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Identification and isolation of *Campylobacter jejuni* and *C. upsaliensis* from bovine local milk, milk products, and human stool samples by molecular technique in Karbala province

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Abstract

This study aimed to determine the prevalence of Campylobacter species in milk and its product samples in Karbala province and detect the antibiotic sensitivity of these isolates. One hundred samples were aseptically collected as follows: 50 samples of bovine raw milk and 50 samples of bovine milk products from different local shops and farms distributed in Karbala province, in addition to, 100 samples from children suffering diarrhea, fever, and abdominal pain from Kerbala (Iraq) General Children's Hospital. The pathogen was found using biochemical testing after samples were immediately inoculated into enriched and sub-cultured on selected media. *Campylobacter* spp. At 24 to 48 hours, colonies on the plate are small, often grayish in appearance, although some have to be creamy grey, slightly raised and moist, and often generated separate colonies of bacteria, flat with irregular borders, and non-hemolytic. This was confirmed by using the polymerase chain reaction method, 11 isolates (5.5%) of 200 samples were determined to be *Campylobacter* species. The 11 isolates were subjected to sequencing to detect the species of Campylobacter which found two species (*C. jejuni* and *C. upsaliensis*). Results showed that Campylobacter spp. 11 positive isolates, *C. jejuni* recorded 9 (81,8%) positive results while *C. upsaliensis* recorded 2 (18.18%) positive results. Antibiotic_ susceptibility_ test was cultured by using disk diffusion assay, campylobacter spp. showed complete sensitive to ciprofloxaci , gentamycin, and Imipenem.

Keywords: *Campylobacter jejuni* and *C. upsaliensis*, infection rate, Antimicrobial susceptibility, Karbala retail points.

Introduction

A gram-negative, tiny, curvy or S-shaped bacterial disease called campylobacter is zoonotic. The majority well-known milk-borne illnesses caused by bacteria, it is generally accepted. The genus *Campylobacter* has grown to contain a variety of significant illnesses that affect both people and animals since its original taxonomic validation. It has consistently been a pathogen that has been isolated in outbreaks over the past few decades in countries that are both developed and developing , Pasteurization is a common practice in the dairy industry, and is primarily intended for the reduction of the milk pathogenic bacteria load, which has to be below the admissible level. Heat treatment translates into an increased shelf-life of milk and limits proliferation and activity of microorganisms detrimental for cheese processing. Common milk pasteurization techniques comprise heating at either 63 °C for 30 min (low-temperature long-time, LTLT), 72 °C for 15 s (high-temperature short-time, HTST) or any other equivalent thermal treatment [1,2].

Campylobacter are common in dairy and cow ranches. Fecal contamination of milk from the known Campylobacter infection reservoir was observed during the documented outbreaks. was the most frequent cause of milk contamination, followed by improperly cleaned machines, bovine illnesses (mastitis), and poorly cleaned machinery. Infections with campylobacter are typically brought on by drinking or eating contaminated food. *Campylobacter* is frequently found in raw milk and dairy products, which raises the possibility that it could spread zoonotic diseases to people[3].

The primary result of consumption certain *Campylobacte*r species can result in infection is campylobacteriosis, a zoonotic infection that is typically diagnosed as gastroenteritis[4]. According to reports, certain Campylobacter species have the capacity for attach to and attack human intestinal epithelial cells epithelial cells of the gut using their flagellum, which reduces the capacity of the intestinal barrier, produces toxins, and purposefully inhibits immunological responses [5].Abdominal pains, diarrhea (usually bloody), vomiting, headache, nausea, dizziness, lethargy, and fever are only a few of the many symptoms [6].

The epidemiology of the *Campylobacter* infection varies greatly across industrialized and underdeveloped nations. In contrast to campylobacteriosis epidemics in developed countries, *campylobacter* enteritis shows no predilection for seasonality in underdeveloped countries[7].

Along with the burden of illnesses brought on The development of resistant *Campylobacte*r species is another cost to public health caused by these bacterial pathogens, and it might turn worse in developing countries where the use of antibiotics is generally unregulated [8 ,9].

