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Original Research Article

Antimicrobial Resistance of *Escherichia Coli* in Cow's Milk Samples from Farm Milk, Milk Vendor and Shops Milk in Babylon City

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Abstract: Cow's milk is a whole food product that is sensitive to many bacterial contamination. In this study sixty cow milk samples were collected from farm milk, milk vendor and shops milk on Babylon city from October to December 2023, all milk samples were obtained from three source for bacterial availability analysis. All collected samples are processed in laboratory for estimation viable bacterial count and phenotypic detection of *Escherichia* by plated them on MacConkey agar, and show greenish metallic sheen on colonies in Eosin Methylene Blue. Consequently, antibiotic resistance profile of isolates done by Kirby-Bauer technique on Muller Hinton agar plats, analysis according to recommended by criteria of Instructions of Clinical Laboratory Standards Institute (CLSI), and biofilm formation detection by staining with methylene blue on tissue plate assay. The results showed detection of 53.2% (n=32) positive of *Escherichia coli* out of sixty cow milk samples and It was confirmed by Vitek system and indole test; farm milk 13.3 % (n=8) with average count 6.7×10^7 cfu/ml, milk vendor 23.3 % (n=14) with average count1.8×10^8 cfu/ml, and shops milk 16.6 % (n=10) average count 1.3×10^8 cfu/ml, and the susceptibility rate of *Escherichia coli* were tested in the following order: Ciprofloxacin 10 mcg (25.0%), Ciprofloxacin5 mcg (31.2 %), Ampiclox (28.1%),Ampicillin (31.2 %), Trimethoprim (37.5%), Gentamycin (25.0%), Tobramycin (43.7%), Streptomycin (15.6%), Amikacin (28.1%), Doxycillin (31.2 %),Cefaxitin (28.1%),Ceftazidime (25.0%), and Aztreonam(31.2) %. In conclusion, data showed higher contamination of Cow's milk in Babylon city from different source with *Escherichia coli*, in which biofilm producers and resistance to many antibiotic were notice, Therefore, we recommend periodic monitoring of producers and sellers of raw milk and its products and imposing strict laws to limit the spread of foodborne illnesses and antibiotic-resistant bacteria.

Keywords: Escherichia Coli, Cow's Milk, Antimicrobial Resistance, Biofilm.

INTRODUCTION

Despite the scientific development and technical improvements that the world is witnessing, food-borne pathogens are the main cause of many diseases, and health, medical, and social care costs continue to cost countries high amounts(Fufa *et al.*, 2022). Milk is considered an important food for humans and an ideal medium for the growth and transmission of many pathogens, including *Escherichia coli*, which causes spoilage of milk and its products and thus causes many diseases to consumers, which represents a major challenge to public health in many countries. (Haftay *et al.*, 2018).

Escherichia coli is a facultative anaerobe and one of the natural plants in the digestive system of humans and animals. It helps in digestion processes and the manufacture of some vitamins. It has been proven at present that these bacteria have many harmful effects serotypes that cause many diseases such as urinary tract infections, hospital-acquired pneumonia, gastrointestinal infections, sepsis, hemolytic-uremic syndrome and meningitis (Jolanta *et al.*, 2019).

The main source of *Escherichia coli* is dairy cows, which can contaminate products and tools, and through contact with dairy cows, strains of *E. Coli* O:157: H7 is highly pathogenic to humans, capable of

surviving in fresh, processed milk and its products, and causing diseases in low doses through the consumption of fresh milk or its products (Ariyanti *et al.*, 2022).

There is widespread global interest in the quality of milk intended for consumption. However, the transportation and circulation of unpasteurized raw milk can be a reason for the transmission of many diseases from these tanks to humans, including *Escherichia coli* infection, and this milk taken from these tanks can be a source of harboring microorganisms through mastitis or external contamination (LeJeune *et al.*, 2004). Moreover, there are many sources of contamination which helps the bacteria to enter the milk, such as unclean animals during milking, air, feed, milk transport equipment, milking equipment, milk storage containers, milking personnel and transportation (Mohammed and Fawzi, 2023).

