DETECTION AND IDENTIFICATION OF TETRACYCLINE-RESISTANT E.COLI ISOLATED FROM READY TO EAT VEGETABLES

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Background: There have been rising outbreaks of food infections which are associated with Ready to Eat (RTE) vegetables consumption. Recently, antimicrobial resistance in human becomes a worldwide problem. Antibacterial resistance such as to agent like tetracycline inhibits the prevention and effective treatment to infectious diseases. Therefore, it is important to detect characteristics of tetracycline-resistant Escherichia coli isolated from RTE vegetables.
Objectives: This study aimed to isolate tetracycline resistant E. coli from Ready to Eat Vegetables and also determine its resistance to other common antibiotics.
Materials and Methods: The study took place in Microbiology Laboratory, University of Brawijaya, Malang City, Indonesia. A total of forty-eight samples (Lettuce, Lemon basil, Yard long bean and Cabbage) were collected from five different places (Pasar Besar, Pasar Belimbing, Giant supermarket, Lai Lai supermarket and Loka supermarket). Subsequently, using media of Eosin Methylene Blue Agar which was supplemented with 15mg/ml of Tetracycline, these samples were analyzed to detect the presence of tetracycline-resistant E. coli. Identification and characterization of E. coli isolates were performed using a biochemical test followed by PCR to detect 16S rRNA and antibiotic resistance of the isolates in disk diffusion.
Results: Eighteen samples (37.5%) showed a positive result of the present of E. coli resistant to antimicrobial tetracycline. This included 6 isolates from Lettuce, 8 isolates from Basil, 3 isolates from Yard long bean and 1 isolate from Cabbage. The higher number of tetracycline resistant isolates was recorded in the samples from Pasar Besar and Pasar Belimbing while the lower number was recorded in the samples from LOKA supermarket.
The results indicated that 18 isolates (100%) were resistant to one or more antimicrobials. Moreover, the highest levels of resistance were observed for tetracycline and streptomycin by 100%, followed by Kanamycin by 77.8%. Mere 11.1% and 5,6 % isolates were resistant to Chloramphenicol and Ciprofloxacin.
Conclusion: To sum up, this study showed that 37.5% of RTE vegetable samples contained tetracycline resistant E. coli in and the isolates highly resistant to tetracycline and streptomycin (100%) could be hazardous for public health.

Keywords - E.coli, Multidrug resistance, Ready to Eat Vegetables, tetracycline.

I. INTRODUCTION

Ready to eat (RTE) vegetables provide highly essential human nourishment that consists of carbohydrates, protein, minerals, vitamins, and fiber. In spite of its health advantages for humans, consuming RTE vegetables brings risks to consumers (Beuchat, 2002). Consumption of RTE is associated with the increasing outbreaks of infectious diseases (Pezzoli, et al., 2007). Garg et al., (2010) discovers that the epidemics resulted from infectious bacteria such as E. coli are epidemiologically associated with the consumption of various kinds of vegetables. The contamination of the vegetables, especially the leafy green ones, by E. coli causes a hazardous health threat to humans (WHO / FAO, 2008). Products consumed in fresh are possibly contaminated with pathogens through exposure to sources of contamination either in the production process on the farms or in the selling places such as the markets (Aycicek et al., 2006). The reported sources of contamination include: soil (e.g. manure, faeces, soil and microorganisms), dust, water, and handling during the pre or postharvest steps (Eni et al., 2010). The primary contamination sources during the postharvest stage involve the containers utilized to transport the crops, the way of human handling, the produce processing, and the storing places (Rediers et al., 2009). Eluding decontamination steps prior to consumption also make fresh products liable to be contaminated with potentially pathogenic bacteria (Burnett and Beuchat, 2001). In addition, less-processed fruits and vegetables are highly likely to be susceptible to contamination since copping and slicing damage the natural protection of whole products, release nutrients and cause the microorganism growth (Abadias et al., 2008; Harris et al., 2003; de Oliveira et al., 2012).
E. coli is a generally found bacterium in the intestinal tract of humans and animals. Majority of E. coli do not harm humans, but particular kinds of it may trigger diarrhoeal diseases or a more dangerous sickness. The serotype of enterohemorrhagic E. coli (EHEC) O157: H7 was denounced to be linked to the outbreaks of infectious diseases associated with various types of foods such as spinach leaves (Pennington, 2010). At present, the E. coli serotype O104: H4 of the Shiga toxin producer (STEC) has become a significant, public interest due to its relation with the German epidemics caused by sprout consumption (Muniesa et al., 2012). It has been observed that leafy vegetables are consumed as raw dishes most often associated to E.coli infections (Heaton and Jones, 2008). Antimicrobial resistance in

humans and animals has become a global problem. This kind of resistance hinders the effective prevention and treatment for infectious diseases (Cizman, 2003; Lee et al., 2001). Some study results reveal that inappropriate choices and extensive administration of antimicrobials have resulted in the evolution and spread of resistant bacteria, especially bacteria that are resistant to some drugs. Moreover, newer compounds, such as antimicrobial of tetracycline classes are no longer effective to inhibits bacteria growth (Levy and Marshall, 2004).