This study's objectives were to determine the rates of infection and molecular identification of *Campylobacter jejuni & Campylobacter upsaliense* in bovine milk products and human feces at Kerbala retail points.

Materials and Methods

Study design and Sample collection

Two hundred samples from bovine milks .milk products and human stool sample , were collected as part of a cross-sectional investigation. These samples came from various areas 100 sample of milk collected from different farm ,field and local store distributed in the province of Karbala, and finally about 100 stool samples from people with diarrhea from General Children's Hospital. Then they were cultivated in the proper media according to accepted techniques for the bacterial identification and cultivating .Following the first bacterial isolation process using the unique and particular Campylobacter culture media, such as campylobacter agar, propagation then took place [10].

Campylobacter isolation and identification

Collected samples were immediately inoculated onto C&S adapted Carry Blair put media, then grown on specific agar (campylobacter agar boosted with 5-10% blood), supplemented, and incubated to 48 hours at 42°C under conditions that were microaerophilic .Gram staining, microscopy examination, and Oxidase, catalase, and indoxyl hydrolysis biochemical tests were used to confirm the identity of the suspected colony[11].

Finally, the positive results were confirmed In order to extract DNA, 1 ml of fresh 24-hour culture the broth (Genaid/Korea) was prepared and used directly in accordance with the manufacturer's instructions. The growth of *Campylobacter jejuni* and *Campylobacter upselienes* infection was verified using PCR test. *Campylobacter ssp*. sodA gene primer was detected using oligonucleotide primers, forward primer GGATGACACTTTTCGGAGC and reverse primer CATTGTAGCACGTGTGTC, which resulted in amplified 812 bp PCR products. This was employed to identify the presence of bacteria in the samples, the procedures with these species-specific primers were carried out as described by [12] After 5 min of denatured state at 94 degrees Celsius, there are 35 cycles of the annealing at 50 Celsius for 30 s, expansion at 72 C for a minute, and the last extension at 72 Celsius for 5 m [11].

all positive isolates were transferred to Macrogen for Sanger sequencing. Using SnapGene version 5.2.5 (www.ncbi pgene.com), the 16SrRNA gene's nucleotide sequences were examined and manually modified for quality trimming.

Antibiotics susceptibility testing

In accordance with CLSI recommendations, 12 different Campylobacter isolates were tested for susceptibility to 12 different antibiotics using a modified Kirby-Bauer diffusion disk method [13]. The inhibition zones surrounding antibiotic disks were interpreted utilizing the Enterobacteriaceae published by CLSI breakpoints. Azithromycin (15 mg), ciprofloxacin (5 mg), ceftriaxone (30 mg), nalicdixic acid (30 mg), gentamicin (10 mg), penicillin (10 mg), erythromycin (10 mg), tetracyclin (30 mg), imipenem (10 mg), ampiciline (10 mg), amoxicillin (25 mg), and streptomycin (10 mg) were the 12 antibiotics that were tested and placed on the agar surface. A zone of clearing following the incubation period indicated the presence of resistance to ciprofloxacin, gentamycin, and imipenem.[2]

Statistical Analysis

The statistical program utilized for this investigation was SPSS (version 21). Differences between the one group were identified using a study of Chi-square scores. Five percentage points ($P \leq 0.05$) were deemed to be a significant difference[14]

Results and Discussion

Culture and biochemical test

On Campylobacter agar base selective medium, a Campylobacter spp. colony that has been successfully isolated presents as a tiny, mucoid colony that is normally grayish in color, while some colonies must be creamy grey. Additionally, these colonies create discrete colonies that are flat with uneven borders and non-hemolytic at 24-48 hours that are slightly elevated, moist, and slightly raised.

