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Prevalence, Population Structure and Antibiotic Resistance Patterns of Ceftiofur Resistant *Enterobacteriaceae* from Chicken Meat and Fish

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ABSTRACT

Few studies have been conducted to investigate the influence of extensive use of ceftiofur by veterinarians on the spread of antibiotic resistant *Enterobacteriaceae* in food of animal origin. The aim of this study was to determine the prevalence and antibiotic susceptibility of ceftiofur resistant *Enterobacteriaceae* in retail chicken meat and fish samples. A total of 80 samples, 40 each of chicken thigh and Nile tilapia (*Oreochromis niloticus*) fish samples were analyzed by enrichment in *Enterobacteriaceae* enrichment broth containing ceftiofur (8 µg/ml) and plated on violet red bile glucose agar plates with ceftiofur (8 µg/ml). 87.5% of chicken meat samples and 80% of fish samples had ceftiofur-resistant *Enterobacteriaceae*. The majority of ceftiofur resistant isolates recovered from chicken meat exhibited multidrug resistance characteristics with higher resistance rates to ceftiofur (100%), ceftriaxone (97.5%), cefepime (97.5%), cefotaxime (95%), than ceftazidime (47.5%). Ten antibiotic resistance patterns were identified from chicken meat and fish isolates. Similarly, all isolated strains from fish showed resistance to ceftiofur, cefotaxime, ceftriaxone, and oxytetracycline and most strains (95.6%) were resistant to cefepime. Interestingly, 8.7% of fish isolates showed resistance to meropenem. In conclusion, the high prevalence of ceftiofur resistant *Enterobacteriaceae* reported in this study raise serious concerns about the public health and safety of retail fish and chicken meat, which might serve as a reservoir for these multidrug-resistant germs and could be passed on to humans via the food chain. Under One Health perspective, the monitoring and surveillance of fish and poultry should be encouraged to better control antimicrobial resistance.

Keywords: Antibiotic resistance, Ceftiofur, Chicken, Egypt, *Enterobacteriaceae* and Fish.

INTRODUCTION

Enterobacteriaceae is a diverse group of Gram-negative bacterium family that are found everywhere in the environment, including soil, water, plants, and the gastrointestinal tracts of animals and humans (Farmer et al., 2005; Octavia and Lan, 2014). Because of the fast progress of microbial taxonomy, the family has grown significantly over the last four decades to include 68 genera and 355 species (Janda and Abbott, 2021). Clinically relevant strains are classified into ten genera including *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Proteus*, *Salmonella*, *Serratia*, *Shigella*, and *Yersinia* (Farmer et al., 2005; Octavia and Lan, 2014). Not only the members of this family have a wide spectrum of disease-causing potential that may cause severe morbidity and death in immunocompromised persons, but they are also important causes of foodborne intestinal infection and zoonotic diseases (Janda and Abbott, 2021). However, some are considered opportunistic and not all are actually pathogenic.

Multidrug-resistant *Enterobacteriaceae* that has rapidly emerged over the last 20 years poses a substantial public health and socioeconomic impact. Different genera of this family have acquired genes that can produce plasmid-mediated AmpC β-lactamases and/or extended-spectrum β-lactamases, which give resistance to the new generations of cephalosporins (Wilson and Torok, 2018). Additionally, co-occurrence of additional resistance genes to quinolones and aminoglycosides is common. WHO, (2017) classified third generation cephalosporin and carbapenem resistant *Enterobacteriaceae* as a critical priority category in need of novel antibiotic development. It is worth noting that mortalities from antimicrobial resistant pathogens are expected to exceed 10 million per year in the healthcare sector by 2050, but infection with resistant bacteria will also cost the global economy trillions of dollars (O'Neill, 2016).