Tetracyclines are a group of antibiotics produced using Streptomyces spp. and active against various gram-positive and gram-negative bacteria. The broad spectrum and the lack of main side effects have caused them to be globally used in therapy and infection controls in pets. (Chopra and Roberts, 2001; Bryan et al., 2004; Miles et al., 2006).

Therefore, the objectives of this study were to isolate tetracycline resistant E.coli (TRE) from RTE vegetables collected from traditional markets and supermarkets in Malang city, Indonesia and to determine the isolate's resistance to other common antibiotics.

II. MATERIAL AND METHODS

Place of study

The study was conducted from March to August 2018 and the analysis of isolates took place at Microbiology and Animal Physiology Laboratories of University of Brawijaya, Malang City, Indonesia.

Sample collection and microbiological analysis of vegetables

Forty eight samples including Lettuce, Lemon basil, Yard long bean and Cabbage were collected from three different places (Pasar besar, Pasar Blimbing, Giant supermarket, Lai Lai supermarket and Loka supermarket). They were then aseptically chopped into smaller pieces using a sterile scalpel. Each 25 grams of the leafy vegetable samples was aseptically transferred to 225 ml of sterile buffered peptone water (BPW) 0.1% (w/v) using sterilized forceps and shacked for 3 min by a shaker (Shalini, 2010). Serial dilutions were prepared in normal saline (0.85% NaCl) from original homogenized, and then the appropriate dilutions (in duplicate) were placed on Eosin Methylene Blue Agar (EMB) which supplemented with 15mg/ml of tetracycline (TCI, Korea) in order to isolate TRE and the plates were incubated at 37°C for 18 to 24 hrs. Similaly, the left washed leaves were also incubated and shacked for overnight using a shaker at 37°C.

Following that, subculture EMB was supplemented with tetracyclines and incubated at 37°C for 18 to 24 hrs. After that, all colonies exhibiting a typical dark purple red colour with a metallic sheen as the TRE characteristic with a pure culture on the EMB agar were subcultured on the MacConkey agar supplemented with 15mg/ml Tetracycline to identify the lactose fermenting colonies.Five or ten colonies of each pure growing TRE on the previous media from the direct plating or enrichment cultures were picked up and inoculated in 1 ml Luria-Bertani (LB) broth containing 15% glycerol and then stored at -70°C for further investigation.

Biochemical tests including Catalase, Oxidase, Triple sugar Iron Agar, Indole, citrate, and MR-VP tests were conducted on all lactose fermenting colonies of TRE.

Susceptibility test

All 18 isolates of TRE were subjected to antibiotic susceptibility testing in which the Kirby-Bauer disc diffusion method was used. Chloramphenicol (30µg), Gentamycin (10µg), Streptomycin (10µg), Tetracycline (30µg), Ciprofloxacin (5µg) and Kanamycin (30µg) (Oxoid, England) were tested during the study period.

The susceptibility test was administered with Mueller-Hinton agar using McFarland 0.5 from overnight cultures, followed by incubation at 36°C for 18-20 hours. Following that, the inhibition zone diameters were determined with a millimetric ruler and recorded. The inhibition zone diameters were interpreted according to the EUCAST and CLSI guidelines.

Genomic DNA extraction

Presto™ Mini gDNA Bacteria Kit, Geneaid, Taiwan was employed to extract DNA total from the isolates. Protocol was done according to the manufacture's instruction. The DNA quality and quantity were evaluated and measured by an absorbance spectroscopy at 260 and 280 nm and then the extracted DNA was stored at -20^{o}C.

16S rRNA gene PCR

PCR reaction was performed in a Master cycler (Eppendorf, Germany). The PCR program was operated in 50µL reaction volumes which contained 2 µL DNA template, 2 µL of each primer, 25 µL PCR MyTaq 2x, and 19 µL of ddH_2O.

