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Autoaggregation ability of diarrheal causative bacteria isolated from Iraqi children

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Abstract

Stool samples were collected from 100 children patient aged between 3 days to 11 years who suffered from diarrhoea to isolate diarrheal causative bacteria. Fifty-one isolates were isolated and identified by cultural, microscopical, biochemical tests, and Vitek 2 system. The isolates were distributed as: 46 isolates belonged to *Escherichia coli*, 4 isolates belonged to *Shigella sonnei*, and one isolate belonged to *Providencia alcalifaciens*. All isolates were tested for antibiotic susceptibility to 10 different antibiotics and autoaggregation ability. All isolates were revealed resistant to Metronidazole while they were sensitive to Imipenem except one isolate of *E. coli* showed resistance to it. In the case of autoaggregation ability, all isolates showed their ability to auto aggregate at 24h, while some of them have the auto aggregation ability at 4h and 9h. The highest auto aggregation percentage at 4h was recorded 86% for *E. coli* among *S. sonnei* and *P. alcalifaciens* isolates which gave percentage rates (57.14 and 33) % respectively. The current study aimed at the isolation, and identification of diarrheal causative bacteria, antibiotic susceptibility, and detection of auto-aggregation ability of diarrheal causative bacteria.

Keywords: Diarrhoea, Diarrheal causative bacteria, Auto aggregation, Antibiotic susceptibility

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Introduction

Diarrhoea is the second leading cause of death in children under 5 years old worldwide. It takes three to seven days of infection with frequent, watery stools as recorded by WHO [1]. Diarrheal causative bacteria transferred by the faecal-oral pathway can be waterborne, foodborne, or direct transmission via hands, fomites, or dirt that young children can swallow [2]. diarrhoea may be bacterial, viral, or parasitic, there are a numeric of bacterial species that cause diarrhoea including *E. coli, Vibrio cholera, Shigella spp., Vibrio cholerae, Listeria monocytogenes, Enterohemorrhagic Escherichia coli (EHEC)*, *Clostridioides difficile*, and Salmonella spp [3] .

In Iraq, incorrect breastfeeding, water supply, poor hygiene and sanitation, low income, crowding, and low mother education are risk factors for diarrhoea [4]. *E. coli* is regarded as a part of intestinal microbiota, because some variations of this bacteria they had the capacity to exhibit virulence, causing different appearances of gastrointestinal illness and causing the most common childhood diarrhoea [5]. Most diarrheal infections are caused by pathogenic bacteria such as *E. coli*, which causes GIT infection in developed countries including symptoms such as stomach pain, bloating, and moderate diarrhoea [6]. It can cause illness to humans and animals and contain pathogenic genes; it can cause an infection after an intestinal adherence by fimbria [7]. An estimated 160,000 deaths per year across all age categories are attributed to Shigella spp., a major cause of diarrhoea among young children in developing nations, and the strain *S. sonnei* cause shigellosis disease in developed nations.[8]

The current study aimed to isolation, and identification of diarrheal causative bacteria, and detection of their antibiotic susceptibility and auto-aggregation ability.

Materials and Method

Isolation and identification of diarrheal causative bacteria

Stool samples were collected from 100 children patient aged between 3 days to 11 years who suffered from diarrhoea visited the following hospitals: Child's Central Teaching Hospital, Al-Kadhimiya Teaching Hospital, and Al-Kindy Teaching Hospital in Baghdad, for the period between the middle of September 2020 to the end of December 2020, to isolate diarrheal causative bacteria. All samples were examined by the naked eye to observe the density, mucus, color, and blood in the stool. After that, the samples were cultured on different selective agar plates included MacConkey agar, Eosin Methylene Blue (EMB), *Salmonella-Shigella* agar (SS), and Xylose Lysin Deoxy Cholate agar (XLD). All plates were incubated at 37°C for 24 h [9]. All bacterial isolates were identified by cultural, microscopical, biochemical tests, and Vitek 2 system.

Antibiotic susceptibility test

Antibiotic susceptibility testing for ten different antibiotics was conducted using the Kirby-Bauer method, as described by the World Health Organization (WHO) included: Ampicillin, Ciprofloxacin, Cefotaxime, Ceftriaxone, Ceftazidime, Imipenem, Nalidixic acid, Tetracycline, Trimethoprim/Sulfamethoxazole, and Metronidazole.

