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PHENOTYPIC DETECTION OF EXTENDED SPECTRUM BETA LACTAMASES *ESCHERICHIA COLI* IN MILK AND SOFT CHEESE WITH ITS WHEY SAMPLES FROM BABYLON PROVINCE

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Abstract

Milk is considered as whole food and a significant part of human nutrition everywhere the world including Iraq. In this study collection and processing 200 samples (100 milk samples, 75 soft cheese samples and 25 whey samples) for isolation and identification of *E. coli* producing (ESBL) during six months from different reign of Babylon province, in which they collected and processed in veterinary public health laboratory by used MacConkey agar and then streaked on EMB agar plates for showing greenish metallic sheen. Congo red stain assay are used for detection of biofilm and Kirby-Bauer technique for detection of antibiotics susceptibility pattern by Muller-Hinton agar and McFarland opacity tubes for checking resistance profile of isolates. ESBL production isolates was studied by phenotype method as Clinical and Laboratory Standards Institute (CLSI) guidelines by Oxoid Cefpodoxime Combination Kit. The results showed detection of 118(59%) positive samples of *E. coli*. In vitro Among 118(59%) *E. coli* isolates only 24(20.33%) samples phenotypically *E coli* producer (ESBL) and 70(59.32%) biofilm production.

***Keywords*:** *Escherichia coli*, ESBL, milk, soft cheese, whey

Introduction

Escherichia coli one of the greatest significant opportunistic gram negative bacteria establish in the environment, foods, and intestines of people and animals. It's can be transmitted through the contamination of food or contact with infected animals or people (Central for Disease and Prevention Control, 2018). Its resistant to numerous antimicrobial agents and cause diverse infections and it's have numerous virulence genes coding to some significant virulence factors (Baker *et al*., 2017). Existence of pathogenic bacteria in milk is significant public health worry, especially when persons who drink raw milk without pasteurization (Nalband, 2015). Many factors which cause cheese contamination with multidrug resistant pathogens like nutritious component, spongier in nature, high water activity and polluted of raw milk by infected or carriers individual (Mohamed and Al-shammary, 2017).

Antimicrobial resistance is rising threat worldwide in human and veterinary medicine, which emerge from random used of antimicrobial agents among pathogenic and commensal bacteria predominant in food and environment cause amplified morbidity, mortality and rise cost of treating infections. Now greatest chief resistance mechanism in gram-negative bacteria such as *E. coli* production β-lactamase, which effectiveness of current cephalosporins and monobactams (Wright, 2016).

Extended-spectrum beta-lactamases (ESBLs) are plasmid-encoded enzymes provided resistance to 3rd generation(but not cephamycins or carbapenems). The activity of ESBL can be inhibited by beta-lactamase inhibitors such as clavulanic acid (Rawat and Nair 2010; Shaikh *et al*., 2015). Biofilm formation one of the significant virulence agent shown in *E. coli* stain, which reduction the susceptibility of bacteria to antimicrobial agents by enclosing them in an extracellular matrix by great production of polysaccharides, and decrease multiplication rate of organisms, which may share in emergence of chronic infections (Tayal *et al*., 2015). Many factor like virulence agent, modify the host immune response and genetic modifications assist bacterial cell to secreted of biofilm and became more resistance to antibiotics (Mittal *et al*., 2014). However, *E. coli* product biofilm strains become more resistant to antibiotics, and compare with non-biofilm strains (Ito *et al*., 2009).

Data on *E. coli* ESBL strain in Iraq are very limited. Therefore, the current study was conducted on different reign in Babylon province to estimate the prevalence of ESBL-producing and biofilm formation of *E. coli* from milk and soft cheese with its whey and to assess the antibiotic sensitivity pattern.

Materials and Methods

Two hindered samples milk, soft cheese and its whey were collected from different reign of Babylon province and were brought to laboratory of veterinary public health in Al-Qassim Green University. All the probable precautions were taken cautiously for less external contamination from collection until processing. Milk samples was inoculated on double strength power modified Tryptone soy yeast extract broth as 1 part sample (15ml) to 9 parts (135ml) broth. Stomacher with two percent buffered tri sodium citrate (2%) were emulsified soft cheese samples inside non-durable clean bags. Processed soft cheese samples were diluted and inoculated in buffered Tryptone soya yeast extract broth one part sample (10gm) to nine part diluent (90 ml) broth with processed milk samples were incubated at 37 ℃ for 24 hours, then inoculated (10 µL) in McConkey agar by loop, at 35-37°C for 24 hours (Bacteriological Analytical Manual, 2017). Showing pure smooth, circular dark pink to red colors colonies, re cultured in Tryptone soya yeast extract broth to reproduce and refresh isolates, then streaked on EMB agar plates and incubated for 37°C for 24 hr. for showing metallic sheen green colonies, confirmed *E. coli* isolates were stored in brain heart infusion broth contain 30% glycerol, as pure culture for standard morphological and biochemical tests as well as preserved for further studied.