The primers used in this study were 27F (5' AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (James, 2010). At first, the PCR amplification was set for initial DNA denaturation at 95°C for 5 min. This was followed by 35 cycles of denaturation at 95°C for 30 sec and annealing at 52°C for 30 sec. Next, the extension at 72°C for 90 sec was perfomed and followed by a final extension at 72°C for 5 min. In the analysis step, 5 µL PCR product was analyzed by electrophoresis (Mupid-exU) in 1% agarose gel at 100 volts for 30 minutes.

It was then stained with 1% solution of ethidium bromide (50 µL/L) and destained with TBE 1x for 10 min. The gel was visualized by UV transillumination and recorded using a digital camera.

III. RESULTS AND DISCUSSION

A total of 18 (37.5 %) of TRE were isolated from 48 samples of TRE from which highest percentage (44.4%) was isolated from Lemon basil followed by Lettuce (33.3%) , while the lowest percentage (5.5%) was isolated from Cabbage sample. Pasar besar market and Pasar blimbing (open markets) were recorded to show the highest percentage (38.9% each) while no TRE was detected from the vegetables from LOKA supermarket (Fig. 1). In this study all colonies isolated on EMB plates and MacConkey agar supplemented with 15mg/ml of Tetracycline were identified.

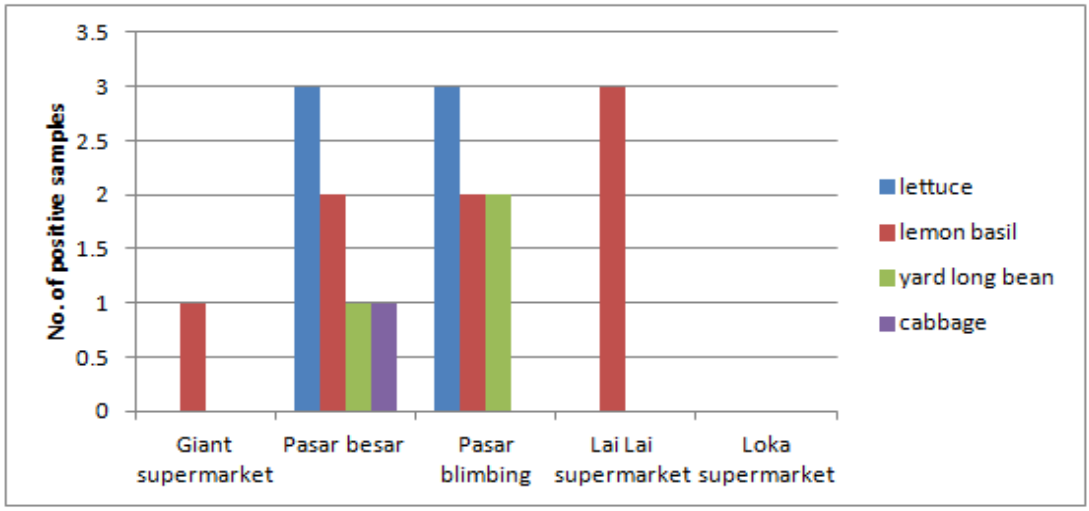


Fig.1. Number of TRE isolated from different vegetables and places.

The result of this study indicates the highest TRE found in Lemon basil and Lettuce samples which are commonly freshly consumed in salad cuisines. This can possibly pose higher risks of microbial contamination to humans and become a health hazard to the community. Lemon basil and Lettuce are easily contaminated by soil and they provide wide surface areas for bacterial colonization. Infiltration of pathogens into cracks, wounds, crevices, stomatal cavities and intercellular spaces of fruits and vegetables has been reported in many studies (Harapas et al., 2010; TabanandHalkman, 2011; Saldaña et al. 2011). Moreover, the pre and postharvest stages that usually make use of contaminated water can also trigger the contamination. In addition, it was frequently observed that the fresh vegetables had to be transported and distributed to the markets over long distances from the production areas. Due to the lack of cold storage facilities, certain types of microorganisms especially the mesophilic ones may continue to grow and proliferate during distribution processes from the farms to the markets. Another possibility was due to the more handling steps done to the fresh vegetables in the supermarkets such as sorting, grading, and trimming. Consequently, they were more frequently exposed to the hands, which could further increase the microbial loads.

On the other hand, it was hypothesized that samples obtained from supermarkets would have less prevalence and microbial loads. This can be caused by the cleanliness of the place, implementation of supporting instruments such as cooling system on the display shelves and adequate hygienic practices such as the use of gloves by the handlers.