Approximately (1.5×10^8) CFU / ml was obtained by choosing 4-5 colonies of each bacterial isolate from the original culture and suspending them in a test tube with 4 ml of normal saline (0.5 MacFarland standard solution). A bacterial suspension was carefully placed onto

Mueller-Hinton agar with a sterile cotton swab and then allowed to dry at room temperature. In order to establish proper contact between the antibiotic's discs and the agar, they were carefully inserted there using sterile forceps. After that, the plates were inverted and left them to dry, incubated for 18-24 h at 37℃. The diameters of the inhibition zones formed around the discs were measured as recommended by the Clinical Laboratory Standards Institute. The isolates were recorded as susceptible or resistant to tested antibiotics, according to CLSI (2021) [10].

Auto-aggregation assay of diarrheal causative bacteria

Visual assay

One percent of bacterial isolates were grown in 9ml of glassy tubes contained nutrient broth medium in which the turbidity was adjusted with McFarland standard solution (0.5) 1.5×10^8 CFU / ml, then incubated at 37°C in a shaker incubator for 24h and vortexed for fifteen seconds, after that left steadily for 4, 9, and 24h at 37℃, after that the results were observed [11,12]. The positive results show sedimentation at the bottom of the tubes while the negative results show turbidity.

Spectrophotometry Assay

The bacterial growth culture was prepared as mentioned at the visual assay, after that 0.1 ml of the upper portion was transferred to a tube contained 3.9ml of phosphate buffer saline (PBS) solution then Shaked and the O.D at 600 nm was recorded. The other part left statically for 4 h at 37℃. After the four hours 0.1 ml of the clear upper part was transferred to other tubes that contained 3.9 ml PBS and the O.D600 recorded after shaking for each tube [13]. According to Janosikova *et al.* (2021) [14] the auto-aggregation percentage was recorded:

(Auto-aggregation) % = $[A_0 - A/A_0] \times 100\%$

Where,

(A_0) = O.D initial of each isolate recorded before stagnation.

(A) = O.D final of each isolate recorded after 4, 9, and 24h.

Results and Discussion

Identification of diarrheal causative bacteria

Cultural characterization for the cultivated Gram-negative enteric pathogenic bacteria in this study was investigated by culturing on their selective media. The colony morphology for 46 isolates of *Escherichia* spp. on MacConkey agar appeared as large, pink colonies on agar, and on Eosin Methylene Blue (EMB) agar colonies appeared as distinctive green

metallic sheen, while the colonies appeared on Xylose Lysine Deoxycholate agar (XLD) appeared as yellow colonies, and on *Salmonella-Shigella* agar (SS) are somewhat pinkish smooth. Another isolate showed black colonies on the *Salmonella-Shigella* agar, pale color on MacConkey agar, green metallic sheen, and purple with a smooth edge on EMB. *Providencia* spp. appeared on MacConkey agar in pale color, and colorless on EMB agar, and SS agar as shown in (Table 1).

Shigella spp. appeared on MacConkey agar also smooth colorless (non-lactose fermenter), also colorless on Eosin Methylene Blue and *Salmonella Shigella* agar, while on Xylose Lysine Deoxycholate agar appeared as pink-red colonies with no black center this agrees with Carroll *et al*. (2015) [15]. The *E. coli* isolates appeared with pink color on MacConkey agar, green metallic color on EMB, and pink-red color on SS agar due to lactose fermentation. The yellow-colored colonies on XLD agar are due to the fermentation of carbohydrates, which leads to the production of acids. The utilization of carbohydrates prevents lysine decarboxylation [16].

The H_2S-producing *E. coli* isolate showed the same characteristics as other studied *E. coli* isolates, except the black-colored colonies appeared when cultivated on SS agar due to the utilization of sodium thiosulfate for H_2S production. *Shigella* spp. colonies appeared colorless on MacConkey, EMB, SS agar, and red color colonies on XLD agar because it doesn't ferment lactose, [17]. Providencia spp. colonies appeared colorless on the enteric media and did not ferment the lactose, this is compatible with Mahon *et al*. (2018) [16].

Gram stain was used for each pure bacterial colony of enteric pathogenic bacteria. Microscopically, examination showed a Gram-negative, rod-shaped, arranged singly. While bacteria appeared as Gram-negative, short rods arranged singly, in pairs and chains, suggested characters of *Shigella* spp., and these results were compatible with Carroll *et al*. (2015) [15]. *Providencia* spp. appeared as bacilli, long cells, negative for gram stain, and this result was similar to Holt *et al*. (1994) [18].