The growing need for animal protein has resulted in a major modernization of agriculture, which includes the routine use of antibiotics in feed to enhance animal growth in addition to their medicinal application. Of note, current poultry farming and handling practices in low-income countries result in emergence of multidrug resistant chicken commensals that might possibly colonize the human intestine (Murray et al., 2021). Similarly, the use of antibiotics in aquaculture for treatment and prevention of diseases has resulted in the spread of antibiotic resistant bacteria in fish microbiota (Pepi and Focardi, 2021). Foodborne dissemination of antibiotic-resistant bacteria from contaminated food has been identified as a significant risk to human health in recent decades. Therefore, antimicrobial resistance monitoring and surveillance in bacteria from food animals is currently crucial for understanding the antimicrobial resistance epidemiology in food of animal origin and tracking the impact of antibiotic use in animals (FAO, 2019).

Ceftiofur is a cephalosporin belonging to the third generation of this group of antibiotics with a broad range of action. It has only been approved for usage in veterinary medicine. Interestingly, some authors have connected the emergence and dissemination of third-generation cephalosporin-resistance in human pathogens such as *Escherichia coli and Salmonella species* to the use of ceftiofur in veterinary medicine (Zhao et al., 2001; Tragesser et al., 2006). Therefore, the World Health Organization has designated ceftiofur as a critically important antimicrobial (WHO, 2018). Of note, on a global basis, there are very few studies that addressed the prevalence of ceftiofur resistant *Enterobacteriaceae* in animal and food of animal origin (Dutil et al., 2010; Fan et al., 2021). This study aimed to determine the prevalence, population structure and antibiotic resistance patterns of ceftiofur resistant *Enterobacteriaceae* in retail chicken and fish samples.

MATERIAL AND METHODS

Sample collection and preparation

A total of 80 samples, 40 each of chicken thigh and Nile tilapia (*Oreochromis niloticus*) fish samples, were randomly purchased from various sources, including chicken shops, local markets and small-scale supermarkets in Menoufia governorate, Egypt, from March to August 2022. Samples were promptly placed in aseptic containers with ice and delivered to the University of Sadat City, Faculty of Veterinary Medicine, Food Hygiene and Control Laboratory.

Bacterial isolation and identification

Chicken thigh samples were rinsed in sterile bags with 0.1% sterile peptone water (400 mL) and shaken for 1 minute (Cox et al., 2010). Then 1 mL of the rinsed samples was placed in 9 ml *Enterobacteriaceae* enrichment broth (Oxoid, UK) supplemented with ceftiofur (8 µg/ml) and incubated in shaking water bath at 37°C for 24 hours. Fish samples were decontaminated first by immersing them in ethyl alcohol and then being gently flamed. Twenty-five grams of fish were aseptically excised using sterile scissors and forceps, placed in a sterile bag containing 225 mL of 0.1% sterile peptone water, and stomached in stomacher for 3 minutes. After that, 1 ml of the stomached and diluted samples was placed in 9 ml *Enterobacteriaceae* enrichment broth (Oxoid, UK) supplemented with ceftiofur (8 µg/ml) and incubated in shaking water bath at 37°C for 24 hours. Loopfuls of enriched broth were then spread onto violet red bile glucose agar plates (Merck, Germany) containing 8 µg/ml of ceftiofur and incubated for 24 hours at 37°C. Colonies showing different morphological characters were purified on MacConkey agar. All presumptive *Enterobacteriaceae* colonies were subjected to Gram staining and a panel of biochemical tests including lysine iron agar, triple sugar iron agar, kligler's iron agar, indole, methyl red, Voges Proskauer, hydrogen sulfide, citrate utilization, ornithine decarboxylase, urease, phenylalanine deaminase, beta-glucuronidase, oxidase, catalase, and nitrate reduction as described by (Brown and Smith, 2017; Procop et al., 2017). Bacterial cultures were kept in glycerol stock (25%) and stored for further analysis at -80°C.

