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Virulence characteristic and antibiotic susceptibility of Aeromonas salmonicida isolated from patients with gastroenteritis and urinary tract infections in Kirkuk City- Iraq

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Abstract

Aims: Aeromonas salmonicida is the causative agent for furunculosis, which is a superficial skin infection in fish, especially in Salmonids; hence named salmonicida. It is a rare but potential pathogen for human infection; it has been associated with cases of blood, skin, eye, endocardial, gastrointestinal, and urinary tract infections. Therefore, the present study aimed to determine the prevalence of: virulence, biofilm formation, and resistant patterns and to detect beta-lactamase enzyme production phenotypically.

Methodology and results: A total of three Aeromonas isolates were included in this study. Two isolates recovered from stool samples of patients with gastroenteritis, and one isolate recovered from patients with urinary tract infections. These three isolates were identified as Aeromonas salmonicida using a GNID card in the Vitek 2 compact system. The antibiotic susceptibility testing was performed using the disc diffusion method. A double disc synergy test was applied to determine the presence of extended-spectrum B-lactamase (ESBL) production. Carbapenemase was screened using the modified Hodge test (MHT) and boronic acid test. Five virulence factors, including protease, lipase, esterase, hemolysins, and biofilm production, were studied. All three isolates exhibited multiple drug resistance patterns (MDR). ESBL and carbapenemase enzymes were seen in stool A. salmonicida isolates only. All three isolates showed multifactorial virulence factor activity.

Conclusion, significance, and impact of the study: ESBL and carbapenemase-producing A. salmonicida emerged as relevant human intestinal pathogens with a variety of pathogenicity factors, so this study emphasizes on the importance of active surveillance plans to control infection and the spread of this pathogen.

Keywords: Aeromonas salmonicida, β-lactamase enzymes, extracellular enzyme production.

INTRODUCTION

Aeromonas salmonicida is Gram-negative bacillus, facultative anaerobic, non-spore-forming bacteria. It was first discovered in the Bavaria brown trout hatchery by Emmerich and Weibel in 1984 (Bora et al., 2016). It inhabits a variety of aquatic environments, including fresh water, sea water, and sewage, it has been isolated from drinking water, hospital wastewater, and aquatic animals, especially fishes (Oladele and Temitope, 2016; Drk et al., 2023). It has been well known for decades to be a fish pathogen infecting cold-blooded vertebrates living at low temperatures (22-25°C), mainly salmonid fish, hence named salmonicida(Kim et al., 2011; Vincent et al., 2019). Therefore, it may be transmitted to humans by all those sources. A. salmonicida has been reported to cause various diseases in humans, such as septicemia, furunculosis, gastroenteritis, and eye infections (Tewari et al., 2014; Kamble., 2015; Varshney et al., 2017).

Pathogenicity of Aeromonas species is associated with several virulence factors, such as adhesions, extracellular hydrolytic enzymes, toxins, and biofilm formation, which allow them to colonize, invade, and infect numerous hosts (Tomás, 2012; Bernabè et al., 2023).

The serious concern reported in Aeromonas spp. and other Gram-negative bacteria is the rapid incidence of resistance to diverse groups of commonly used antibiotics, which have critical importance to human health (Shareef and Ghareeb, 2017; Al-kakai and Shareef, 2022).For instance , isolates of Aeromonas have shown relatively high resistance to β- lactamase antibiotics such as cephalosporins and carbapenems (Piotrowska et al., 2017; Zdanowicz et al, 2020). So, this study aimed to isolate and identify Aeromonas spp. from clinical samples, identify the factors that contribute to their virulence, determine the isolates resistance pattern, and phenotypic screening of β -lactamase enzymes and investigate the ability to form biofilm.

MATERIALS AND METHODS

Bacterial isolation and identification

From December 2023 to March 2024, a total of 49 clinical specimens, including stool (13) and urine (36), were collected from patients attended hospitals in Kirkuk city. All clinical samples were cultured on MacConkey agar, blood agar, and nutrient agar. Plates were incubated at 37°C for 24 hours. *Aeromonas salmonicida* was identified by Gram staining, culture characteristics of the colonies on agar plates, and by) biochemical tests using catalase, oxidase, IMViC, and urease. The identifications of the *A. salmonicida* isolates were confirmed by using the VITEK 2 Compact GN card system (Biomerieux, France), according to Australian and New Zealand Standard Diagnostic Procedures (ANZSDP) for *Aeromonas salmonicida* identification (Buller et al., 2021).