Biochemical tests for all Gram-negative isolates in this study (*Shigella* spp., *Escherichia* spp., *Providencia* spp.) were shown positive results for the catalase and negative results for the oxidase test, these results are similar to Murray *et al*. (2015) [19]. For Kligler Iron Agar *Shigella* spp. appeared with Alkaline slant, Acid butt with no gas and no H_2S production, *Escherichia* spp. gave Acid slant, Acid butt with gas, and no H_2S production, and these results are compatible with the results of Winn, (2006) [20]. Only one isolate, the slant appeared with yellow color, gas production, and black bottom. The black color indicated the production of H2S, and the yellow acidic slant is due to the fermented sugars Glucose and Lactose, this is identical to Kosilova *et al.* (2020) [21].

The bacteria produce H_2S that play role in protection as an anti-oxidation, this is organized enzymatically, and encoded genetically. The reducing ability of the Cysteine level is done by Sulfides that lowering the oxidation when sequestrating the unconjugated Irons from the Fenton reaction [22,23]. The identification of isolates was attributed to the result of the Vitek 2 GN ID card. Results showed that 46 isolates belonged to *E. coli*, 4 isolates belonged to *Shigella sonnei*, and one isolate belonged to the *Providencia alcalifaciens*.

Table (2) showed that *E. coli* with an isolation percentage (90.1) % was the predominant isolates in collected diarrheal samples the in current study followed by 7.8% of *S. sonnei* and 1% of *P. alcalifaciens*.

Table 1.

Diarrheal causative isolates appearance on media after 24h at 37ºC.

Media / **Isolates**	**MacConkey Agar**	**Eosin Methylene Blue Agar**	***Salmonella-Shigella* Agar**	**Xylose Lysine Deoxycholate Agar**
***Escherichia* spp.**	Large pink colonies	Distinctive green metallic sheen colony	pinkish smooth colonies	Yellow colonies
***Shigella* spp.**	Colorless colonies and smooth	Colorless	Colorless	Red colonies, with no black center
***Providencia* spp.**	Colorless	Colorless	Colorless	Red colonies

Table 2.

Percentage of diarrheal bacteria isolates from diarrheal samples.

Bacterial isolates	No. of isolates	Percentage of isolation (%)	
		*Total sample	**Total isolates
Escherichia coli	46	46	90.1
Shigella sonnei	4	4	7.8
Providencia alcalifaciens	1	1	1.9

*Percentage from (100) sample. **Percentage from (51) isolate.

Vitek 2 system could be an automated diagnostic apparatus designed for rapid identification of Gram-negative bacteria, which showed that *E. coli*, and *Shigella* spp. are very commonly associated with enteric disease in developing countries. The recovery of some pathogenic bacteria such as *E. coli* is a serious public health concern because it is a source of food poisoning which can cause severe illness and even death in developing countries. Abdulhameed *et al.* (2020) [24] mentioned that the *E. coli* is the forefront of bacteria isolated from diarrhea in children. *Shigella sonnei* was predominant, Shigellosis is commonly associated with mild watery diarrhea [25]. *Shigella* species is the major diarrheal causative bacteria, about 200 million *Shigella* infections and (3–5) million deaths occur annually in developing countries, most of which affect children under 5 years of age [26].

Infantile gastroenteritis of microbial origin is still a serious concern for public health, especially in low-income countries, and accounts for significant Mortals [27]. Diarrhea is a major health problem and is considered a leading cause of morbidity and mortality, especially among children in developing countries [28].

Providencia spp. is the causative of diseases that are transported by food such as diarrhea [29].

Antibiotic Susceptibility Test

Antibiotic Susceptibility of 51 isolates of enteric pathogenic bacteria which included: *E. coli*, *S. sonnei*, *P. alcalifaciens*, against 10 antibiotics from different classes which include: Cephalosporines (Cefotaxime, Ceftazidime, Ceftriaxone), Fluoroquinolones (Ciprofloxacin), Quinolones (Nalidixic acid), Tetracyclines (Tetracycline), Folate pathway

(Trimethoprim/Sulfamethoxazole), Penicillins (Ampicillin), Carbapenems (Imipenem), and Metronidazole. The results were interpreted according to the recommendation of CLSI (2021) [10].

