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The Antibiotic Resistance-Related Indicators In *Campylobacter* Species Isolated From Water And Retail Fresh Milk Samples In Southern Iraq

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Abstract: *Campylobacter* species are prominent bacteria associated with human gastrointestinal diseases and are primarily present in the faeces of domestic animals, sewage discharge and agricultural runoff. These viruses have been linked to disease outbreaks resulting from the intake of contaminated water and milk in many regions of the world. However, there is a lack of detailed reports on this issue in the Misan Governorate. Therefore, this study assessed the prevalence, pathogenicity and antibiotic resistance markers of *Campylobacter* species isolated from water and milk samples. A combined total of 70 raw milk samples and 57 water samples were gathered. These samples were subjected to enrichment in Bolton broth and then incubated for 48 h at 42 °C in a 10% CO_2 environment with limited oxygen. Subsequently, the cultures that had been enriched were subjected to additional processing and purification. *Campylobacter* colonies that were likely to be present were separated and verified by PCR using specialised primers designed to identify the *Campylobacter* genus, specific species and genes linked with virulence. The antimicrobial resistance profiles of the isolates were examined using the disc diffusion method against a panel of 12 antibiotics. The presence of important genotypic resistance genes was assessed by PCR. A total of 309 presumptive *Campylobacter* isolates were collected, of which 180 were confirmed to be from the *Campylobacter* genus. Among these, 40.37% were found in water samples and 35.47% were found in milk samples. The isolates displaying significant phenotypic resistance were examined for relevant resistance genes. Among these isolates, the highest prevalence of resistance was seen for the catII gene (93%), whereas the presence of VIM, Ges, KPC, tetD, tetK, tetC, bla-OXA-48-like, IMI and catI genes was not detected. The presence of this pathogen and the identification of genes associated with virulence and antibiotic resistance in *Campylobacter* isolates obtained from water / milk samples indicate a potential threat to human health.

Keywords: Resistance-Related, *Campylobacter*, Water, Fresh Milk.

Introduction

Campylobacter species are common gastrointestinal pathogens that induce diarrhoea. These pathogens are highly relevant to public health due to the escalating number of species associated with human infections (Igwaran & Okoh, 2019). The majority of campylobacteriosis infections occur as a result of ingesting food, such as contaminated food, unpasteurised milk and polluted water (Ammar et al., 2021). Water is vital for sustaining life, although many individuals lack access to potable and uncontaminated water (World Health Organization, 2019). As a result, numerous individuals succumb to waterborne bacterial diseases (García-March et al., 2020). Waterborne infection is a global problem that is estimated to result in millions of deaths each year and everyday instances of illness, including systemic diseases, gastroenteritis and diarrhoea (Magana-Arachchi & Wanigatunge, 2020). Water sources, such as ponds, streams, lakes and rivers, can be contaminated by various causes. These causes include animal droppings on pasture, direct pollution by wild birds in the surrounding areas and the release of inadequately treated wastewater or untreated sewage (Wato et al., 2020). In South Iraq, the inhabitants are at risk of contracting a number of waterborne diseases, including cholera, typhoid fever, viral hepatitis, and gastroenteritis, among others. According to Todd (2017), one of the most prominent signs of campylobacteriosis is abdominal pain and discomfort. Over the past few years, the amount of unpasteurized milk consumed has risen rather than pasteurized milk (Fusco et al., 2020) Newborns, pregnant women, the elderly, and people with weakened immune systems are especially susceptible to dangerous illnesses from raw milk. Nevertheless, belief in its

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health benefits, taste and better nutrients (Aprea & Mullan, 2022) are reasons to consume unpasteurized raw milk. The risk of adverse health effects is much higher with unpasteurised raw milk, and outbreaks can occur. Raw milk may be contaminated with pathogenic bacteria that usually come from infected animals or the environment (Fusco et al., 2020) The Andrzejewska et al. (2019) and Jaakkonen et al. (2020). Campylobacter species, one of the pathogens (Admasie et al., 2023) that can contaminate raw milk. Outbreaks of illnesses due to Campylobacter species have been attributed in various regions. Infections with Campylobacter are associated with a range of severe symptoms, including abdominal pains, vomiting, nausea, fever and diarrhoea (Joensen et al., 2020) (Fig. 1). In extreme cases, the first part of a Campylobacter infection can be followed by complications like Guillain–Barré syndrome or death. Many of Campylobacter species including C. foetus, C. coli, C. jejuni, and C. lari have been reported to be responsible for various diseases in human (Mousavi, Bereswill & Heimesaat, 2020). Another public health concern is the spread of antibiotic resistance Campylobacter strains, especially in less developed countries where antimicrobials abuse is rampant (Kariuk, 2019). On the world stage we are now facing the emergence and global spread of antibiotic-resistant bacteria and genes, which is classed as a major One Health problem (Hernando-Amado et al., 2019). Releasing animal waste, human excreta and wastewater effluents to the environment are the major sources of antibiotic-resistant bacteria (ARB) dissemination. Bacteria exposed to these may play a role in the proliferation of antibiotic resistant genes (ARGs) (Amarasiri, Sano & Suzuki, 2020). Antibiotic resistance appears as a result of vertical gene transfer, or exchange between and within bacterial species. Antibiotic resistance genes (ARG) have appeared as a novel type of environmental contaminants. In particular, it is known that aquatic ecosystems are hotspots of ARB and ARG sinks (Zainab et al., 2020). As a result, the primary objective of this research was to determine the existence of zoonotic Campylobacter species isolates from water and milk samples in the Misan Governorate, which is located in South Iraq, as well as the degree of antibiotic resistance that these isolates had.