Phenotypic detection of some virulence factor

production Detection of extracellular enzyme

production

Lipase production

A. salmonicida were grown on the egg-yolk agar medium, which was made by (melting the brain heart infusion agar (BHIA), cooling to 55 C, and adding the egg yolk suspension). After 24 hours of incubation at 37°C, Copper II Sulphate ($CuSO_4$) solution was poured into plates and left for 10 minutes; then, the excess solution was removed and the plates were dried in the incubator. The formation of a blue-green color halo around the colonies was considered an indicator of lipase production by the isolates(Wai *et al*., 2016).

Protease production

Protease production was detected by streaking *A. salmonicida* isolates on nutrient agar plates containing 10% skim milk agar, and then the plates were incubated at 37°C for 24 hr. A clearance halo zone around the streaks has been confirmed to be positive for protease production (Ayoub *et al.*, 2024).

Esterase production

Tween 80 agar medium was prepared according to Salm and Said (2019). *A.salmonicida* isolates were inoculated on Tween 80 agar plates and incubated at 37°C for 24-48 hrs. formation of precipitates around bacterial spots considered positive results (Noor *et al.*, 2020).

Hemolysin production

The hemolytic activity was detected by inoculating the *A.salmonicida* isolates on blood agar plates; the plates were incubated at 37c for 24 hours and then tested for hemolysis around the colonies. The results were recorded as follows: α-hemolysis (greenish zone), β-hemolytic (clear zone), or γ-hemolysis (no hemolysis) (Oladele and Temitope, 2016).

Biofilm formation

It was detected using Congo red agar (CRA) and broth (CRB) assays, which were prepared as previously described by Cho et al.(2022). A loopful inoculum of the bacterial isolates from overnight broth culture was streaked onto Congo red agar plates, and one colony of isolates on

CRA was inoculated in the CRB for the broth test. Brown to black colours after 24 hrs at 37°C were considered positive for biofilm formation.

Antibiotic susceptibility test

The test was performed by the disc diffusion method using commercially available discs according to CLSI. (2023). Antibiotic discs include piperacillin (100 µg), ceftazidime (30 µg), ceftriaxone (10 µg), cefotaxime (10 µg), cefepime (10 µg), gentamicin (10 µg), amikacin (10 ug), aztreonam (30 µg), meropenem (10 µg), imipenem (10 µg), tetracycline
(10 µg), levofloxacin (5 µg), and ciprofloxacin (10 µg).

Screening of β -lactamase enzymes:

-Screening for Extended Spectrum Beta-Lactamase Production (ESBL)

The Double Disc Synergy Test (DDST) method was used for the detection of ESBLs that are inhibited by beta-lactamase inhibitors such as amoxicillin/clavulanic acid. Mueller-Hinton agar (MHA) plates were inoculated with a 0.5 MacFarland bacterial suspension using a sterile cotton swab, and an amoxicillin/clavulanic acid disc (20 µg amoxicillin
+ 10 µg clavulanic acid) was placed at the center of the plates. Then discs of ceftazidime, imipenem, ceftriaxone, cefotaxime, cefepime, and aztreonam were placed around the center amoxicillin/clavulanic acid disc, and the plates were incubated at 37°C for 24 hours. The DDST is considered a positive result for ESBL production if synergy with clavulanate occurred with any one of the antibiotics (enlargement of the zone of inhibition) (Georgios *et al*., 2014; Drk *et al.*, 2023).

-Screening for carbapenemase production

Two methods were used to reveal carbapenemase production:

Modified Hodge test

This test was used to detect the presence of cabapenemase only without discriminating between the type of carbapenemase. The test was achieved by inoculating the study isolates together with a carbapenem -susceptible indicator strain, such as E. coli, and evaluating the distortion of the indicator strain inhibition zone because of carbapenemase production by the study isolates. MHA was inoculated with a 0.5 McFarland suspension of E. coli, and a meropenem disc was placed at the center of the plate. Then 3-5 colonies of test isolates were streaked from the center to the periphery of the plate in a straight-line direction, and the plates were incubated at 37°C for 24 hours. The presence of a distorted inhibition zone due to the growth of the E. coli toward the meropenem disc is interpreted as a positive result (Pasteran *et al.*, 2011; Georgios *et al*., 2014).