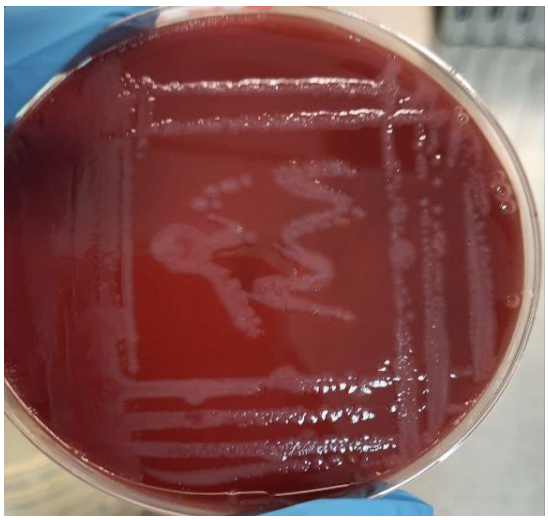


Figure (1): *Campylobacter* colony structure on blood-containing Campylobacter Base Agar

Campylobacter spp. prevalence based on sample sources

Campylobacter species prevalence in milk and milk products

Out of 100 samples of bovine milks and milk product, 3 % positive existence of Campylobacter spp.(table 1). Based on statistician inference, it is foredooming that (2%) for raw milk and (5%) of milk products at Karbala is contaminated by Campylobacter species. Regarding There are wide variations in the prevalence of Campylobacter in milk and milk products at holly Kerbala provenience.

Table (1): prevalence of *Campylobacter spp*. of milk and milk product

TYPE	NO .sample	Positive	percentage
Raw milk	50	1	2%
Cheese	20	1	5%
Cream	10	0	0%

Yogurt	20	1	5%
Total	100	3	3%
Statistical analysis	Chi square= 0.621 P=0.01		

According to the current findings, there is a connection between the presence of bacteria and the kind of sample where there was a demonstrable rise in milk when compared to milk-derived products (p-value 0.01).

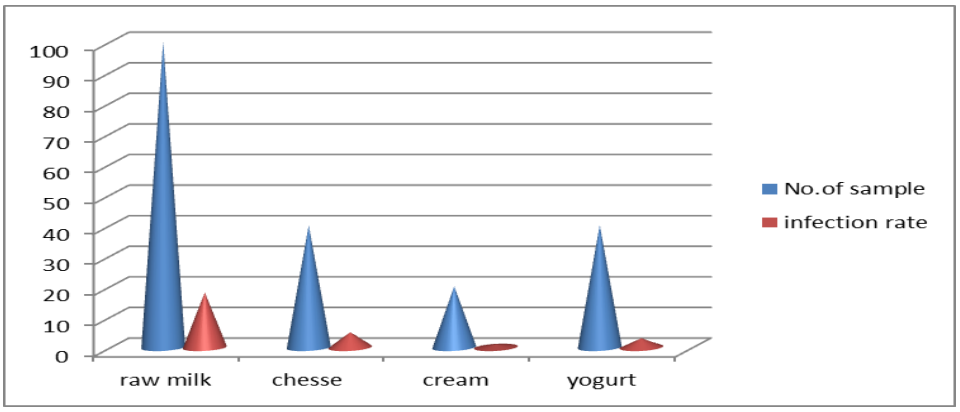


Figure (2): infection rates of *Campylobacter spp.* In milk and milk product

According to the Global_Enteric_Multicenter_Study(GEMS) and the European_Centers_for_Disease_Control_and_Prevention (ECDC), *Campylobacter* is a significant gastrointestinal pathogen that is responsible for outbreaks all over the world. The current investigation yielded 3 (3%) favorable findings, which were dispersed among bovine milk and milk-related products. These findings are concur with a prior study conducted in Pakistan, which discovered that butter and raw milk had the greatest concentrations of *Campylobacter*. [15]. Additionally, similar prevalence rates were recorded in Italy research [16],Tanzania[17], and in Erbil (Iraq) [2] . while, other Iranian [18], Turkey [19], Egypt [20], and India [21] research revealed lower rates.

These variances could be explained by a number of factors, including regional differences, the sensitivity of the detection method, levels of hygiene, eating practices, and the presence of natural *Campylobacter* reservoirs [22].

