Research Article

First Isolation of *Vibrio* spp. from Birds and Their Owners: Study of Virulence Factors of *Vibrio cholerae*

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Abstract | This research aimed to isolate *Vibrio* spp. from birds and owner samples collected randomly in Baghdad city. The samples underwent standard bacteriological examinations for the isolation of *Vibrio* spp. A total of 400 samples were included, comprising 160 cloacal swabs, 40 fresh feces samples from birds, 150 stool samples, and 50 hand swabs from owners. In the present study, *V. cholerae* accounted for 54 out of 400 samples (13.5%), *V. parahaemolyticus* for 38 out of 400 samples (9.5%), *V. vulnificus* for 14 out of 400 samples (3.5%), and *V. alginolyticus* for 23 out of 400 samples (5.75%). Four virulence factors were studied for nine isolates of *V. cholerae*. Phospholipase activity showed a positive result in 6 out of 9 isolates (66.66%), proteinase activity in 7 out of 9 isolates (77.77%), while hemolytic activity and biofilm formation were positive in all *V. cholerae* isolates, with a 100% rate. For the first time in Iraq, isolation of *Vibrio* spp. has been reported in birds and owners. This study concludes that the high percentage of *Vibrio* spp. in birds is linked to the significance of zoonotic transmission. Further investigation is required to assess the risks of *Vibrio* spp. colonization in other animals and humans in close contact.

Keywords | *Vibrio* spp, Birds, Virulence factors, Owners, Phospholipase Activity, Proteinase Activity.

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INTRODUCTION

Vibrio is a gram-negative bacterium that is motile due to polar flagella. It can exist singly or in chains forming S-shapes or spirals, and is commonly found in marine coastal waters (Abdul et al., 2017; Nayyef et al., 2017). All species of Vibrio exhibit growth stimulation from sodium ions, with oxidase-positive species being predominant. Many virulence factors in *Vibrio* spp. like outer membrane protein (OMP), lipopolysaccharide (LPS), thermostable direct hemolysin (TDH), TDH-related hemolysin (TRH), vibrioo vulnificus cytolysin (VVC), and zonula occludens toxin (Zot), caseinase, gelatinase, amylase, phospholipase, collagenase, cholera toxin (CT), toxin coregulated pilus (TCP), protease, hemolysin, and biofilm formation which were detected in many studies in *V. cholerae.* Serious illnesses are caused by some species in both humans and other animals. *V. cholerae* causes cholera, and *V. parahaemolyticus* causes acute gastroenteritis and septicemia that characterized by watery diarrhea, sometimes bloody diarrhea, together with headaches, fever, abdominal pain, nausea, vomiting, and wound infection while *V. vulnificus* cause severe gastroenteritis and potentially a deadly infection. Moreover, *V. alginolyticus* may contribute to eye and wound infections (Al-Obaidi, 2012; Abbas, 2017). *V. cholerae* can cause cholera by infecting the mucosal epithelium, which often happens in developing countries around the world. Cholera can cause death from dehydration (Abdulrazzaq, 2010; Al-thwani et al., 2010; Qamar et al., 2022).

Birds could carry pathogenic bacteria, spreading by excretion, so they considered a reservoir of *Vibrio*, and fecal-food-mouth transmission with intestinal environments.

Vibrio is transmitted to humans or animals through water sources contaminated with fecal material from infected individuals. Birds reservoirs could lead to changes in the number of bacteria in the environment, while climatic and/or environmental changes can potentially be responsible for the cholera in human populations (Zgheir et al., 2019). The aim of the present study was the isolation and identification of *Vibrio* spp. from birds and owners from different areas of Baghdad city with studying specific virulence factors like phospholipase, protease, hemolysin, and biofilm formation activity in *V. cholerae*.

MATERIAL AND METHODS

Ethical Approval

The animal care and use committee of the College of Veterinary Medicine, Baghdad University, approved the study protocols (No. 2452/PG on November 5, 2023).

Sampling

Four hundred (400) samples, including n=150 stool samples from patients (workers of bird shops and chicken restaurants) with diarrhea and n=50 hand swabs from owners of birds, and n=200 samples from birds (160 cloacal swabs and 40 fresh feces) were randomly collected from different places in Baghdad city. *Samples* were collected in containers and transported to the laboratory of microbiology in the college of veterinary medicine.