Material and Methods

Description of Study Area

The study was carried out in Misan Governorate, South Iraq.

Collection of Samples

This study gathered 127 isolates, comprising 15 water samples from the Tigris River, 15 water samples from lakes, 5 water samples from marshes, 46 milk samples from cow/bulk milk tanks on farms, 24 milk samples from vehicles/roadside, 16 milk samples from retail markets, and 6 milk samples from butcheries. A total of 1 L of water and 250 ml of milk were collected in sterile 1 L and 250 mL polypropylene containers, respectively. Samples were collected from different districts and sub-districts of Misan Governorate, southern Iraq. Then, specimens were cooled and quickly transported in ice water in a refrigerated container and analyzed no longer than 6 hours post-collection.

Isolation of *Campylobacter* Species from Water Samples

Campylobacter isolation method from Van Dyke et al. (2010) was used. In conclusion, each water sample of 1000 mL was filtered by using nitrocellulose membrane filter (pore size <0.45 µm), and filter sheet was carefully picked up by sterile forceps and in-place put in 20 mL Bolton selective enrichment broth. Bolton broth selective supplement and 5% (v/v) defibrinated equine blood were incorporated into the broth.

The solution was then incubated in an (HF151UV CO2)-(10% CO2) incubator at 42 °C for 48 h. The incubation was carried out in an oxygen-depleted environment. An antibiotic selective supplement, which consisted of cefoperazone and amphotericin, was applied to mCCDA plates, and a fraction of the enhanced cultures was distributed that way. After this, the plates containing the mCCDA were introduced into the incubator in accordance with the procedures that were detailed before. Defibrinated horse blood at a concentration of seven percent (v/v) was used to restreak the Campylobacter colonies that were selected. Following that, these plates were incubated in the same manner that was described more earlier.

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Isolating *Campylobacter* species from milk samples

In accordance with the procedures described by Bianchini et al. (2014), milk samples were performed for analysis. At a volume-to-weight ratio of 1:10, twenty millilitres of milk samples were mixed to two hundred millilitres of Bolton selective enrichment broths. This was supplemented with a Bolton antibiotic supplement that was defibrinated horse blood at a concentration of five percent (volume/volume). After that, the mixture was placed in an HF151UV CO2 incubator and allowed to wait for forty-eight hours at a temperature of forty-two degrees Celsius. Incubation was carried out in an environment that was microaerophilic and included ten percent carbon dioxide. Next, the isolates were subjected to purification.

DNA Extraction

Bacterial extraction of DNA was done by the boiling method according to Sierra–Arguello et al. Somewhat adapted from (2018) Briefly, single colonies of *Campylobacter* were isolated onto blood agar plates and cultured in 5 mL of Tryptone Soya Broth (TSB). An (HF151UV CO2) incubator provided cultivation at (42 °C) for (48) hours with (10%) carbon dioxide. Then, 1 ml of the soup was centrifuged at (12,800) rpm for (5) minutes, and the supernatant was poured off. They were then transferred to sample Eppendorf tubes (1.5 mL), mixed with (400 µL) sterile distilled water. A heating block was used to heat the suspensions to (100 °C) for (10) minutes. Subsequently, the suspensions were centrifuged at (12,800) revolutions per minute for (5) minutes to remove the cell debris. After being decanted, the supernatant that included the particles that were suspended was kept at a temperature of -20 degrees Celsius until it was needed.

Confirmation, characterization, and amplification of virulence genes at the molecular level.

The PCR technique, which targeted a 439-bp section of the 16S rRNA gene, was used to authenticate each of the Campylobacter isolates from the study. It was also reported by Igwaran and Okoh (2020) that this had occurred. C. foetus, C. jejuni, C. lari, and C. coli were the four identified strains of Campylobacter that were isolated and confirmed.

For this classification, the primer sets used were directed at cj0414 (*C. jejuni* 029 status c, up), glyA (*C. jejuni* 029 status c, midway), and cstA and asK (*C. jejuni* status a, right) genes. Furthermore, a polymerase chain reaction (PCR) analysis was performed on virulence characteristics by employing primers for the invasion gene (iam), the invasion protein gene (ciaB), the colonisation gene (flaA), the adhesion gene (cadF), the toxin production gene (cdtB), and the flgR gene, which code for flagella synthesis and modification (flgR) (Liu et al., 2023). This year (2019). A total of 25 µL of reaction volume was utilised for the PCR studies. The volume of the reaction was composed of 1.0 microlitres of each PCR primer, 12.5 microlitres of master mix, 5.0 microlitres of extracted DNA, and 5.50 microlitres of water that was free of nucleases. After the PCR products were amplified, they were identified using gel electrophoresis on a 1.5% (w/v) agarose gel in 5× TAE buffer. The gel was stained with ethidium bromide thereafter.