Prevalence of *Campylobacter spp.* According to the Months:

The prevalence of *Campylobacter spp.* infections in our study applied to certain months, the study period undertaken of months from October 2022 to the end of march 2023 as shown in figure (3).

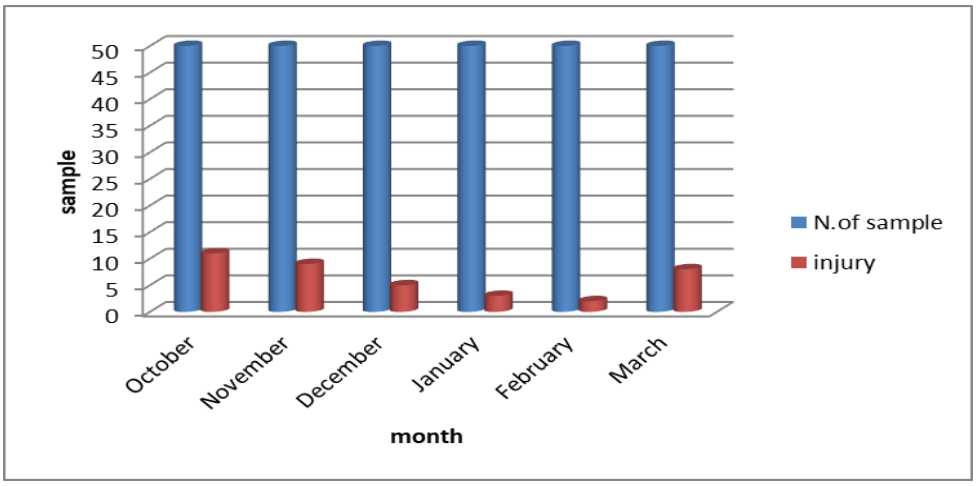


Figure (3): *Campylobacter* species distribution by month

According to study months, the spread of *Campylobacter spp.* showed a significant difference ($p<0.05$), the lowest rates of isolation in February 2 (4%), while the highest positive result was in October 11 (22%).

The current study involved detection the distribution of the infection by *Campylobacter* Species, according for months of the study, and the result showed in (figure 2) The lowest prevalence rate of isolation was observed on February 2 (1%), while the greatest was observed on October 11 (5%), with significant variations ($p<0.05$) being reported. In relation to the *Campylobacter spp*. found in this study, early spring and early summer had the highest recorded occurrence of *Campylobacter* Winter had the lowest rate in terms of based on time prevalence. These results are in line with a study conducted in Nigeria, which discovered that the occurrence of campylobacteriosis increases in the summer and then decreases throughout the winter [23] .

Numerous research had linked warm seasons to increased *Campylobacter* prevalence in Germany [24,25,26], Egypt [27] , Lebanon [28]and Iraq [11]. Although the underlying cause of this seasonality is yet unknown, it may point to a potential correlation between temperature and *Campylobacter* survival and infection spread [2]. The risks of campylobacteriosis occur in the summer in temperate locations where the frequency is clearly seasonal, but it is much less so in tropical areas [29].

Campylobacter spp. prevalence in human stool samples

From a total of (100) stool samples taken from infected patients who had diarrhea and some of them also had other symptoms including fever, colic, and vomiting at educational children's hospitals in Karbala city, Human infections with *Campylobacter spp.* were eight percent (8%); see Figure (4**).**

