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ANTIMICROBIAL RESISTANCE OF *Escherichia coli* ISOLATED OF *ULAM* FROM SUPERMARKETS AND WET MARKETS IN KUALA TERENGGANU, MALAYSIA

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ABSTRACT

Raw vegetables were highly exposed to microbial contamination by handling at harvest or during postharvest processing. Nowadays, emerging issues threatening public health are bacterial resistance to antibiotics due to the excessive usage and misuse of antibiotics in agriculture. In this study, antibiotic susceptibility profiles of 23 *Escherichia coli* strains were tested by the standard disk diffusion method. Sixteen antimicrobial agents namely amikacin, amoxycillin/cluvanic acid, ampicillin, ampicillin/sulbactam, chloramphenicol, ceftriaxone, ciproflaxacin, ceftazidime, cephalotin, cefoperazone, gentamicin, kanamycin, nalidixic acid, streptomycin, tetracycline and trimethoprim were included in this study. In this study, 78.3% of the *E. coli* isolates were found to be resistant to cephalotin and it was the highest compared with the other antibiotics. It was found that 87% of isolates exhibited resistance to at least one antibiotic. *E. coli* showed high resistance to ampicillin (52.2%) and tetracycline (52.2%). In contrast, ceftriaxone and ceftazidime were found to be (100%) effective in restraining the growth of *E. coli* isolates. The highest multiple antibiotic resistance index (MAR) index was 0.48. Multiple resistance was observed in 47.8% of isolates with resistance to three to seven antibiotics. In conclusion, *ulam* could be the potential source of this antibiotic resistance of *E. coli*, and it may pose health threats to consumers.

Key words: Antimicrobial resistance, raw vegetables, disk diffusion test, *Escherichia coli*

INTRODUCTION

In Malaysia, about 120 species of herbs and trees are recognised as Malay traditional vegetables (consisting of plants or parts of plants). It is known locally as *ulam* and become important in food intakes, especially among the Malays. In Malaysia, the per capita consumption of vegetables has increased significantly from 7.25 kg in 1982 to 40.58 kg in 2001 (Tey *et al*., 2009). There is an increasing pattern of leafy green vegetable consumption because of their nutritional value (Beuchat, 2002). As a result, the foodborne outbreaks associated with fresh produce have also increased. This increment would promote the risk of foodborne diseases if consumed raw or minimally cooked vegetables because pathogens can be part of the native microflora of vegetables (Qadri *et al.*, 2015).

Antimicrobial-resistant (AR) pathogenic bacteria strains occurrence, propagation, accumulation and maintenance have become public health issues worldwide (Anderson, 1999; Levy & Marshall, 2004; Salleh *et al.*, 2017). Emerging issues threatening the public health related to bacterial resistance to antibiotics due to the abusive and misuse of antibiotics without proper prescription for the treatment of human, animal and plant diseases. World Health Organization (WHO) has confirmed antimicrobial resistance is a growing public health threat and as an emerging public health problem because pathogenic bacteria withstand therapy that causes main problems in the disease control (WHO, 2014).

Antibiotic resistance in *E. coli* is of particular concern because it is the most common Gram-

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negative pathogen in humans. *E. coli* is a frequent cause of life-threatening bloodstream infections and other common infections, such as urinary tract infections, diarrhea, peritonitis, septicemia, meningitis and Gram-negative bacterial pneumonia (Aso *et al*., 2017). The *E. coli* foodborne outbreak from the contaminated fenugreek sprout in May 2011 in Europe was reported to be the deadliest and the third largest in the history (CDC, 2013). By the emergence of resistance to the most first-line antimicrobial agents, the treatment for *E.coli* infection has been increasingly complicated (Van *et al.*, 2007). It is crucial to implement surveillance programme to determine the effectiveness of antibiotic therapy and to monitor the antibiotic resistance profiles of important pathogens, hence providing the necessary foundation for effective mitigation strategies (Kuan *et al.*, 2017).