The results revealed that all the bacterial isolates showed variable resistance levels to the ten tested antibiotics. All isolates were resistant to Metronidazole, they varied in the levels of resistance to other antibiotics. All four isolates of *Shigella sonnei* isolates showed resistance to Ampicillin and Cefotaxime. Also, results revealed that the resistant rate was of *S. sonnei* isolates (3) from (4) were resistant to tetracycline, Trimethoprim/Sulfamethoxazole, Ceftazidime, Ceftriaxone, and Ciprofloxacin but sensitive to Imipenem and Nalidixic acid. One isolate showed susceptibility to Ciprofloxacin. The *P. alcalifaciens* isolate showed resistance to Ceftazidime, Ceftriaxone, and Ciprofloxacin, Nalidixic acid, Cefotaxime, and Trimethoprim/Sulfamethoxazole.

On the other hand, the results revealed that (43) isolates from (46) isolates of *E. coli* were resistant to Cefotaxime and Ceftazidime at resistance (93.47%), and 39 (84.78%) isolates were resistant to Ceftriaxone.

The results showed 43 (93.47%) isolates reveal resistance to Ampicillin, while 33 (71.74%) isolates were resistance to the Nalidixic acid, Ciprofloxacin, and 34 (73.91%) were resist to Trimethoprim/Sulfamethoxazole, and the resistance of 27 (58.69%) isolates to the Tetracycline, while 3 isolates of *E. coli* (9), (19), (43) showed resistant rate (6.52%) for Imipenem Figure (1). Three isolates from 45 were sensitive to Ceftazidime, Ampicillin, and Cefotaxime, (7) isolates were sensitive to Ceftriaxone, and (13) isolates were sensitive to Nalidixic acid and Ciprofloxacin. While (12) isolates showed sensitivity to Trimethoprim/Sulfamethoxazole and (19) have been sensitive to Tetracycline from (45) isolates of *E. coli*, while all the isolates were sensitive to Imipenem accept *E. coli* (9), (19), (43).

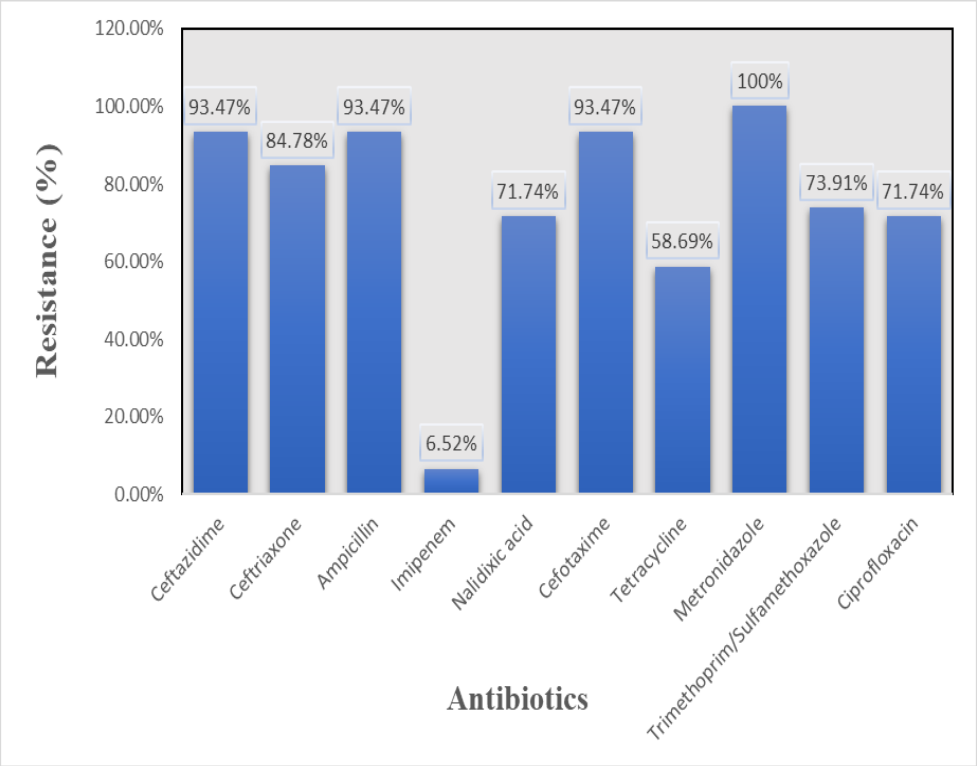

Figure 1.