Isolation of *Vibrio* Spp.

Samples were cultured in Peptone water and incubated at 35–37 °C for 18–24 hours. After incubation, subcultures were maintained in selective media including thiosulfate citrate bile salts sucrose agar (TCBS), and Hicrome Vibrio agar. Plates were incubated under aerobic conditions at 37°C for 24 hours and examined for detection of the pure isolates. The isolates were further examined and confirmed by Grams' staining and Vitek 2 biochemical techniques (Swapna, 2017).

Detection Of Virulence Factor For *V.cholerae*

This investigation included the study of the four main types of virulence factors associated with V. cholerae, focusing on nine isolates of V. cholerae confirmed by PCR. The virulence factors assessed were phospholipase activity, protease activity, hemolytic activity, and biofilm formation ability (AL-Hadrawi et al., 2019).

Statistical Analysis

The Statistical Analysis System (SAS (2018) program was used to detect the effect of different factors on study parameters. A chi-square test was used to compare significant differences between percentages (0.05 and 0.01 probability) in this study

RESULTS

The present study reported 34 isolates with 17% as *Vibrio* spp. *V. cholerae* constituted 14 (7%) of the samples, among 150 stool samples and 50 hand swabs, followed by *V. parahaemolyticus* 8 (4%), *V. vulnificus* 7(3.5%) and *V. alginolyticus* 5(2.5%) as shown in Table 1.

The present investigation revealed that from 200 samples of birds represented by 160 cloacal swabs and 40 fresh feces, 95 isolates (47.5%) exhibited *Vibrio* spp. These results revealed Vibrio spp. infection in 40 ducks (20%), 23 chickens (11.5%), and 7 pigeons (3.5%). Additionally, 10 pet birds (5%) and 15 samples of fresh feces were found positive. Specifically, *V. cholerae* accounted for 40 cases (20%), *V. parahaemolyticus* for 30 cases (15%), *V. alginolyticus* for 18 cases (9%), and *V. vulnificus* for 7 cases (3.5%), as shown in the Table. 2. *V. cholerae* identified as curved bacilli, +ve in mucoid string test, and they also formed yellow shiny colonies on TCBS and purple color colonies on Hicrome agar, as shown in Figure 1,2,3.

Detection Of Virulence Factors Of *V. Cholerae*

The four primary types of virulence factors associated with *V. cholerae* were investigated in this investigation, and it indicated that the isolates' potential for pathogenesis may be reflected in the bacteria's capacity to create these factors. A number of virulence factors are required to be produced by *V. cholerae* during infection in order to colonize and infect hosts. Nine isolates of *V.cholerae* were employed in this investigation, depending on the results of PCR to identify the virulence factors.

Phospholipase Activity

When *V. cholerae* isolates were cultivated on egg yolk agar, the outcome of their phospholipase activity (PLA) was a zone of precipitation around the colony. Accordingly, six *V. cholerae* isolates tested gave a positive result for PLA with a 66.66% positive percentage (Table 3). In this investigation, the average Pz value among the isolates that generated phospholipase was 3.60, with different scores as shown in Figure 4.

Protease Activity

The study results revealed that 77.77% (7/9) *V. cholerae* gave a clear zone around the colony when cultured on protease media. This finding shows that these isolates had protein hydrolysis activity to varying degrees, as indicated in Table 4 and Figure 5.

Detection Of Haemolytic Activity

Investigation results demonstrated *V. cholerae* isolates had hemolytic activity of 100%, and all these isolates produced beta-haemolysis, as shown in Figure 6.

