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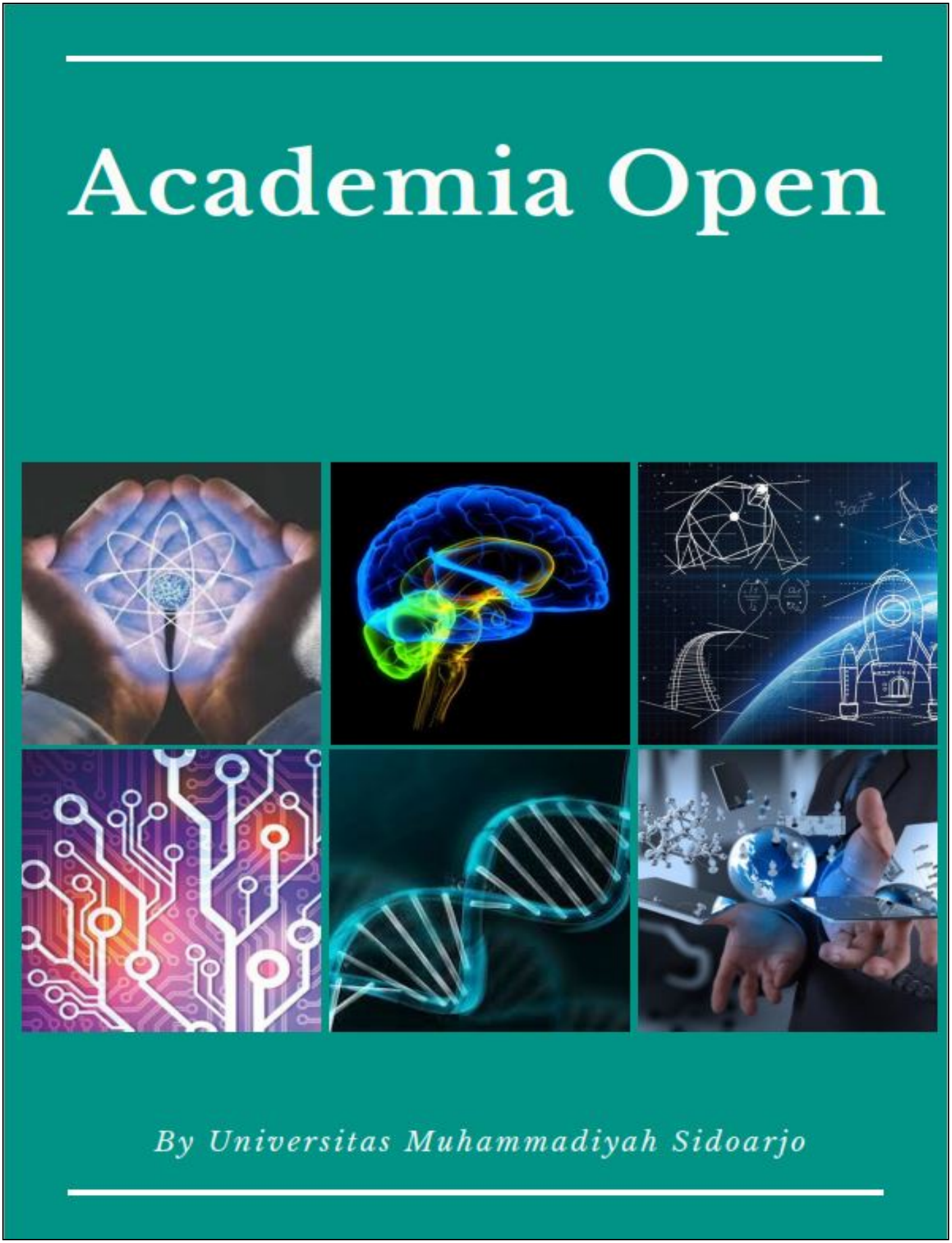

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Watery diarrhea between Vibrio cholera and Aeromonas hydrophila during Iraqi cholera outbreaks

Diare berair antara Vibrio cholera dan Aeromonas hydrophila selama wabah kolera di Irak