The global incidence of antimicrobial resistance in *Escherichia coli* has been increasing over the years, which raises concern and underscores the immediate need to take rapid and appropriate measures to prevent the increase and transmission of infection (Salleh *et al.*, 2022). Many studies in many countries of the world have proven that the food chain containing animal bacterial reservoirs is the easiest route for the passage of *Escherichia coli* bacteria that are resistant to commonly used antibiotics (Abebe *et al.*, 2023). Moreover the incorrect and indiscriminate use of therapeutic agents as a preventive or therapeutic means for humans and animals has led to the emergence of resistant strains and makes it difficult to treat various disease conditions. *Escherichia coli* resistance has been reported, and its treatment has become very difficult and extremely complex due to the emergence of its resistance to most first-line antibiotics, such as ampicillin, amoxicillin, ceftriaxone, chloramphenicol, ciprofloxacin, cotrimoxazole and tetracycline (Frehiwot *et al.*, 2023).

Biofilms are a way of life of unicellular organisms that can provide nutrients, exchange information, and protect them from harsh environmental conditions. However, *Escherichia coli* biofilms are more resistant than their planktonic cells because *Escherichia coli* are able to switch their cells between planktonic and planktonic forms, and *Escherichia coli* showed the ability to colonize and form biofilms on surfaces. The bacteria must be very close to the biofilm to form the membrane (about 10–20 nm distance from the surface), and this mechanical attachment to surfaces variety of surfaces is thanks to fimbriae and flagella (Milojević *et al.*, 2017). Moreover, there are many isolates that have the ability to produce biofilms in the body and in vitro. In fact, *Escherichia coli* is among the dominant anaerobic species found in the digestive system that have the ability to form films in an environment with the structural characteristics of multiple types of biofilms (Beloin *et al.*,2010).

In Babylon, milk and its products are consumed in many areas of the governorate in significant quantities. However, the quality of milk and its products is not monitored in most areas of the governorate, which leads to an increase in the poor quality of milk and its products. Moreover, most breeders and milk sellers sell the milk directly without taking any preventive measures to prevent contamination. This study was conducted to determine the extent of milk contamination with *Escherichia coli*, and measure its resistance to antibiotics, and examine the ability of this pathogen to form biofilms.

MATERIAL AND METHODS

The current study was conducted in ideal conditions, and all necessary circumstances were taken into account to conduct the experiment in the period from October to December 2023 in Babylon governorate.

Collection and Preservation of Milk Samples

Samples were collected from many source form different regions of Babylon city. 60 samples were collected in each study, with each sample consisting of 25-50 ml. samples were taken in the sterile cup and collected in refrigerated and thermally insulated containers and transported directly to the public health laboratory of veterinary medicine.

Estimation of Total Bacterial Counts

1 ml of cow milk was homogenized into 9 ml of serial peptone water by pipette. Then serial dilutions were prepared and taken 1 ml from the first tube and added to the next tube, in order to obtain dilutions1\100, 1\1000, 1\10000, and 1 ml of $10^{-4}10^{-5}$ 10^{-6}, 10^{-7} and 10^{-8} dilutions was cultured on standard plate count agar and incubated at 37°C for 24 hrs. The counts expressed of the suspension as colony-forming unit (cfu/ml) (Haftay *et al.*, 2018).

Isolation of Bacteria

All milk samples was inoculated as 1 part milk sample (10ml) to 9 parts (90ml) on Tryptone soy broth and inoculated at 37 °C for 24 hours, then inoculated (10 µL) by loop on McConkey agar at 35-37°C for 24 hours (Bacteriological Analytical Manual, 2017).

Showing dark pink to red colors circular colonies, re-cultured in Tryptone soya broth and inoculated over night at 37 °C, then streaked on EMB agar plates and incubated over night at 37°C. for showing metallic sheen green colonies, after confirmed *E. coli* isolates stored as pure culture in brain heart infusion broth contain (glycerol 30%),as preserved for further studied.

Indole Test

A pure single colony was incubated with tryptone broth in test tube at 37°C for 24 (as part of the IMViC procedures) then Add 0.5 ml of Kovács reagent to the tube after inoculated, development of a brown-red

to purple-red color as oil layer shape at the top of the broth within twenty sec. indicates the presence of indole- (Acharya Tankeshwar, 2023).

