Prevalence and Characterization of Extended-Spectrum β-Lactamase-Producing *Escherichia coli* and *Klebsiella pneumoniae* Isolated from Raw Vegetables Retailed in Southern Thailand

Chonticha Romyasamit[1+*], Phoomjai Sornsenee[2+], Siriphorn Chimplee[3], Sitanun Yuwalaksanakun[1], Dechawat Wongprot[1], Phanvasri Saengsuwan[3]

[1]Department of Medical Technology, School of Allied Health Sciences, Walailak University, Nakhon Si Thammarat 80161, Thailand
[2]Department of Family and Preventive Medicine, Faculty of Medicine, Prince of Songkla University, Songkhla 90110, Thailand
[3]Department of Biomedical Sciences and Biomedical Engineering, Faculty of Medicine, Prince of Songkla University, Hat Yai, Songkhla, 90110, Thailand
[+]Co-first author

Corresponding Author:
Chonticha Romyasamit
222 Thaiburi, Tha Sala, Nakhon Si Thammarat, 80161, Thailand
Email address: chonticha.ro@wu.ac.th

Abstract

Background. The increasing prevalence of broad-spectrum ampicillin-resistant and third-generation cephalosporin-resistant Enterobacteriaceae, particularly *Escherichia coli* and *Klebsiella pneumoniae*, has become a global concern, with its clinical impacts on both human and veterinary medicine. This study examined the prevalence, antimicrobial susceptibility, and molecular genetic features of extended-spectrum β-lactamase (ESBL)-producing *E. coli* and *K. pneumoniae* isolates from ten types of raw vegetables.

Methods. In total, 305 samples were collected from nine markets in Nakhon Si Thammarat, Thailand, in 2020.

Results. Fourteen ESBL-producing *E. coli* (n=?) and *K. ~~pneumoniae~~pneumonia* (n=?) isolates (1.6% and 3.0%, respectively) were highly sensitive to β-lactam/carbapenem antibiotics (imipenem, 100%), while eight were sensitive to non-β-lactam aminoglycosides (amikacin and gentamicin, 85.71%). ESBL producers were most resistant to β-lactam antibiotics including ampicillin (85.71%) and the cephalosporins cefotaxime and ceftazidime (64.29%). The bla_{SHV} gene was the most frequently detected in ESBL-producing *E. coli* and *K. pneumoniae*. However, two ESBL-producing *E. coli* producers also carried three other ESBL-encoding genes, bla_{TEM},

Commented [DEDP1]: The title of the manuscript mention prevalence of ESBL producing *E. coli* and *K. pneumoniae.* In the first section of the results the following statement was made:ESBL-producing *E. coli* and *K. pneumoniae* isolates were found in 14 of the 305 samples obtained from seven out of ten types of vegetables (4.6% of the total).

It is important to incorporate information on prevalence in the vegetables, before describing the genes screened for.

Commented [DEDP2]: How many isolates of *E. coli* were obtained and how many Klebsiella isolates?

Commented [DEDP3]: Here you mention the number if isolates and previously only percentages. Please be consistent.

Commented [DEDP4]: Were 85.71% resistant to amikacin and and 85.71 % resistant to gentamicin

bla$_{CTX-M1}$, *bla*$_{GES}$ *and bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M9}$, which may be due to their association with food chains and humans.

Discussion. Indeed, our results suggest that raw vegetables are an important source of ESBL-resistant *E. coli* and *K. pneumoniae*, which are potentially transmittable to humans via raw vegetable intake.

Introduction

Extended-spectrum β-lactamase (ESBL)-producing microorganisms, particularly *Escherichia coli* and *Klebsiella pneumoniae*, produce nosocomial infections in patients and can also affect communities of heathy people (1-3). The increasing prevalence of ESBL-producing strains has enhanced the possibility of the development of β-lactam antibiotic resistance (2, 4). This will exacerbate global public health problems and thus requires resolution. Outside of nosocomial infections, ESBL-producing *E. coli* and *K. pneumoniae* have emerged as community infection concerns, causing severe infections, such as urinary and respiratory tract infections and bacteremia (2, 4, 5). It is, therefore, crucial to identify~~disseminate~~ the sources of ESBL-producing *E. coli* and *K. pneumoniae*.

