ANTIMICROBIAL RESISTANCE AND BIOFILM FORMATION PATTERNS OF Escherichia coli ISOLATED FROM MARKET RAW MILK AT ZAGAZIG CITY

Aya R. Mohammed*, Esmat I. El-Said, S.F. Abd El-Aal and Rania M. Kamal
Food Control Dept., Fac. Vet. Med., Zagazig Univ., Egypt

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ABSTRACT: In the present study, one hundred samples of raw cow milk were collected randomly from different dairy shops and markets in Zagazig city for isolation and identification of Escherichia coli which is considered a reliable indicator for fecal contamination and an important cause of food poisoning. Identification was done microscopically, biochemically by different biochemical tests (IMVIC) and serologically. The incidence of E. coli in raw milk samples was 47%. Also, the serological identification of E. coli isolates revealed that O26 is the most predominant serogroup by percentage of 21.3%. E. coli pose the greatest threat to human health because of its growing resistance to antibiotics. Antimicrobial susceptibility testing (AST) was done by disc diffusion method against 10 antimicrobials and the results revealed that E. coli isolates were highly resistant to amoxicillin-clavulanate, ampicillin, cefotaxime and ceftazidime with percentages of 89.4%, 89.4%, 100.0% and 100.0%, respectively. However, they were highly sensitive to chloramphenicol, ciprofloxacin and tetracycline with percentage of 100.0%, 100.0% and 93.6% respectively. In addition, 89.4% of E. coli isolates showed multi drug resistance (MDR). The ability of bacteria for adherence to food surfaces and biofilm formation is a source of food contamination that affect food safety and industry. Micro titer plate assay used for testing biofilm formation and represented that 78.7% of E. coli isolates were non-biofilm producers, 6.4% were weak biofilm producers, 14.9% were moderate biofilm producers and none of isolates was strong biofilm producers.

Key words: E. coli, raw milk, antimicrobial resistance, biofilm.

INTRODUCTION

Milk ranks high among other foods and is considered as the most perfect food for human from birth to senility as it is not only has good sensory properties and all nutrients required for the body for rapid growth but also could prevent or reduce risks of many nutritional deficiency diseases (Kalkwarf et al., 2003; Marshall et al., 2003).

Raw milk is still used by large number of farm families and workers and by a growing segment of the general population who believe that the milk is not only safe but also imparts beneficial health effects that are destroyed by pasteurization (Angulo et al., 2009).

Due to its high nutritious content, milk allows the growth of a copious number of microorganisms. Therefore, apart from its endogenous microbiota, diverse and numerous other microorganisms originating from the teat canal, udder skin, milking machines, tanks, and containers used to store it, reflecting the farm and the pasture environment as well, might colonize the milk as soon as it has been milked (Addis et al., 2016).

Among all micro-organisms Escherichia coli is frequently contaminating organism in food and is reliable indicator of fecal contamination and generally present due to insanitary conditions of water, food, milk and other dairy products (Jayarao and Henning, 2001).
Antibiotic resistance is a seemingly growing problem in both developing and developed countries of the world. The rise in the use of antibiotics in human medicine, veterinary medicine and agriculture has been contributory to the rapid rise of antibiotic resistance among bacterial species especially and this has increased the challenge of multidrug-resistant bacteria in the environment (Reinthaler et al., 2010; Xu et al., 2014).

The emergence and dissemination of multidrug-resistant (MDR) bacterial species have been associated with the extensive use of broad-spectrum antimicrobials in treating human and animal infections (Rzewuska et al., 2015). In animal husbandry, antibiotics are used as animal growth promoters and are regularly abused for the prevention and treatment of animal infections (Nepal and Bhatta, 2018). All these together contribute to the development of bacterial resistance to antibiotics. Genetic factors such as horizontal gene transfer and clonal expansion of resistant isolates also play a crucial role in the acquisition of antibiotic resistance traits in bacteria (Peterson and Kaur, 2018).