Antimicrobial susceptibility testing

The disc diffusion test (The Clinical and Laboratory Standards Institute, 2020) was used to determine the antibiotic resistance of isolated strains against ten antibiotics (Oxoid, Hampshire, UK). The antibiotics tested were as follows: cefepime (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), ceftiofur (30 µg), ceftriaxone (30 µg), ciprofloxacin (5 µg), colistin (10 µg), gentamicin (30 µg), meropenem (10 µg), and oxytetracycline (30 µg).

Visualization of data

A heat-map showing resistance and susceptibility to antibiotics was plotted using Complex Heatmap (v2.6.2) (Gu et al., 2016). The incidence of ceftiofur resistance strains was visualized by Excel program.

RESULTS AND DISCUSSION

1. Prevalence of ceftiofur-resistant Enterobacteriaceae in fish and chicken samples:

1.1. Chicken samples

The findings of this investigation show that the analyzed chicken samples are very contaminated with *Enterobacteriaceae* that are ceftiofur-resistant (87.5%, 35/40). Seven genera were detected namely *Serratia*, *Klebsiella*, *Citrobacter*, *Escherichia*, *Enterobacter*, *Proteus*, and *Providencia*. The present work recovered non-repetitive 40 isolates, the most common species identified was *Serratia marcescens* (42.5%, 17/40) (Figure 1). Similarly, Schwaiger et al., (2012) reported a high prevalence of *Serratia* spp. (41.5%) in retail chicken samples in Germany. However, the high incidence of *Serratia marcescens* is unlikely to be due to contamination during evisceration of chicken, as members of this genus are not natural intestine dwellers, but rather water, plant, and soil inhabitants (Grimont and Grimont, 2015). Consequently, the source of contamination might be the water used to wash chicken. On the other hand, other enteric pathogens such as *Enterobacter cloacae*, *Enterobacter aerogenes*, *E. coli*, *Citrobacter freundii*, *Citrobacter diversus* and *Citrobacter amalonaticus* were detected in percentages of 7.5%, 2.5%, 2.5%, 7.5%, 12.5%, and2.5%, respectively, suggesting exposure of analyzed samples to different sources of contamination during slaughtering and cleaning of chicken.

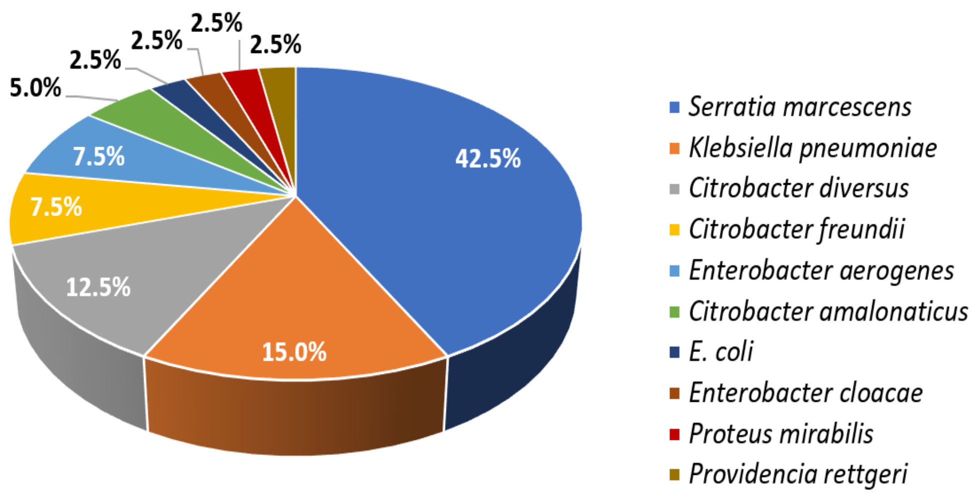

Figure (1): Prevalence of ceftiofur-resistant *Enterobacteriaceae* among the examined chicken samples.