Resistance to Antibiotics in *Campylobacter* Isolates

Testing for antimicrobial susceptibility was performed on Mueller-Hinton agar plates using the disc diffusion technique. The tests were performed on *Campylobacter* isolates. According to Lazou and Chaintoutis (2023), the plates were augmented with 5% defibrinated horse blood on top of the blood that was already there. Finally, 1-2 colonies of the bacterial growth in TSB that was incubated for 48 h at 42 °C in 10% CO2 were diluted in steril normal saline and adjusted such that the turbidity was equivalent to that of a 0.5 McFarland standard. Then the solution was softly swabbed to the whole surface of the Mueller Hinton agar plates. Plates were saturated with antibiotic discs and incubated in a CO2 incubator at 42 °C for 24 h under microaerobic conditions. Doxycycline (30 µg), ampicillin (10 µg), and meropenem (20 µg) were the antimicrobials that were utilised in the in vitro study. Additionally, antiseptics such as 2% chlorhexidine for 1 minute and 10% povidone-iodine were also utilised among the antimicrobials. 10 microgrammes of erythromycin, 15 microgrammes of azithromycin, 30 microgrammes of tetracycline, 10 microgrammes of gentamicin, 2 microgrammes of clindamycin, 30 microgrammes of ceftriaxone, 5 microgrammes of ciprofloxacin, 5 microgrammes

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of levofloxacin, 30 microgrammes of chloramphenicol, and 10 microgrammes of imipenem. In accordance with the recommendations provided by the Clinical and Laboratory Standards Institute (CLSI), the identification of inhibitory zones for tetracycline, doxycycline, ciprofloxacin, and erythromycin was carried out (Ge et al., 2013). Due to the limited information concerning specific breakpoints for *Campylobacter* for ampicillin, gentamicin, azithromycin, ceftriaxone, chloramphenicol, levofloxacin, clindamycin and imipenem, the results were compared using CLSI breakpoints for Enterobacteriaceae (Chibwe, Odume & Nnadozie, 2023).

MAR Index as a Measure of Multiple Antibiotic Resistance

The multiple antibiotic resistance (MAR) index for each Campylobacter isolate was calculated using the formula: MAR = a/b, where a indicates the number of drugs to which the isolate demonstrated resistance, and b signifies the total number of antibiotics to which the isolate was subjected (Adzitey et al. 2012). Let a signify the quantity of antibiotics resistant to the test isolate, and b represent the total number of antibiotics subjected to susceptibility testing for the isolate.

Identification of Antimicrobial Resistance Genes via Molecular Screening

Molecular screening of isolates exhibiting phenotypic resistance to all tested drugs was conducted by PCR to detect genotypic resistance genes. The primer sets utilised in this investigation were derived from the analysis of prior research. The primer sets identified by Salehi et al. TetA, tetC, tetB, and tetD were utilised in the present investigation. The TetK and tetM gene primer sets from Ong et al. were sourced in 2017. Liu et al. (2022) provided the primer set for the gyrA gene, as referenced by Severgnini et al. The article from 2021 presented the primer pair for the ermB gene. Igwaran and Okoh (2020) supplied the primer sets for the catI and catII genes. The primer set for the aac(3)-IIa-(aacC2) gene was supplied by Onohuean and Nwodo (2023). Han et al. (2021) produced primer sets for the VIM, bla-OXA-48-like, Ges, KPC, and IMI genes.