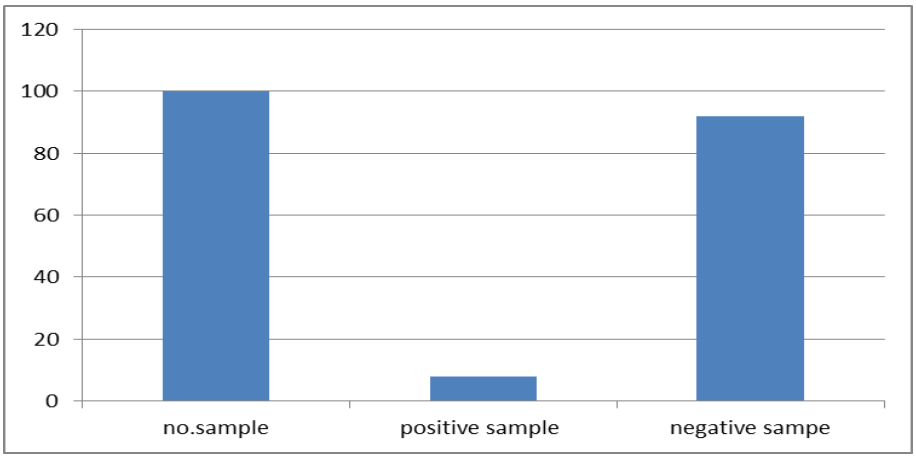


Figure (4): *Campylobacter spp.* prevalence in Human Stool The specimens.

According to this study, 8 (8%) of 100 stool samples produced *Campylobacter species.* (Table 2) indicates that there is significant value (P≤0.05) link between age and Campylobacter spp. infection, with the incidence rate being lowest in children under five years old. This result was agree with [30],who identified *Campylobacter spp*. from stool samples by PCR assay, while much higher than that found by [31], who demonstrated that PCR was more sensitive (100%) than direct bacterial culture (49%) and who discovered *Campylobacter spp.* in patients with diarrhea (16.6%) by direct Real-Time PCR by hipo gene primers. The difference in detection rate between this study and other research may be influenced by a variety of variables, including age, season, region, and human immune status [32].

This overall *Campylobacter spp*. recovery rate was consistent with a 2011 study by AL-Hamadani and Saleh in the al Diwanyiah governorate, which indicated that the percentage of *C.jejuni* in children was 9%.

5.4% of children with diarrhea were reported to have an isolated *Campylobacter* species in Turkey. [33] India's research mentioned the percentage 7% [34] and In Lebanon, 11.1% [35]. According to WHO and FAO reports from 2012, Europe and North America had a 9.3 percent incidence of Campylobacteriosis, One of the four primary causes of diarrheal diseases worldwide is *Campylobacter* and thought to be one of the most typical and gastroenteritis is primarily caused by bacteria and affects people everywhere [36].

Due to the fastidious nature of the bacteria, *Campylobacter's* self-limitation, and possibly other difficult-to-control factors, the culture method has a low recovery rate. These factors include contamination, intestinal flora, loss of organism viability during transportation, and consumption of proton pump inhibitors (PPI), which have an indirect antibacterial effect[37]. All of these could be to blame for a campylobacter culture's negative predictive value. [38,39, 37,11].

Distribution of Campylobacter isolates according to age:

According to the results of the current investigation, which found 8 (8%) positive results, there is a significant ($p<0.05$) link among age and *Campylobacter spp*.

infection, as the prevalence rate is lowest in children under the age of five and rises with the age ,table (2).

Table (2): the frequency of *Campylobacter spp.* in stool samples from patients, broken down by age

Age rang (years)	Number of sample	Number of positive culture	Percentage%
2 month-1year	35	5	71 % a
1-5 year	33	2	40% ab
5-10 year	30	1	16% a
Total	100	8	8%

***Similar litters refer to the significant differences between different litters while age groups show no significant differences ($p<0.05$).**

There was significant difference between percentage of age groups where the first group (2month - 1year) has significant difference with third age group (5-10 year) while second group (1-5 year) has no significant differences with both first and third age groups

Due to their immature immune systems and the fact that *Campylobacter* infection is regarded as a self-limited disease dependent on immune system, the results of this study and others like it demonstrated that the most effective age with campylobacter is under five years [40,41].

These findings were agree with that founded by other researchers in some points in Brazil, [42] revealed a substantial correlation between the prevalence of *C. jejuni* in infants and toddlers aged 0 to 12 months.. In Québec /Canada ,the incidence rate in children less than 4 years was significantly higher than other ages for both rural and urban area [43]. Furthermore, [44] Children under the age of two have been reported to frequently have campylobacter infections, which can occasionally be fatal. The fact that children are typically the only ones affected by Campylobacter in underdeveloped countries where the disease is frequent suggests that early exposure may lead to the development of a protective immunity[45,46,47].

