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Research paper

Extended-Spectrum Beta-Lactamase Enzyme (ESBL) Production from Antimicrobial-Resistant Escherichia coli Isolates and their Attachment on Stainless-Steel Surface

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Abstract

Food contact surfaces may pose a threat of becoming vector for antimicrobial-resistant transmission of bacteria along the food chain. Twenty-four isolates of *Escherichia coli* were investigated to determine the antimicrobial resistance, production of Extended-Spectrum Beta-Lactamase Enzyme and their attachment ability on stainless-steel surface. The antimicrobial resistance and enzyme production tests were carried out according to standard disc diffusion assay, while attachment was simulated on stainless steel discs. All 24 isolates were resistant to Amoxycillin and Penicillin, while 50% and 37.5% were resistant to Ceftriaxone and Cefotaxime, respectively. Three of 24 isolates (12.5%) produced the enzyme against cefotaxime, ceftazidime and ceftriaxone. The enzyme production was further confirmed by the expansion of cefotaxime, ceftriaxone and ceftazidime inhibition zone towards amoxicillin-clavulanate disc. All 3 enzyme-producing isolates (EC-6, EC-7 and EC-12) exhibited their ability to attach to stainless-steel disc. Attachment was significantly increased ($p<0.05$) with prolonged incubation times with the highest attachment (6.07 ± 0.05 $\log_{10}$ cfu/ml) by isolate EC-6 at 72h. The attachment ability indicates that resistant *E. coli* can be potentially transmitted into the food chain via contaminated food contact surfaces. Our data could be used to develop research to link the spread of antimicrobial resistance towards effective intervention strategies.

Keywords: *antimicrobial resistant; attachment; E. coli; extended spectrum beta lactamase enzyme (ESBL); stainless steel*

1. Introduction

Escherichia coli is a gram-negative bacterium in Enterobacteriaceae family. It is the main aerobe of the gastrointestinal flora in humans and other animals (Tenaillon et al., 2010), and has been widely studied as an indicator of antimicrobial selection pressure (Gronvold et al., 2010). The transmission of *E. coli* to human was primarily via contamination of foods, raw materials or food contact surfaces. Food contact surfaces have been highlighted as one of important vehicles in *E. coli* contamination to foods, as they can be found on any surfaces such as human hands, equipment and utensils. These surfaces may hold sufficient nutrients if effective cleaning and sanitization process is not in place and may favor the growth of *E. coli*. The ability of *E. coli* to attach onto various food contact surfaces have been extensively reported (Mahyudin et al., 2018; Beauchamp et al., 2012; Goulter-Thorsen et al., 2011; Adetunji & Isola, 2011; Ryu & Beuchat, 2005; Ryu et al., 2004; Aliyu et al., 2016; Dourou et al., 2011; Tschudin-Sutter et al., 2014).

Antimicrobial resistance (AMR) among microorganisms has been one of the major public health concerns (WHO, 2014) as their resistance makes infections more complicated to be treated (Sabaté et al., 2008). The most common mechanisms among the Enterobacteriaceae (eg: *E. coli)* to acquire AMR is by enzymatic inactivation using extended-spectrum beta-lactamases (ESBL) that confer them with resistance to β-lactam antibiotics (eg: penicillins, cephalosporins, and the monobactam aztreonam).

Over the years, resistance to cephalosporins among members of Enterobacteriaceae has increased, mainly due to the growing prevalence of extended-spectrum β-Lactamases (ESBL)-producing *E. coli* worldwide (Cantón et al., 2008; Castanheira et al, 2008). Since the first transfer of antibiotic-resistant genes from *E. coli* was described (Smith, 1969), the findings have been confirmed by numerous studies (Aaerestrup and Wegener, 1999; Winokur et al., 2001; Angulo et al., 2004; Wang et al., 2006).

The spread of ESBL has been reported in food-chain transmission and has been frequently associated with *E. coli* strains isolated from food-producing animals (Trott, 2013; Ojer-Usoz et al., 2017). Cross-contamination of food by *E. coli* has been reported that when food contact surface become contaminated, pathogens can survive and even multiply on the surface, and are readily transferred to other surfaces or foods in numbers sufficient to represent an infection hazard. Therefore, it is important to understand the ability of ESBL-producing *E. coli* to attach on food contact surface (Wachtel et al., 2003).