Detection of Biofilm Production by Micro Titer Plate Assay

Micro titer plate assay (MtPA) is a quantitative method used to measure and determine biofilm production in lab by using micro plate reader. Firstly, Prepared suspension of bacteria in Mueller-Hinton Broth (MHB) with glucose 1% as supplement and adjusted to 0.5 McFarland, then inoculated into 96-well flat-bottomed sterile poly styrene micro plate 20 µl of bacterial suspensions and the plate inoculated at 24 h at 37°C in static condition. (Sahra Kırmusaoğlu, 2019).

After incubation the plate was washed three times with phosphate buffered saline (PBS) to remove non-adherent cells, then left the plate to dry and fixed the adherent biofilm with 2% sodium acetate then stained with 0.2% (w v–1) crystal violet solution for 30 min. finally, rinsed the plate with deionized water for remove excess stain and plate was left 2-3 hours for dryness then stained layers in bottom and around internal edge of wells were photographed (Bhakti *et al*., 2017).

Antimicrobial Susceptibility Test

Antibiotic susceptibility analysis was performed according to (CLSI, 2021), and by using available commercial antibiotic disks by Kirby Bauer method on Muller Hinton agar plats. The antibiotic disks were used, Ciprofloxacin, Ciprofloxacin, Ampiclox, Ampicillin, Trimethoprim, Gentamycin, Tobramycin, Streptomycin, Amikacin, Doxycillin, Cefaxitin, Ceftazidime, and Aztreonam. 4-5 colonies grown on McConkey plates at 37°C were selected and inoculated with 4-5 ml of nutrient medium for two hours at 37°C and compared with 0.5 McFarland standards tubs. Muller Hinton agar plats were prepared and antibiotic discs were carefully placed after wiping the medium with the prepared inoculum with a sterile cotton. The results was analysis after incubation for 18-24 hours at 37°C (Jan, 2009).

RESULTS AND DISCUSSION

A total of 60 milk samples from Babylon city were cultured for estimation of total bacterial count. It was found that 85% (n=51) of the samples had bacteria growth while nine samples (15%) had no growth, and these were careless in the analysis of data.

The overall estimation total viable bacterial count of cow milk from deference source (Table 1).

Table 1: Estimation total viable bacterial count from farm milk, milk vendor and shops milk

Source of sample	No	Mean ±SE of Total viable bacterial count (x 10^8) CFU/ml
Shops milk	20	1.3 ±0.07 b
Milk vendor	20	1.8 ±0.11 a
Farm milk	20	0.67 ±0.04 c
LSD (P-value)	---	0.427 * (0.00298)
Means having with the different letters in same column differed significantly, ** (P≤0.01).		

The isolation results showed the detection of thirty-two (53.2%) positive isolates gram negative *Escherichia coli* out of 60 milk samples from three different source: farm milk (n=8), shops milk (n=10) milk vendor (n=14) and (Table 2).

Table 2: Number and percentage % of *Escherichia coli* in cow milk of all samples

Source of sample	No. of Examined samples	No. of Positive sample	No. of Positive (%) of samples (60)
Shops milk	20	8	13.30%
Milk vendor	20	10	16.60%
Farm milk	20	14	23.30%
Total	60	32	53.20%
Chi-Square (P-value)	---	---	1.770 NS (0.412)
Non-Significant.			

In general, it is possible to use agar plates counts to estimate the number of bacteria present in milk, and according to sources, the minimum and safe limits are 10^5(cfu/ml), and less than 1.5×10^4 (cfu/ml) considered desirable (Milk Quality, 2017).

The study finding a higher estimation viable bacterial count from farm milk (average count: 6.7×10^7 CFU/ml), milk vendor (average count:1.8×10^8 CFU/ml) and shops milk (average count 1.3×10^8 CFU/ml), and higher than previous reports in Al-hilla state (Babylon city) (Mohammed *et al.*, 2019) and Karbala city (Saleh *et al.*, 2022), and Dohuk (Mohammed and Fawzi 2023).

This difference may be due to the lack of culture among most farmers, as well as unsanitary treatment

when collecting milk and the use of unclean tools, as well as transporting milk in unrefrigerated cars, in addition to the environment contaminated with many microbes, including *E. coli*, which helps these microbes grow at a high speed.