The studies on the occurrence of resistant bacteria particularly in fresh vegetables in developing countries and especially our country are still very limited and lag behind the increasing pace of its danger (Faour-Klingbeil *et al.*, 2016). Khalid *et al*. (2015) reported the antimicrobial resistance of *Campylobacter jejuni* in *ulam* at farms and retail outlets in Terengganu, but there is still limited studies on *E. coli*. The objective of this study was to examine the antibiotic resistance profiles of *E. coli* isolated from ulam, collected from supermarkets and wet markets in Terengganu.

MATERIALS AND METHODS

Isolation of *Escherichia coli* from fresh vegetables

Thirty samples of fresh vegetables were collected from different sampling sites in Kuala Terengganu during June-August 2016. The sampling sites included supermarkets, wet markets and mini markets/groceries. Each sample taken from sampling site was put separately to avoid cross contamination, kept in the polystyrene box containing ice packs and transported to the laboratory. No additional washing steps were applied to the samples after collection. Samples were stored at 4-8°C and were processed within 2 hours of collection. A 25 g of each cut of *ulam* was weighed into sterile stomacher bag. Then, the 225 mL of sterile buffered peptone water (BPW) was added and then stomached for 2 minutes using a stomacher (BagMixer). The samples were subjected to a serial dilution. A loopful of the culture enrichment was streaked onto MacConkey (from Oxoid, UK), Violet Red Bile Agar (VRBA) (from BD, France) and Eosin Methylene Blue (EMB) (from Lab M, UK). The plates were incubated at 35°C for 24 hours. *Escherichia coli* colonies grown on VRBA appeared pink to red colonies with a red precipitate around colonies. While for MacConkey agar, *E. coli* appeared pink to red with bile salt precipitate surrounding colonies. Colonies appeared blue-black centred colonies with green metallic sheen when grown on EMB agar (USDA, 2001). Those positive colonies were selected and screened biochemically using triple sugar iron (TSI) agar or lysine iron (LI) agar slopes in conjunction with urease and sucrose/lactose media. The TSI and LI agar were incubated at 37°C for 24 hours. Typical strains of *E. coli* produce an acid (yellow) butt and slope in TSI agar. It showed an alkaline (purple) reaction throughout the LI medium.

Identification by API 20E System

Before performing API20E (bioMerieux), these isolates must be confirmed a Gram-negative rod (Gram staining and oxidase test). The tests were performed according to the manufacturer's instruction (Holmes *et al*., 1978). Three-quarter water was added to fill all the honeycombed wells of the tray. A loop was flamed until it was red hot then allowed it to cool for 20 seconds. A single isolated colony was selected and emulsified in ampule of API NaCl 0.85% Medium. A sterile pipette was used to transfer the isolate into the well. For (CIT, VP and GEL tests), both the tubes and cupules were filled with the isolate solution. For the remaining tests, only the tubes were filled. For (ADH, LDC, ODC, H_2S and URE), the tubes were overlaid with mineral oil to create anaerobiosis. The strips were incubated at 37°C for 18-24 hours. After the incubation period, the strip was read by referring to the Reading Table. If three or more tests (GLU test + or -) were positive, the strip required the addition of a reagent. One drop of TDA reagent was added to the TDA test. One drop of JAMES reagent was added to the IND test. One drop each of VP 1 and VP 2 was added to the VP test. After 10 minutes, the colours against the chart were compared and API record sheet recorded. If the number positive tests (including the GLU test) before adding the reagents was less than 3, the strip was re-incubated for further 24 hours without adding any reagent. The test reveals requiring the addition of reagents. The identification of unknown isolates was performed by using *apiweb*™ identification software through online.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was carried out by the Kirby-Bauer disk diffusion method on Mueller-Hinton agar plates as recommended by the Clinical and Laboratory Standards Institute (Barry, 2017). A total of sixteen (16) antibiotics were selected to determine the susceptibility to *E. coli*. The antimicrobial disks tested were amikacin (AK; 30 µg), amoxicillin-clavulanic acid (AMC; 30 µg), ampicillin (AMP; 10 µg), ampicillin-sulbactam

(SAM; 20 µg), cefoperazone (CFP; 75 µg), ceftazidime (CAZ; 30µg), ceftriaxone (CRO; 30 µg), cephalothin (KF; 30 µg), chloramphenicol (C; 30 µg), ciprofloxacin (CIP; 5 µg), gentamicin (CN; 10 g), kanamycin (K; 30 g), nalidixic acid (NA; 30 g), streptomycin (S; 10 g), tetracycline (TE; 30 g), and trimethoprim (W; 1.25/23.75 µg). These antibiotics are commonly used in food animal production.