A biofilm is defined as an organized collection of surface attached microbial communities of cells that are embedded into a self-produced exopolymeric matrix mainly composed of proteins, polysaccharides and sometimes DNA (Hall-Stoodley et al., 2004). The formation of biofilm is a result of different stress condition(s) where biofilms acts as a defense mechanism enhancing the survival rate of microorganism. They play an important role in microbial pathogenesis and persistence as well as serve as grounds for genetic exchanges. It acts as shield protecting the microbial community from action of various antimicrobial agents such as antibiotics, preservatives, chemical sanitizers, thermal treatment etc. that are traditionally used in food industry, thus making them robust and hard to eradicate (Monte et al., 2014). The biofilm formation for many bacterial species including E. coli occurs as early as two hours and they can survive up to ten years in food industries despite the regular cleaning and sanitation treatment (Corcoran et al., 2014).

This study was undertaken to determine the occurrence and characteristics of E. coli strains in market raw cow milk at Zagazig city and to estimate the potential of these sources acting as vehicles of antimicrobial resistance.

MATERIALS AND METHODS

Samples Collection and Preparation

One hundred random samples of raw milk were collected from different dairy shops and markets in Zagazig city, Sharkia Governorate, Egypt under hygienic condition during the period from February to August 2020. Approximately 500 ml of the samples were transferred to the laboratory of Food Control Department, Faculty of Veterinary Medicine, Zagazig University in an insulated icebox with a minimum of delay to be examined microbiologically.

Isolation and Identification of E. coli

Eleven ml of well-mixed samples were aseptically transferred into a sterile bottle containing 99 ml of sterile buffered peptone water (BPW) to make a dilution of 1:10 and incubated at 37°C for 24hrs. A loopful of the BPW enrichment was streaked on Eosin Methylene Blue agar (EMBA; Oxoid) and then incubated at 37°C for 24hrs. The agar plates were examined for growth of E. coli. To get pure cultures, a single colony was further subcultured on EMBA according to (Ngaywa et al., 2019)

Films of pure suspected cultures were stained with Gram’s stain and examined microscopically. Cultures analyzed by using the following biochemical tests:

- Indole Motility Test
- Methyl Red
- Voges-Proskauer Tests
- Simmons Citrate Agar Test according to APHA. (2004)

Serological Identification of Isolated E. coli

The isolates were serologically identified by slide agglutination test according to Kok et al. (1996) using rapid diagnostic E. coli antisera sets (DENKA SEIKEN Co., Japan) to identify O antigen.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing (AST) was done using the Kirby-Bauer disc diffusion
method on Mueller-Hinton agar (Oxoid, Hampshire, England) as described by the Clinical and Laboratory Standards Institute (CLSI, 2017), with an inoculum equivalent to 0.5 McFarland standards. Incubation was done at 35±2°C, ambient air, for 16–18 hrs. The following 10 antimicrobials were tested:

Anoxicillin/clavulanic acid, ampicillin, cefotaxime, ceftriaxone, chloramphenicol, ciprofloxacin, kanamycin, Nalidixic acid, streptomycin and tetracycline

The sensitivity/resistance was interpreted based on the diameter zone of inhibition, inclusive of margins, following the Clinical Laboratory Standards Institute guidelines (CLSI, 2017). The isolates were classified as intermediate, susceptible or resistant (CLSI, 2017). In addition, we calculated the number of E. coli isolates that were considered multidrug resistant (MDR), that is, resistant to three or more antimicrobials of different classes (Frye and Fedorka-Cray, 2007) and the Multiple Antimicrobials Resistance index (MAR) for each E. coli isolate according to the methodology described by (Krumperman, 1983). This index shows the relationship between the number of resistant E. coli to each antimicrobial and the total number of classes tested.

