How to Cite:
Awad, N., & Al-saadi, M. J. (2022). Isolation and molecular diagnosis of Citrobacter freundii in raw meat (beef, mutton and fish) in AL-Rusafa district of Baghdad city. *International Journal of Health Sciences*, *6*(S8), 1482–1491.
https://doi.org/10.53730/ijhs.v6nS8.10026

Isolation and molecular diagnosis of Citrobacter freundii in raw meat (beef, mutton and fish) in AL-Rusafa district of Baghdad city

Noor Awad
Department of Public Health, College of Veterinary Medicine, University of Baghdad, Iraq
Correspondence author email: Nanynoor7@gmail.com

M. J. Al-saadi
Department of Public Health, College of Veterinary Medicine, University of Baghdad, Iraq

Abstract*---Citrobacte freundii*** one of the important foodborne contaminated bacteria in meat and fish, while in Iraq there are limited researches about this bacterium, for that, the objectives of this study include isolate, diagnosed and molecular characterization of *Citrobacter freundii* in raw meat (beef, mutton) and carp fish samples collected from local markets and different butcher's shop in a AL-Rusafa district of Baghdad city. One hundred and fifty samples were collected aseptically as 50 samples from each of meats type, there were submitted to laboratory examinations, to culture on routine bacteriological media as a first step for bacterial diagnosis. Bacterial identification done by biochemical test and confirmed by Vitek-2 and Molecular assays (polymerase chain reaction (PCR)). Result showed there are 23 from 150 samples having *Citrobacter freundii*. Highest *C. freundii* percentage found in carp samples at (36%), flowed by mutton and beef (6%, 4%) respectively. We conclude that carp fish represent one of the important sources for bacterial foodborne disease where it used as a maine source of proteins and minerals in Iraq.

***Keywords*---**Citrobacter freundii, raw meat, fish, Vitek-2.

Introduction

Meat at all kinds (large animals, poultry and aquatic animals) is one of the most valuable and beneficial food sources, where, it consists of protein, amino acids, mineral salts, fats, fatty acids, vitamins in addition to carbohydrates (Mansour *et*

al., 2019). Raw meat contributes by 90% to the transmission of pathogens،which responsible of food-borne illness in humans especially bacterial agents due to its chemical characteristics leading to expansion of a wide range of microbial communities (Bughti *et al.*, 2017; Nagarajan *et al.*, 2018; Rasheed, 2012). Typically, meat of healthy animals is sterile; so microbiological quality of any meat kinds depends on the health and physical status of the animal. Bacterial contamination of raw meat can come from both endogenous and exogenous sources, endogenous gets from blood, gastrointestinal contents, feet, hide or skin, at the point exogenous contamination of meat carcasses during slaughtering and processing is an inevitable process. Through slaughtering procedures dressing, evisceration, processing, and storage, hands, clothes of employees, equipment, and water used for washing carcasses, as well as dust and air (Sallam *et al.*, 2021; Uzeh *et al.*, 2021). On the other hand, fish contaminated with bacteria can easily come through surrounding environment like water, sediments and feeding behavior, also infected during careless handling at landing sites, storage and transportation thus leading to lower quality of fish and it related products. Gills and skin, as well as , digestive tract and internal organs acting as one of the major vehicles for the transmission of pathogenic bacteria (Chatreman *et al.*, 2020; Sheng & Wang, 2021). *C. freundii* one of the foodborne pathogens associated with several opportunistic infections, such as severe diarrhea, pediatric diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS), urinary tract infections, pneumonia, neonatal meningitis and brain abscesses in humans, related infections infants and immunocompromised patients (Bunyan, 2020; Mohammed & Al-Samarraae, 2021; AL-Nassry, 2011). as well as, its responsible of nosocomial outbreaks involving clonal drug-resistant isolates have also been observed in healthcare settings (Pletz *et al.*, 2018). Depending on the previous researches *Citrobacter freundii* have medical and economical important for that this study aimed to investigate contamination percentage in raw meat (beef, mutton, fish) with *C.freundii* in Al_Rusafa district of Baghdad.