E. coli from working stock was cross-streaked on Mueller-Hinton (MH) agar (from Oxoid, UK) plate and incubated at 35°C for 16-24 hours. Then, 4-5 of well isolated colonies were suspended in normal saline tubes using a sterile cotton swab. To prepare the inoculum, the turbidity of bacterial suspension was adjusted to match the standard McFarland 0.5 (approximately 10^8 CFU/mL). The prepared inoculum should be used within 30 minutes. Prior to use, MHA plates were allowed to dry under laminar flow for 10 minutes to let the excess moisture be absorbed on the agar surface. A sterile cotton swab was dipped in the inoculum and pressed firmly on the inside wall of the tube above the culture level to remove excess inoculum from the swab. The swab was streaked over the entire surface of the agar three times, each time at a 60° angle to the previous streaking. The inoculation was completed by running the swab around the rim of the agar. After air drying, antibiotic discs were placed 30 mm apart and 10 mm away from the edge of the plate by using sterile forceps (Rasheed *et al*., 2014). Four antimicrobial disks were dispensed onto each plate sufficiently separated from each other so as to avoid overlapping of inhibition zones (Learn-Han *et al*., 2009). Gently pressing down each test disk with the tip of the forceps to ensure complete contact with the agar. All the plates were then incubated at 35°C for 16-24 hours. *Escherichia coli* ATCC 29522 was used as a reference strain for antimicrobial disc control. Antibiotic susceptibility profile of a bacterial isolate was identified by measuring the size of the growth inhibition zone to the nearest millimeter (Kuan *et al.*, 2017). The inhibition zones were interpreted as sensitive (S), intermediate susceptibility (I) and resistant (R), according to the breakpoints recommended by the Clinical and Laboratory Standards Institute (CLSI) (Agent & Code, 2013).

Multiple antibiotic resistance (MAR) index

Antibiotic resistance pattern of each bacterial isolate was determined by calculating the MAR index, as described by Kuan *et al.* (2017):

$$\text{MAR index} = a/b$$

a = Number of antibiotics to which the particular isolate was resistant

b = Total number of antibiotics tested

RESULTS AND DISCUSSION

Out of 30 samples collected, 19 samples were detected with *E. coli*. A total of 57 presumptive *E. coli* were isolated and subsequently tested by using API20E. A total of twenty-three (23) (40.35%) *E. coli* isolates were positively tested and retrieved for antimicrobial resistance profiling. The antimicrobial resistance profiles of *E. coli* isolates was performed by using standard disc diffusion method and the results were interpreted based on the updated breakpoint provided by the Clinical and Laboratory Standards Institute (CLSI) (Agent & Code, 2013).

Table 1 shows the distribution of 23 *E. coli* isolates by type of samples and location. 17 (74%) strains of *E. coli* isolates were isolated from supermarkets and 6 (24%) strains of *E. coli* isolated from wet markets. Higher prevalence of *E. coli* was found in the supermarket than in wet markets. Probably, the cross contamination occurs during handling procedures and unhygienic practice by the customers during the raw vegetable selection process at the display unit. Plastic containers used for storage and transportation of fresh produce may contribute to cross contamination and foodborne infection. Biofilm formation on the plastic container cannot be removed by sanitizer and can cause

Table 1. The distribution of 23 isolates of *Escherichia coli* isolates by type of samples and location

Location	Types of Sample	Total number of isolates	Isolates Coding
Supermarkets	*Ketumbar*	5	SMKB2, SMKB3, SMKB4, SMKB5, SMKB8
	Kangkung	4	SMKG1, SMKG2,SMKG3, SMKG4
	Kesum	2	SMK2, SMK3
	Daun Sup	3	SMDS1, SMDS2, SMDS4
	Pegaga	1	SMP1
	Ulam Raja	1	SMU2
	Taugeh	1	SMT4
Wet Markets	*Salad Kampung*	4	WMSK1, WMSK4, WMSK6, WMSK7
	Daun Sup	1	WMDS
	Pucuk Putat	1	WMPP2