Table 1: Percentage of *Vibrio* spp. isolates from owners

Type of sample Type of *Vibrio*	Stool samples (n=150)	%	Hand Swabs (n=50)	%	Chi-Square: χ^2 (P-value)	Total number of *Vibrio* spp. isolates	%
V. cholerae	4	2.6	10	20	9.437 (0.0042)**	14	7
V. parahaemolyticus	4	2.6	4	8	2.507 (0.216)NS	8	4
V. alginolyticus	4	2.6	1	2	0.772 (0.902)NS	5	2.5
V. vulnificus	4	2.6	3	6	1.694 (0.361)NS	7	3.5
Total	16	10.6	18	36	11.67 (0.0072) **	34	17
Chi-Square: χ^2 (P-value)	---	0.00 (1.00) NS	---	10.451 (0.0079) **	---	---	

** (P≤0.01), NS (not significant).

Table 2: Percentage of *Vibrio* spp. isolation from birds

Type of birds Type of *Vibrio* spp.	Duck (60) cloacal swab	Chicken (50) cloaca swab	Pigeon (20) cloacal swab	Pet bird (30) cloacal swab	Fresh feces (40) from birds	Total number of *Vibrio* spp. isolates	%
V. cholerae	15	5	2	5	13	40	20
V. parahaemolyticus	10	8	5	5	2	30	15
V. alginolyticus	10	8	-	-	-	18	9
V. vulnificus	5	2	-	-	-	7	3.5
Total percentage (%)	20	11.5	3.5	5	7.5	-	47.5
Chi-Square: χ^2 (P-value)	11.285 (0.0013) **					---	10.833 (0.0022) **

** (P≤0.01).

Table 3: Phospholipase activity of *V. cholerae* isolated from owners and birds

Isolates of *V. cholerae*	Pz value *	Score of isolates	% of isolates that produced phospholipase	
1	-ve	-		
2	0.67	++		
3	0.68	++		
4	-ve	-		
5	0.69	++	(6/9)	66.66%
6	0.81	++		
7	0.70	++		
8	-ve	-		
9	0.50	+++		
Mean of Pz value	3.60			

*: Pz = Phospholipase activity zone

Table 4: Protease activity of *V. cholerae* isolates from owners and birds

Isolates of *V. cholerae*	Zone of hydrolysis (mm)	% of isolates that produced Proteinase	
1	+		
2	-ve		
3	+		
4	+	(7/9)	77.77%
5	+		

6	-ve	
7	+	
8	+	
9	++	

Diameter of lysis zone: + (1-2 mm lysis zone) = Mild protease activity, ++ (3-5 mm lysis zone) = Strong protease activity

Table 5: Biofilm formation ability of *V.cholerae* isolated from owners and birds

Isolates of *Vibrio cholerae*	Score of biofilm			% of isolates that formed biofilm
	Strong	Moderate	Weak	
1		0.692		(9/9) 100%
2		0.607		
3		0.699		
4			0.480	
5		0.617		
6		0.699		
7		0.727		
8		0.747		
9	0.853			

The biofilm formation was defined as none, weak (<0.480), Moderate (0.480-0.800) and strong (>0.800).

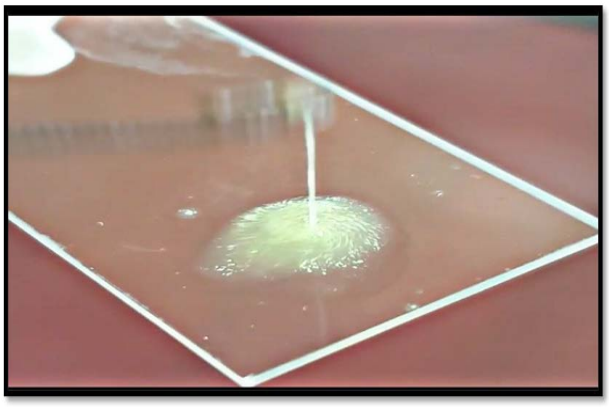

Figure 1: Positive result of string test showed a mucoid string when an inoculating loop was used to pick the suspension slowly away from the slide.

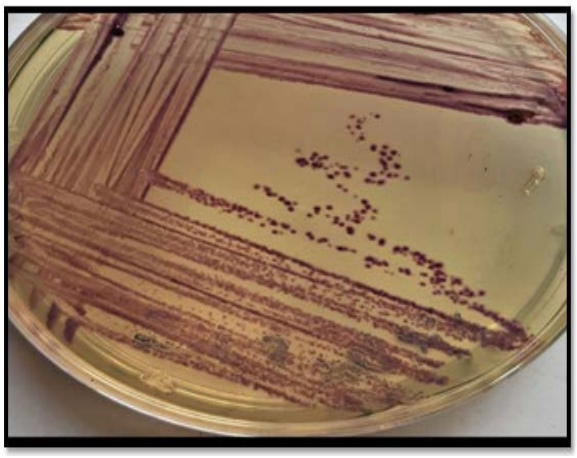

Figure 3: *V. cholerae* was cultured on Hicrome agar shows purple colored colonies after 24-48 hrs at 37°C.