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Abstract

Cholera remains a significant health challenge globally, especially in regions with poor infrastructure and healthcare. This study aimed to detect Aeromonas hydrophila in clinical samples from diarrhea patients during the 2022-2023 outbreaks in Iraq. Archived samples from Baquba General Teaching Hospital were analyzed using culture media, biochemical tests, and the VITEK 2 system. Both Vibrio cholerae and A. hydrophila isolates produced virulence factors such as hemolysin, protease, lipase, phospholipase, and biofilm. Antibiotic susceptibility testing showed A. hydrophila was susceptible to cefepime, ceftazidime, amikacin, imipenem, meropenem, and piperacillin-tazobactam but resistant to aztreonam, levofloxacin, and ciprofloxacin. V. cholerae was susceptible to amikacin, imipenem, meropenem, piperacillin-tazobactam, doxycycline, and chloramphenicol but resistant to trimethoprim-sulfamethoxazole and ampicillin. The findings highlight the need for better recognition of Aeromonas as a gastrointestinal pathogen and emphasize the importance of improved diagnostics and public health initiatives to reduce diarrheal diseases.

Highlight:

- **Detection Methods:** Culture media, biochemical tests, VITEK 2 system used.
- **Virulence Factors:** Hemolysin, protease, lipase, phospholipase, biofilm production identified.
- **Antibiotic Resistance:** Specific resistance observed in A. hydrophila and V. cholerae.

Keyword: Cholera, Aeromonas hydrophila, Antibiotic Susceptibility, Diarrhea, Iraq Outbreak

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Pendahuluan

Vibrio cholerae is oxidase-positive, Gram-negative curved bacilli that are motile due to the presence of polar flagellum belong to the family, Vibrionaceae. They are non-capsulated, non–spore-forming, and they can grow under aerobic or anaerobic conditions (Sastry *et al*., 2016). It is widely distributed in aquatic environments such as estuarine, freshwater and marine. In addition, *V. cholera* has been associated with outbreaks of the most feared epidemic diarrheal disease, called cholera (Almagro-Moreno *et al*., 2015). Cholera is a severe diarrheal illness which transmitted by contaminated water and food (Pande *et al*., 2018). When this organism enters the body it travels to the gut lining, where it releases the cholera toxin causing rapid loss of fluid and electrolytes which may progress to severe dehydration, hypovolemic shock and death if left untreated (Kanungo *et al*., 2022). Symptoms such as watery diarrhea, vomiting, lethargy, and dehydration appear 12 hours to five days after the organism's incubation (Cho *et al*., 2010). Most infected people with *V. cholera* are asymptomatic, but the bacteria can live in their stool between 1 to 10 days after infection to be shaded into the environment, where they can infect others. Most people who get sick have only mild to moderate symptoms, while a few get extremely sick with acute watery diarrhea and severe dehydration, shock and finally death If left without treatment (Legros 2018).

A lack of clean drinking water in Iraq represents a major health problem, as cholera is transmitted by contaminated water. This diarrheal stool which was contaminated by *V. cholerae* will release in large quantities into the environment, resulting in the spread of this bacterium and its transmission via the fecal–oral route. Within 1 to 3 days of infection, without suitable treatment, 25% to 50% of patients may die as a result of circulatory collapse or a steep drop in blood pressure. The highest death rate has been recorded among children, the elderly and immunocompromised people (Sack *et al*., 2004; Chowdhury *et al*., 2016). This bacterium has a high capacity to adapt to varying conditions of salt concentration, pH, osmolarity and bile salts prevailing in the environment, and in human host. It is classified into more than 200 somatic O antigen serogroups (Yamai *et al*., 1997). The O1 serogroup has two biotypes, classical and El Tor, both could individually be serotyped as either Ogawa or Inaba (Ramamurthy *et al*., 2003).

The Gram-negative bacilli *Aeromonashydrophila* species of the genus Aeromonas, which belongs to the family aeromonadaceae that received increasing attention opportunistic pathogens because of its association with both dysenteric diarrheal and extra-intestinal infections in human disease especially in children and persons with impaired immune system (Naharro *et al*., 2009; Uche and Johnkennedy, 2014). Aeromonas bacteria are linked to two types of gastroenteritis, the first type is a disease similar to cholera, which causes rice-watery diarrhea, and the other type of disease is dysenteric gastroenteritis that causes loose stools filled with blood and mucus. The dysenteric gastroenteritis is the most severe out of the two types and distributed of *A. hydrophila* is widely in fresh and salt water also frequently found in chlorinated and no chlorinated drinking water (Galindo and Chopra, 2007). The objectives of this study are to Isolation of *V. cholerae* and *A. hydrophila* among the clinical cases during cholera outbreak and detection the virulence factors of both isolates.