Materials and Methods

Sampling, isolation and identification

A total of 150 samples (50 raw beef, 50 mutton and 50 carp fish) collected randomly from butcheries and markets around Al-Rusafa, transported to Meat Hygiene laboratory, College of Veterinary Medicine, University of Baghdad. Under serial condition chopped meat, pick pieces in 10 gm, also flash muscle of fish. All samples socked in Nutrient Broth for bacteria growth at 37°C for 24h. Next streaking on Nutrient agar, clear and pure colony cultured on different selective media (MacConkey, XLD, EMB and Simmons citrate agar), inoculated at 37 °C for 24 h**,** and biochemical test (MacFaddin, 2000).

Identified by Vitek2

Vitality Index of Traditional Environmental Knowledg 2 (Bio Mérieux, France). The automated Vitek-2 bacterial identification susceptibility testing system with a designed identification card was used in this study for confirmation of suspected bacteria. The 64-well card contains 43 colorimetric substrates for phenotypic identification of bacterial species (O'Hara & Miller, 2003).

Molecular diagnosis

Molecular diagnosis for *C. freundii* was by Polymerase chain reaction (PCR) used to identity of the most common bacteria. Used nutrient broth tube have pure colony toke it from nutrient media, were inoculated aerobically at 37°C for 24h while shaking. Total genomic DNA of cultivated isolates was extracted following the manufacture's recommendation Presto ™ Mini gDNA Kit (Geneaid, Taiwan). Amplification of the 16S rDNA gene was carried out by polymerase chain PCR technique using universal primers (Macrogen, Korea) forward 27F (5`-AGA GTT TGA TCC TGG CTC AG-3) and reverse primer 1492R (5`-TACGGTTACCTTGTTACGACTT-3`). The protocol for PCR condition was initial denaturation 95°C for 5 min., denaturation 95°C for 30 sec., annealing 60 °C for 40 sec., extension 72 °C for 1 min., and final extension 72 °C for 7min hold at 10°C for 10 min (Aris *et al.*, 2013).

Statistical analysis

Detect the effect of difference in study percentage. Chi-square test was used to significant compare between percentages in this study (SAS, 2012).

Result

Colony morphology & biochemical test

(Figure 1 & 2) appear morphology of *Citrobacter freundii* colonies on nutrient agar generally 2-4 millimeters in diameter translucent to opaque, smooth, low, convex and moist entire edge, on MacConkey bacterial colonies are medium -sized with glossy surface pink colonies (lactose ferment). Colonies grown on Xylose lysine Deoxycholate (XLD) agar were yellow with an intensive yellow center and a surrounding zone of yellow precipitation. On EMB form flat brown colonies of 2-3 mm in diameter after 24hr incubation. On the other hand, on Simmons citrate agar colony change the medium color to blue due to its ability to use citrate as a carbon source. Biochemical test of *C. freundii* it Oxidase (-ve), while it (+ve) Catalase.

Figure 1-A) *C. freundii* colonies on Nutrient agar translucent to opaque, smooth, convex and moist edge. B) MaCkonkey agar, glossy surface pink colonies. C) (XLD) agar yellow center yellow with an intensive