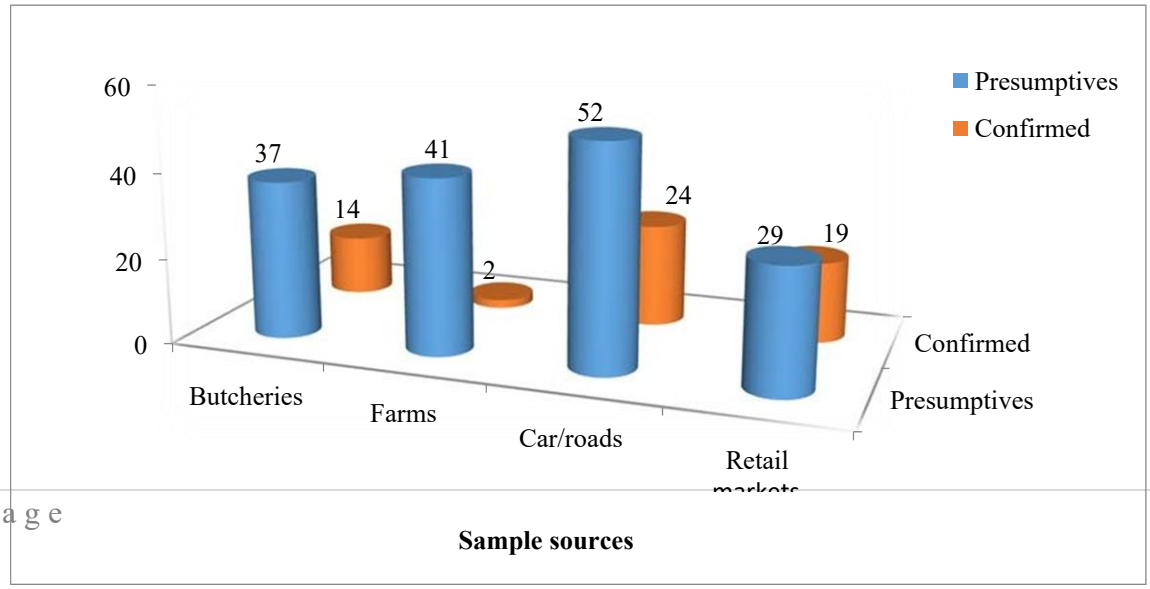
Statistical Analysis

Microsoft Office tools carried out statistical analysis.

Results

Molecular Identification of the Genus *Campylobacter*

Isolates of *Campylobacter* were gathered from a total of 438 potential isolates, of which 162 were recognised as *Campylobacter* with a certainty rate of 36.99%. *Campylobacter* was discovered to be present in 103 of the 279 potential isolates that were recovered from water samples taken from dams and rivers. This represents a percentage of 36.92%. Moreover, 33 out of the total number of water samples from 56, which is about 58.93%, were found to be *Campylobacter.* Finally, among the 159 isolates of potential milk samples, 59 or 37.11% of them were found to be *Campylobacter*. Furthermore, out of the 72 milk samples obtained from different sources including butcheries, retail markets, farms, and car/roads, 19 samples (26.38%) were found to be positive for *Campylobacter* (Figure 1).



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Molecular Detection of *C. coli*, *C. jejuni* and *C. foetus*

Among the 162 isolates that were confirmed to belong to the *Campylobacter* genus, they were further categorized into *C. fetus, C. coli,* and *C. jejuni.* However, *C. lari* was absent from the group.

Table 1: Patterns of distribution of *Campylobacter* species detected in the sample components.

Sample Sources	*C. foetus* (%)	*C. coli (%)*	*C. jejuni (%)*	*C. lari* (%)	No. of Isolates That Belong to Other *Campylobacter* Species (%)
Water	6 (5.83)	8 (7.77)	40 (38.83)	0	49 (47.57)
Milk	6 (10.17)	6 (10.17)	4 (6.78)	0	43 (72.88)

Virulence Gene Molecular Detection in the Species of *Campylobacter* Identified

Virulence genes associated with toxin production (cdtB), invasion (ciaB and iam), adhesion (flaA and cadF), and regulation of flagella synthesis (flgR) were found via a PCR-based assessment of virulence genes. The iam gene (32.86%) was the most prevalent among the (6) virulence genes that were screened in the 70 isolates identified as *C. coli*, *C. foetus, C. jejuni* (Table 1). This was followed by the cdtB and cadF genes (5.71%) and flgR gene (20%). The percentage occurrence of virulence-associated genes identified in *C. jejuni*, *C. coli* and *C. foetus* varied, with the exception of the ciaB gene, which was not detected in any of the isolates. *C. jejuni* isolates contained a high prevalence of the iam gene (35%) compared with a low prevalence of the flgR gene (4.55%), as determined by PCR. A higher frequency of detection of virulence-associated genes was noted in *C. coli* than in *C. jejuni* and *C. foetus*. Out of the four *C. coli* isolates, 5.71% of them had both the iam and flaR genes. Similarly, both the iam and flaR genes were found in only one isolate of *C. jejuni* (1.43%). *C. coli* isolate (1.43%) combined the two genes cadF and iam. Also, we found cadF, cdtB, and iam genes in one *C. coli* strain (1.43%). Out of which, two isolates (2.88%) found together with C. jejuni. Finally, both the iam and cdtB genes were also found in C. coli. The *Campylobacter* species virulence genes identified in water and milk samples showed a widespread pattern of distribution.

Phenotypic Resistance Profiles of *Campylobacter* Isolates to Antibiotics

The effectiveness of twelve different antimicrobial drugs was assessed against a total of 162 *Campylobacter* isolates that were obtained from samples of water and milk. Among the 12 antibiotics that were assessed, *Campylobacter* isolates isolated from milk and water samples exhibited the greatest phenotypic resistance to clindamycin (95.68%). Conversely, the least resistance was detected towards imipenem (21.47%). The isolates also exhibited significant phenotypic resistance to the following drugs: gentamicin (56.17%), levofloxacin (59.88%), ciprofloxacin (77.78%), chloramphenicol (78.27%), tetracycline (83.33%), ampicillin and azithromycin (87.04% each), doxycycline (87.65%), ceftriaxone (93.21%), and erythromycin (95.06%). Figure 2 illustrates these resistance levels. As a result of the majority of the isolates exhibiting resistance to more than three different classes of antimicrobial medicines, the criteria for multidrug resistance (MDR) categorisation were satisfied. CRO-E-CD-AP exhibited the lowest phenotypic MDR rate among CD-T-CRO-E-DXT-AP in *C. foetus*, *C. coli* isolates, and mCD- E-ATH-T-DXT-AP in *C. jejuni* (Table 3). A significant proportion of the isolates exhibited resistance to over 2–9 classes of antimicrobial agents. Among these, *C. foetus, C. jejuni*, and *C. coli* exhibited the highest resistance profiles to LEV-CRO-C-CIP-E-ATH-CD-T-GM-DXT-AP (22.86%). Table 3 presents comprehensive images of the multiple resistance patterns demonstrated by *C. foetus, C. coli*, and C. *jejuni*.