There has been no comprehensive study regarding the occurrence of ESBL-producing *E. coli* in food or food contact surfaces in Malaysia. Therefore, in this study, resistant *E. coli* isolates were examined for their ESBL production and the ability to attach to stainless steel surfaces.

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2. Materials and method

2.1. Test isolates

Twenty-four *E. coli* isolates were obtained from culture collection at the Microbiology Research Laboratory, Faculty of Food Science and Technology, Universiti Putra Malaysia. The isolates were isolated by swabbing from cutting board surfaces from selected restaurants in Serdang, Selangor from our previous study. The isolates have been identified according to standard procedures. The isolates were subjected to Gram's staining for classification using various biochemical tests such as lactose fermentation, urease, citrate utilization, Indole production, methyl red, Voges-Proskauer, motility, growth on Eosin Methylene Blue (EMB) agar, hydrogen sulphide production and gas production. They were further confirmed by PCR using specific *Escherichia coli* 16S rRNA primers. All bacteriological media and antibiotic discs used in the study were purchased from Merck, Germany and Oxoid, England, respectively.

2.2. AMR test

Susceptibility testing to detect resistance to 11 antibiotics was carried out on the 24 *E. coli* isolates using Kirby-Bauer agar-disk diffusion technique on Mueller-Hinton agar in accordance with the Clinical and Laboratory Standard Institute criteria (CLSI, 2014) and the resistance was examined and recorded.
The antibiotics tested against the isolates with their varying concentrations based on CLSI guidelines alongside their respective abbreviations were; Ciprofloxacin (CIP) 5 µg, Amoxycillin (AML: 10 µg), Gentamicin (CN: 10 µg), Penicillin G (P: 10 µg), Ceftriaxone (CRO: 30 µg), Sulphafurazole (SF: 300 µg), Streptomycin (S: 25 µg), Ceftazidime (CAZ: 30 µg), Cephalothin (KF: 30 µg), Nalidixic acid (NA: 30 µg) and Cefotaxime (CTX: 30 µg).
The diameters for their inhibition zones were recorded and determined as resistant, intermediate or susceptible (see Table 1). Multidrug resistant isolates were those resistant to antibiotics belonging to two or more classes of antibiotics were observed.

Table 1: Disc diffusion criteria for AMR (CLSI, 2014)

Antibiotic	Inhibition zone diameter (mm)		
	Resistant	Intermediate	Susceptible
CIP	≤15	16-20	≥21
AML	≤13	14-16	≥17
CN	≤12	13-14	≥15
P	≤13	14-16	≥17
CRO	≤19	20-22	≥23
SF	≤12	13-16	≥17
S	≤11	12-14	≥15
CAZ	≤17	18-20	≥21
KF	≤14	15-17	≥18
NA	≤13	14-18	≥19
CTX	≤22	23-25	≥26

Ciprofloxacin (CIP: 5µg), Amoxycillin (AML: 10µg), Gentamicin (CN: 10µg), Penicillin G (P: 10µg), Ceftriaxone (CRO: 30µg), Sulphafurazole (SF: 300µg), Streptomycin (S: 25µg), Ceftazidime (CAZ: 30µg), Cephalothin (KF: 30µg), Nalidixic acid (NA: 30µg) and Cefotaxime (CTX: 30µg).

2.3. ESBL production

A lawn culture of all test isolates (0.5 McFarland) was prepared on a Mueller Hinton Agar (MHA) (Merck, Germany) plate. ESBL screening and confirmation tests were carried out according to CLSI (2014) method with some modifications. Control strains used for the ESBL production were *Klebsiella pneumoniae* 700603 (positive) and *E. coli* 25922 (negative).
All isolates were initially screened for ESBL production using disc susceptibility test against three indicator cephalosporins (Oxoid, England), namely cefotaxime (30 µg), ceftazidime (30 µg), and ceftriaxone (30 µg). Isolates that showed a diameter of inhibition against cefotaxime (≤27 mm), ceftazidime (≤22 mm) and ceftriaxone (≤25 mm) were considered as resistant and were further tested using double disc synergy test (DDST) to confirm the ESBL production.
The cephalosporins (cefotaxime (30 µg), ceftazidime (30 µg), and ceftriaxone (30 µg)) and amoxicillin-clavulanate (20/10 µg) discs were placed at a distance of 30 mm from center to center on lawn cultures on the MHA plates (Harwalkar et al., 2013) and the plates were incubated overnight at 37 °C. The zone of inhibition enhancement of the cephalosporins towards the amoxicillin/clavulanic acid disc indicated as positive for an ESBL production.