1.2. Fish samples

The results showed in (Fig. 2) revealed that fish samples had a significant amount of ceftiofur-resistant *Enterobacteriaceae*. Thirty-three (82.5%) of the 40 fish samples tested positive for *Enterobacteriaceae*, suggesting a possible risk of antimicrobial-resistant bacteria exposure through consumption of fish. Totally, 46 isolates could be detected. Six genera were detected namely *Klebsiella*, *Escherichia*, *Serratia*, *Enterobacter*, *Citrobacter*, and *Providencia*. The most isolated species was *Klebsiella pneumoniae* at percentage of 47.5% (19/46). *E. coli* was the second most often recovered ceftiofur-resistant *Enterobacteriaceae* (32.5%, 13/46). Similar findings were reported by Alttai et al., (2023) who detected *E. coli* in 35.9% of samples collected from local market in Iraque. Of note, this finding supports an earlier study that found fish to be the primary source of *E. coli* in streams of warm water (Guillen and Wrast, 2010). Other species identified were *Serratia marcescens* (15%, 6/46), and *Enterobacter cloacae* (2.5%, 5/46), as shown in Figure (2).

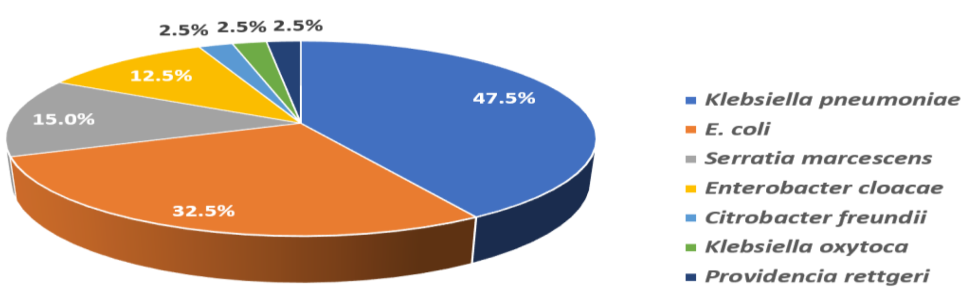

Figure (2): Prevalence of ceftiofur-resistant *Enterobacteriaceae* in the examined fish samples.

2- Antimicrobial resistance among Enterobacteriaceae:

2.1. Chicken samples

As illustrated in (Figure 3A), the majority of ceftiofur resistant isolates recovered from chicken exhibited multidrug resistance characteristics. Our analysis demonstrated that the isolated *Enterobacteriaceae* were more resistant to the third generation cephalosporines, ceftiofur (100%), ceftriaxone (97.5%), cefepime (97.5%), cefotaxime (95%), than ceftazidime (47.5%). Similarly, high level of resistance to the third generation cephalosporines was detected in chicken meat in Egypt (Abdallah et al., 2015). Notably, a significant proportion of isolates (87.5%) demonstrated resistance to colistin, which is routinely used to treat enteric illness in chickens. However, lower percentage of colistin resistance (8%) was reported in *Enterobacteriacea*e isolated from healthy chicken in Egypt (Moawad et al., 2018). Fortunately, none of the isolated strains showed resistance to meropenem, a carbapenem antibiotic, commonly used to treat life-threatening infections (Steffens et al., 2021).

As illustrated in Figure (4), the isolated strains represent 10 antibiotic resistance patterns. Two common patterns were determined. The first was detected in 17 strains "EFT (ceftiofur) + CTX (cefotaxime) + CFM (cefepime) + CAZ (ceftazidime) + CRO (ceftriaxone) + CIP (ciprofloxacin) + CT (colistin) + OT (oxytetracycline)" The second one was observed in 11 strains and included all antibiotics tested except ceftazidime (CAZ) and meropenem (MEM). The remaining strains showed diverse resistance to different antibiotics. To the best of our knowledge, this is the first study conducted to determine the prevalence and to characterize ceftiofur resistant *Enterobacteriaceae* isolated from chicken meat.