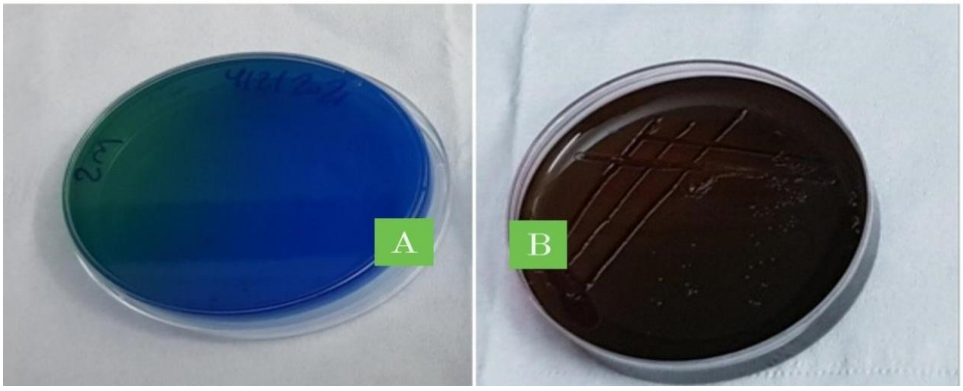

Figure 2- A) Simmons citrate agar citrate (+ve) *Citrobacter freundii* color change to blue. B) EMB form flat brown colonies

Vitek 2 compact result

To confirm diagnosis of *Citerobacter freundii* the result of Vitek-2 was 98% identical as shown in (figure 3).

Source: **Collected:**

Comments:	

Identification Information	**Analysis Time:**	3.80 hours	**Status:** Final
Selected Organism	98% Probability **Bionumber:**	**Citrobacter freundii** 4415610655520210	
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	lLATk	+	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	lMLTa	-	62	ELLM	-	64	lLATa	-			

(Figure 3) *C. freundii* 98% identical diagnosed by Vitek-2.

Molecular result

PCR products were loaded directly. For PCR product, 5µl was directly loaded to well. Electrical power was at 100v/mAmp for 75min. DNA moves from Cathode to plus Anode poles. The Ethidium bromide-stained bands in gel were visualized using Gel imaging system (Figure 4). Sequences and confirmation by Macrogen

Corporation, Korea. The results analyzed using geneious software of *C. freundii* data National Center for Biotechnology Information (NCBI) (Figuer 5).

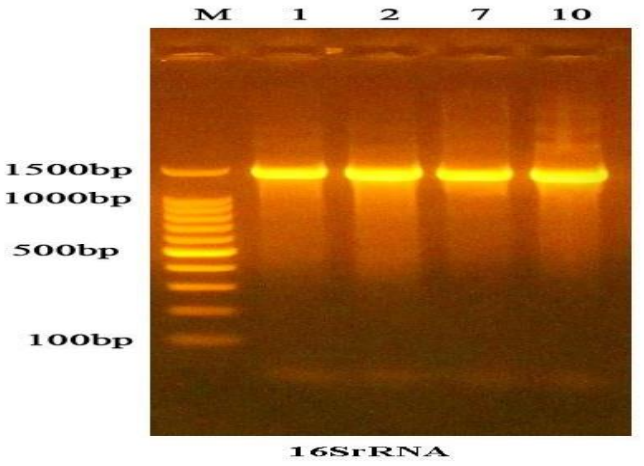

Figure 4: Amplified of 16SrRNA gene of *Citrobacter freundii* fractionated on 1% agarose gel electrophoresis stained with Eth., (1500bp), M: (100bp) DNA molecular weight marker