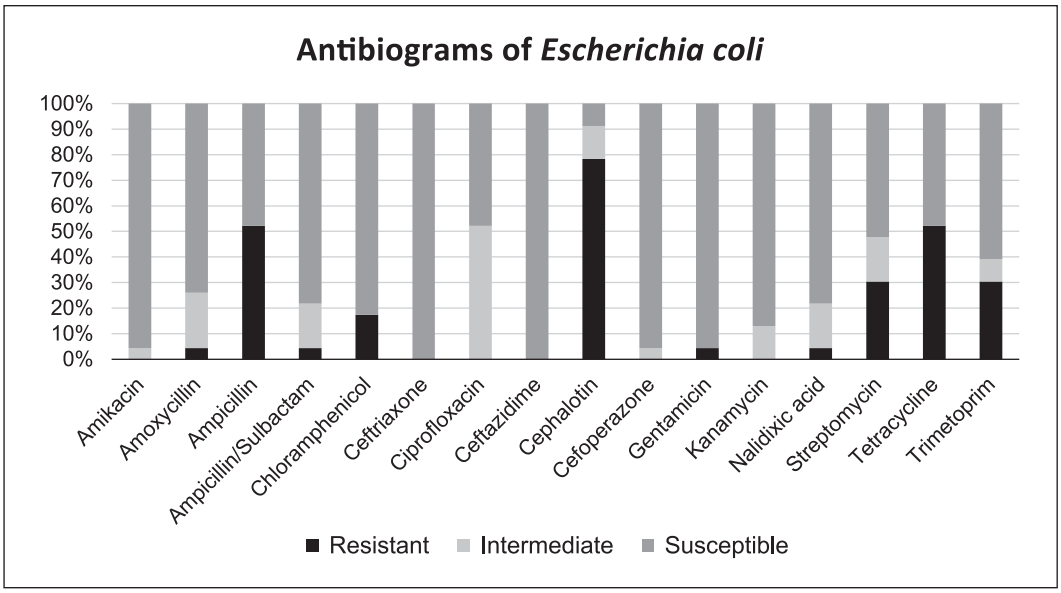

Fig. 1. The antibiotic resistant pattern of *Escherichia coli* in raw vegetables (*ulam*) in Terengganu.

widespread contamination after frequent reuse (Shi *et al.*, 2016).

Figure 1 shows the detailed percentage of antibiograms of *E. coli* isolates which were resistant, intermediate resistant and susceptible against 16 antibiotics. 87% of *E. coli* isolates showed resistance to at least one antibiotic. These findings are in agreement with previous studies that showed high resistant rates among bacterial population isolated from vegetables (Hamilton-Miller & Shah, 2001; Hassan *et al*., 2011; Viswanathan & Kaur, 2001). Antibiotic resistant in *E. coli* isolate was the highest to cephalothin (78.3%), followed by ampicillin (52.2%), tetracycline (52.2%), streptomycin (30.4%), trimethoprim (30.4%), chloramphenicol (17.4%), amoxycillin/cluvanic acid (4.3%), ampicillin/ sulbactam (4.3%), gentamicin (4.3%) and nalidixic acid (4.3%). The bactericidal activity of cephalothin results from the inhibition on the inner membrane of *E. coli* cell wall via the bind and inactivates to penicillin-binding proteins (PBP). As the first-generation antibiotic, cephalothin is known to be the least effective against *E. coli* (Faour-Klingbeil *et al*., 2016). It has been marketed in 1964 and continues to be widely used. Our result (78.3%) was in line with the previous result from (Abakpa *et al.*, 2015), who reported that 100% *E. coli* isolated from vegetables and environmental samples showed resistant to cephalotin.

Ampicillin is a beta-lactam antibiotic that attacks Gram-positive and some Gram-negative bacteria including *E. coli*. Ampicillin would inhibit cell wall biosynthesis, by binding to one or more of the penicillin binding proteins (PBPs) located inside the bacterial wall. It would inhibit the final transpeptidation step of peptidoglycan synthesis in the bacterial cell wall and eventually leads to cell lysis (Lawrence & Anthony, 2013). Based on this study, a high level of ampicillin resistance (52.2%) in *E. coli* isolates is alarming since this antibiotic is known as one of the regular traditional antibiotic treatment. Based on the previous study, the resistant rate of *E. coli* isolates to ampicillin reported by (Faour-Klingbeil *et al.*, 2016) is 69%.