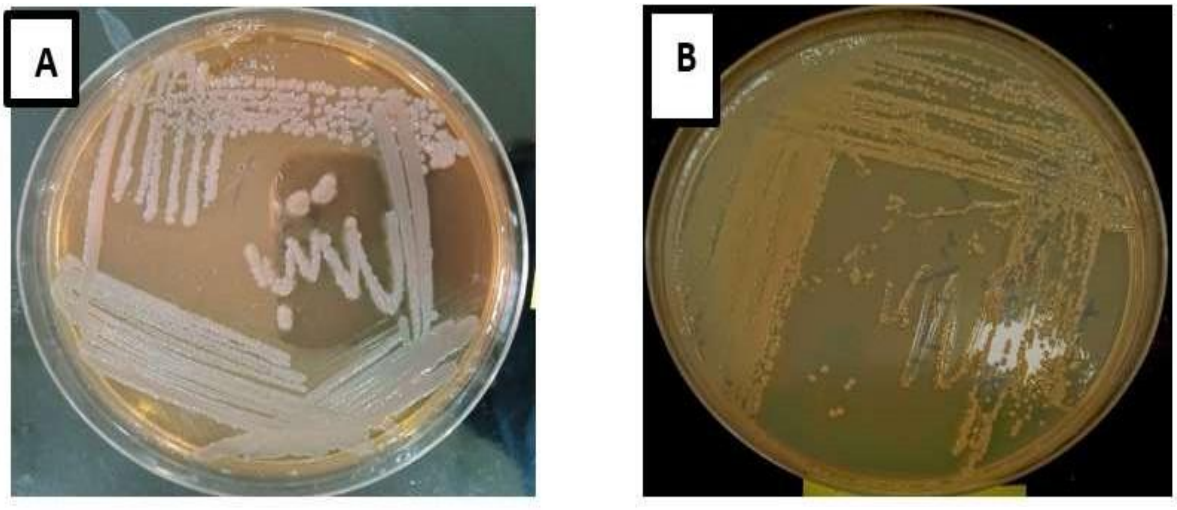
Phenylboronic acid test

This test is used for detection of KPC /carbapenemase production by the study isolates. MHA plates were inoculated with a 0.5 McFarland suspension of *A. salmonicida* isolates, then Two discs of meropenem were placed on the plate; 20 µL of phenylboronic acid (20 g/L) was added to one of the meropenem discs; then the plates were incubated at 37
°C for 24 hours. The test is considered positive when the inhibition zone of the meropenem + phenylboronic acid is ≥ 5 mm larger than the inhibition zone of meropenem alone (Georgios et al., 2014; Drk *et al.*, 2023).

RESULTS AND DISCUSSION

Isolation and Identification

total of 3 isolates (2%) of A. salmonicida were isolated (two isolates from stool samples and one isolate from a urine sample). All isolates were characterized regarding macroscopic and microscopic examination. On MacConkey agar, A. salmonicida appears as small, pale, and lactose nonfermented colonies (Figure 1A). As well as producing pigmented brown colonies on nutrient agar medium (Figure 1B). All isolates were Gram-negative, rod-shaped bacteria. These findings match those of (Dallaire-Dufresne et al., 2014; Buller et al., 2021; Bakiyev et al., 2023).



Non-lactose fermenter colonies of *A. salmonicida* on MacConkey agar.

Gray colonies of *A.salmonicida* on nutrient agar

Figure1: Cultural characteristics of *A.salmoncida* on media

Regarding biochemical characterization, Table 1 shows all isolates were positive for catalase, oxidase, and methyl red tests, while they showed negative results for indole, Voges-Proskauer, citrate

utilization test, urease production, and the motility test. This result is in line with the finding of Buller et al. (2021) and Bakiyev et al. 2023. The isolates were identified as A. salmonicida with 93-94% probability and excellent identification confidence using the GNID card in VITEK 2 automated identification as shown in Figure 2.