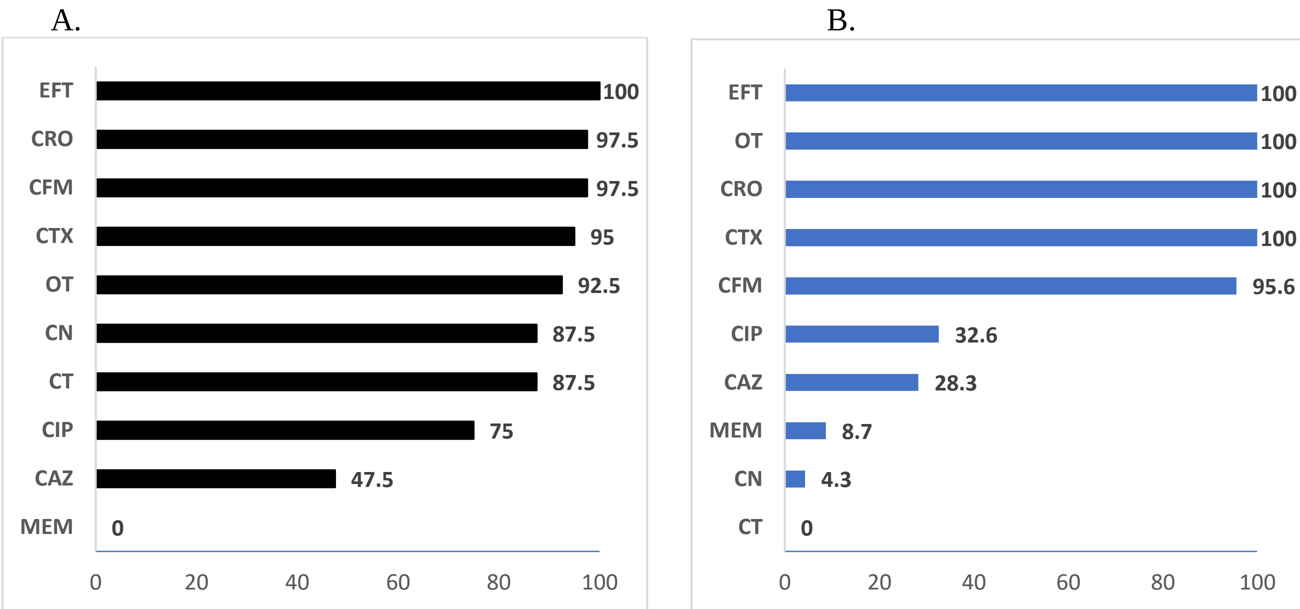

Figure (3): Percentages of resistance to antibiotics in strains isolated from (A) chicken and (B) fish. EFT, ceftiofur; CAZ, ceftazidime; CFM, cefepime; CIP, ciprofloxacin; CN, gentamycin; CTX, cefotaxime; CAZ, ceftazidime; CRO, ceftriaxone; CT, colistin; CIP, ciprofloxacin; MEM, meropenem; OT, oxytetracycline.

It is worth noting that *Serratia marcescens*, the most prevalent species identified in chicken isolates, showed resistance to more than three classes of antibiotics. *Serratia marcescens* is an opportunistic pathogen that mostly affects people who have had antibiotic therapy in the past or patients who have a weakened immune system (Tavares-Carreon et al., 2023). It causes several illnesses including pneumonia, meningitis, endocarditis, peritonitis, arthritis, keratitis, osteomyelitis, and urinary tract infections (Zivkovic Zaric et al., 2023). Additionally, it is characterized by high ability to resist the third generation cephalosporines, including ceftiofur which was incorporated in the isolation media (Sandner-Miranda et al., 2018). The present study highlights the importance of application of strict hygienic measures during food processing and thus minimize the danger for transmission of such bacteria to the customer.