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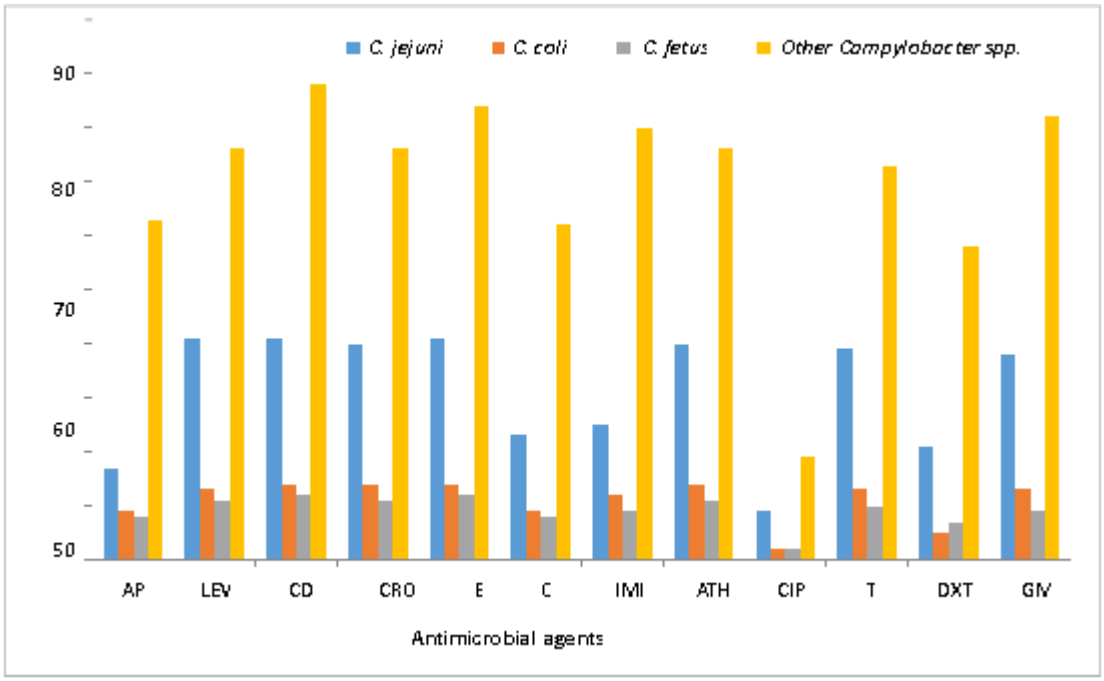
Table 2: Prevalence of identified virulence genes in species of *Campylobacter* in water samples.

		Virulence Genes Screened (%)					
***CaMpylobacter* spp.**	**No of Isolate**	***iam***	***cadF***	***flaA***	***flgR***	***ciaB***	***cdtB***
C. foetus	6	1 (16.7)	-	-	1 (16.7)	-	-
C. jejuni	40	14 (35)	-	-	2 (5)	-	3 (7.5)
C. coli	8	4 (50)	3 (37.)	-	1 (12.5)	-	1 (12.5)

Table 3: Show the prevalence of identified virulence genes in different species of *Campylobacter* in milk samples.

		Virulence Genes Screened (%)					
***CaMpylobacter* spp.**	**No of Isolate**	***iam***	***cadF***	***flaA***	***flgR***	***ciaB***	***cdtB***
C. foetus	6	-	1 (16.7)	-	4 (66.7)	-	-
C. jejuni	4	-	-	-	-	-	-
C. coli	6	4 (66.7)	-	-	6 (100)	-	-



C. foetus, C. coli, C. jejuni, and other *Campylobacter* species that were isolated from water and milk samples were determined to have varying degrees of resistance to twelve different antimicrobial drugs, as shown in Figure 2. Ciprofloxacin (CIP), levofloxacin (LEV), azithromycin (ATH), ampicillin (AP), tetracycline (TET), imipenem (IMI), clindamycin (CD), doxycycline (DXT), erythromycin (E), gentamicin (GM), ceftriaxone (CRO), and chloramphenicol (C) were the antibiotics that were used to treat the infection under investigation.

Table 4. The Show phenotypic resistance profiles of *Campylobacter* isolates to antibiotics in water.