Table 1: The biochemical profile of *A.salmonicida* isolates

Biochemical tests	***A.salmonicida* isolates and its source**		
	***A. salmonicida*-1 (stool)**	***A. salmonicida*-2 (stool)**	***A. salmonicida*-3 (urine)**
Catalase	+	+	+
Oxidase	+	+	+
IMVIC			
Urease	-	-	-
Indole	-	-	-
Methyl red	+	+	+
Voges-proskauer	-	-	-
Citrate utilization	-	-	-

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Patient Name: 46 b, . Patient ID: rturtuj
Location: Physician:
Lab ID: 51 Isolate Number: 1

Organism Quantity:
Selected Organism : Aeromonas salmonicida

Source: **Collected:**

Comments:	

Identification Information	**Analysis Time:**	9.87 hours	**Status:**	Final
Selected Organism	94% Probability **Bionumber:**	**Aeromonas salmonicida** 0001000000000200		
ID Analysis Messages				

Biochemical Details

2	APPA	-	3	ADO	-	4	PyrA	-	5	lARL	-	7	dCEL	-	9	BGAL	-
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	-	15	OFF	-
17	BGLU	-	18	dMAL	-	19	dMAN	-	20	dMNE	-	21	BXYL	-	22	BAlap	-
23	ProA	-	26	LIP	-	27	PLE	-	29	TyrA	-	31	URE	-	32	dSOR	-
33	SAC	-	34	dTAG	-	35	dTRE	-	36	CIT	-	37	MNT	-	39	5KG	-
40	ILATk	-	41	AGLU	-	42	SUCT	-	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	-	48	LDC	-	53	lHISa	-	56	CMT	+	57	BGUR	-
58	O129R	-	59	GGAA	-	61	IMLTa	-	62	ELLM	-	64	lLATa	-			

Figure 2: Results of biochemical characteristic details of A.salmonicida by Vitek 2 compact system

To the best of our knowledge, this is the first report of gastroenteritis and urinary tract infection (UTI) caused by A. salmonicida in Kirkuk City, Iraq. The results showed that out of 13 stool samples, 2 isolates (15.4%) of A. Salmonicida were recovered from stool samples of gastroenteritis patients with watery diarrhea, our results agree with those reported by Oladele and Temitope. (2016) in Nigeria, who noticed a higher rate of A. Salmonicida isolation from

diarrhoeagenic stool samples. Also, the study recovered 1 isolate (2.7%) from out of 36 urine samples from patients with UTI infection. Exposure to a contaminated water source, either through drinking water or consumption of fish, appears to be the most important risk factor associated with A. salmonicida infection (Moore et al., 2017; Salehi et al., 2019) . However, in the current study cases, the source of infection is unknown.

Generally, A. salmonicida Causes furunculosis and sepsis in the fish, the recent enhanced isolation of this organism from various human infections may be attributed to increased availability of automated identification systems such as the Vitek 2 compact system, matrix-assisted laser desorption ionization time of flight (MALDI-TOF), and PCR probes, along with increased awareness about rare organisms, especially in immunocompromised cases(Moore et al., 2017; Lodha et al., 2022).

Previously numerous cases of human infection caused by A. salmonicida were reported. In India, it was recovered from blood, skin, postoperative endophthalmitis, and urinary tract infection in AIDS patients (Tewari et al., 2014; Kamble, 2015; Varshney et al., 2017; Lodha et al., 2022). In Iran, a case of endocarditis was reported (Salehi et al., 2019). In Spain, two clinical strains of A. salmonicida were isolated from human patients, one that suffered from acute gastroenteritis and the other that had cellulitis in the right foot after trauma (Vincent et al., 2019). In China, isolated from HIV patients with acute febrile illness (Yang et al., 2020). In the USA, cases of bacteremia in patients with diabetes and chronic kidney disease and from colonic polyps and benign prostatic higher plasia patients due to well water consumption (Katz et al., 2015; Moore et al., 2017).