Molecular identification

16S rRNA gene PCR-based molecular identification

The molecular test used for identification and confirmation of *Campylobacter spp*. as genus by PCR technique, the culture appeared 19 suspected samples, but the PCR gave 11(5.5%) positive isolates showed to be *Campylobacter spp.*

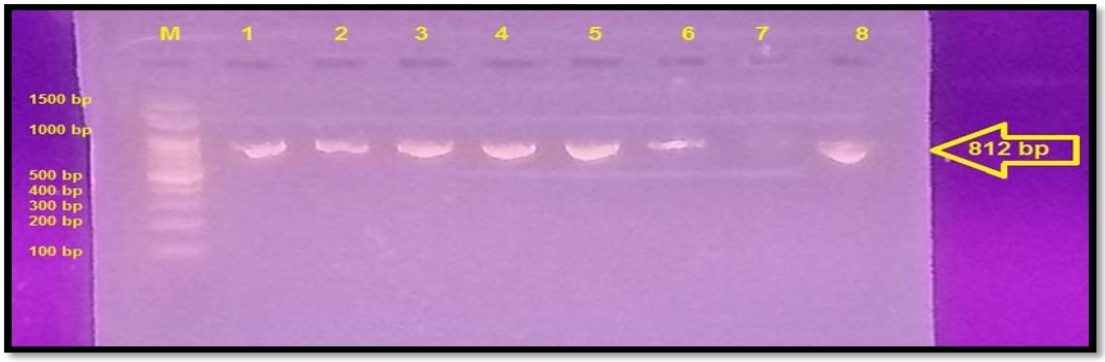


Figure (5): Amplification of the 16S rRNA gene specific to the Campylobacter species

The availability of a trustworthy species isolation strategy, which gives data that can be compared across laboratories and over time for all strains is essential to achieving this goal [48].

Typing by Sequence Method

Sequence Typing of Partial 16S rRNA Gene from *Campylobacter spp.*

Molecular identification of Campylobacter was applied using the 16 S rRNA gene. The amplicons from the isolated samples were delivered to MACROGEN® for Sanger sequencer sequencing. Nucleotide sequences of high quality (forward or reverse) were found, compared to archives in the National_Center_for_Biotechnology_Information (NCBI), and classified using bioinformatics programs and algorithms designed for this kind of study.

The results of the isolates showed a 100% match rate with the global isolates, and it was considered the first record of the isolates in the Genome Bank and was given special numbers as shown in Table (3).

Table (3):special number of isolates colonies

No.	Analysis	Accession number	Code	Source
1	*C. jejuni*	OQ253478.1	S1	Milk
2	*C.jejeni*	OQ318488.1	IQ4	Milk
3	*C. jejuni*	OQ283981.1	S3	Milk
4	*C.jejuni*	OQ287043.1	S4	Human
5	*C. jejuni*	OQ253511.1	S7	Human
6	*C .jejeni*	OQ284054.1	S10	Human
7	*C.jejuni*	OQ318512.1	IQ12	Human
8	*C.jejnui*	OQ331024.1	IQ16	Human
9	*C.upsaliensis*	OQ338009.1	IQ 17	Human
10	*C. upsliensis*	OQ338139.1	1Q18	Human
11	*C.jejuni*	OQ329425.1	IQ14	Human

the analysis of the 11 isolates, showed matching 9 (81%) sequences as a *C. jejuni* and 2 (18%) of strains as a *C. upsalinses* among the sequenced isolates in table (4).

Table (4): prevalence of *Campylobacter jejuni _C. upsaliensis* according to the type of samples

Type	No. sample	*c.jejeni*	*c. uplsalinsis*
Milk& Milk product	3	3 (1%)	0
Human	8	6(75%)	2(25%)
Total	11	9(81%)	2(25%)
Statistically analysis	P=0.012 Chi =7.62		

The 11 *Campylobacter* strain isolates used in this study's bioinformatics analysis displayed very high similarity (approximately 99%) with *Campylobacter spp*. isolates previously found in Europe and America [49].