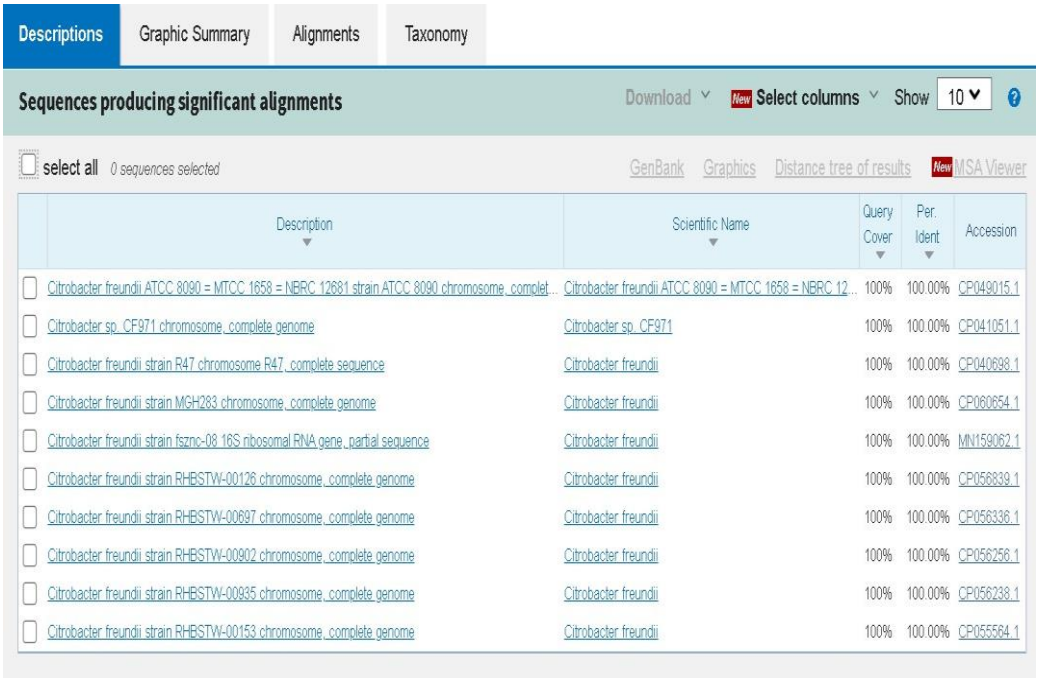

Figure 5: *Citrobacter freundii* showed 100% alignment with NCBI sequence library

150 samples collected from raw meat types (mutton, beef, carp fish), only 23(21.33%) contaminated with *Citrobacter freundii* at significant variation (P< 0.01),where, the highest percentage (36%) reported in carp fish, at isolation

number 18 from 50, followed with mutton and beef contamination ratio were 3(6%) and 2(4%) respectively (Figur6). From above results notice that there were significance variation when compare between meat kinds and contamination value under($P< 0.01$).

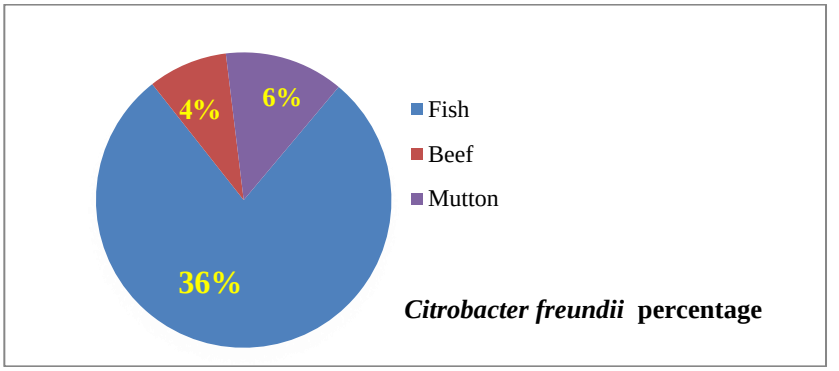

Figure 6: Distribution of *C. freundii* rate according to meat type, fish have the highest contamination rate, followed by mutton and beef minimal rate

Discussion

In this study, we isolated *Citrobacter freundii* from contaminated raw meat and fish, were all isolated 23 sample (21.33%) diagnosed successfully. From recent results found the highest *Citrobacter freundii* contamination percentage (36%) reported in carp fish 18 of 50 samples diagnosed. It's come from bad environmental condition, loss of sanitation behavior in local markets all of those causes beside internal organs they have bacterial agents their are major vehicles for the transmission of bacterial contamination from flesh to consumers (Begum *et al.*, 2019; Chatreman *et al.*, 2020; Sheng & Wang, 2021). *Citrobacter freundii* is responsible of skin lesions, hemorrhagic internal organs, and cause systemic infection in common carp, able to cause injury and high mortality (Yang *et al.*, 2021; Pan *et al.*, 2021).