Biofilm production assay

Poovendran *et al*. (2013) and Nanis (2017) described examination method for detection of biofilm formation, the medium was composed of (8 gm/l) Congo red stain as concentrated aqueous solution autoclave for 15 minutes at 121°C segregated from other medium ingredient then added to sterilized brain heart infusion agar was supplemented with sucrose (5 gm/100 ml) after cooled to 55°C. After inoculation of the organisms and incubated for 24 to 48 hours at 37°C appearance of black colonies with a dry crystalline shown that bacteria are producing biofilm and non-producers stay pink color. Foundation change of color medium which indicator the amount of production of biofilm by the organisms.

Test for detection of ESBL in *E.coli*

In food laboratory extended spectrum beta lactamase enzymes determined by easy performance and its understanding straightforward method (Oxoid Combination Disk Method). The principle of this method is measure difference of inhibition zone around the disk of cephalosporin and the same disk cephalosporin plus clavulanate. The kit contains Cefpodoxime/ clavulanic acid (CD01) 10/1µgm and Cefpodoxime (CPD10) 10µg, the test is acceptable for *E. coli* producing *ESBL* ≤ 2-mm increase in zone diameter for antimicrobial agent tested in combination of Cefpodoxime/ clavulanic acid (CD01) 10/1µgm vs the zone diameter when the Cefpodoxime (CPD10) 10µg tested alone (Oxoid, 2019; CLSI, 2019). (Muller-Hinton agars) are use with sufficient distance (20 mm) between two discs to permit the development of visibly zones of inhibition and combination between of them (Drieux *et al*., 2008).

Antimicrobial susceptibility test

A total of 118 isolates of *E. coli* (64 from milk,43 from cheeses and 11 from whey) were subjected to antibiotic susceptibility analysis by available commercial antibiotic disks according to recommended by criteria of Instructions of Clinical Laboratory Standards Institute by Kirby Bauer disk method on Muller Hinton agar plats. The following antibiotic disks were used, ampicillin (25 µg), ceftazidime (30 µg), cefepime (30 µg), aztreonam (30 µg), imipenem (10 µg), amikacin(30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), norfloxacin (10 µg), and clarithyromycin (15 µg), after prepared inoculum nutrient broth (4-5ml) with (4-5) colonies of *E. coli* isolated from inoculated overnight MacConkey agar plats then incubated 2 hr. at 37°C to reach of 0.5 McFarland standards turbidity tubs and Muller Hinton agar plats were prepared for streaking surface with cotton swab after dipped with adjusted of inoculum suspension then chosen of antimicrobial disks and putting carefully on the surface of the Muller Hinton agar to guarantee contact with the agar and distributed moderately and interpretation the results after incubator at 37 °C for (18-24) hrs. Length of a central line which extend from one side to opposite side around each of the antibiotic disks were measured in millimeters which indicate the susceptibility of organism to each drug as per (CLSI, 2015) guideline.

Results

The test was done after morphological and biochemical characterization indicated that a total of 118 isolates of *E. coli* (64 of milk, 43 soft cheese, and 11 in whey) from 200 samples (100 milk samples, 75 soft cheese and 25 whey samples). Phenotypically positive for ESBL-producing *E. coli*. 13(11.01%) from milk, 9(7.62%) from soft cheese, 2(1.69%) in whey and 70(59.32%) biofilm production isolates.

Table 1: Isolation of *E. Coli* from difference sources

Source (type)	Total sample	Isolate No	Percentage (%)
Milk	100	64	64.00
Cheese	75	43	57.33
whey	25	11	44.00
Total	200	118	59.00
Chi –Square (χ^2)	---	---	7.850 **
P-value	---	---	0.0063
** (P<0.01).			