Metode

Sample collection and identification

Archived clinical isolates (watery diarrhea specimens) from 2022 and 2023 outbreaks used in this study were obtained from presumptive cholera cases attending Baquba General Teaching Hospital, in Diyala Governorate, Iraq. The samples came from people aged 20 to 60 years who were suspected of having cholera by a specialist clinical physician. Samples were transported in Cary-Blair transport medium to the laboratory and inoculated in alkaline peptone water for 4 to 6 hours at 35°C, then cultured on blood agar, MacConkey agar and selective thiosulfate citrate bile salt sucrose agar (TCBS) at 37°C for 24 hours. The isolation and presumptive diagnosis of isolates relied on the protocol provided by the Central Public Health Laboratory (CPHL) of the Iraqi Ministry of Health. The VITEK 2 system (bioMérieux) was used to confirm the diagnosis. O1 polyvalent anti-sera and monovalent anti-sera were used to define isolates as Ogawa or Inaba serotype. Biochemical tests and string test were examined according to (MaccFadin, 2000). Drug susceptibility testing was tested by VITEK 2 system (bioMérieux)

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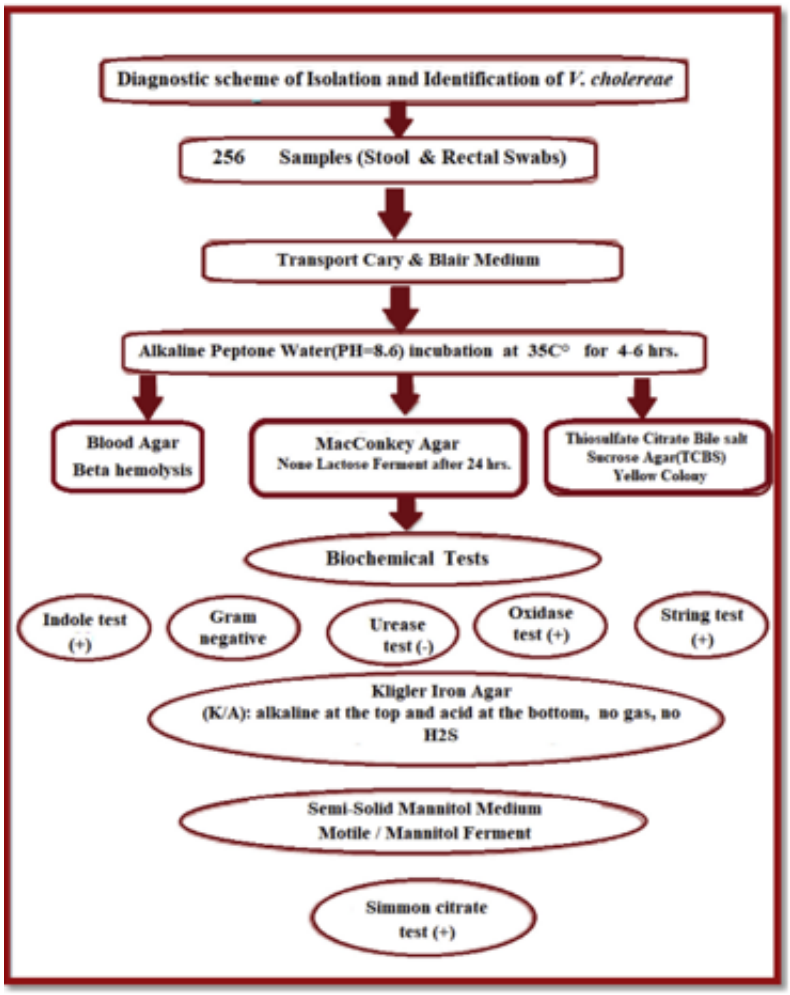

Figure 1. ***Diagnosis scheme of isolation and identification of Vibrio cholerae in present study depending on protocol provided by Central Public Health Laboratory (CPHL), Iraqi Ministry of Health (Al- Sa'ady et al. 2020) .***

Phenotypic Detection virulence factors

Hemolysin Detection

The strains were tested for β-hemolytic activity on base agar (Himedia, India) supplemented with 7% sheep erythrocytes (Collee *et al*., 1996).