It is common practice to identify between different *Campylobacter spp*. using 16S rRNA gene sequences [50]. Our findings provided significant evidence in favor of the approach to that mentioned and recommended by [21]. The findings did, in fact, concur with [48] Typing by Sequence Method, this method of high-resolution bacterial genotyping has been helpful in molecular research [51].

The identification two isolates with high efficiency in the production of gasteroenitierites It was recorded in the Global Gene Bank, and it was found that the use of traditional methods is insufficient in most cases, due to the heterogeneous phenotype and polymorphism as well as the different environmental conditions. It was diagnosed using the polymerase chain reaction (PCR) technique, depending on primers prepared for the purpose of molecular diagnosis[48].

The results showed that the registration of this bacteria was recorded in the global gene bank, as shown in the registration information and the study of the affinity and similarity between the registered bacteria. Traditional diagnosis and genotype determination are important in the classification of bacteria. The SSU region has been widely used in classification and molecular diagnosis due to its ease of amplification and its wide range of variability, even in highly related species[52].

DNA sequencing to ensure the sequence of nucleotides and then compare it with other international strains, and the NCBI-BLAST program was used and gave accurate results by comparing it with international strains, and the genetic analysis program was used Molecular Evolutionary (BLAST) is an analysis application designed to compare similar gene sequences, evolutionary relationships, and the pattern of DNA and protein evolution [53]

The reason for the wide spread of this bacteria in different places around the world is due to the possibility of its transmission through the import and export of various foodstuffs and goods, as well as the possibility of its transmission through people who carry it [54].

Evaluation of antibiotic susceptibility test.

The susceptibility of 11 isolates to 12 antibiotics was examined, and they were then classed as susceptible, intermediate and resistant, as shown in figure [6].

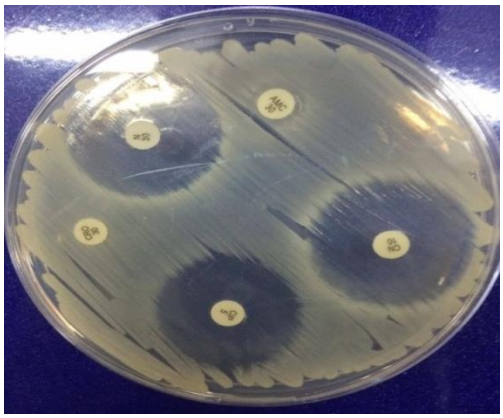


Figure (6): According to an antibiotic susceptibility test, this figure indicates the extraordinary resistance of several *Campylobacter spp.* isolates