No		**Sample**	**No of Isolates**

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	Antimicrobial Resistance Patterns	Water	*C. foetus*	*C. coli*	*C. jejun*	Total	MAR Index
1	E-ATH-T-CD-DX-AP	2	-	1	1	2	0.5
2	CRO-C-E-CD-AP- ATH	1	-	1	-	1	0.5
3	LEV- E-ATH-C-CIP-CD	1	-	-	1	1	0.5
4	LFV-CRO-ATH- -CIP-F-CD-AP	1	-	1	-	1	0.58
5	CRO-CD-T-E-ATH-DXT-AP	3	-	3	-	3	0.58
6	E-ATH-GM-DXT-AP- CD-T	1	-	1	-	1	0.58
7	CRO-E-ATH-GM-AP-CD-T	1	-	1	-	1	0.58
8	CRO-E-ATH-DXT-AP-CD-T	1	-	1	-	1	0.58
9	CRO-E-ATH-GM-DXT-AP-CD-T	3	1	3	1	5	0.67
10	CRO-C-E-ATH-DXT-AP-CD-T	3	-	3	-	3	0.67
11	CRO-E-ATH-IMI-DXT-AP-CD-T	2	-	2	-	2	0.67
12	CRO-CIP-E-ATH-DXT-AP-CD-T	1	-	1	-	1	0.67
13	CRO-E-ATH-IMI-GM-AP-CD-T	2	-	2	-	2	0.67
14	CRO-CIP-E-ATH-DXT-AP-CD-T	1	-	1	-	1	0.67
15	CRO-CIP-E-GM-DXT-AP-ATH-CD-T	2	1	1	-	2	0.75
16	LEV-CRO-CIP-DXT-AP-E-ATH-CD-T	1	-	1	-	1	0.75
17	LEV-CRO-C-CIP- DXT-AP-E-ATH-CD-T	1	-	1	2	3	0.83
18	LEV-CRO-CIP--GM-DXT-AP-E-ATH-CD-T	4	1	1	2	4	0.83
19	CRO-C-CIP-E-GM-DXT-AP-ATH-CD-T	1	1	-	-	1	0.83
20	CRO CIP-E-ATH-GM-DXT-AP-IMI-CD-T	1	1	-	-	1	0.83
21	LEV-CRO-C-CIP-E--GM-DXT-AP-ATH-CD-T	11	5	10	1	16	0.92
22	CRO-C-CIP-E-ATH--GM-DXT-AP-IMI-CD-T	1	-	1	-	1	0.92
23	LEV-CRO-C-CIPE-T-GM-DXT-AP--ATH-IMI-CDH	7	3	3	1	7	1

Table 5. Show the phenotypic resistance profiles of *Campylobacter* isolates to antibiotics in milk.

No	Antimicrobial Resistance Patterns	Sample Milk	No of Isolates				
			C. foetus	*C. coli*	*C. jejuni*	Total	MAR Index
1	**CRO-E-AP-CD**	1	1	-	-	1	0.35
2	**CRO-E-DXT-AP-CD-T**	1	-	-	1	1	0.5
3	**CRO-E- GM-ATH-DXT-AP-CD-T**	2	1	3	1	5	0.68
4	**CRO-C-E-GM-AP-CD- T**	1	-	-	1	1	0.68

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5	**CRO- E-DXT-C-CIP-AP-CD-T**	3	-	1	-	1	0.68
6	**LEV-CIP-CRO-C-E-ATH-CD-DXT-CD-T**	1	1	-	-	1	0.68
7	**CRO-C-E-IMI-ATH -DXT-AP-CD-T**	1	-	-	1	1	0.76
8	**LEV-E-ATH-C-CIP-CD-T-GM-AP-CD-T**	1	-	-	1	1	0.76
9	**C-CIP -IMI-E-ATH-DXT-AP-CD-T**	1	-	1	-	1	0.76
10	**LEV-C-CIP-CRO -E-ATH-GM-DXT-AP-CD-T**	5	5	10	1	16	0.93

Identification of Genotypic Resistance Genes in *Campylobacter* Isolates using Molecular Techniques

In Campylobacter isolates, the presence of genotypic resistance genes was determined through the use of polymerase chain reaction (PCR), with a particular emphasis placed on the existence of the catII gene, which is linked to resistance to chloramphenicol.

The catII gene was discovered in the *Campylobacter* isolates, with 38 (95%) of the identified isolates being *C. coli*, *C. jejuni* and *C. foetus*. The prevalence of tetracycline resistance genes was high in *C. coli*, *C. jejuni* and *C. foetus*, with A-tet, B-tet and M-tet being discovered in (27.42%), (32.26%), and (88.71%) of the samples, respectively. The presence of additional ARGs in *C. foetus*, *C. coli*, and *C. jejuni* was observed. These genes included B-erm (associated with erythromycin resistance), A-gyr (associated with gentamycin resistance), aac(3)-IIa-(aacC2) a (also associated with gentamycin resistance), and C-amp (associated with ampicillin resistance). The prevalence of these genes in the respective species was found to be (15.38%), (39.13%), (81.54%), and (84.85%). None of the *C. foetus*, *C. coli*, and *C. jejuni* strains isolated from the milk and water samples were positive for the KPC, Ges, VIM, D-tet, C-tet, K-tet, IMI, bla-OXA-48-like, and catI genes. The PCR results revealed that the majority of the isolates contained multiple resistance genes. Among the *C. jejuni* isolates, the highest numbers of resistance genes detected were A-tet, A-gyr, C-amp, M-tet, catII, and aac(3)-IIa-(aacC2) a genes. In the case of *C. coli* isolates, the resistance genes observed were A-tet, B-erm, C-amp, M-tet, catII, and aac(3)-IIa-(aacC2) a genes. Similarly, the *C. foetus* isolates were found to harbour A-tet, B-erm, C-amp, M-tet, catII, and aaac(3)-IIa-(aacC2) a gene (Table 5). The presence of multiple resistance genes in the isolates suggested that they possessed two or more categories of ARGs concurrently. Table 4 presents a comprehensive overview of the various ARGs found in *C. foetus*, *C. coli,* and *C. jejuni* isolated from milk and water samples. The gel electrophoresis pictures that illustrate the amplified PCR products are shown in Figure 2.