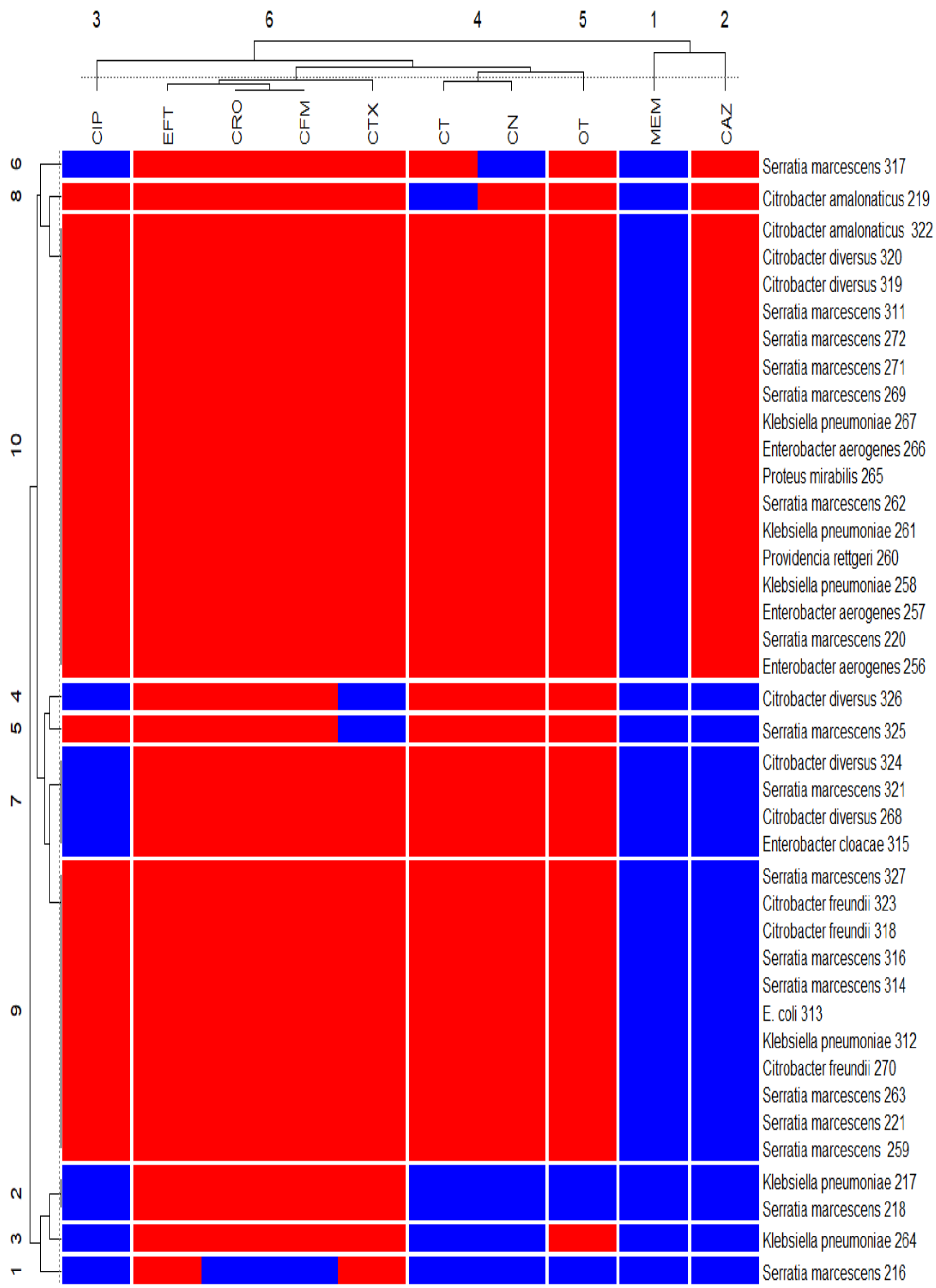

Figure (4): Heat map showing antimicrobial resistant patterns of *Enterobacteriaceae* strains isolated from chicken samples. Red color indicates resistance to antibiotic and blue color indicates susceptibility to antibiotic. EFT, ceftiofur; CAZ, ceftazidime; CFM, cefepime; CIP, ciprofloxacin; CN, gentamycin; CTX, cefotaxime; CAZ, ceftazidime; CRO, ceftriaxone; CT, colistin; CIP, ciprofloxacin; MEM, meropenem; OT, oxytetracycline.