The difference in the counting and isolation of microorganisms may be due to incorrect preparation of the udder, as well as the presence of microorganisms in the udder ducts, in addition to external contamination resulting from tools, transportation, human workers, and sellers. All of these and other reasons cause an increase in the bacterial load of raw milk.

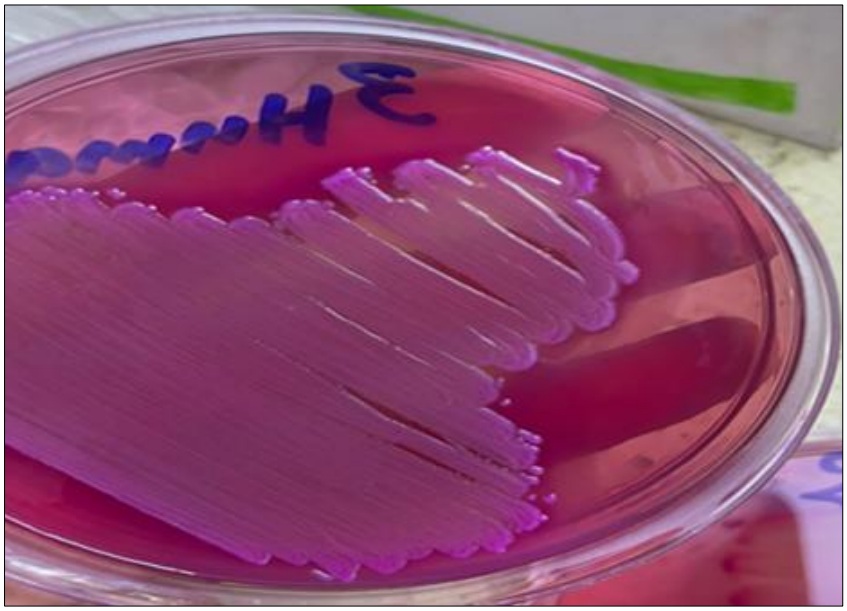
A table of resistance to antibiotics has been organized for *Escherichia coli* isolates, isolated from cow's milk. These bacteria have shown resistance to many antibiotics, as shown in (Table 3).

Table 3: Antibiotic resistance Pattern, intermediate and susceptible of *Escherichia Coli*

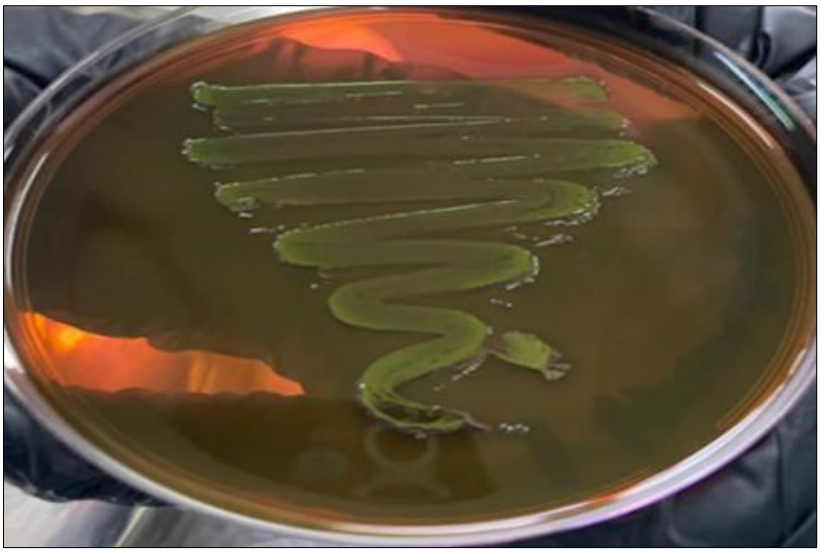
Antibiotics	Resistance	Intermediate	Sensitive	P-value
Ciprofloxacin (CIP) 10 mcg	15.60%	21.80%	62.50%	0.0001 **
Ciprofloxacin (CIP) 5 mcg	31.20%	40.60%	28.10%	0.0419 *
Ampiclox (APX) 30 mcg	28.10%	25.00%	46.80%	0.0276 *
Ampicillin (AM) 10 mcg	31.20%	28.10%	40.60%	0.0411 *
Trimethoprim (TM) 10 mcg	37.50%	31.20%	31.20%	0.217 NS
Gentamycin (GN) 10 mcg	15.60%	21.8%%	62.50%	0.0001 **
Tobramycin (TOB) 10 mcg	43.70%	37.50%	18.70%	0.0001 **
Streptomycin HLS 300 mcg	15.60%	15.60%	71.80%	0.0001 **
Amikacin (AM) 30 mcg	28.10%	28.10%	43.70%	0.0348 *
Doxycillin (D) 30 mcg	31.20%	31.20%	37.50%	0.217 NS
Cefaxitin (CX)30 mcg	28.10%	31.20%	40.60%	0.0419 *
Ceftazidime (CAZ) 30 mcg	25.00%	28.10%	46.80%	0.0276 *
Aztreonam (ATM) 30 mcg	31.20%	15.60%	53.10%	0.0001 **
P-value	0.0001 **	0.0001 **	0.0001 **	---
* (P≤0.05), ** (P≤0.01).				