Table 2 : Isolation of(ESBL) enzyme from *E. Coli* from difference sources

Source (type)	**Total sample**	**Isolate No**	**Percentage (%)**
Milk	64	13	20.31
Cheese	43	9	20.93
Whey	11	2	18.18
Total	118	24	20.33
Chi –Square (χ^2)	---	---	0.873 NS
P-value	---	---	0.3719
NS: Non-Significant.			

Table 3 : Isolation of Biofilm from *E. Coli* from difference sources

Source (type)	Total sample	Isolate No	Percentage (%)
Milk	64	41	64.06
Cheese	43	22	51.16
Whey	11	7	63.63
Total	118	70	59.32
Chi –Square (χ^2)	---	---	4.902 *
P-value	---	---	0.0417
* (P<0.05).			

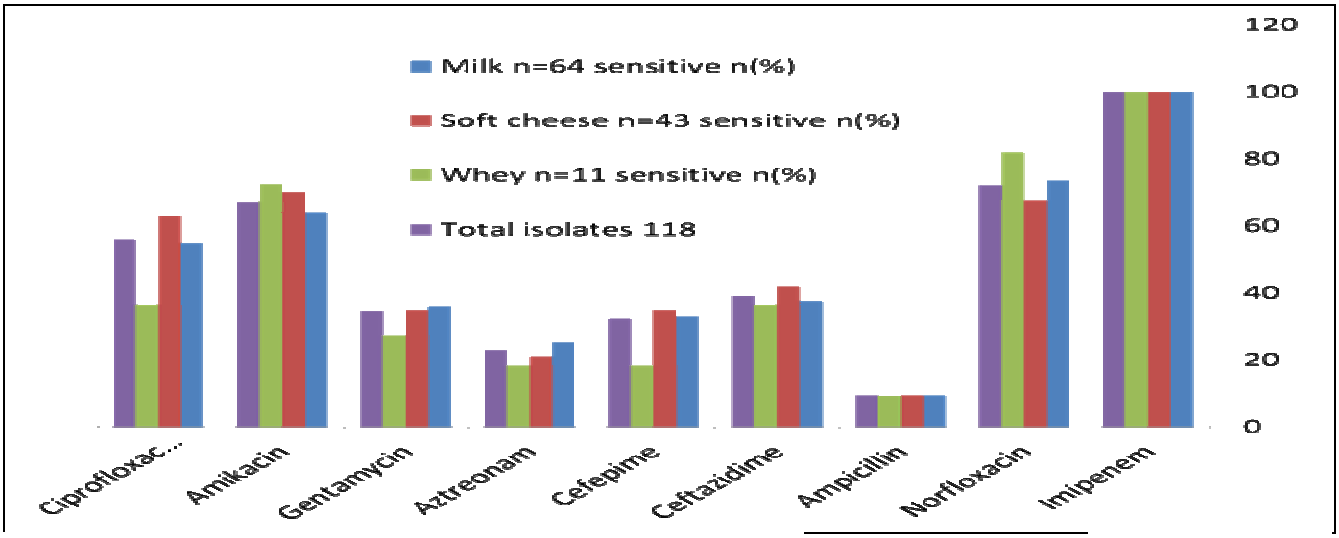
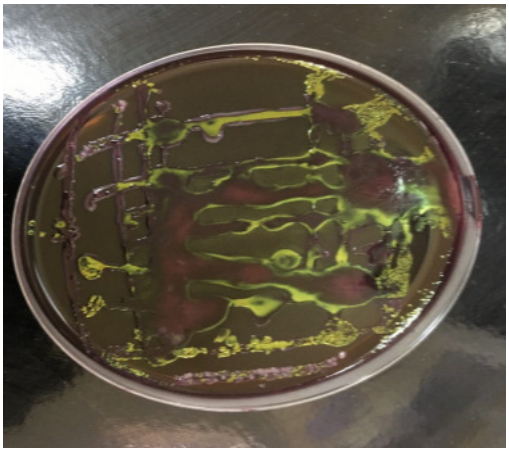
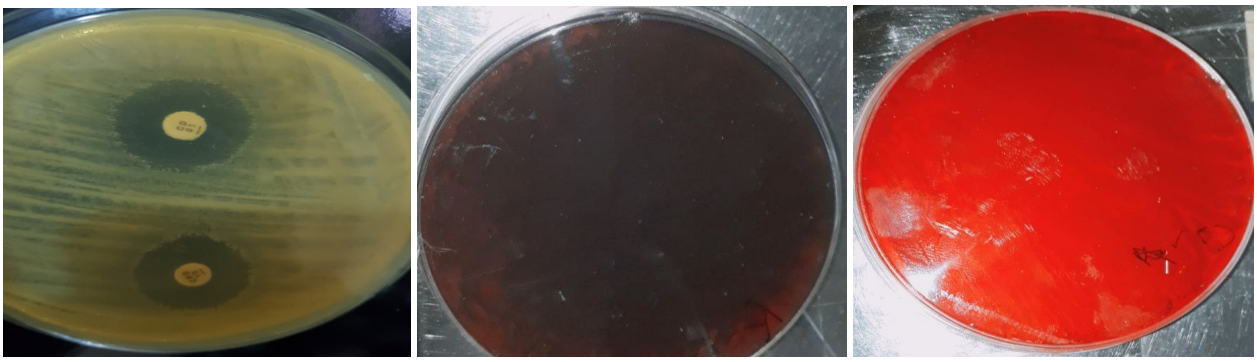


