality of life[30,31]. The result revealed that in total of 417 fecal samples, 30 grew *S. aureus* (7.19%), and of 417 fecal samples 96 yielded bacterial pathogens (23.02%), *S. aureus* was the most common bacterial pathogen found in 30/96 cases (31.25%).

The PCR results showed that 7/30 (23.33%) of the *S. aureus* isolates possessed the *sea* gene, and 3/30 (10 %) had the *seb* gene. In total of 30 isolates Staphylococcal enterotoxin genes *sea* and *seb* were more prevalent among methicillin resistant *S. aureus* (MRSA) were 6/11 (54.54%), 3/11 (27.27%) respectively, then in multidrug resistant *S. aureus* (MDR) which were 6/12 (50%), and 3/12 (25%) respectively, and in Extensively drug resistance (XDR) *S. aureus sea* was1/3 (33.33%) and there is no *seb genes*, while in methicillin sensitive *S. aureus* (MSSA) *sea* was 1/19 (5.26%), and there is no *seb genes*, and there was no Staphylococcal enterotoxin genes *sea* and *seb* in penicillin sensitive *S. aureus* (PSSA). *S. aureus* resistance against a wide range of antimicrobials is another important factor in spread of infections by this bacterium that is increasing worldwide.

Different results have been reported in other countries for the prevalence of *sea* and *seb* genes in *S. aureus.* isolated from fecal samples.

Our results consistent with the findings of other studies, in Seoul, Korea; Kim *et al.,* 2017, they reported that out of 664 stool samples taken from children with acute gastroenteritis from age (1-18), 183 (27.6%) yielded bacterial pathogens, and *S. aureus* was the most common bacterial pathogen, found in 72 cases (39.3%)[6]. In a similar study in China hospitals the overall colonization prevalence of *S. aureus* and MRSA were 20.0% and 4.5% respectively, in fecal samples from patients with age 1day to 17 years old[22].

Other studies reported different prevalence of *S. aureus*, in 2007, Flemming and Ackermann, reported that in German out of 2,727 stools investigated, 198 grew *S. aureus* (7.265%). A total of 114 *S. aureus* strains (57.58%), produced the following enterotoxins in vitro: A, 36 (31.58%); B, 20 (17.54%). Twenty-nine (14.6%) *S. aureus* strains were identified as methicillin-resistant[32].

In 2014, Kadhim in Iraq detected *S. aureus* in (6.7 %) diarrhea samples, and (66.7%) were found to be positive for production of enterotoxin B[21]. In United States of America out of 625 human stool samples, 58 were positive for *S. aureus* (9.3%). A high number of isolates were resistant to multiple antibiotics including oxacillin (43.1%), and erythromycin (51.7%), all isolates were susceptible to vancomycin[33]. In developed countries, Salmonella was the most frequently detected bacteria found in German patients with acute gastroenteritis AGE, followed by enterotoxin gene-carrying *S. aureus* and *Campylobacter spp.* Enterotoxin-encoding *S. aureus* was 1.7% [34].

Infection with *S. aureus* was detected in 64/345 fecal samples (18.6%), taken from Iranian patients with gastroenteritis. In total, 50% of the strains (32/64) carried the enterotoxin genes; the most common was *sea* (56.1%). These genes were more prevalent among MDR-MRSA (58.8%) compared with methicillin-sensitive *S. aureus* (15.4%) strains. *Staphylococcus aureus* should be considered an etiological agent of gastroenteritis. Infection with MRSA resistant to multiple antimicrobial classes (MDR-MRSA) is common among gastroenteritis patients[35]. In Iranian outpatients with underlying inflammatory bowel disease *S. aureus* were detected in 15.8%. In *S. aureus* isolates, only positivity for the presence of *sea* enterotoxin was detected[36]

The prevalence of *S. aureus* and genes encoding enterotoxins in diarrhea samples in other studies were higher or lower than our results, these variation between our results and other studies, can be attributed to several factors, including the size of the samples tested, the season in which the sampling was done, and geographical difference. Our result revealed that vancomycin is still the main antibiotic of choice for treatment of infection caused by methicillin resistant *S. aureus* (MRSA) and extensively drug resistance (XDR) *S. aureus.* Also, some strains of *S. aureus* remain susceptible to penicillin. Thus, this antimicrobial agent remains the treatment of choice for patients infected with penicillin sensitive *S. aureus* (PSSA).

In China Liang and his colleague did research in 2018, in their result the highest rate of resistance was detected for penicillin (PEN) (93.9%), followed by erythromycin (ERY) (58.8%), clindamycin (CLI) (55.7%), tetracycline (TCY) (31.3%), sulfamethoxazole-trimethoprim (SXT) (7.6%), ciprofloxacin (2.3%), rifampicin (1.5%), nitrofurantoin (2.3%), and gentamicin (0.8%). All of the isolates were susceptible to vancomycin[37]. In Italy, Rifampicin is one of the major drugs used on its own and also in combination to treat numerous infections sustained by methicillin-resistant Staphylococcus aureus (MRSA), but rifampicin resistance (RIF-R) is increasing in multidrug-resistant-MRSA isolates (16.4%), with respect to Europe (5.7%)[31]. Some *S. aureus* strains remains susceptible to penicillin. Thus, this antimicrobial agent remains the treatment of choice for patients infected with penicillin susceptible isolated[38,27]. In Guangzhou, China Ai and his colleague did research in 2020, they found that all MRSA strains were resistant to cefoxitin screening, also all MRSA strains were resistant to penicillin. The *S. aureus* strains exhibited highest rate of resistance to penicillin (PEN, 84.2%), followed by erythromycin (ERY, 38.8%), the resistance rates of antibiotics were lower for gentamicin (GEN, 2.7%), ciprofloxacin (CIP, 1.9%), and rifampicin (RIF, 0.7%); however, all isolates were susceptible to vancomycin[22].

Patients with MRSA colonized diarrheal stool impact significantly on environmental contamination[39]. The epidemiology of prevalent MRSA is changing dynamically and geographically[37]. Furthermore, having a gastrointestinal condition would increase the risk of intestinal *S. aureus* carriage [33].

Conclusions

These results confirmed the involvement of *S. aureus* in the occurrence of gastroenteritis, and the enterotoxigenic genes *sea* and *seb* were more prevalent among methicillin resistant *S. aureus* (MRSA), then multidrug resistant *S. aureus* (MDR), and extensively drug resistance (XDR) *S. aureus*. Vancomycin is still the main antibiotic of choice for treatment of infection caused by methicillin resistant *S. aureus* (MRSA) and extensively drug resistance (XDR) *S. aureus*. Our findings suggest that PCR is a rapid, specific, and inexpensive method for detecting SE.

Conflict of Interests: None.

Source of Funding: Self.

Ethical Clearance: The Research Ethics Committee of Erbil Polytechnic University approved the study proposal and approved also by Erbil Teaching Hospital.

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