Similarly, a study by Soendjojo (2012) discovered a much higher number of a bacterial colony on some fresh vegetables sold at the farmers' markets compared to the vegetables obtained from the grocery stores. Thus, it was concluded that the effective sterilization treatments applied by the grocery stores in order to increase the shelf life of the produces had resulted in the reduction of the microbial population on the perishables. On the contrary, another study by Vital et al. (2014) comparing the microbial qualities of fresh produces obtained from open air markets and supermarkets in Philippines revealed relatively higher incidence levels and microbial loads observed in the samples obtained from the supermarkets compared to those obtained from the open air markets, although the difference was statistically not significant. In general, it can be concluded that microbial contamination on fresh produces can occur at any stages of supply chain, from production, harvesting, distribution, selling point and domestic preparation, as what observed in the present study.

Biochemical characterization

All the TRE isolates were confirmed using biochemical tests including Catalase, Oxidase, indole, Triple sugar Iron Agar, citrate, and MR-VP tests (Tab.1).

Tab. 1: Biochemical test for TRE.

Samples code	Oxidase	Catalase	Triple sugar Iron Agar	indole	citrate	MR-VP
L.L1.No.1	(-)	(+)	Acid (Slant and Butt), Gas (+) H_2S (-)	(+)	(-)	(+) (-)
L.L1.No.2	(-)	(+)	Acid (Slant and Butt), Gas (+) H_2S (-)	(+)	(-)	(+) (-)
L.L1.No.3	(-)	(+)	Acid (Slant and Butt), Gas (+) H_2S (-)	(+)	(-)	(+) (-)
B.L1.No1	(-)	(+)	Acid (Slant and Butt), Gas (+) H_2S (-)	(+)	(-)	(+) (-)
B.L1.No2	(-)	(+)	Acid (Slant and Butt), Gas (+) H_2S (-)	(+)	(-)	(+) (-)
Y.L1.No1	(-)	(+)	Acid (Slant and Butt), Gas (+) H_2S (-)	(+)	(-)	(+) (-)
Y.L1.No3	(-)	(+)	Acid (Slant and Butt), Gas (+) H_2S (-)	(+)	(-)	(+) (-)
B.L2.No1	(-)	(+)	Acid (Slant and Butt), Gas (+) H_2S (-)	(+)	(-)	(+) (-)
B.L2.No2	(-)	(+)	Acid (Slant and Butt), Gas (+) H_2S (-)	(+)	(-)	(+) (-)
B.L2.No3	(-)	(+)	Acid (Slant and Butt), Gas (+) H_2S (-)	(+)	(-)	(+) (-)
L.L3.No1	(-)	(+)	Acid (Slant and Butt), Gas (+) H_2S (-)	(+)	(-)	(+) (-)
L.L3.No2	(-)	(+)	Acid (Slant and Butt), Gas (+) H_2S (-)	(+)	(-)	(+) (-)
L.L3.No3	(-)	(+)	Acid (Slant and Butt), Gas (+) H_2S (-)	(+)	(-)	(+) (-)
B.L3.No1	(-)	(+)	Acid (Slant and Butt), Gas (+) H_2S (-)	(+)	(-)	(+) (-)
B.L3.No3	(-)	(+)	Acid (Slant and Butt), Gas (+) H_2S (-)	(+)	(-)	(+) (-)
Y.L3.No3	(-)	(+)	Acid (Slant and Butt), Gas (+) H_2S (-)	(+)	(-)	(+) (-)
C.L3.No1	(-)	(+)	Acid (Slant and Butt), Gas (+) H_2S (-)	(+)	(-)	(+) (-)
B.L5.No1	(-)	(+)	Acid (Slant and Butt), Gas (+) H_2S (-)	(+)	(-)	(+) (-)

Where L- Lettuce, B= Lemon basil, Y- Yard long bean, C- Cabbage, L1- Pasar blimbing, L2- Lai Lai supermarket, L3- Pasar besar, L4=-Loka supermarket, L5- Giant supermarket, No- Number of sample.

Susceptibility test

Antibiotic-resistant bacteria or their corresponding resistance determinants are known to spread from animals to humans via the food chain (Boehme et al., 20014; Tenover, 2001). Vegetables that are eaten raw may contribute to this phenomenon because epiphytic bacteria can develop antibiotic resistances due to the large amount of antibiotics used in agriculture, soil treated with organic fertilisers, such as sewage sludge and manure, and contaminated irrigation water. These factors may lead to vegetal contamination with resistant bacteria from animal and/or human sources (De la and Davies, 2000; Boehme et al., 2004). Consequently, all TRE (n: 18) isolated from RTE (Table 4) were found resistant to five common antibacterial chemotherapeutic agents. All isolates (18 isolates of 100%) were resistant to both Tetracycline and Streptomycin, followed by as many as 14 isolates (77.8%) resistant to Kanamycin. Additionally, 2 and 1 isolates were resistant to other agents such as Chloramphenicol (11.1%) and Ciprofloxacin (5.6%), respectively. No bacteria were found resistant to Gentamycin. Fifteen isolates (83.3%) were susceptible to Chloramphenicol followed by Ciprofloxacin 13 (72.2%) and Gentamycin 4 (22.2%) (Tab2).