Raw vegetables are considered exceptional human food due to their convenience for uncooked consumption. In addition, consumers deem vegetables more advantageous to health; thus, there has been a noted increase in the consumption of vegetables instead of foods produced from animals. However, uncooked vegetables can have high microbial contamination, which, in turn, could lead to a high rate of cross-contamination events (microbes-vegetables-human) (3, 6-8). In fact, it has been reported that ESBL-producing bacteria could be present in fresh vegetables such as iceberg lettuce, spinach, and tomato (9, 10). However, studies on the incidence of ESL-producing bacteria in fresh vegetables, which are commonly found and consumed in tropical countries such as Thailand, remain to be limited.

Thus, this study aimed to examine the prevalence and characteristics of ESBL-producing *E. coli* and *K. pneumoniae* in common raw vegetables found in Southern Thailand. The associations between ESBL-encoding genes and ESBL-producing strains were characterized to correlate data with those previously obtained in isolates from other food and clinical sources.

Materials & Methods

Vegetable collection

In total, 305 samples derived from ten common types of edible vegetables were included in this analysis: Thai yardlong beans (34), Thai eggplant (44), winged bean (25), young cashew leaves (20), Thai basil (36), cabbage (21), cucumber (36), tomato (31), long coriander (32), and lettuce (26). These were bought randomly between September 2020 and October 2020 from four and five local retail markets in Tha Sala and Mueang districts, respectively, in Nakhon Si Thammarat, Thailand. After purchase, the vegetable samples were collected in sterile containers, maintained under 4°C, and tested within 24 h.

Commented [DEDP5]: It is important to mention that the organisms/genes cold be transmitted, however it Is not guaranteed to happen.

Commented [DEDP6]: In the abstract you mention Enterobacteriaceae and here microorganisms. It is important to mention that they belong to the Enterobacteriaceae family.

Commented [DEDP7]: Add reference

Commented [DEDP8]: Add reference

Commented [DEDP9]: Specify the most important organisms i.e. E. coli and Klebsiella as mentioned in Kim et al (reference 6).

Commented [DEDP10]: It is important to mention Enterobacteriaceae which was the focus of the studies referred to.

Bacteria identification process and ESBL-producing bacteria isolation

About 25 g of each samples was weighed, suspended in 225 mL of 0.1% buffered peptone water (Oxoid, Hampshire, UK), and incubated at 37°C for 24 h. These microbial enrichments were streaked onto ESBL selective agar (HiMedia Laboratories, Mumbai, India), followed by incubation at 37°C for 24 h under aerobic conditions to select for ESBL-producing bacteria. The suspected Enterobacteriaceae colonies of *E. coli* (pink to purple) and *K. pneumoniae* (bluish green) were sub-cultured onto nutrient agar (Oxoid) at 37°C for 24 h, followed by biochemical identification using Vitek2 (bioMérieux, Marcy 1'Etoile, France). All the *E. coli* and *K. pneumoniae* isolates were then identified and confirmed by matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MADLI-TOF MS) (11). All the confirmed isolates were stored at 2°C–8°C until antimicrobial drug susceptibility testing and ESBL production investigation.

Screening of presumptive ESBL-producing isolates

The standard disk diffusion method was performed by placing β-lactam ring drugs ceftazidime (CAZ) (30 µg) and cefotaxime (CTX) (30 µg) on disks. The amount of bacteria was adjusted by 0.5 McFarland standard, and the suspension was inoculated onto Mueller-Hinton agar (Oxoid) by sterile cotton swab. Thereafter, the drug disks were placed on inoculated plates and were incubated at 37°C for 24 h. Isolates presenting an inhibition zone to CTX (≤27 mm) and CAZ (≤ 22 mm) around the disks were regarded~~implied~~ as presumptive~~β-lactam ring drug-resistant or~~ ESBL-producing strains, based on Clinical and Laboratory Standards Institute (CLSI) guidelines.