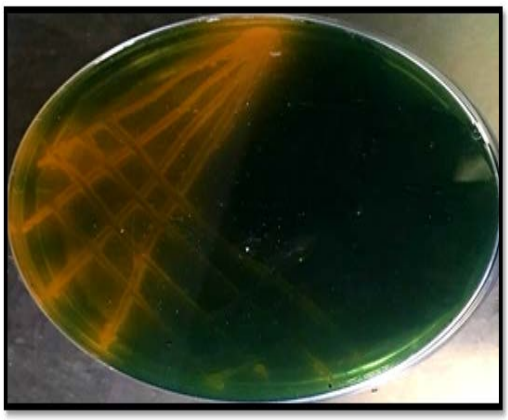

Figure 2: Cultivation of *V. cholerae* on Thiosulfate citrate bile salts sucrose agar at 37 °C for 24hrs showed clear yellow shiny colony.

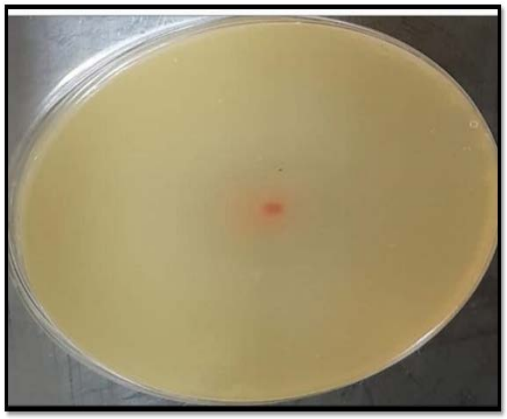

Figure 4: Activity of phospholipase of *V. cholerae*. Arrow showing precipitating zone around the colony

Figure 5: Protease activity of *V.cholerae* on BSA medium at 37°C for 48-72 hrs. Arrow showing clear zone around the colony.

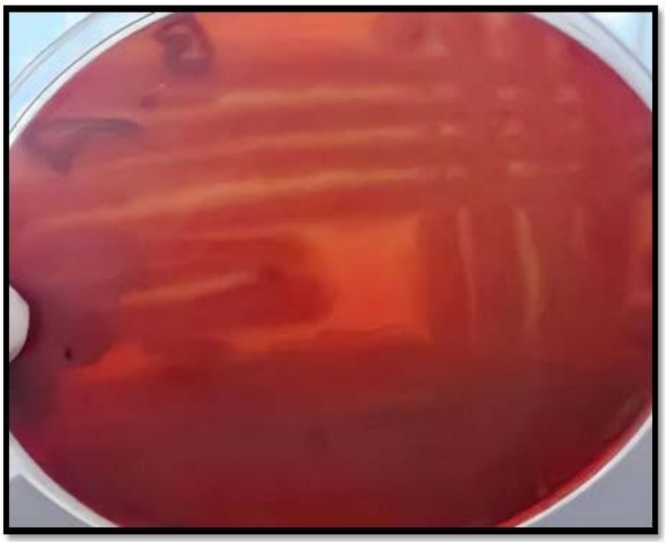

Figure 6: Haemolysin activity of *V.cholerae* when cultured on blood agar at 37°C for 48hrs. Arrow shows clear β haemolysis.

Biofilm Formation

According to Table 5, the current study proved isolates had the ability to form biofilm, with different scores ranging from weak to moderate to strong in 100% of cases.