2.2. __Fish samples__

All isolated strains from fish showed resistance to ceftiofur, cefotaxime, ceftriaxone, and oxytetracycline. Also, almost all strains were resistant to cefepime (95.6%) (Figure 3B). Since carbapenems are thought to be the first-line therapy for severe infections brought on by extended spectrum β-lactamase-producing bacteria, the discovery of carbapenem-resistant isolates in this study in percentage of 8.7% raises major concerns about public health. Of note, higher incidence of carbapenem resistance was reported by Hamza et al., (2020) from fish farms in Egypt. They found that 34 of 66 isolates were resistant to both cephalosporin and carbapenem groups and 26 isolates were resistant to carbapenems alone. Interestingly, the collected fish samples in this study seemed to be healthy, indicating that they may serve as reservoirs for multidrug resistant bacteria in humans. Surprisingly, none of the identified strains were colistin resistant, in contrast to findings from other nations that found significant percentages of colistin resistant enterobacterial isolates from fish (Binsker et al., 2022). As illustrated in (Figure 5), the fish isolates showed 10 antibiotic resistance patterns. The most common pattern identified in 29 isolates was "EFT (ceftiofur) + CTX (cefotaxime) + CFM (cefepime) + CRO (ceftriaxone) + OT (oxytetracycline)."

Recently, zoonoses of fish have attracted increased attention, owing mostly to the discovery of novel fish-borne zoonotic pathogens (Gauthier, 2015; Vaneci-Silva et al., 2022). Although the role of *K. pneumoniae* as a fish pathogen and source of fish-borne zoonosis is less well recognized, it poses a substantial hazard to both animal and human health. It is a well-known opportunistic human pathogen capable of causing major infectious disorders such as urinary tract infections, pneumonia and bacteremia (Alharbi et al., 2023) and has been recently linked to outbreaks in aquatic organisms in India (Das et al., 2018) and the United States of America (Jang et al., 2010). Additionally, it plays an important role in transmission to clinically important drug resistance genes to human pathogens (Wyres and Holt, 2018). Interestingly, in this study, *K. pneumoniae* represented about half of fish isolates. Except for *K. pneumoniae* strain 73, all identified strains were resistant to third generation cephalosporines: ceftiofur, cefotaxime, ceftriaxone, cefepime as well as oxytetracycline, constituting almost all of cluster 2 (Figure 5). The present findings stress that because of the extensive use of antibiotics, aquaculture may constitute a risk for the transmission of multidrug resistant *K. pneumoniae* to human pathogens.