Confirmation of ESBL-producing isolates

The double-disk synergy method was performed to confirm ESBL production of the presumptive positive isolates~~after identification of inhibition zones~~. *E. coli* and *K. pneumoniae* suspensions were placed onto Mueller-Hinton agar (MHA). Then, 30 µg of CTX and CAZ disks was placed on the center of the plate, followed by CTX and CAZ plus clavulanic acid (30/10 µg) disks at a distance of 20 mm from the central disk in the same plate (CLSI 2020). All plates were incubated at 37°C for 24 h and was considered as an ESBL-producer when there was a ≥5 mm increase in the zone of inhibition with CTX or CAZ disk with clavulanic acid in comparison to CTX or CAZ alone.

Antibiotic sensitivity test

To assess the antibiotic sensitivity profile of ESBL-producing *E. coli* and *K. pneumoniae* isolated from the vegetables, the~~a~~ Kirby-Bauer disk diffusion technique was used. Isolates were suspended and inoculated onto MHA, and then β-lactam ring antibiotic disks were placed on the cultured disks. These plates contained 10 µg each of ampicillin (AMP), imipenem (IPM), meropenem (MEM), tetracycline (TE), gentamicin (CN), or cefpodoxime (CPD) or 30 µg each of amikacin (AK), CTX, ceftriaxone (CRO), CAZ, or aztreonam (ATM) and were incubated at 37°C thereafter. The inhibited zone was measured by using sliding calipers and interpreted via

Commented [DEDP11]: Please change title to reflect the specific organisms targeted

Commented [DEDP12]: How were these isolates purified?

Commented [DEDP13]: Add details of reference

Commented [DEDP14]: Add original reference for Kirby-Bauer test.

comparison with the Kirby-Bauer inhibited zone chart, as recommended by CLSI guidelines. *K. pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 were used as positive and negative control strains, respectively, to monitor the quality of susceptibility testing and ESBL detection methods. Multidrug resistance (MDR) is defined as a resistance to at least one agent in ≥3 antimicrobial classes (12, 13).

Genotypic c~~C~~haracterization of ~~genotypic~~ ESBL-producing isolates

Standard polymerase chain reaction (PCR) was performed to screen for the presence of seven ESBL genes: blaTEM, blaSHV, blaCTX-M1, blaCTX-M9, blaGES, blaVEB, and blaPER using specific primers described in Table 1. PCR reactions contained 1× buffer, 1.5 mM of MgCl2, 400 µM of dNTPs, 0.2 µM of forward and reverse primers each, and 1 U Taq polymerase and the concentration of DNA template, depending on specific primers. The PCR cycling conditions were dependent on their specific primers of the targeted gene as tabulated in Table 1. After PCR processing, PCR products were analyzed by agarose gel electrophoresis.

DNA extraction

DNA extraction was performed via AccuPrep Genomic DNA Extraction Kit (Bioneer, South Korea). Briefly, the bacterial suspension was centrifuged at 1,200 rpm, and the supernatant was removed. The pellet was washed with phosphate-buffered saline. After centrifugation, the supernatant was removed, and 20 µL of proteinase K was added. Tris-EDTA (TE) buffer was added to the DNA, and the final solution was adjusted for direct use as a PCR template.

Enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) and DNA amplification

Primers of 5'-ATG TAA GCT CCT GGG GAT TCA C-3' (F) and 5'-AAG TAA GTG ACT GGG GTG AGC G-3' (R) were used~~designed~~ for ERIC-PCR amplification (14-16). The total volume was 25 µL for each reaction, including 2 µL of template DNA (*E. coli* or *K. pneumoniae*), 5.2 µL of mastermix, 0.25 µL of forward and reverse primers (100 µM), and 17.3 µL of deionized water. The thermocycler was then run for 30 cycles of denaturation (95°C), annealing (48°C), and extension (72°C) steps for 60 s each. Deionized water and bacterial DNA of *E. coli* and *K. pneumoniae* strains) were then used as negative and positive controls, respectively.