2.4. Attachment on stainless steel disc

Attachment on stainless steel surface was assessed according to Souza et al., 2014 with some modifications. Test cultures were grown overnight at 37 °C in Tryptic Soy Broth (TSB) medium. The overnight cultures were centrifuged at 2500 rpm for 3.5 mins twice. The pellet was retrieved and diluted with Phosphate Buffer Saline (PBS) to obtain load of 10^8 CFU/ml. An amount of 50 µl of the PBS culture was mixed into 100 µl Brain Hearth Broth (BHB) medium. The BHB culture was placed onto the stainless-steel disc (discs were placed in a petri dish) and incubated at 37 °C for 24, 48, and 72 hours.
The discs were then soaked in peptone water for 15 seconds, swabbed with sterile cotton, and the cotton placed into a tube containing peptone water. Serial dilutions were made and 0.1 ml from each dilution was spread on Plate Count Agar (PCA) medium, incubated overnight at 37 °C and colonies were enumerated.

2.5. Statistical analysis

All the data were analyzed using the Minitab 18.0 (Minitab Inc., State College, PA,197 USA) statistical software, using two-way analysis of variance (ANOVA) to identify the significant differences between factors in the present study.

3. Results and discussion

3.1. Identification of isolates

All isolates were confirmed positive for *E. coli* based on biochemical test and sequel to PCR using specific primer that amplified genes of their identification.

3.2. AMR profile

The resistance profile of the 24 *E. coli* isolates against the 11 tested anti-biotics is displayed in Table 2. All isolates (100%) were resistant to Amoxycillin and Penicillin G. A number of 12 (50%) and 9 (37.5%) isolates were resistant to Ceftriaxone and Cefotaxime, respectively. Four (16.7%) and two (8.3%) isolates showed resistance to Cephalothin and Ceftazidime, respectively. No resistance was observed against Ciprofloxacin, Gentamicin, Sulphafurazole, Streptomycin and Nalidixic acid in all test isolates. The exposure of bacterial strains to a multitude of beta-lactams has induced continuous production and mutation of beta-lactamases in these bacteria, resulting in expanding of their activity even against the newly developed beta-lactams antibiotics (Shaikh et al., 2015).
Multiple drug resistance is defined as the complete or intermediate resistance to 3 or more antimicrobial classes and has been common among the isolates of *E. coli*. In this research however, the concept of using intermediate as resistance was avoided and hence the resistance is now considered as the resistance to only 2 different antimicrobial classes. It is now expressed by microorganisms

as the resistance to 2 or more antibiotics from 2 or more different classes.

Table 2: Antimicrobial resistance profile of *E. coli* (n=24) isolates

Antibiotic	Antibiotic resistance profile					
	Resistant		Intermediate		Susceptible	
	n	%	n	%	n	%
CIP	0	0	8	33.3	16	66.7
AML	24	100	00	0	0	0
CN	0	0	15	62.5	9	37.5
P	24	100	0	0	0	0
CRO	12	50	12	50	0	0
SF	0	0	21	87.5	3	12.5
S	0	0	20	83.3	4	16.7
CAZ	2	8.3	18	75	4	16.7
KF	4	16.7	5	20.8	15	62.5
NA	0	0	14	58.3	10	41.7
CTX	9	37.5	0	0	24	100

Ciprofloxacin (CIP: 5µg), Amoxycillin (AML: 10µg), Gentamicin (CN: 10µg), Penicillin G (P: 10µg), Ceftriaxone (CRO: 30µg), Sulphafurazole (SF: 300µg), Streptomycin (S: 25µg), Ceftazidime (CAZ: 30µg), Cephalothin (KF: 30µg), Nalidixic acid (NA: 30µg) and Cefotaxime (CTX: 30µg). Data were obtained from two replications.