Fig. 1 : Shows the antimicrobial susceptibility of all *E. coli* isolated from of milk, soft cheese and whey its observed imipenem (100%) followed norfloxacin 85(72%), Amikacin 79(66.94%), Ciprofloxacin 66(55.93%), Ceftazidime 46(38.98%), Gentamycin 41(34.74%), Cefepime 38(32.20%), Aztreonam 27(22.88%) and Ampicillin 11(9.32%)



Picture (1) : *Escherichia coli* colonies in Eosin Methylene Blue(EMB) Agar (Note: Greenish Metallic Sheen



Picture (3): Appearance of black colonies with a dry crystalline shown that bacteria are producing biofilm (lift) and non-producers stay pink color(right).

Statistical Analysis

Chi-square analysis was used to compare between among data. through Statistical Analysis System- SAS (Statistical Analysis System, User's Guide. Statistical, 2012).

Discussion

Quantity and quality of microbial load in milk and milk products depend on hygienic measure through production of milk and dairy plant (Torkar and Teger 2006).

In this study, Approximately 59% % of total samples screened were find of *E. coli* isolates, All positive isolates showed typically cultural and morphologically, which was also supported by (Batabyal *et al*., 2018; Samanta, 2013).

In this study, Approximately 20.33% *of E. coli* isolates were phenotypically positive to produce ESBL, which was agreement with reported by (Ali *et al*., 2016; Badri *et al*., 2017) but other study showed high prevalence of ESBL-producing *E. coli* in milk samples 54.54% (20) (66.7%) (Kamaruzzaman, 2015). In other study (Bhoomika *et al*., 2016) was observed only 12.32 of *E. coli* isolates ESBL producer in milk samples.

In this study, Approximately 59.32% were phenotypically positive to produce biofilm and resistance to antibiotic were found higher than that of biofilm non-producing, This finding agreement with the findings of other researchers from different parts of the world (Sanjeev *et al*., 2016; Nair *et al*., 2013).

High level of antibiotic resistance as shown in this study but imipenem (100%) to be highly sensitive supported by (Batabyal *et al*., 2018) was found to be sensitive against these pathogens but resistance to gentamycin 58.33% and ceftazidime 91.67%. Also supported by their study reported by (Delveen *et al*., 2016) was found (1.6%) resistant to imipenem, ceftazidime (23%) and aztreonam (23.8%). Also supported by (Rashid *et al*., 2013) was found resistance against drugs such as amoxicillin 56%, ciprofloxacin 40%, amikacin 72% and gentamicin, but sensitive to norfloxacin were (80%).

Conclusion

We have found multiple drug resistant ESBL, *E. coli* produced, in milk samples, cheese with its whey in different regions from Babylon Province. The major findings regarding the spread of these strains are due to illegal used of antimicrobial agents in farming animals. We have denoted that most individuals who transport and collect milk from different regions do not abide by the hygienic qualification. Furthermore, In Babylon Provence lacks a main center for collecting milk. As we know, process of soft cheese made from contaminated milk subsequently increases the food borne pathogens. Such pathogens helps in the emergence of antibiotic-resistant bacteria, which deactivates antimicrobial agents. As a result we can conclude that the environmental agent is the culprit of the resistance of *E coli*.

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