Tetracycline is known as the most frequently used antibiotic on chicken farms (Faour-Klingbeil *et al.*, 2016), the overuse of this antibiotic may lead to a high percentage of tetracycline resistance (52.2%) reported in this study. *E. coli* isolates that show resistance to tetracycline also show resistance to at least two antibiotics. This finding is an agreement with Van *et al*. (2007) findings which showed microorganisms that have become resistant to tetracycline may exhibit resistant to other antibiotics too.

The resistant rate of *E. coli* isolates to gentamicin (4.3%) was interestingly comparable to results reported by Burjaq & Shehabi (2013) (6.6%) on *E. coli* isolated from fresh leafy vegetables presented in Jordanian retail markets. Gentamicin works by "irreversibly" bind to specific 30-S ribosomal protein, interrupting protein synthesis resulting in codon misreading and translocation inhibition (Gill & Amyes, 2004).

Both Ceftazidime and Ceftriaxone were beta-lactam, third-generation cephalosporin antibiotic with bactericidal activity. Cephalosporins exert bactericidal activity by interfering with bacterial cell wall synthesis and inhibiting cross-linking of the peptidoglycan necessary for bacterial cell wall strength and rigidity. The cephalosporins are also thought to play a role in the activation of bacterial

cell autolysins which may contribute to bacterial cell lysis. Compared to the second and first generation, they were more active against Gram-negative bacteria and less active against Gram-positive bacteria. Interestingly, this study showed that ceftriaxone and ceftazidime were found to be (100%) effective in inhibiting the growth of *E. coli* isolates. This is in the support of the findings of (Aso *et al.*, 2017), 78.58% of *E. coli* isolated from a patient in Nigeria showed resistant to ceftriaxone.

These tests revealed that 47.8% of the *E. coli* isolates were classified as MDR (Table 2). We found a much higher percentage for MDR with respect to those reported by Burjaq and Shehabi (2013), a total of 27.8% were resistant to three or more antibiotics. According to Nipa *et al.* (2011), this happens, for instance, when the antibiotics were frequently used in the food of animal origin production and in medicine. Besides, the misuse and overuse of antibiotics also has resulted in the prevalence of *E. coli* strains resistant to antibiotics and presents a risk to human health.

Table 3 shows the MAR index of *E. coli* strains isolated from *ulam.* Overall, *E. coli* isolates in this study demonstrated a Multiple antibiotic resistance index (MAR) index ranging from 0.06 to 0.48. According to Adeshina *et al.* (2012), a MAR index greater than 0.2, signify that the isolates of such bacteria originated from an environment where several antibiotics were used. This study showed that about 30.43% of *E. coli* isolates exhibited MAR index greater than 0.2. In 2016, approximately 3.4 million metric tons of fertilizers were produced in Malaysia. Application of manure as fertilizer is the main reason for agricultural antibiotic contamination. Manure has become a reservoir of resistant bacteria and antibiotic compounds. The application of manure to agricultural soils is significantly increasing antibiotic resistance genes and selection of resistant bacterial populations in soil. Antibiotic-resistance genes from the soil can enter the food chain via contaminated fresh vegetables and later will cause potential consequences for human health (Heuer *et al.*, 2011). It is suggested that organic agriculture practices could reduce the spread of antibiotic resistant bacteria and livestock used are raised without the use of antibiotics unless medically necessary.