Lipase and Lecithinase (phospholipase) production

Lipase and Lecithinaseactivity were determined according to Collee *et al*. (1996).

Protease Detection.

Protease hydrolysis was tested according to Benson (2002).

Biofilm production assay

Biofilm formation was quantified using a microtiter plate test method described by (Foster *et al*., 2020).

Hasil dan Pembahasan

Isolation of *V. cholerae* and *A . hydrophila*

Culture and Biochemical Tests

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The colonies of *V. cholerae* and *A. hydrophila* were yellow shine color on TCBS agar. In addition to those colonies appeared as pale on the MacConkey agar indicated that *V. cholerae* and *A. hydrophila* was unable to ferment lactose sugar. On blood agar *V. cholerae* and *A. hydrophila* produces smooth, convex, rounded and β-hemolytic and pale white to grey color colonies. Microscopically examination has revealed that *A. hydrophila* a Gram-negative bacillus, straight shape, singly or pairs and rarely as short chains, and not spore forming, while *V. cholerae* appear as gram-negative non-spore forming, slightly curved rods arranged as single or double of bacteria and the comma shape or vibriod shape distinguish these bacteria from other gram- negative bacilli. These characteristics were obtained also by previous studies (Carriero *et al*., 2016; Abbott *et al*., 2003).

The results of biochemical tests were adopted as a complementary characteristic of the initial diagnosis of *V. cholerae* and *A. hydrophila*, all isolates were positive result for oxidase test and were characterized by their ability to ferment glucose with no gas formed on kligler iron agar (Alk/Acid), it produces (Alkaline) red color top and bottom (acidic) yellow color with no gas formed (only *A. hydrophila* forming gas on KIA) without H_2S; it gives a positive result for, catalase, Indole, simmon citrate tests (table 1). This result was predicted by previous studies (AL-Fatlawy *et al*., 2017; Jawetz *et al*., 2016).

Tests	Isolates	
	V. cholerae	A. hydrophila
Oxidase	+	+
Catalase	+	+
Indole	+	+
Simmon citrate	+	+
KIA	A/K *, No gas/ No H2S	A/K, gas/ No H2S
Urease	-	-
String test	+	-

Table 1. ***Biochemical tests for both strains***

*** A: Acid, K: Alkaline, KIA: Kligler Iron Agar**

A total of 345 archived clinical isolates (watery diarrhea specimens) suspected cholera cases from 2022 and 378 isolates from 2023 outbreaks that had positive results for V. cholerae, but after confirmation of the identification by the VITEK 2 system, only 24% and 29% isolates of V. cholerae were obtained in 2022 and 2023 respectively. The rest of the isolates were *A. hydrophila* and other Gram-negative bacteria (table 2).

Biotyping and serotyping diagnosis tests were used to identify biotypes, serogroups and serotypes of *V. cholerae*. El Tor biotype had prevailed in all clinical isolates of *V. cholerae,* serogroup O1, with (100%) being Ogawa serotype.

Year	V. cholerae confirmed cases	A. hydrophila	Other bacteria	p-value
2022	83 (24%)	63 (18.3%)	199 (57.7%)	
Total	345			
2023	110 (29%)	49 (13%)	219 (57.9%)	
Total	378			

Table 2. ***Prevalence of diarrheal cases during outbreak***

Results of our study revealed that the number of females infected by *V. cholerae* were more than infected males in both years of outbreak (fig. 2).

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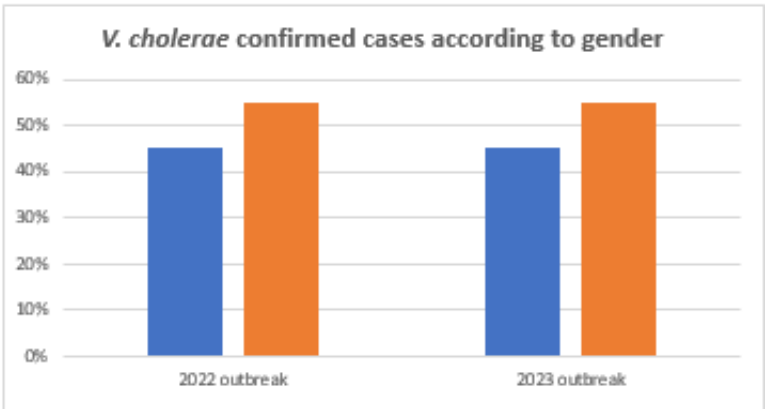

Figure 2. ***distribution of cholera cases between genders***

The result shows that clinical isolates of *A. hydrophila* and *V. cholerae* were able to produce different virulence factors that increase its pathogenicity. All isolates were positive for heamolysin production (100%), (β-heamolysis).