Phenotypic Virulence Properties of A. salmonicida Isolates

Table 4 shows the various phenotypic virulence properties of A. salmonicida identified in this study; stool A. salmonicida isolates were hemolysin, protease, lipase, and esterase producers while urine isolate only produce protease and esterase (Figure 4). Virulence factors associated with extracellular products are crucial for translocation in the epithelium, and the presence of these virulence-positive A. salmonicida isolates poses a serious risk to the public's health. Most studies characterizing virulence factors associated with Aeromonas spp. pathogenicity has been performed in strains isolated from environmental sources such as polluted and drinking water and from human diarrheal samples (Tahoun et al., 2016; Pessoa et al., 2019; Bernabè et al., 2023). The extracellular enzymes, such as hemolysins, protease lipase, aerolysin, and various toxins, have been detected genotypically and phenotypically in many studies on A. salmonicida over the years (Sen and Rodgers, 2004; Beaz- Hidalgo and Figueras, 2013; Vincent et al., 2019; Chen et al., 2022; Bakiyev et al., 2023). In the current study, biofilm formation was reported in all three isolates (Table 2 and Figure 4). Several previous studies demonstrated that Aeromonas is characterized by the ability to form biofilm on the biotic or abiotic surfaces that

increase their virulence and pathogenic Capacity and resistance to antibiotics (Hoel et al., 2017; Dias et al., 2018; Bernabè et al., 2023).

Table2: Phenotypic virulence characteristic of A. salmonicida isolated from stool and urine source

Code of isolate and its source	Protease	Lipase	Esterase	Hemolysins	Biofilm formation
***A.salmonicida* -1(stool)**	+	+	+	+	+
***A.salmonicida* -2(stool)**	+	+	+	+	+
***A.salmonicida* -3(urine)**	+	-	+	-	+

lipase positive test on egg-yolk agar showing blue -green colour zone around colonies

Positive protease test of *A.salmonicida* on skim milk agar showing zone of hydrolysis

Esterase positive test on Tween 80 agar plate showing zone of precipitation

Hemolysine positive test on blood agar showing B-hemolysis

Figure 3: Extracellular enzyme production by *A.salmoncida* isolates on different culture media.

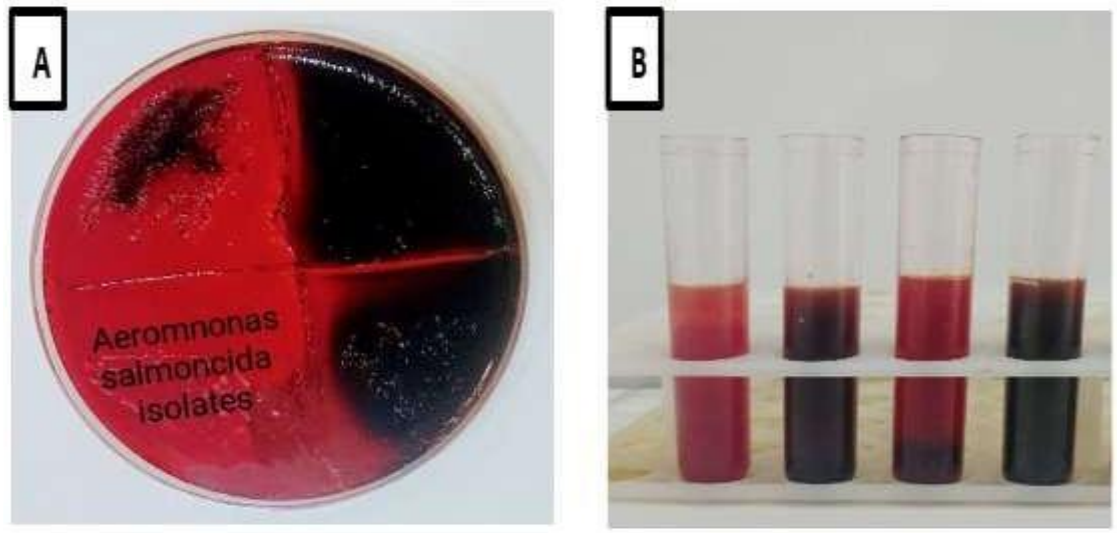

Figure 4: Screening of biofilm formation by *A.salmonicida* with Congo red test .(A)Representative image of Congo red with agar plate.(B)with broth culture.

Antibiotic susceptibility test

The antibiotic susceptibility of *A. salmonicida* isolates to 13 antibiotics (seven classes) was investigated, as shown in (Table 3). The antibiotic to which the three isolates exhibited 100% resistance was piperacillin. (Penicillin class), cefotaxime, ceftazidime, and ceftriaxone (third generation Cephalosporins), and tetracycline, while they were susceptible to cefepime (fourth generation Cephalosporin), aztreonam (Monobactam), amikacin (Aminoglycosidase), ciprofloxacin, and levofloxacin (Quinolones). However, the susceptibility to other antibiotics varied, resistance to imipenem and meropenem (carbapenem antibiotics) was found among two stool isolates (Table 3), while the urine isolate showed resistance to imipenem and gentamicin (Table 3).