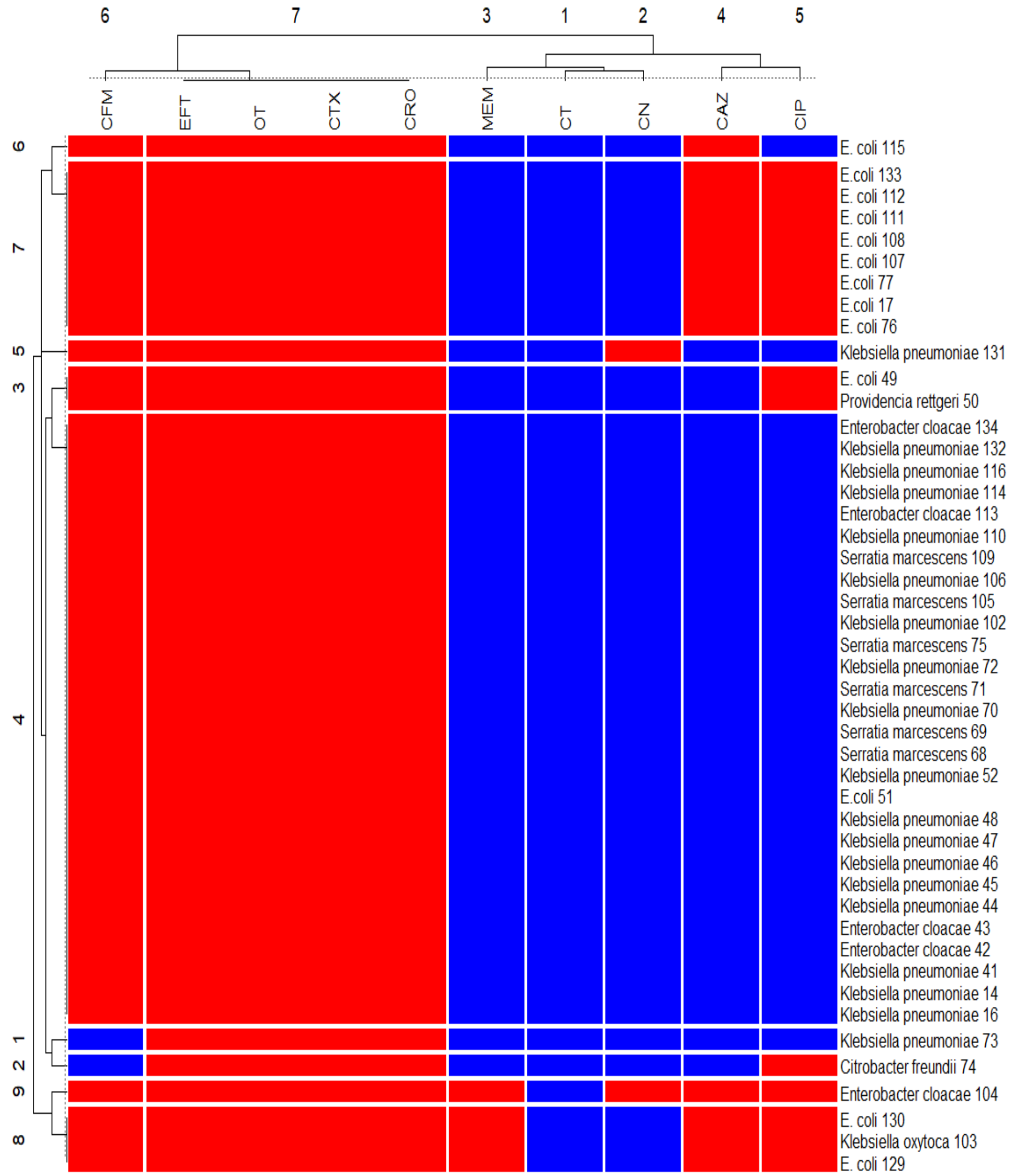

Figure (5): Heat map showing antimicrobial resistant patterns of *Enterobacteriaceae* strains isolated from fish. Black color indicates resistance to antibiotic and white color indicates susceptibility to antibiotic. EFT, ceftiofur; CAZ, ceftazidime; CFM, cefepime; CIP, ciprofloxacin; CN, gentamycin; CTX, cefotaxime; CAZ, ceftazidime; CRO, ceftriaxone; CT, colistin; CIP, ciprofloxacin; MEM, meropenem; OT, oxytetracycline.

In conclusion, this is the first study that addressed the prevalence of ceftiofur resistant *Enterobacteriaceae* in retail fish and chicken. The high prevalence of ceftiofur resistant *Enterobacteriaceae* reported in this study raise serious concerns about the public health and safety of retail fish and chicken, which might serve as a reservoir for these multidrug-resistant germs and could be passed on to humans via the food chain. Under One Health perspective, the monitoring and surveillance of fish and poultry and improved antimicrobial usage for treating animals, should be encouraged to better control antimicrobial resistance.

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