In *A. hydrophila* all isolates had the ability to produce protease by hydrolyze the protein (Casiean) (100%) also the ability to hydrolyze fats by Lipase enzyme (100%), and (80%) of them produce phospholipase. On the other hand, *V. cholerae* produce 75%, 62% and 70% of protease, lipase and phospholipase respectively (table 3).

Isolates	Hemolysin	Lipase	Protease	Phospholipase	Biofilm	p-value
V. cholerae	100% β-hemolysis	62%	75%	70%	46%	
A. hydrophila	100% β-hemolysis	100%	100%	80%	100%	

Table 3. ***V irulence factors of both species***

The results in this study indicate that all *A. hydrophila*isolates (100%) had the ability to produce biofilm, while only 46% of *V. cholerae* isolates can produce biofilm. The biofilm plays an important role in the establishment of infection, enhanced pathogenesis and drug resistance.

Antibiotic susceptibility test

Antibiotic susceptibility testing was done for all *A. hydrophila*and *V. cholerae* isolates. All *A. hydrophila*tested isolates were uniformly susceptible to cefepime, Ceftazidime, Amikacin, Imipenem, Meropenem and piperacillin-Tazobactam and resistant to Aztreonam, Levofloxacin and Ciprofloxacin. While *V. cholerae* isolates were uniformly susceptible to Amikacin, Imipenem, Meropenem, piperacillin- Tazobactam, Doxycycline, and Chloramphenicol show resistant toward Trimethoprim-Sulfamethoxazole and Ampicillin.

Discussion

The results of this study revealed that El Tor biotype, O1 serogroup and Ogawa serotype were the predominant type of *V. cholerae* classification in Diyala province. These results were in agreement with other studies in Babylon and other Provinces of Iraq (Al-Shok and Baiee, 2009; AL-Abbassi *et al*., 1999) who documented that *V. cholerae* El Tor O1 Ogawa was the most common cause of cholera in Babylon and Iraqi provinces, while these results were in disagreement with (Bunyan *et al*., 2019; Malik *et al*., 2015] that revealed that El Tor biotype, O1 serogroup and Inaba serotype were the predominant type of *V. cholerae* classification in Babylon province.

The differences and similarities in this study with others could be related with climate change during the past years in Iraq, where Constantin de Magny& Colwell, 2009 mentioned that climate and environmental changes may result in modification of the population size of *V. cholerae* in the environment and will influence the emergence of cholera disease in human populations. Meanwhile, the quality and availability of drinking water can be changed in unstructured conditions, such as war situation (Zoinlkon, 1996), so the environment may have converted to be more suitable for *V. cholerae* El Tor O1 Inaba in contrast to *V. cholerae* El Tor O1 Ogawa. The predominance of El Tor biotype in Iraq could be related to fact that El Tor biotype known to tolerate a wider range of environmental conditions and also is thought to persist longer in the harsh environment better than the classical biotype, for that reason El Tor O1 biotype has now virtually displaced the classical biotype completely throughout the world (Olson *et al*., 2018).

Regarding cholera isolates, results showed that all the V. cholerae isolates were able to produce the hemolysin. The appearance of clearly hallo zone around the colony on blood agar medium referred to the ability of this organism to

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produce the Hemolysin enzyme type beta (β). Microorganisms evolve a number of mechanisms for the acquisition of iron from their environment. One of them is the production of Hemolysin enzyme, which acts to release iron complex (AL-Fatlawy *et al*., 2017).

Results showed that (75%) of the clinical isolates of *V.cholerae* produced protease (table 3). AL-Hadrawi, (2019) referred to that (83%) of isolates in his study were have ability to protease production. Also, Awuor et al (2022) study showed that 71.42% of the isolates of *V. cholerae* produced protease. Proteases produced by *V. cholerae* had a very important role in its pathogenicity, due to the hydrolysis of several physiologically important protein such as mucin, fibronectin, and lactoferrin (Namdari *et al*., 2000). It could also proteolytically activate cholera toxin A subunit, El Tor cytolycin and hemolysin in *V. cholerae* (Booth *et al*., 1984).