Biofilm Formation

The biofilm formation test was performed as described by Stepanovic et al. (2000), where E. coli strains were incubated in TSB broth at 37 ± 1°C for 24 hrs. Then aliquots were diluted in a new tube until reaching the turbidity of one on the McFarland scale. Subsequently, 200 μL of each suspension in triplicate were inoculated into a 96-well sterile polystyrene micro plates. In the first three wells, only sterile broth was added as a negative control. The plates were incubated without air circulation at 37 ± 1°C for 24 h. Next, the bacterial suspensions were aspirated from each well and washed three times with 250 μL of sterile 0.9% sodium chloride solution. The bacterial cells were then fixed with 200 μL of methanol (PA) for 15 min and dried at room temperature. Later, they were stained with 200 μL of 2% Hucker crystal violet for 5 min, washed in running water and dried at room temperature. Re-solubilization was performed with 160 μL of 33% glacial acetic acid followed by reading using a spectrophotometer (Thermo Scientific® Multiskan GO) at 570 nm. After reading, the optical density value of each strain (OD) was obtained by calculating the arithmetic mean of the absorbance of the three wells, and this value was compared to three standard deviations above the mean of the absorbance of the negative control (ODC). The strains were classified in four different categories as follows: A) non-biofilm producer (OD ≤ ODC); B) weak biofilm producer (ODC < OD < 2 × ODC); C) moderate biofilm producer (2 × ODC < OD ≤ 4 × ODC); and D) strong biofilm producer (4 × ODC < OD) (Stepanovic et al., 2000).

RESULTS AND DISCUSSION

Incidence of E.coli in Market Raw Milk and Serological Identification of Isolates

Milk, a perishable complete nutritious food is considered a good medium of growth for many of the microorganisms (Khayal and Ragia, 2013).

Conditions for contamination of raw milk at different critical points are due to less hygienic practices in pre-milking udder preparation, sub-optimal hygiene of milk handlers, and poor sanitation practices associated with milking and storage equipment, higher environmental contamination during transportation or contamination during waiting along the roadside (Garedew et al., 2012).

There is an increasing in number of people whose consuming raw unpasteurized milk due to enhancing nutritional quality, taste and health benefits in spite of several documented milk born disease outbreaks occurred from consumption of raw unpasteurized milk and dairy products manufactured using raw milk (Leedom, 2006; Oliver et al., 2009).

In recent years, much attention has been paid toward E. coli because of its importance as an organism of true faecal origin with the possible existence of associated enteric pathogens. Commensal E. coli plays a dynamic role in the ecology of intestinal tract. The E. coli genome exhibits a high degree of heterogeneity; therefore, these bacteria can be a commensal organism. On the other hand, it can be also a dangerous pathogen causing intestinal or extra-intestinal infections. It is of major public health
significance related to risk of introducing these bacteria to the food chain (Newell et al., 2010).

Illness caused by entero-pathogenic E. coli can range from self-limited watery diarrhea to life threatening manifestations such as hemorrhagic colitis, hemolytic uremic syndrome, and thrombocytopenic purpura and may lead to death (Alexander and Prado, 2003).

The results presented in Table 1 revealed that, the incidence of E.coli in raw milk were 47%. Meshref (2013) reported nearly similar results where he found E.coli by (52.6%). The contamination rate in raw milk samples was extremely lower than the findings of Sobeh et al. (2002), Somro et al. (2002), Chye et al. (2004) and Altalhi and Hassan (2009) as they found 88, 65, 65 and 66% of their samples were contaminated by E. coli, respectively, but higher than the rate of 32, 27.5, 10 and 3.3% reported by Ahmed and Sallam (1991), Mezyed et al.(2008), Tasci (2011) and El-Prince et al. (2010), respectively.

Table 2 illustrated the serological identification of isolated E. coli. The strains of E. coli isolated from examined raw milk samples were O25, O26, O44, O55, O78, O86, O114, O119, O125, O127, O158 and O168 by percentage of (10.6%), (21.3%), (8.5%), (14.9%), (4.3%), (4.3%), (6.4%), (6.4%), (8.5%), (4.3%), (4.3%) and (6.4%) respectively. While nearly similar results were obtained by El-Nahas et al. (2015) who found O114, O26, and O127 in raw milk samples. Also El-Zamkan et al. (2018) isolated E. coli O26, O55 & O119 from raw milk. Khafagy et al. (2017) isolated O158, O55, and O86 from raw milk samples obtained from dairy farms and shops. Rashid et al. (2013) reported the presence of one milk sample serologically identified as O86. El-bagory et al. (2016) and other researchers as; Lamye et al. (2013) and Abike et al. (2015) recorded for the identification of E.coli strain that belongs to the serogroup O55. Others as Wenz et al. (2006) and Koraney (2016) recorded O158, which is one of the frequent serogroups that isolated from raw milk samples. In addition, AbdEl-Maabud (2014) and Shalaby et al. (2019) isolated E. coli O26 from raw milk samples.