Table3: Susceptibility to antibiotics of A.salmonicida against 13 antibiotics according to CLSI

Class of antibiotic	Antibiotic agent	*A.salmonicida* Isolates		
		1	2	3
Penicillin	**Piperacillin (PIP)**	R	R	R
Cephems -Third generation Cephalosporin -Fourth generation Cephalosporin	**Cefotaxime (CTX)**	R	R	R
	Ceftazidime (CAZ)	R	R	R
	Ceftriaxone (CRO)	R	R	R

	Cefepime (FEP)	S	S	S
Monobactam	**Aztreonam (ATM)**	S	S	S
Carbapenems	**Imipenem (IPM)**	R	S	R
	Meropenem (MEM)	R	R	S
Aminoglycosides	**Amikacin (AK)**	S	S	S
	Gentamicin (GN)	S	S	R
Quinolones	**Ciprofloxacin (CIP)**	S	S	S
(fluoroquinolones)	**Levofloxacin (LEV)**	S	S	S
Tetracyclines	**Tetracycline (TE)**	R	R	R

In agreement with our study, Lodha et al. (2022) reported similar susceptibility to amikacin, ciprofloxacin, and levofloxacin and resistance to piperacillin, imipenem, and meropenem. There is a report of resistance to tetracycline in a patient with A. Salmonicida septicemia (Bora et al., 2016). Resistance of A. Salmonicida, to most antibiotics used in the treatment of human infections, has been recognized as a serious concern owing to their potential health risk to humans and animals (Oladele and Temitope, 2016; Amos, 2018). However, antimicrobial resistance seems to differ between strains isolated from different geographic environments and clinical sources.

In this study, all A. salmonicida isolates were resistant to more than one or three antibiotics. Which recovered that the multiple antibiotic resistance (MAR) index was high, as shown in Table 3; the highest MAR index was recorded for the urine A-salmonicida (0.538) against CTX, CRO, CAZ, Pip, GM, TE, and IPM antibiotic groups, followed by 0.46 against CTX, CAZ, PIP, TE, IPM, and MEM for stool A. Salmoncida-1 and against CTX, CRO, CAZ, Pip, TE, and MEM for stool A-Salmoncida-2 (Table 4). An isolate is considered MDR if it is resistant to at least one agent in three or more antimicrobial classes. In addition, the MAR index was calculated by dividing the number of antibiotics to which the bacteria were resistant by the total number of studied antibiotics (Krumperman, 1983; El- Hossary et al., 2023).

Table 4: Multi-Antibiotic Resistance Index of A.salmonicida and source of it.

Bacterial code and source	No.of resistant antibiotics	Resistance profile	No.of antibiotic classes	MAR index
A. salmonicida-1 (stool)	6	CTX, CAZ, PIP, TE, IPM, MEM	4	0.46
A. salmonicida-2 (stool)	6	CTX, CRO, CAZ, PIP, TE, MEM	4	0.46
A. salmonicida-3 (urine)	7	CTX, CRO, CAZ, PIP, TE, GN, IPM	5	0.538

All A. Salmonicida isolates showed piperacillin and third-generation cephalosporin resistance, which may be attributed to the lactamase enzyme produced by A. Salmonicida. Production of beta-lactamases is the most prevalent mechanism of resistance against β -lactam antibiotics. Three classes of chromosomally mediated β -lactamases are Class B-metallo- β -lactamases (MBLs), Class C- AmpC- β -lactamases, Class C- Penicillinases and acquired Class A. Extended-spectrum β-lactamases (ESBLs) have been increasingly reported in both clinical and environmental Aeromonas spp. (Chen et al., 2012; Bhowmick and Bhattacharjee, 2018; Hilt et al., 2020).In the current study, three phenotypic methods, the modified Hodge test (MHT) and phenylboronic acid test (KPC/carbapenemase), were used to detect carbapenemase β-lactamase production, and the double disc synergy test (DDST) was used for class A ESBLs production in all A-Salmoncida isolates. the result showed that stool isolates tested strongly positive by MHT and urine A-Salmonicida isolate gave a negative result (Figure 5). Our results are in agreement with the work of several previous studies that demonstrated the presence of β - lactmase enzymes phenotypically and genotypically in Aeromans strains from human , animals and in aquatic environments (Wu et al., 2011, 2012; Rosso et al., 2019; Hilt et al., 2020; Drk et al., 2023).