DISCUSSION

In owners, thirty-four isolates (17%) were identified in the current investigation as *Vibrio* spp., among these 14/34 (41.1%) isolates were *V. cholerae* isolates. This finding was lower than the findings of other studies that recorded an isolation ratio of 100% of *V. cholerae* from stool samples (Goel and Jiang 2010; Abdulabbas et al., 2019). While Ramazanzadeh et al. (2015) found 81.2% and Jameel et al. (2016) recorded 62.8% *V. cholerae* infection in humans. Additionally, the results of Awuor et al. (2022) confirmed 80.2% of *V. cholerae.* However, present results were in consistent with findings reported by Hussein et al. (2017) those reported 19.88% of *V. cholerae*, in contrast to recent findings. AL-Hadrawi et al. (2019) discovered *V.cholerae* in 26.66%. On the other side, study of Zhao et al. (2022) revealed that farmer could be infected with *V.cholerae* by contact with contaminated open wounds, and McLaughlin et al. (2005) reported that 24% of patients having wounds contaminated with *Vibrio* and Patel1 et al. (2018) reported 7.40% *prevalence of V. parahaemolyticus* in the hand swabs of handlers. Also Akond et al. (2008) reported 13.2% incidence in the hand swabs.

In the present study, the most often isolates were *V.cholerae*, *V.vulnificus, V.alginolyticus and V.parahaemolyticus*. According to previous studies that were performed on *Vibrio spp. isolation* of various isolates of these species was performed from birds and avian habitat (Ayala and Ogbunugafor, 2023; Islam et al., 2023). Additionally, Other research focused on the *V. cholerae* that was isolated from domestic or wild birds (Aberkane et al., 2015; Cardoso et al., 2018; Hirsch et al., 2020, Zheng et al., 2021).

In birds, the present study showed 47.5% isolation of *Vibrio spp.* This ratio is in close proximity to other findings. According to studies of (Periasamy et al., 2013; Songzhe et al., 2019; Páll et al., 2021) *Vibrio* spp. were isolated from poultry, poultry waste and bird feces with 40%, 40%, and 44.7%, respectively. Whereas other studies showed a lower percent of *Vibrio spp*. isolation when compared with the current research, they discovered 16% to 29.63% *Vibrio spp.* in birds feces (Keshav et al., 2010; Akter, et al., 2022). The lowest ratio was found in the study of Miyasaka, (2006) who recorded 6.66% of *Vibrio s*pp. isolated from avian excrement. In the case of *V. cholerae,* Hirsch et al. (2020) isolated only 4% in duck samples. All of the conventional method of isolation matched those of Balogu et al. (2019) who discovered *Vibrio spp. in* chicken with 59%, and Zheng et al. (2021) who recovered 14.55% *Vibrio* isolates from the feces of birds and water samples. While Azwai et al. (2016) isolated *Vibrio spp.* from chicken burger samples with 5% occurrence rate. According to a study of Al-Shammari et al. (2019) *V. cholerae* was isolated from fish specimens with 89% prevalence percentage. On the other hand, Patel1 et al. (2018) obtained 3.33% *V. parahaemolyticus* from freshwater and shrimp samples. Other studies like Hernández-Díaz et al. (2015) and Velazquez-Roman et al. (2012) reported 2.4% to 7.7% of *V. parahaemolyticus* in stool samples, and 85% of the *V.cholerae* isolates from cases of watery diarrhea were discovered by AL-Hadrawi et al. (2019). Even though these conventional methods are time-consuming and less accurate, misdiagnosis does happen occasionally.

According to the current study, 66.66 percent of the *V. cholerae* were phospholipase producers. The present findings conform to those carried out by Al-Thwani et al. (2005), who discovered that 66% of isolates were capable of producing phospholipase. While other studies reported higher percentage of phospholipase production when compared with the present study. Other workers like Bueno, (2011); Fernández-Abreu et al. (2017); AL-Hadrawi et al. (2019) and Awuor et al. (2022) found phospholipase producers among *V.cholerae isolates* with percentages of 85.7%, 77.6%, 76%, and 73%, respectively. The production of phospholipases is related to the virulence of bacterial isolates. Arachidonic acid is released by this enzyme and has a significant role in the development of diarrhea. So, phospholipases are linked to virulence in bacterial illnesses, as indicated by Oliver and Kaper, (2007). Phospholipases are essential for the pathogenesis of some microorganisms because they help certain bacteria pass the host defense system and can be involved in host cell invasion. According to reports, the substance released by phospholipid hydrolysis by PLs is crucial for host cell penetration and cell lysis, as discussed by Kumari Bandana et al. (2018).