Antibiotics resistance of *E. coli* isolates.

The Multi-drug resistance (MDR) isolates were shown in this study as *S. sonnei*, which are resistant at least to one or two-drug in each class, resistant to Cephalosporines class (Cefotaxime, Ceftazidime, and Ceftriaxone), Fluoroquinolones class (Ciprofloxacin), Tetracyclines class (Tetracycline), Penicillins class (Ampicillin), Folate pathway (Trimethoprim-sulfamethoxazole) and Metronidazole drug. The resistant capability of *Shigella sonnei* to the Tetracyclines, Cephalosporins, Fluoroquinolones, Penicillins, Ciprofloxacin was compatible with Shad and Shad, (2021) [30], in which mentioned in their research, there is several methods utilized by this bacteria such as resistant plasmid to antibiotics which play role in the resistant gene spreading by horizontal gene exchange of the mobile elements, and there is pathogenic islands on chromosomes that make it multi-drug resistant strains. *E. coli* (31) isolate was resistant to all the antibiotics utilized in this study but sensitive to Imipenem.

The 3-mercaptopyruvate sulfurtransferase is a gene in *E. coli* that encode for the production of an enzyme that stimulates Hydrogen Sulfide production, this gives the resistance ability to several antibiotics [23], and resistant genes for various antibiotics such as fluoroquinolones resistance genes *oqxA*, *oqxB*, tetracyclines resistance gene

tet(A), β-Lactams resistance gene *blaTEM*-1B "these genes signified by 3-MST (*sseA*) [31]. The most common means of resistance in bacteria is enzymatic inhibition, transmission, or change in the genetic materials. The diarrheal causative bacteria had resistance to Ampicillin and Metronidazole, increasing resistance to this drug could be the result of Ampicillin usage in the country for a long time and inaccuracy in diagnosis, frequent overuse, and the quality of antibiotics [32].

Antimicrobial resistance is defined as the resistance of microorganisms to an antimicrobial agent to which they were at first sensitive, and this resistance may be generated and transmitted in many ways [33]. The emergence of microbial resistance to multiple antimicrobial agents has become a significant global concern, resulting in adverse effects on patients [33]. The reason of resistance is due to the bad use to antimicrobials [34]. The *P. alcalifaciens* isolate also was recorded as MDR, which had resistance to one or more antibiotics from each class such as Cephalosporines class (Cefotaxime, Ceftazidime, and Ceftriaxone), Fluoroquinolones class (Ciprofloxacin, Nalidixic acid), Folate pathway (Trimethoprim-sulfamethoxazole) and Metronidazole drug, other isolates were recorded MDR *E. coli* which are resistant to Cephalosporines class (Cefotaxime, Ceftazidime, and Ceftriaxone), Fluoroquinolones class (Ciprofloxacin and Nalidixic acid), Tetracyclines class (Tetracycline), Folate pathway (Trimethoprim-sulfamethoxazole), Penicillins class (Ampicillin) and Metronidazole drug.

Multi-drug resistance (MDR) is defined as the resistance of microorganisms to more than two antibiotic groups. The emergence of MDR causes increased rates of morbidity and mortality. Also, the increased costs of treatment have limited the effectiveness of the existing antibiotic which causes the failure of treatment [35,36]. Antibiotics are essential in life-threatening cases, but overuse of antibiotics can adversely affect society [37]. The resistance capability of *E. coli* to the used antibiotics in this study was compatible with Park *et al*. (2022) [34]. The acquirement of genes and the transference of microorganisms that can cause disease between humans and animals, and the random usage of antibiotics as a treatment for diarrhea make some of *E. coli* isolates MDR. Reported that MDR *E. coli* isolates have *cat*A1 and *tet*A genes against Tetracycline and chloramphenicol. The results showed that the most effective antibiotic against gram-negative Diarrheal causative isolates was Imipenem, which works by inhibiting the formation of the bacterial cell-wall [38].

Autoaggregation Assay of Enteric pathogenic Isolates

Visual Assay

The results revealed that all the 51 enteric bacterial isolates in this study had an autoaggregation ability at 24h. The results showed that (3) *E. coli* isolates from 46 isolates showed aggregative ability at 4h while the aggregation of (21) isolates appeared at 9h, and (26) *E. coli* isolates their sedimentation appeared at 24h.