As an important aspect of hazard assessment, it is a crucial need for monitoring and reviewing the antimicrobial resistance regularly, especially multidrug resistance of foodborne pathogens (Kuan *et al.*, 2017). These generated data can be used to recommend some preventive measures in order to

Table 2. The Distribution of multidrug-resistant (MDR) *Escherichia coli* isolated from supermarkets and wet markets

Source	Label	*E. coli* isolates, count (%)		
		N	MDR	Non-MDR
Supermarket	SM	17	6	11
Wet market	WM	6	5	1
Total (%)		23	11 (47.8)	12 (52.2)

Table 3. Antibiotic resistance profiles and MAR index of *Escherichia coli* strains isolated from *ulam-ulaman*

MAR Index	Antibiotic resistance profile[a]	Source and Isolate's code[b]	Percentage of Isolate (%)
0.48	AMPCKFSSAMTEW	WMSK1	4.3
0.38	AMCAMPKFSTEW	WMDS	4.3
0.38	AMPCKFSTEW	WMSK7, SMK2, SMK3	13.0
0.31	AMPCNKFTEW	WMSK6	4.3
0.31	AMPKFNASTE	SMKB3	4.3
0.19	AMPKFTE	WMSK4,SMKB2, SMKB4	13.0
0.19	AMPTEW	SMKB5	4.3
0.13	KFS	SMP1	4.3
0.13	AMPTE	SMKB8	4.3
0.06	KF	WMPP2, SMKG1, SMKG2, SMKG4, SMDS2, SMDS4, SMT4	30.4

[a]AMC: Amoxicillin-clavulanic acid; AMP: Ampicillin; SAM: Ampicillin-sulbactam; C: Chloramphenicol; KF: Cephalothin; C: Chloramphenicol; CN: Gentamicin; NA: Nalidixic acid; S: Streptomycin; TE: Tetracycline; W: Trimethoprim.
[b]SM: Supermarket; WM: Wet market; DS: Daun sup; KG: Kangkung; K: Kesum; KB: Ketumbar; P: Pegaga; PP: Pucuk putat; SK: Salad kampung; T: Taugeh.

APPENDIX

Antibiotic susceptibility profiles of 23 *E. coli* strains isolated from vegetable samples tested by disc diffusion method

Antibiotic	Antimicrobial profile of *E. coli*		
	Susceptible (%)	Intermediate (%)	Resistant (%)
Amikacin (30 µg)	22 (95.7)	1 (4.3)	–
Amoxycillin/Cluvanic acid (30 µg)	17 (73.9)	5 (21.7)	1 (4.3)
Ampicillin (10 µg)	11 (47.8)	–	12 (52.2)
Ampicillin/Sulbactam (20 µg)	18 (78.3)	4 (17.4)	1 (4.3)
Chloramphenicol (30 µg)	19 (82.6)	–	4 (17.4)
Ceftriaxone (30 µg)	23 (100)	–	–
Ciprofloxacin (5 µg)	11 (47.8)	12 (52.2)	–
Ceftazidime (30 µg)	23 (100)	–	–
Cephalotin (30 µg)	2 (8.7)	3 (13)	18 (78.3)
Cefoperazone (75 µg)	22 (95.7)	1 (4.3)	–
Gentamicin (10 µg)	22 (95.7)	–	1 (4.3)
Kanamycin (30 µg)	20 (87)	3 (13)	–
Nalidixic acid (30 µg)	18 (78.3)	4 (17.4)	1 (4.3)
Streptomycin (10 µg)	12 (52.2)	4 (17.4)	7 (30.4)
Tetracycline (30 µg)	11 (47.8)	–	12 (52.2)
Trimethoprim (1.25/23.75 µg)	14 (60.9)	2 (8.7)	7 (30.4)

The inhibition zone was interpreted as sensitive, intermediate and resistant, according to the breakpoints recommended by the Clinical and Laboratory Standards Institute (CLSI) (Agent & Code, 2013).

revise dosing of antibiotics, prevent or reduce environmental spread, and inform the suitable medical treatments for the illnesses caused by the pathogens. Therefore, biosafety issues regarding antibiotic resistance are sustainably safeguarded (Roca *et al.*, 2015).

CONCLUSION

The presence of antibiotic resistant of *E. coli* in raw vegetables (*ulam*) from wet markets and supermarkets demonstrate the role of fresh produce as a reservoir of resistant pathogenic bacteria. The high percentage of cephalotin resistance, in particular, shows the extensive use of this antibiotic. Cephalotin may no longer be ideal for treating fresh produce associated infections, especially those caused by *E. coli*. The high prevalence of MDR *E. coli* to antibiotics, resulting in bacterial infection much more difficult to treat with current antibiotics and later it may infect human populations. In order to solve these problems, it is necessary to implement stricter hygienic practices and good agricultural practices.

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