E. coli were reported to have been resistant to various antibiotics ranging from Amoxycillin, Penicillins to quinolones or fluoroquinolones which are responsible for inhibition of the synthesis of nucleic acid. Other studies on the multidrug resistance of *E. coli* that correspond with this including isolates obtained from food handlers in Malaysia (Tan et al., 2014) and various surfaces (Mahyudin et al., 2018; Beauchamp et al., 2012; Goulter-Thorsen et al., 2011; Adetunji & Isola, 2011; Ryu & Beuchat, 2005; Ryu et al., 2004; Aliyu et al., 2016; Dourou et al., 2011; Tschudin-Sutter et al., 2014).

In this study, none of the isolates showed resistance to the members of the class Aminoglycosides (Gentamicin and Streptomycin), Quinolones (Ciprofloxacin and Nalidixic acid) and Sulphonamides (Sulphafurazole). The resistance was only against two classes of Penicillins (Amoxycillin and Penicillin G) and Cephalosporins (Cephalothin, Cefotaxime, Ceftazidime and Ceftriaxone) which were all beta lactam antibiotics.

3.2. ESBL production

Three of 24 isolates (12.5%) were found to be potential ESBL producers (Table 3). These three isolates; namely EC-6, EC-7 and EC-12 demonstrated a diameter of inhibition of ≤27mm, ≤22mm and ≤25mm against cefotaxime, ceftazidime and ceftriaxone, respectively. Positive confirmation was obtained in the DDST test for ESBL confirmation, indicated by the expansion of cefotaxime, ceftriaxone and ceftazidime inhibition zone towards the amoxicillin-clavulanate disc (Figure 1).

Emergence and wide dissemination of ESBL-producing E. coli have been reported from various food-producing animals (Michael et al., 2017; Angulo et al., 2004), agricultural environment (Blaak et l., 2014), food-chain transmission (Zogg et al., 2016) or healthy fecal carriers (Fernández-Reyes et al., 2014). Hence, it can be concluded that any contact with the raw food materials or food contact surfaces may have implication to spread of ESBL.

There is limited study on screening and confirmation ESBL producer from food contact surfaces in Malaysia. This initial study showed only 12.5% were ESBL producers. This is in contrast with other studies that were carried out on poultry in Turkey (Gundongan & Avci, 2013), which reported a high incidence (44.4%) of ESBL producing *E. coli*, while Oyinloye and Ezekiel (2011) reported about 20.7% of their resistant-strains of enterobacteria (*E. coli*, Salmonella and Klebsiella species) in Nigeria. Recently, a study in broiler chicken in Germany revealed the prevalence of ESBL-producing Enterobacteriaceae on the skin (55%), filet (28%) and environmental samples (28%) (von Tippelskirch et al., 2018). A high prevalence of ESBL-producing Enterobacteriacea, ranging from 53.3 - 92.2% was also reported in poultry from Madagascar, Reunion and Mayotte (Gay et al., 2018).

commercial farms

Table 3: Screening of ESBL production in *E. coli* isolates (n=24)

Isolate	Diameter of inhibition (mm)		
	CTX 30	CAZ 30	CRO 30
EC-1	37.76	32.33	34.19
EC-2	39.28	32.65	33.60
EC-3	30.98	28.28	31.62
EC-4	31.85	29.58	31.52
EC-5	32.80	30.19	31.65
EC-6	22.67	18.65	22.62
EC-7	13.35	8.20	19.11
EC-8	31.11	28.22	28.79
EC-9	30.87	28.60	29.79
EC-10	29.43	30.07	28.39
EC-11	30.44	27.35	28.62
EC-12	11.79	14.20	21.66
EC-13	29.76	28.25	28.78
EC-14	31.55	28.61	29.35
EC-15	31.39	28.80	29.18
EC-16	31.54	29.07	27.55
EC-17	28.81	28.40	30.56
EC-18	28.50	28.02	28.01
EC-19	29.69	27.75	28.37
EC-20	32.00	28.20	29.89
EC-21	29.35	27.91	28.73
EC-22	30.55	29.07	29.65
EC-23	30.12	28.63	29.22
EC-24	30.12	28.63	29.22

CTX 30 = cefotaxime 30µg, CAZ 30 = ceftazidime 30µg, CRO 30 = ceftriazone 30µg. Data were obtained from two replications.