Table 6: Show the multiple antibiotic resistance genes in C. fetus, C. coli, and C. jejuni isolates in Water Sample.

No	**Multiple Resistance Genes Harboured**	***Campylobacter* Species**		
		C. foetus	***C. coli***	***C. jejuni***
1	**A-tet, cat II**	-	2	-
2	**A-tet, A-gyr, C-amp**	1	-	-
3	**C-amp, K-tet, cat II**	-	1	-
4	**A-tet, A-gyr, cat II,**	-	1	-
5	**A-tet, B-tet, C-amp**	-	6	-
6	**A-tet, C-amp, cat II**	-	4	-
7	**A-gyr, C-amp, M-tet**	-	-	1

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8	**A-tet, C-amp, M-tet, cat II**	2	-	-
9	**A-tet, B-tet, C-amp, aac(3)- IIa-(aac C2) a**	-	2	-
10	**A-tet, B-erm, B-tet, C-amp**	-	1	-
11	**A-tet, B-tet, C-amp, catII**	-	1	-
12	**C-amp, M-tet, caţII, aac (3)- IIa-(aacC2) a**	-	-	1
13	**A-tet, B-erm, caţII, aac (3)- IIa-(aacC2) a**	-	1	-
14	**A-tet, A-gyr, C-amp, aac (3)- IIa-(aacC2) a**	1	-	1
15	**A-tet, A-gyr, B-tet, C-amp, catII**	-	2	-
16	**A-tet, A-gyr, C-amp, catII, aac(3)-IIa-(aacC2) a**	-	4	3
17	**A-tet, A-gyr, B-erm, C-amp, M-tet, caţII,**	-	1	-
18	**A-tet, A-gyr, B-tet, C-amp, caţII**	-	1	-
19	**A-tet, C-amp, M-tet, caţII, aac(3)- IIa-(aacC2) a**	2	2	
20	**A-tet, A-gyr, C-amp, M-tet, catII**	1	1	1
21	**A-tet, B-tet, C-amp, B-erm, aac(3)- IIa-(aacC2) a**	-	3	-
22	**A-tet, A-gyr, B-tet, B-erm, C-amp**	-	2	-
23	**A-tet, B-erm, C-amp, M-tet, caţII, aac(3)-IIa-(aacC2) a**	-	-	1
24	**A-tet, A-gyr, C-amp, M-tet, caţII, aac(3)-IIa-(aacC2) a**	-	2	-

Table 7: Show the multiple antibiotic resistance genes in *C. fetus, C. coli, and C. jejuni* isolates in Milk Sample.

No	**Multiple Resistance Genes Harboured**	***Campylobacter* Species**		
		C. foetus	***C. coli***	***C. jejuni***
1	**B-erm, caţII**	-	1	-
2	**A-tet, C-amp, M-tet**	1	-	-
3	**A-tet, C-amp, caţII**	-	4	-
4	**A-tet, C-amp, aac(3)- IIa-(aacC2) a**	-	-	2
5	**A-tet, caţII, aac(3)- IIa-(aacC2) a**	1	-	-
6	**A-tet, C-amp, M-tet, catII**	2	-	-
7	**A-gyr, C-amp, M-tet, caţII, aac(3)- IIa-(aacC2) a**	-	-	1
8	**A-tet, C-amp, M-tet, caţII, aac(3)- IIa-(aacC2) a**	2	2	

Discussion

There is a growing global trend in the identification of *Campylobacter* species, with reports coming from Asia, Europe, America, and Africa. This phenomenon poses a significant public health problem (Rossler et al., 2019; Endtz, 2020; Zbrun et al., 2020). Additional data on the risk of consuming unpasteurised milk and unchlorinated water are necessary to enhance existing findings regarding the involvement of *Campylobacter* species in waterborne and milkborne diseases. Thus, this study aimed to evaluate the occurrence, as well as the phenotypic and genotypic profiles associated with antimicrobial resistance, of *Campylobacter* species in water and milk samples available to the consumer. *Campylobacter* species in milk and water samples were detected by PCR techniques and culture-based. However, there was little information available on this in the Eastern Cape Province, even though the province has the largest livestock population in the country. According to the