SL No.	Antimicrobial agent	Symbol	Disc Content (µg)	Susceptibility		
				Resistant No. (%)	Intermediate No. (%)	Sensitive No. (%)
1	Kanamycin	k	30	14 (78)	2 (11)	2 (11)
2	Ciprofloxacin	Cip	5	1 (6)	4 (22)	13 (72)
3	Gentamycin	CN	10	0 (0)	14 (78)	4 (22)
4	Chloramphenicol	C	30	2 (11)	1 (6)	15 (83)
5	Streptomycin	S	10	18 (100)	0 (0)	0 (0)
6	Tetracycline	Tet	30	18 (100)	0 (0)	0 (0)

Tab 2: Antimicrobial susceptibility pattern of TRE isolated from RTE vegetables.

As above mentioned, the TRE isolated from RTE vegetables include several opportunistic human pathogens, which may cause a variety of infectious diseases in humans and, in addition, antibiotic resistances can be horizontally disseminated, after ingested by the consumers, to other gut commensal or pathogenic bacteria. To add, the RTE perishables may contribute to an additional source of bacterial food contamination during inappropriate handling at homes. Therefore, consumption of fresh vegetables may trigger a potential risk factor for the consumer health, particularly in debilitated or immune compromised individuals. Thus, microbial contamination of RTE produce can be considered as a food safety concern. For this reason, the hygienic quality of fresh vegetables should be improved and controlled to minimize the foodborne disease risks. Better methods of contamination prevention on the farms and during packing and processing stages and the use of a terminal control such as irradiation can be an alternatve to reduce the disease transmission risks from fresh perishables (Lynch et al., 2009; Hamilton et al., 2006; Tyler and Triplett, 2008; Tauxe,1997; Sivapalasingam et al., 2004; DuPont, 2007; Aarestrup and Wegener, 2008 ; Walsh and Fanning, 2008).

16S rRNA gene PCR

PCR analyses were subjected to the isolated microbes presumed to be E. coli based on microbiological phenotyping studies to confirm the species. The indicates that all of the 18 bacteria were confirmed to be E.coli according to the E.coli 16S rRNA-PCR which showed ~1500bp bands. (Fig2).

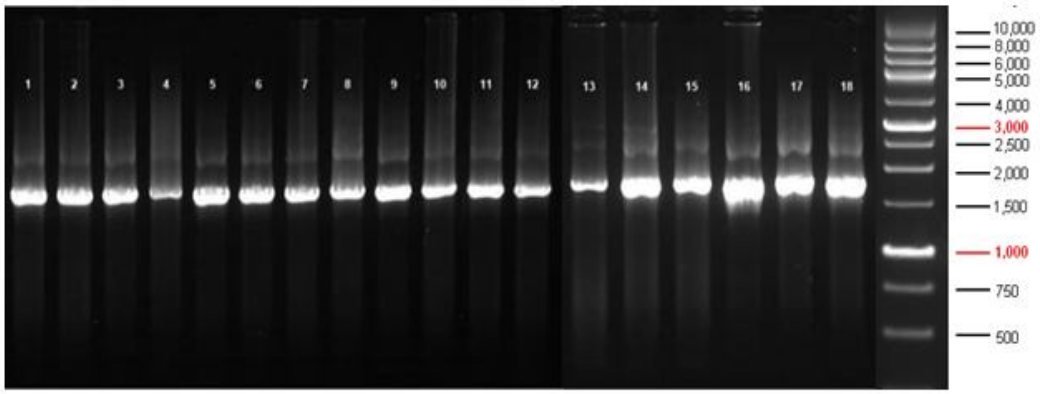


Fig.2. PCR amplification results for TRE 16S rRNA; Lanes 1-18 (microbial isolates from 18 samples.

CONCLUSION

From the 48 RTE vegetable samples examined in this study, 37.5% of the vegetables were contaminated with TRE and some isolates were found to be resistant to multi-drugs and presented high risk factors for public health. Contamination itself reflects poor hygiene quality that may be related to sub-standard agriculture, handling, packaging, and marketing practices. Better monitoring programs are necessary to prevent the potential epidemics as fresh perishables are now substantially linked to bacterial infection.

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