In the this study, 77% of the *V. cholerae* displayed protease activity, which is consistent with the findings of studies carried out by Al-Thwani et al. (2005) and Abbass, (2006) who discovered that 72.7% of *V. cholerae* had this activity, and Awuor et al. (2022) discovered that 71.42% of *V. cholerae* isolates generated protease. While 50% of the isolates were recorded by Al-Khafaji, (2007) as being protease positive. Additionally, according to, AL-Hadrawi et al. (2019) 83% of the *V. cholerae* appeared to generate enzymes. Based on the hydrolysis findings of many studies, the variations are attributed to varying virulence factor activities of *V. cholerae* isolates, which are crucial to the pathogenicity of the disease, act as disease-causing toxins and may be necessary for bacterial survival, as pointed out by Namdari et al. (2000) and Zheng et al. (2021).

The results of this study may suggest that this organism's capacity to produce haemolysin is related to the hallo zone around the colony on media, as mentioned by Pulungsih, et al. (2006) and AL-Fatlawy et al. (2017). The findings of this study concurred with those reported by (Namdari et al., 2000; Al-Thwani et al., 2005; AL-Hadrawi et al., 2019; Awuor, et al., 2022) who reported 100% heamolytic activity. While 40% of *Vibrio* isolates from seafood samples that exhibited β-heamolytic activity was recorded by Sirijan Santajit et al. (2022).

The development of biofilm formation by *V. cholerae* has a role in the pathogenesis and transmission of diseases that can be fatal to humans and develop resistance to antimicrobial drugs. The development of biofilms during an infection may speed up *V. cholerae* in the small intestine and spreeding by the fecal-oral pathway (Silva and Benitez, 2016). Numerous studies were conducted to determine *V. cholerae* ability to produce biofilms like Nandita et al. (2019).

The current result supports a recent study that found that all isolates of *V. cholerae* that were isolated could produce biofilm. On the other hand, some studies documented lower percentage of biofilm producers. *V.cholerae* isolates of some studies compared with the present study such as Balogu et al. (2019) and Sirijan Santajit et al. (2022) who found 44.6%, and 36.4% of *V.cholerae* had the ability to form biofilm respectively. *V.cholerae* biofilm increase resistance against stress (osmotic and oxidative) and protection from predation by phages and protozoa. Biofilm formation played a role in outbreaks of cholera as mentioned by (Matz et al., 2005; Alam et al., 2006; Faruque et al., 2006; Zgheir et al., 2019). The agreement or discrepancy between previous studies and the current study may be attributable to prior exposure, and an unknown degree of immunity, location and habitat type. Infections occur in areas contaminated by the waste of humans or animals, potentially spreading *Vibrio* spp. to other regions. The seasonality of research, sample collection, and diagnostic methods all have a relationship with the fluctuation in isolation rates. These variations may be linked to climatic changes such as high temperatures, hurricanes, heavy rains, floods, salinity fluctuations, and organic nutrient contamination. This could be attributed to a lack of health awareness, as well as interactions among the samples from the animals, the owners, and the environment (such as stool). The presence of *Vibrio* spp. may be linked to the use of water from contaminated sources, or to a lack of awareness among bird owners and individuals in contact with them regarding the importance of controlling the spread of epidemics and the transmission of diseases from birds to humans. Consequently, environmental contamination with bird feces may contain *Vibrio* spp. It is spread by eating or drinking food or water contaminated by the feces of birds or the stools of infected humans (Miyasaka et al., 2006; Dijk et al., 2019).

CONCLUSIONS

This study found that *Vibrio spp.* are prevalent in birds and owners. The study further exhibited that *Vibrio spp. are* widespread in Baghdad city with high percentage prevalence in birds. Keeping in view the importance of zoonosis of *Vibrio* infections, further investigation is necessary to evaluate the hazards of *Vibrio* spp colonization in other animals and handlers.

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NOVELTY STATEMENT

The novelty of the study is the focus on isolation of *Vibrio spp.* from birds and owners which is the first isolation in Iraq and studying virulence of *V. cholerae* isolates.

AUTHORS CONTRIBUTION

These authors each contributed equally

CONFLICT OF INTEREST

The authors have declared no conflict of interest

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