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, sponger 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 cefamycin or carbapenem). 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 tr sodium citrate (2%) were emulsified soft cheese samples inside non-durable clean bags. Processed soft cheese samples were diluted and inoculated in buffered Tryptone soy yeast extract broth one part sample (10gm) to nine part diluent (90 ml) broth with processed milk samples were incubated at 37°C 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 and 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 clarithromycin (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 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

<table>
<thead>
<tr>
<th>Source (type)</th>
<th>Total sample</th>
<th>Isolate No</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>100</td>
<td>64</td>
<td>64.00</td>
</tr>
<tr>
<td>Cheese</td>
<td>75</td>
<td>43</td>
<td>57.33</td>
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<tr>
<td>Whey</td>
<td>25</td>
<td>11</td>
<td>44.00</td>
</tr>
<tr>
<td>Total</td>
<td>200</td>
<td>118</td>
<td>59.00</td>
</tr>
<tr>
<td>Chi –Square (χ²)</td>
<td>---</td>
<td>---</td>
<td>7.850 **</td>
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<tr>
<td>P-value</td>
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<td>---</td>
<td>0.0063</td>
</tr>
</tbody>
</table>

** (P<0.01).

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

<table>
<thead>
<tr>
<th>Source (type)</th>
<th>Total sample</th>
<th>Isolate No</th>
<th>Percentage (%)</th>
</tr>
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<tbody>
<tr>
<td>Milk</td>
<td>64</td>
<td>13</td>
<td>20.31</td>
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<tr>
<td>Cheese</td>
<td>43</td>
<td>9</td>
<td>20.93</td>
</tr>
<tr>
<td>Whey</td>
<td>11</td>
<td>2</td>
<td>18.18</td>
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<tr>
<td>Total</td>
<td>118</td>
<td>24</td>
<td>20.33</td>
</tr>
<tr>
<td>Chi –Square (χ²)</td>
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<td>---</td>
<td>0.873 NS</td>
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<tr>
<td>P-value</td>
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<td>0.3719</td>
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</tbody>
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NS: Non-Significant.

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

<table>
<thead>
<tr>
<th>Source (type)</th>
<th>Total sample</th>
<th>Isolate No</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>64</td>
<td>41</td>
<td>64.06</td>
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<tr>
<td>Cheese</td>
<td>43</td>
<td>22</td>
<td>51.16</td>
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<tr>
<td>Whey</td>
<td>11</td>
<td>7</td>
<td>63.63</td>
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<tr>
<td>Total</td>
<td>118</td>
<td>70</td>
<td>59.32</td>
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<tr>
<td>Chi –Square (χ²)</td>
<td>---</td>
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<td>4.902 *</td>
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<tr>
<td>P-value</td>
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<td>0.0417</td>
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* (P<0.05).
Fig. 1: Shows the antimicrobial susceptibility of all *E. coli* isolated from milk, soft cheese and whey its observed imipenem (100%) followed norfloxacin 85(72%), Amikacin 79(66.94%), Ciprofloxacin 66(55.93%), Cefazidime 46(38.98%), Gentamycin 41(34.74%), Cefepime 38(32.20%), Aztreonam 27(22.88%) and Ampicillin 11(9.32%)

![Escherichia coli colonies in Eosin Methylene Blue(EMB) Agar (Note: Greenish Metallic Sheen)](image)

![Appearance of black colonies with a dry crystalline shown that bacteria are producing biofilm (lift) and non-producers stay pink color(right).](image)

**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*.

**References**