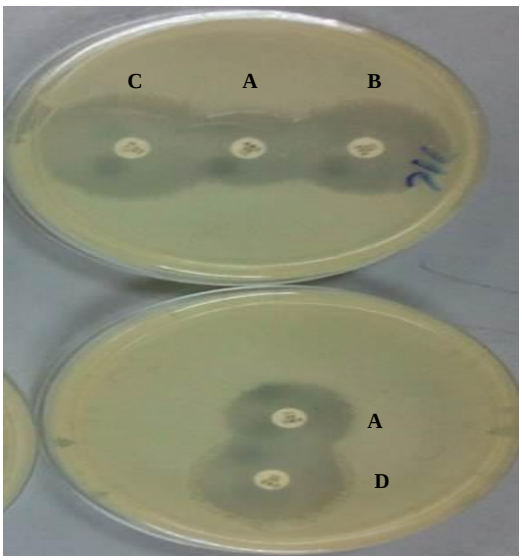

Fig. 1: Double disc synergy test (DDST) for detection of ESBL production (represented by *E. coli* isolate EC-7) showing increase in zone of inhibition surrounding cefotaxime (B), ceftazidime (C) and ceftriazone (D) discs towards amoxicillin-clavulanate disc (A).

3.3. Attachment on stainless steel disc

All tested isolates adhered on stainless steel disc and the adherence was significantly increased ($p<0.05$) with prolonged incubation times (Table 4). The highest count was demonstrated by isolate EC-6 (6.07 ± 0.05 $\log_{10}$ cfu/ml) at 72h while EC-7 had the lowest (3.49 ± 0.04 $\log_{10}$ cfu/ml) at 24h incubation. The ability of the three test isolates to attach to the surface, however was found to be insignificant ($p<0.05$). These were in-agreement with studies by several authors (Mahyudin, et al. 2018). This study also indicated that isolates EC-6, EC-7 and EC-12 were potential biofilm formers at 72h incubation as their attached cells ranged from 10^5 to 10^7 cfu/ml (Zotolla & Sasahara, 1994; Wirtanen et al., 1995).

Table 4: Attachment of *E. coli* onto stainless steel surface after incubation at 37˚C for 24, 48 and 72 hours

Isolate	Attachment (log_{10}CFU/ml±SD)[a]		
	24h	48h	72h
EC-6	3.61±0.07	4.97±0.28	6.07±0.05
EC-7	3.49±0.04	4.05±0.25	5.66±0.36
EC-12	3.67±0.05	4.59±0.10	5.40±0.67

[a]Values were the mean of two replications.

4. Conclusion

In conclusion, all isolates were confirmed as *E. coli* and all were resistant to Amoxycillin and Penicillin. A number of 12 (50%) and 9 (37.5%) were resistant to Ceftriaxone and Cefotaxime, respectively. The isolates that were resistant to Cephalothin and Ceftazidime were 4 (16.7) and 2 (8.3%), respectively. No resistance was observed against Ciprofloxacin, Gentamicin, Sulphafurazole, Streptomycin and Nalidixic acid in all test isolates. Three (12.5%) produced the enzyme against cefotaxime, ceftazidime and ceftriaxone. All 3 enzyme-producing isolates (EC-6, EC-7 and EC-12) exhibited their ability to attach to stainless-steel disc with significant attachment increment with prolonged incubation times. The attachment ability indicated that resistant *E. coli* can be potentially transmitted into the food chain via contaminated food contact surfaces. Although the study demonstrated low resistant and ESBL production among the isolates, the isolates were easily attached on the stainless-steel surface. Although it is impossible to ensure there is zero contamination of *E. coli* on the cutting board, several measures can be taken to minimalize the contamination such as improving hygiene practices among the food handlers, proper utilization of cutting board. Our data could be used to develop research to link the spread of antimicrobial resistance towards effective intervention strategies.

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