Polymerase chain reaction products and gel electrophoresis

The ERIC-PCR products were electrophorized on a 1.5% agarose gel in loading buffer. A 100-base pair (bp)-DNA marker (Fermentas) was used as a standard measuring means. The gel bands were observed by UV light.

Dendrogram and phylogenetic relationships

Commented [DEDP15]: Add reference

Commented [DEDP16]: Genes should be written in italics

Commented [DEDP17]: Were these primers for Group 9? Please look at the following article which clarifies the groups and variants of genes very nicely: Njage, P.M.K., Buys, E.M. (2014). Pathogenic and commensal Escherichia coli from irrigation water 512 show potential in transmission of extended spectrum and AmpC β -lactamases determinants to 513 isolates from lettuce. Microbial Biotechnology, 8. 462–473. doi: 10.1111/1751-7915.12234.

Commented [DEDP18]: References of the primers

Commented [DEDP19]: How was the DNA concentration determined? What was the range of DNA concentrations used for PCR analysis. Also how was the DNA template concentration dependant on the primers used?

Commented [DEDP20]: Add reference for the primers used to amplify the genes.

Commented [DEDP21]: Which methodology was used for confirming the sequence identities of the amplicons obtained?

Commented [DEDP22]: What was the concentration of the DNA used?

The DNA band pattern from the *E. coli* and *K. pneumoniae* samples from gel electrophoresis regarding ERIC-PCR products was used as a generational structure for dendrogram analysis using the GelClust free software. For constructing a computerized dendrogram, the presence and absence of bands were presumed as 1 and 0, respectively. The dendrogram was then designed using the unweighted pair-group method with arithmetic mean, which is categorized in clustering methodologies and is based on clustering analysis.

Results

Prevalence of ESBL-producing *E. coli* and *K. pneumoniae* bacteria

ESBL-producing *E. coli* and *K. pneumoniae* isolates were found in 14 of the 305 samples obtained from seven out of ten types of vegetables (4.6% of the total) (Table 2). The highest frequencies of both ESBL-producing bacterial species were found in Thai yardlong beans (3/34, 8.8%) and Thai basil (3/36, 8.3%), followed by winged beans (2/25, 8%), tomato (2/31, 6.5%), and cucumber (2/36, 5.6%), with minimal detection in long coriander (1/32, 3.1%) and Thai eggplant (1/44, 2.3%). ~~Meanwhile,~~ ESBL producers were not detected in young cashew leaves, cabbage, or lettuce (Table 2 and 3).

ESBL-producing *K. pneumoniae* isolates were more frequently found than *E. coli* strains at 9/14 (64.29%) and 5/14 (35.711%), respectively. Nine *K. pneumoniae* isolates that produced ESBL were variously distributed in several vegetables in this study. Only one out of nine *K. pneumoniae* isolates (11.11%) was obtained from each of ~~represented in~~ five types of vegetables, i.e., Thai eggplant, winged beans, Thai basil, cucumber, and long coriander, and four of the nine isolates (22%) were found in Thai yardlong beans and tomato (two each). Five ESBL-producing *E. coli* isolates were found in four types of vegetables. Two isolates were most frequent in Thai basil (40%), and one isolate (20%) was detected in each vegetable including Thai yardlong beans, winged beans, and cucumber (Table 2 and 3).