Globally, the unsupervised use of antimicrobial agents in the treatment of animal and human infections have been contributed to the emergence of antimicrobial resistance (Van Boeckel et al., 2015). The antimicrobial resistance mainly originates from the transfer of resistance genes across microbes enabling them to survive in the presence of antimicrobial agents that eventually resulted in failure of antibiotic therapeutic protocols (Blair et al., 2015). Furthermore, the overuse of antibiotics in animal husbandry as growth promoters could be a potential source of bacterial resistance through dissemination of resistant microbes from intestinal microbiota of livestock that contaminate the surrounding environment and enhance the transmission of resistant genes to autochthonous bacteria (resident microbes) (McEwen and Collignon, 2018).

**Antimicrobial Resistance and Biofilm Formation of E. coli Isolates**

The development of antimicrobial resistance among the pathogenic bacteria poses a problem of high concern. Table 3 shows that E. coli isolates were highly resistant to amoxicillin-clavulanate, ampicillin, cefotaxime and ceftazidime with percentages of 89.4%, 89.4%, 100.0% and 100.0% respectively. In addition, the isolates were highly sensitive to chloramphenicol, ciprofloxacin and tetracycline with percentage of 100.0%, 100.0% and 93.6% respectively. Nearly similar results were obtained by Nobili et al. (2016) where he reported a significantly higher percentage (100%) resistance to amoxicillin-clavulanic acid. Thaker et al. (2012) recorded higher resistance rate to ampicillin (100 %). While Bhardwaj et al. (2021) found complete resistance against amoxicillin and ampicillin. Shalaby et al. (2019) found that all E.coli isolates exhibited susceptibility to chloramphenicol, ciprofloxacin, and tetracycline (except for one isolate that was resistant to ciprofloxacin). In addition, the results reported by Tadesse et al. (2018) were relatively similar to this study where the in vitro growth E. coli was restrained by ciprofloxacin and tetracycline.

The multiple antibiotic resistance (MAR) index, an index describing the resistance of isolates to different antibiotics, was calculated for each isolate as described in the materials and methods section. Values for the MAR index were 0.2 (i.e., an isolate being resistant to two
Table 1. Incidence of *E. coli* in examined market raw milk samples

| No. of samples | Positive samples | % of total |
|----------------|------------------|------------|
| 100            | 47               | (47.0%)    |

Table 2. Serological identification of *E. coli* strains isolated from market raw milk samples

| Serogroup | No. | % of total |
|-----------|-----|------------|
| O25       | 5   | (10.6%)    |
| O26       | 10  | (21.3%)    |
| O44       | 4   | (8.5%)     |
| O55       | 7   | (14.9%)    |
| O78       | 2   | (4.3%)     |
| O86       | 2   | (4.3%)     |
| O114      | 3   | (6.4%)     |
| O119      | 3   | (6.4%)     |
| O125      | 4   | (8.5%)     |
| O127      | 2   | (4.3%)     |
| O158      | 2   | (4.3%)     |
| O168      | 3   | (6.4%)     |
| Total     | 47  | (100.0%)   |

Table 3. Antibiogram pattern of identified *E. coli* isolates (n=47)