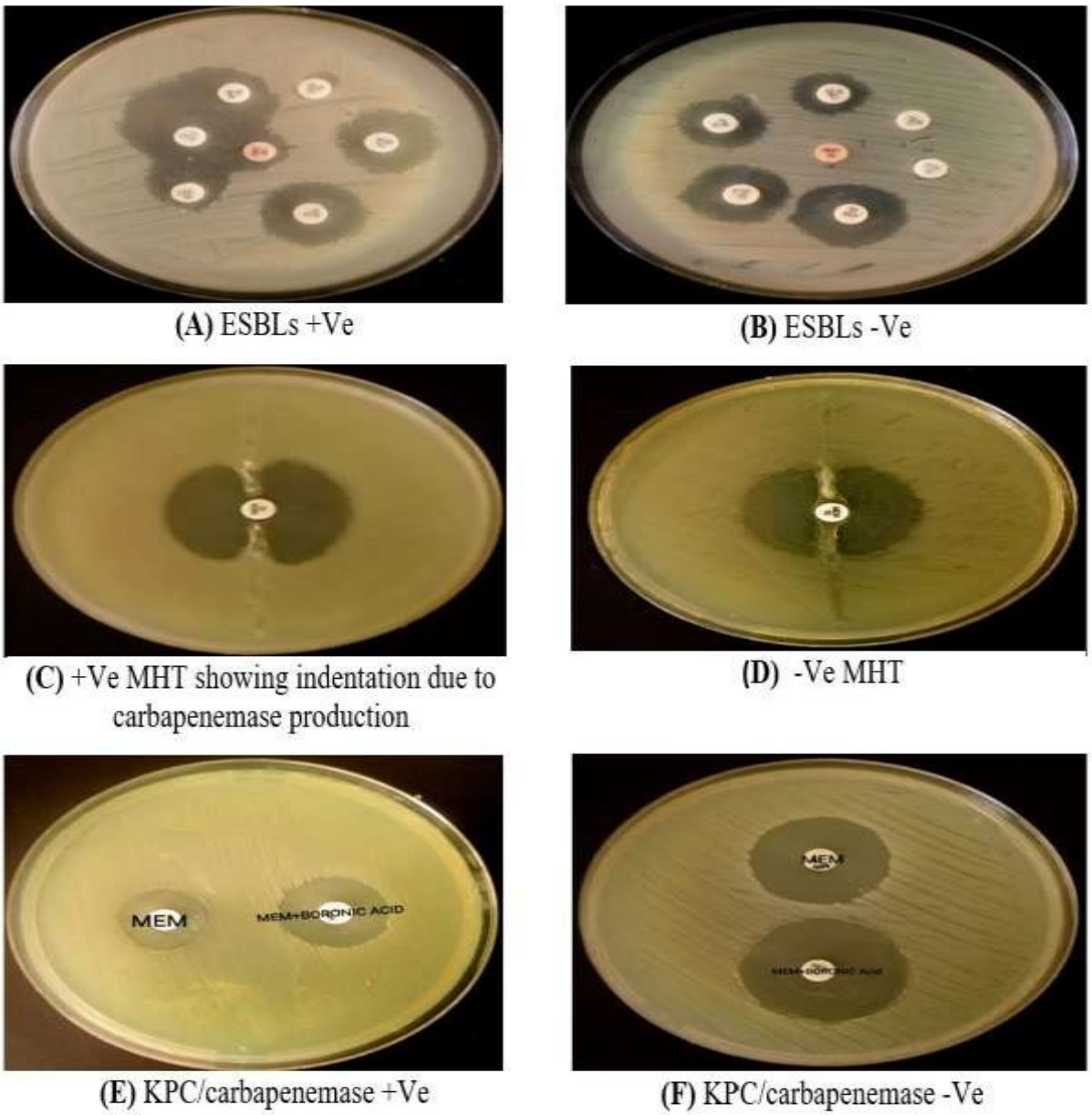

Figure 5: Phenotypic results of β-lactamase test of *A. salmonicida* isolates

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