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research results, *Campylobacter* was isolated from 103 (36.92%) isolates from water samplesOut of the 56 water samples that were examined, 33 (58.93%) were found to be positive for Campylobacter levels. I am twenty-six points. Among the 72 samples of whole milk, 38 percent were confirmed to be positive for Campylobacter. This is a percentage of 26.38 percent per sample. Additionally, out of the total of 72, 59 isolates from milk samples were identified as Campylobacter. That accounts for 37.11 percent of the total. The study revealed up to tenfold higher levels of Campylobacter in water samples compared to the levels detected in the milk samples. These findings align with the research results of Mulder et al. (2020), Chukwu et al. (2019), Hyllestad et al. (2020), and Heydarian et al. (2023) which highlighted an increased prevalence of river water samples containing Campylobacter species. Prior reports confirmed our findings. In another research carried out by Andrzejewska et al., Campylobacter species were detected in samples of unpasteurised milk. (2019), Hansson et al. (2020), Jaakkonen et al. (2020), based on Igwaran and Okoh (2020) and Kenyon et al. (2020). The results of this study agreed with their results. Milk that has never been heat-treated, typically known as unpasteurised milk, has recently been a focus of attention due to its association with outbreaks of campylobacteriosis. The global burden of campylobacteriosis is substantial, as reported by Igwaran and Okoh in 2020. Igwaran and Okoh (2019) related that the consumption of raw cow's milk has been associated with the occurrence of epidemics of campylobacteriosis in Europe and the United states. It is generally accepted that Campylobacter species are the main etiological agents of gastroenteritis in children in Iraq. This includes the identification of these pathogens from residence purchased raw milk and water samples (Ali & Bunyan, 2021) C. jejuni was the most prevalent bacterium detected in water samples with a detection rate of 38.83% (Table 1). C. foetus was detected in 5.83% of the samples, while C. coli was detected in 7.77% of the samples. Results similar to those produced by Almashhadany (2021), Kanaan and Mohammed (2020) and Al-Mawla, Al-Dalla Ali, and Al-Ani (2008) were found in this research. Abdul-Rahman (2019) reported a significant presence of C. foetus and C. coli in the milk samples, which is consistent with the present study but not in coherent with the finding of Almashhadany (2021). The fact that pathogenic Campylobacter species were isolated from river water and non-pasteurised milk samples emphasises the importance of these sources as possible reservoirs of Campylobacter speciesThe virulence genes iam, cdtB, flgR, and cadF were the four most prevalent genes across the 70 C. foetus, C. coli and C. lari isolates (Fig. 2). As a result of the investigation, the patterns of distribution of the iam gene were discovered among the many different Campylobacter species. A virulence marker that is involved in the invasion of host cells is designated by the iam gene. The iam gene was found in every single one of the C. coli and C. jejuni isolates that were first isolated from the milk and water samples. This observation was consistent with the results of Igwaran and Okoh, (2020) and Selwet and Galbas (2012). Whereas Lopes et al. (2021) got. CdtB is a toxin-producing gene that has been detected in strains of C. jejuni and C. coli obtained from diverse sources such as beef, raw milk, chicken, humans, and bovine cervical mucus. Previous research by Zishiri and Zowalaty (2020) on beef and raw milk; Ramatla et al. Murawska et al. (2022) on poultry. This finding that was consistent with their findings for people (2007) and then bovine cervical mucus (2022). In this work, the virulence-associated gene flgR was examined. This was observed in 25% of C. coli, 46.7% of C. foetus and 5% of C. jejuni. According to the data shown in Table 2, the flgR gene was present in 5.56% of water and 62.5% of milk isolates. We also could see the difference in the presence of flgR genes between water and milk samples. Our results were in accordance with the research of Hamal (2021), as they also found flgR gene in C. coli (Hamal, 2021). The presence of the flgR gene has never been documented in any of the studies that have previously been performed in this region for the various species of Campylobacter. Moreover, to the best of our knowledge, we report, for the first time, the absence of data about detection of flgR gene in Campylobacter isolates from dairy and aquatic sources. Regulated phase variation of the flgR gene This mechanism allows bacteria to alter the surface antigens for adaptation to new hosts (Igwaran & Okoh, 2020). According to Talukdar et al. In a study by et al., in 2020, cadF was identified as cosumes as a virulence gene that the virus can help the virus attach to gut cells. Detection of CadF Gene The

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cadF gene was detected in C. foetus and C. coli at a frequency of 5.71 percent in water and milk samples. Results of Hamal (2021), Salazar (2020), and West (2019) indicated that the cadF gene is present in Campylobacter species. It was also detected in a group of Peruvian children, and in meat and Campylobacter isolated from dogs. Our studies had similar conclusions with this study. Campylobacter genomes include one or more virulence genes, as stated by Redondo, Carroll, and McNamara (2019). These genes are responsible for the transmission of the infection to humans. The results of our analysis revealed that some of the Campylobacter species harbored a high number of virulence genes. Further research carried out by Hull et al. (2021), García-Sánchez et al. (2019), Kim et al. (2019), and Han et al. This idea was further evidenced by (2019) They detected numerous virulence genes distributed across various species of Campylobacter. Our investigation supports and thereby corroborates their reports.

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