| Antimicrobial         | Concentration (µg) | Resistant | Intermediate | Susceptible |
|-----------------------|--------------------|-----------|--------------|-------------|
| Amoxicillin-clavulanate | 20/10 µg           | 42(89.4%) | 0(0.0%)     | 5(10.6%)    |
| Ampicillin            | 10 µg              | 42(89.4%) | 0(0.0%)     | 5(10.6%)    |
| Cefotaxime            | 30 µg              | 47(100.0%)| 0(0.0%)     | 0(0.0%)     |
| Ceftazidime           | 30 µg              | 47(100.0%)| 0(0.0%)     | 0(0.0%)     |
| Chloramphenicol       | 30 µg              | 0(0.0%)  | 0(0.0%)     | 47(100.0%)  |
| Ciprofloxacin         | 5 µg               | 0(0.0%)  | 0(0.0%)     | 47(100.0%)  |
| Kanamycin             | 30 µg              | 9(19.1%) | 25(53.2%)   | 13(27.7%)   |
| Nalidixic acid        | 30 µg              | 0(0.0%)  | 29(61.7%)   | 18(38.3%)   |
| Streptomycin          | 10 µg              | 20(42.6%)| 20(42.6%)   | 7(14.9%)    |
| Tetracycline          | 30 µg              | 3(6.4%)  | 0(0.0%)     | 44(93.6%)   |
of the 10 antibiotics tested), 0.4 (resistance to 4 out of the 10 antibiotics tested), 0.5 (resistance to 5 out of the 10 antibiotics tested) and 0.6 (resistance to 6 out of the 10 antibiotics tested) with percentages of 10.6%, 53.2%, 23.4% and 12.8%, respectively.

*E. coli* has also been shown to be a significant reservoir of genes coding for antimicrobial drug resistance and therefore is a useful indicator for resistance in bacterial communities (Arsene-Ploetze *et al.*, 2018; Katakweba *et al.*, 2018).

In the present study, 89.4% of *E. coli* isolates showed resistance to more than three classes of antimicrobials (Table 4).

Biofilm formation is one of the most important virulence factors that protect microbes from antimicrobial drugs and treatment (Olsen, 2015). The ability of spoilage and pathogenic bacteria to adhere onto food surfaces and form biofilms serve as a persistent source of food contamination that threatens food safety and causes huge losses to the food industry (Tezel and Şanlıbaba, 2018).

Table 5 revealed that 78.7% of *E. coli* isolates were non-biofilm producers, 6.4% were weak biofilm producers, 14.9% were moderate biofilm producers and none of isolates was strong biofilm producers. Nearly similar studies of Milanov *et al.* (2015) who recorded that 19 (76%) isolates of *E. coli* did not produce biofilm and 6 (24%) were classified as weak biofilm producers.

Musa *et al.* (2019) found that 6 out of 15 (40%) of *E. coli* were strong biofilm producers, 2 out of 15 were moderate biofilm producers (13.3%), 4 out of 15 (26.7%) were weak biofilm producers and only three isolates (20%) were non-biofilm producers.

Bhardwaj *et al.* (2021) reported that out of 32 *E. coli* isolates tested, 4 were strong formers, 11 were moderate, 15 were weak producers and 2 non-producers.

Da Silva Chagas *et al.* (2017) recorded that 11 (55%) of 20 biofilm producing strains were identified as *E. coli*, and all strains were classified as strong biofilm producers.

Cruz-Soto *et al.* (2020) found that 26 (76.5%) of the isolates formed biofilm to some degree, while the remaining 8 (23.5%) did not form biofilm. Of the biofilm-forming isolates, 7 were classified as strong and moderate biofilm producers and 12 as a weak one.

This study provides further evidence that raw cow milk is a potential source of *E. coli*, some of which are associated with serotypes clinically significant bearing biofilm formation ability and multiple antibiotic resistance that may raise public health concern due to the potential human infection and antimicrobial resistance dissemination throughout food system.

**Table 4. Multi drug resistance pattern and multiple antibiotic resistance index of identified *E. coli* isolates (n=47)**

| No. of isolates | Multi drug resistance (MDR) | 0.2 | 0.4 | 0.5 | 0.6 |
|-----------------|-----------------------------|-----|-----|-----|-----|
|                 | No. | %   | No. | %   | No. | %   | No. | %   |
| 47              | 42  | (89.4%) | 5   | (10.6%) | 25  | (53.2%) | 11  | (23.4%) | 6   | (12.8%) |

**Table 5. Ability of *E. coli* strains isolated from market raw milk samples to form biofilm**

| No. of isolates | None | Weak | Moderate | Strong |
|-----------------|------|------|----------|--------|
|                 | No. | %    | No. | %    | No. | %    | No. | %    |
| 47              | 37  | (78.7%) | 3   | (6.4%) | 7   | (14.9%) | 0   | (0.0%) |
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