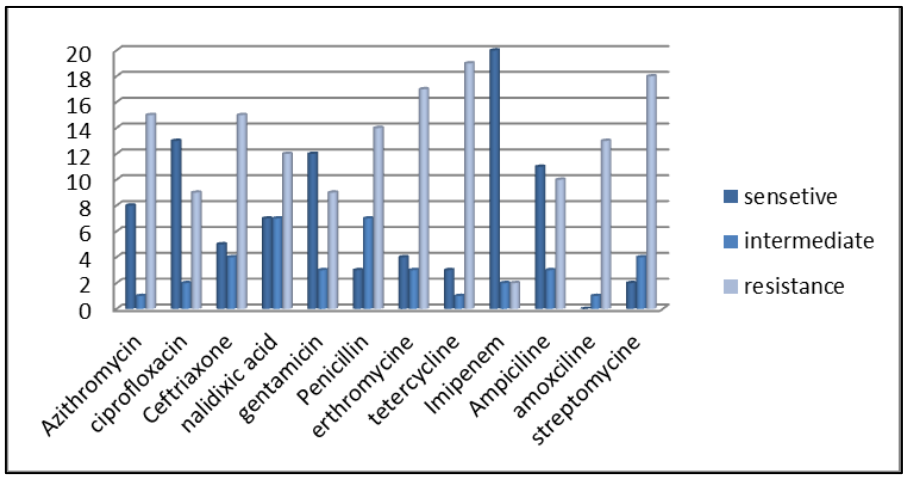
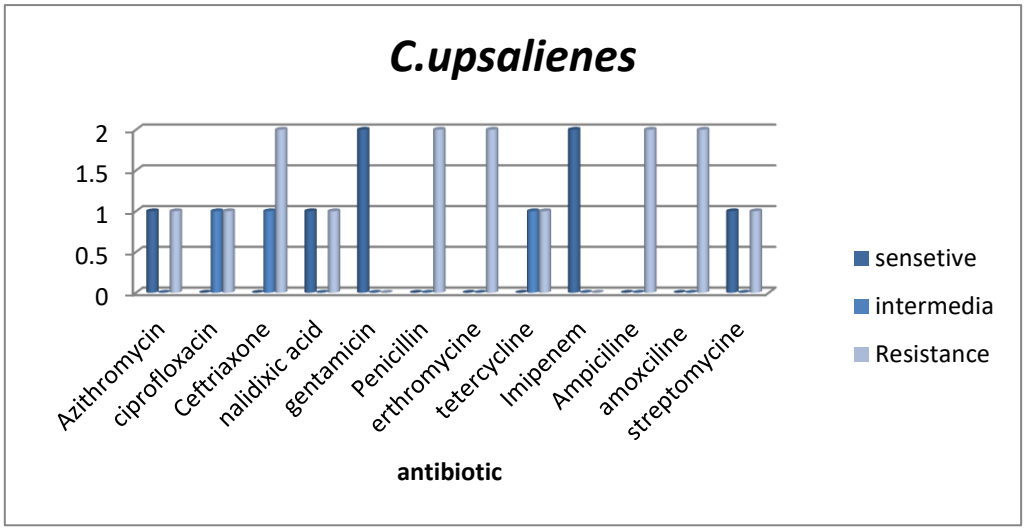


Figure (7): Effect of antibiotics on *Campylobacter jejuni*

Campylobacter jejuni showed in our study which high sensitive to Imipenem (83%) , gentamycin (50%) and ciprofloxacin (54%).While 100% resistance to tetracycline, ampicillin, and streptomycin was noted (Figure 8). In published research from various nations throughout the world, the pattern of antibiotic resistance of Campylobacter isolates varies greatly.



Figure(8):effect of antibiotic on *Campylobacter upsalienis*

Campylobacter upsaliensis showed in our study which high sensitive to Imipenem (100%) and gentamycin (100%) While 100% resistance to erthromyicine, amoxicline, and ampicillin was noted (Figure 9). In published studies from several nations throughout the world, Significant regional diversity can be seen in the pattern of Campylobacter isolates' antibiotic resistance.

In published research from various nations throughout the world, *Campylobacter* isolates exhibit a wide range of antibiotic resistance patterns. [55] .

The current study's finding of ciprofloxacin sensitive was agree with levels previously reported in Iran (30.77% and 34.4%) [56,57]. As well as, higher than that reported in India [21]. This study's high sensitivity to ampicillin, erythromycin, and gentamicin is consistent with the high sensitivity to gentamicin and streptomycin discovered in a recent Tanzanian study of cattle corpses and unpasteurized milk samples [17].

This is the study reporting prevalence and estimating impact of some risk factors of *Campylobacter* infection in milk and milk products, and use molecular test to confirm the diagnosis which show that PCR technique represent the rapid more accurate method to diagnose the pathogen, and according to the sequence registration of local isolates appeared two *Campylobacter spp.* (*C. jejuni* ,*C.upsaliensis*). The present data showed an increase prevalence rate in October and November. The infection rates were inversely recorded with age and were higher at younger ages. on other side, the antibiotic susceptibility test used to investigate of *campylobacter* resistance to antibiotics, To reduce the risks to public health posed by the spread of pathogens with multiple drug resistance, randomly use of antibiotics should be prohibited.

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