All *Citrobacter freundii* in this study isolated from fish flash (muscle), (kumar *et al.*, 2015) isolated three species of bacteria *Citrobacter freundii* on of them, found it in muscle and fins of carp, but (Salgueiro *et al.*, 2020) in his study showed *Citrobacter freundii* (only in muscle samples). While, *C. freundii* isolated from brain of Nile tilapia fish (El Asely *et al.*, 2020) at rate 3.4%. Our study result near from (Elsherief *et al.,* 2014) they reported rate of *Citrobacter freundii* (24%) in fish. Didn't agreement with (Zaky *et al.*, 2017) *Citrobacter freundii* represented only 7%. Also far from (Rawash *et al.*, 2019) study of contamination bacteria in fish *C. frundii* (4%) in Benha city. Locally, our result didn't agree with (Al-Obaidi & Al-Dabbagh, 2012) isolated *Citrobacter freundii* at 3(3.7%), from intestine of C. carpio which collected from Tigris river passing through Mosul city, Iraq, (Abid & Al-Hamdani, 2016) isolated *Citrobacter freundii* at rate 6(19.35%) from skin ulceration in Sulaimani province, Iraq. (Al-Hisnawi, 2016) identified it in the intestinal mucosa and gills of L. santifrom a local river in Kerbala city, Iraq. (Hammood & Ibrahim, 2018) fish in Tigris River passing through Shawaka, Baghdad showed contaminated with *C.freundii* at 8%, also don't match result of

(Haider *et al.*, 2019) *C. freundii* was(8.5%) isolated from skin, gills and intestines of common carp in Babylon province. Closer to (Jassim *et al.,* 2019) *C. freundii* was 19 (29.23%) isolated from the gills and intestine in Basrah Province, Iraq.

Mutton sample are 3 of 50 contaminated with *Citrobacter freundii* (6%) percentage in this study which its far from (Mohammed, 2006) in Khartoum, he found *Citrobacter spp* at 25.1% came from intestine during the preparing of carcass Where, far accordance with (Sharma *et al.*, 2015), they assessed bacterial contamination of raw mutton *Citrobactor Spp* at (44%), samples collected from open markets of the city of Kolkata, almost near from (Mohammed, 2015) study in Khartoum, it was 8(10.81%) *Citrobacter freundii* from retail meat Market. Also didn't match to study of (Mansour *et al.*, 2019) percentage of *Citrobacter spp.,* (20%) isolated from sheep meat in Benghazi market. (Uzeh *et al.*, 2021) in Nigeria, *Citrobacter freundii* isolated near in isolated number (5) but far from percentage its (25%).

Finally, depending on the statistical analysis in this study found only 2 of 50 beef samples have *Citrobacter freundii* at (4%). Locally, didn't agree with (Al-Iedani *etal.*, 2015) in Al-Basrah *C. freundii* isolate was 15 from 27 muscular tissue with percent at 55.5%, while Al-Shammary *et al.*, 2015 *C .freundii* was 1(2.3%) isolated from vealmincemeat (beef minced meat) in Baghdad, but closer from (Hadi & Jabbar, 2020) recorded C. *freundii* 1(3.2%) collected beef meat and beef product. This result didn't match with (Mboto *et al.*, 2012) *C. freundii* was (13.9%) isolated from fresh meats sold in Nigeria. Match (Nossair *et al.*, 2015) 2(4%) from cattle meat in Behera province, Egypt. Didn't agreed with (Milhem *et al.*, 2016), determined the prevalence *C. freundii* in beef samples with contamination ratio (1.7%) in Syria. (Sallam *et al.*, 2020) reported opposite to our study bacteria isolated from thigh beef surface were *Citrobacter* spp., (25%).

Conclusion

Carp fish represent one of the important sources for bacterial foodborne disease caused by *Citrobacter freundii* where it used as a maine source of proteins and minerals in Baghdad.

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