The current study showed that *Escherichia coli* isolated from cow milk possesses resistance to many antibiotics, varying between high and moderate, and this arises from the indiscriminate use of veterinary medicines to treat some recurrent cases of mastitis, in addition to the contamination of milk from people with chronic diseases as a result of incorrect handling. Many studies have indicated the presence of resistance to this bacteria isolated from milk (Hassan *et al.*, 2022) and (Satwik *et al.*, 2021)

Regarding biofilm production, this study was in agreement with many researchers on the ability of this isolated bacteria to adapt and produce biofilm, which may have a role in the resistance of this bacteria to antibiotics (Muslim Musa, 2020) and (Milanov *et al.*, 2015).

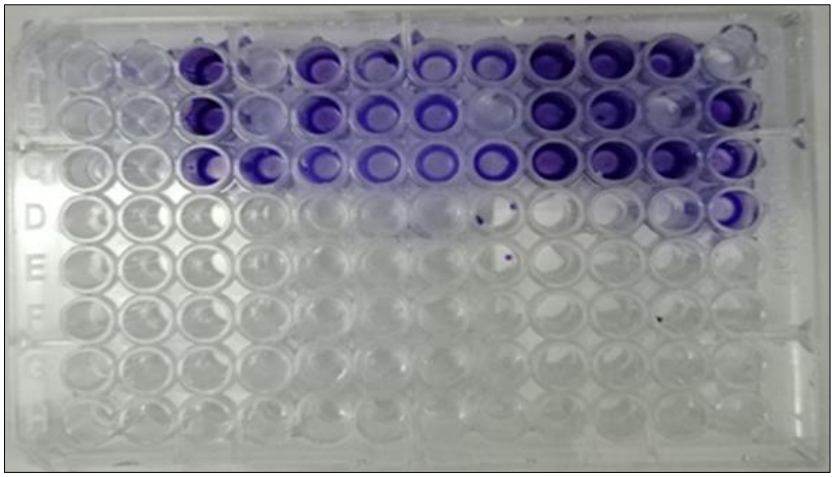


Show circular pink colonies On MacConkey agar



Metallic sheen colony on Eosin-methylene-blue agar

Positive Indole test of *Escherichia coli*



Biofilm formation on microtiter plate

Antibiotic susceptibility test

Statistical Analysis

The Statistical Analysis System- SAS (2018) program was used to detect the effect of difference factors in study parameters. Least significant difference –LSD test (Analysis of Variation-ANOVA) was used to significant compare between means. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability) in this study.

CONCLUSION

The current study demonstrated the high contamination of cow's milk samples taken from several sources with *Escherichia coli* due to Unhygienic handling of raw milk and lack of health control in collecting and transporting milk, as well as raw milk is also considered a source of antibiotic-resistant of *Escherichia coli*. Which gives an indication of the increase food borne diseases that transmitted through the milk. Therefore, we recommend taking into account healthy methods in dealing with food materials and not using indiscriminate antibiotics in treating humans and animals.

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