The result showed that (62%) of isolates were have the ability to produce lipase. AL-Hadrawi, (2019) referred to that (73%) of isolates were have the ability to lipase production. Lipases enzymes catalyse the hydrolysis of the ester bonds of triacylglycerols and may have a critical role in V. cholerae pathogenicity or nutrition acquisition. The production of excesses amount of lipases allow bacteria to penetrate fatty tissue with the consequent formation of abscesses. Several studies showed that environmental strains of V. cholerae may produce a variety of enzymes including proteases, lipases, and hemolysin and other which are necessary for their survival in environment (Sakib et al., 2018). The result showed that the isolates were (70%) produced phospholipases. These results were agreed with AL-Hadrawi, (2019) who referred that (76%) of isolates were have the ability to phospholipase production. Oliver and Kaper, (2007), mentioned to the role of this enzyme in the cholera disease by the release of Arachidonic acid from the phospholipid found in the cell membranes of the lumen cells, this play an important role in the prostaglandin E2 (PGE2) which is responsible for the increase of liquids secretion from the lumen cells and this led to watery diarrhea occurs.

In *A. hydrophila*, results showed that all isolates had ability to produce β-hemolysin (100%) which cause complete hydrolysis of RBCs on blood agar, this results similar to Bunyan & Obais (2018). As shown to be cytolytic for the erythrocytes and mammalian cells in culture. All isolates are able to lysis of erythrocytes. As researchers indicate that clinical isolates had high pathogenicity were responsible for diseases occurs in human because they secrete different toxins (Yogananth *et al*., 2009). Our results reveal that *A. hydrophila* isolates were able to hydrolyze the protein by protease enzyme (100%) when tested on skim milk agar, and these results were agreed with Bunyan & Obais (2018), who indicates that *A.hydrophila* is producing protease enzyme. Protease enzyme secreted outside of the cell through a process of growth as they accumulate significantly in the phase stability of the bacteria, and it is one of virulence factors important for *A.hydrophila* bacteria (Trower et al., 2000).

All isolates were lipase producer (100%). Lipase is able to catalyze both the hydrolysis and synthesis of ester bonds of triacylglyceride, (Yang *et al*., 2011). The clinical isolates produce (80%) phospholipase. Phospholipase produced by bacteria is involved in different pathogenic process associated with intestinal damage (Scoaris *et al*., 2008).

Regarding biofilm formation, the results revealed that all *A.hydrophila* isolates were biofilm former (100%), in different degrees while in *V. cholerae* only (46%) were biofilm producer. Result agrees with Bunyan & Obais (2018), who indicates that all *A. hydrophila* isolates under study were biofilm producer. Biofilm plays an important role in the establishment of *A. hydrophila* infection, enhanced pathogenesis and drug resistance. One of the pathogenicity mechanisms of these bacteria is the formation of biofilms in their hosts, which contribute to an increase in the virulence of these microorganisms and in their resistance to antibiotics, consequently, in their survival. Bacteria succeed in forming a biofilm within the human host when the infection often turns out to be untreatable and will develops into a chronic state (Bunyan *et al*., 2017).

Biofilms formation is highly associated with resistance to environmental stresses such as starvation, desiccation, disinfectants and antimicrobial agents (Jahid and Ha, 2012).

Kesimpulan

Cholera remains one of the most prominent diseases affecting many parts of the globe, and its causative agent, Vibrio Cholerae, infects individuals of all ages. As Cholera cases once again rise all over the world, Iraq continues its struggle against this disease after its occurrence in 2015. Many factors add to the reason for Cholera’s prevalence, including poor infrastructure, lack of efficient healthcare, poor sanitation and hygiene, malnourishment, and the war-torn state of the country. For long-term improvements in health outcomes, awareness campaigns must be implemented and improvements in infrastructure and public healthcare facilities should be achieved. Further insight and research are required to develop techniques to reduce the incidence of Cholera and decrease its global prevalence.

Aeromonas is an important, often neglected pathogen capable of causing a variety of gastrointestinal tract symptoms such as acute diarrhoea and dysentery and may even mimic cholera. It is, therefore, pertinent to recognize this pathogen as an important agent in the causation of severe diarrhoea. The importance of *Aeromonas* as a gastrointestinal pathogen must be recognized, and therefore, it must be actively sought in a diarrhoea case.

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