On the other hand, the results revealed that all *S. sonnei* isolates had high auto-aggregation at 9 and 24h only except *S. sonnei* (1) showed aggregation ability at 4, 9, and 24h. Whereas the auto-aggregation of *P. alcalifaciens* isolate appeared at 4, 9, and 24h, as shown in Table (3).

Table 3.

Auto-aggregation ability of diarrheal causative bacteria isolates (visual assay)

Bacterial isolates	No. of isolates	No. of positive isolates of autoaggrwgation		
		4h	9h	24h
Escherichia coli	46	3	21	26
Shigella sonnei	4	1	4	4
Providencia alcalifaciens	1	1	1	1

Nwoko and Okeke, (2021) [39] reported that the auto-aggregation is the most frequent indirect method for detecting and quantifying auto-aggregation based on the fact that bacterial sedimentation was faster in liquid culture, and the projections fimbriae were specialized structures located on the *E. coli* cell surface that play role in many purposes such as adhesion.

The *E. coli* uses these projections for sticking to the epithelial cell, which helps to form tiny bacterial cellular colonies tighten by a hair-like appendages pilus, and there is another form IV fimbriae that play role in the auto-aggregation, which is regarded as a protein secretion. Another study by Schembri *et al.* (2001) [40], mentioned to the *Fim*H gene with different changes in its amino acids that aid in auto-aggregation of *E. coli*. The *E. coli* have many mechanisms of auto-aggregation one of them is Ag43 which an auto-

transporter protein that aids in auto-aggregation in the broth, which is expressed to form a shape of crimpy colonies, due to the external projections formed by the deposition of protein into fine fibers. *S. sonnei* regarded as an enteric invasion that origins inflammation and damage epithelial tissue this leads to sever severe colitis causing death [41].

Spectrophotometry Assay

Five diarrheal causative isolates were chosen for detection of autoaggregation percentage included (3) isolates of *E. coli*, one isolate of *S. sonnei*, and one isolate of *P. alcalifaciens*. The results revealed low percentage of autoaggregation was recorded for *E. coli* isolates except *E. coli* (38) recorded higher percentage rates (86%) among *S. sonnei* (1) and *P. alcalifaciens* isolates which gave percentage rates (57.14 and 33) % respectively as shown in Figure (2).

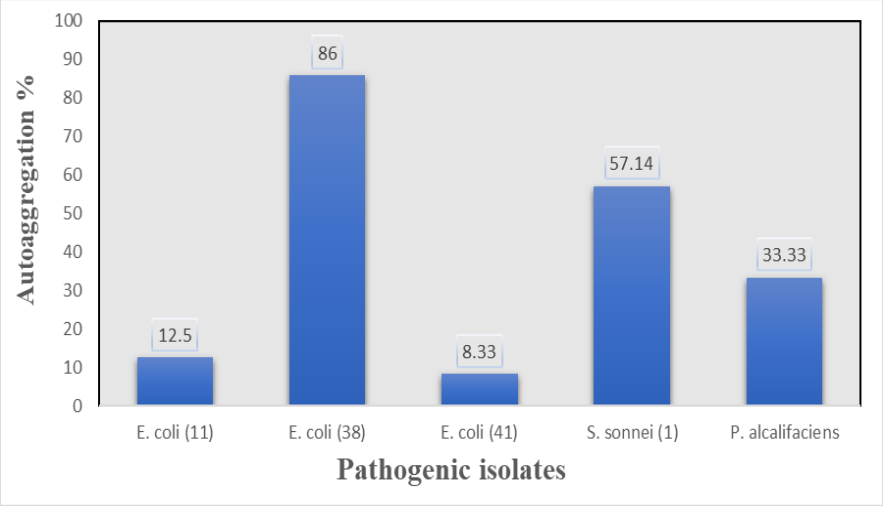

Figure 2.

Auto-aggregation percentage of diarrheal causative bacteria after 4h of incubation.

Conclusions

The diarrheal causative bacteria in the current study had the auto-aggregation ability and resist to a wide range of antibiotics.

Ethical Approval

The study was approved by the Ethical Committee.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

All authors shared in conception, design of the study, acquisition of data, and manuscript writing, the critical revising and final approval of the version to be published.

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