Interestingly, both ESBL-producing *E. coli* and *K. pneumoniae* isolates were present among four specific vegetables, namely, Thai yardlong beans, Thai basil, winged beans, and cucumber (Table 2). There was an equal proportion (1:1) of ESBL-producing *E. coli* and *K. pneumoniae* isolates in both winged beans and cucumber. Moreover, ESBL-producing *E. coli* and *K. pneumoniae* were frequently found in Thai basil and Thai yardlong beans. More ESBL-producing *E. coli* isolates were detected in Thai basil than in Thai yardlong beans (2:1), which was in contrast to the proportion found in these vegetables (1:2) with another ESBL-producing *K. pneumoniae* (Table 2).

Indeed, both the ESBL-producing *E. coli* and *K. pneumoniae* were widely distributed in seven out of ten edible vegetables and were more frequent in Thai yardlong beans and Thai basil samples than in other vegetables. Individually, the most frequent ESBL-producing *E. coli* was found in Thai basil, whereas the most frequent ESBL-producing *K. pneumoniae* was found in Thai yardlong beans and tomato.

Antibacterial susceptibility phenotype

Commented [DEDP23]: Which version was used?

Commented [DEDP24]: Sentence construction changed to avoid confusion. At first looked as if the same isolate was obtained from each of the vegetables tested.

Commented [DEDP25]: If I look at Table 3, E. coli was not as prevalent in Mueang and it is perhaps worth mentioning

Eleven antibiotic agents of five classes (both of β-lactam and non-β-lactam ~~ring~~) were used to assess the antimicrobial susceptibility of ESBL-producing *E. coli* and *K. pneumoniae* isolates from ten common edible vegetables in Southern Thailand by disk diffusion method. All 14 isolates (100%) were strongly sensitive to IPM, followed by AK/CN, MEM/TE, ATM, CRO, CPD, CTX/CAZ, and AMP (Table 4).

All ESBL-producing *E. coli* isolates were sensitive to not only IPM but also CN with 100% susceptibility (5/5). The frequency of sensitivity in ESBL-producing *E. coli* isolates decreased in AK, TE/ATM, and AMP/MEM/CRO/CPD by 80% (4/5), 60% (3/5), and 40% (2/5), respectively. Furthermore, only one isolate (20%) exhibited intermediate activity against AK and CPD (Table 4). ESBL-producing *K. pneumoniae* isolates were also determined to be all susceptible to carbapenem IPM and MEM antibiotics (100%, 9/9). These strains were also highly sensitive to TE/AK (88.89%, 8/9), CN/ATM (77.78%, 7/9), CRO (66.67%, 6/9), and CTX/CAZ/CPD (55.56%, 5/9), respectively. Intermediate sensitivity was showed with AK and CRO (1/9 isolate of each) against K. pneumoniae, representing 11.11% of the total sample (Table 4).

The presence of IPM and AK sensitivity against ESBL-producing *E. coli* and *K. pneumoniae* is promising; however, these strains were also resistant to several antibiotics (Tables 4 and 5). In total (n = 14), both ESBL producers (12) were found to be resistant to AMP (85.71%), followed by CTX/CAZ (9, 64.29%), CPD (6, 42.86%), CRO (5, 35.71%), ATM (4, 28.57%), MEM/TE (3, 21.43%), and CN (2, 14.29%).

The antibiotic-resistant profile of ESBL-producing *E. coli* (n = 5) was established as 100% of CTX/CAZ (5/5), 60% of AMP/MEM/CRO (3/5), and 40% of TE/CPD/ATM (2/5). All isolates of ESBL-producing *K. pneumoniae* (n = 9) were completely resistant to AMP (100%), followed by CTX/CAZ/CPO (4, 44.44%), CN/CRO/ATM (2, 22.22%), and TE (1, 11.11%) (Table 4).

Multidrug resistance patterns

MDR is defined as bacteria's resistance to antibiotics or to at least one agent in three or more antimicrobial classes (12). Here, seven out of 14 (50%) of *E. coli* and *K. pneumoniae* isolates were MDR. There were three *E. coli* MDR isolates (60%) and four *K. pneumoniae* MDR isolates (44.44%) (Tables 5, 6). Interestingly, the *E. coli* isolate (A60301) obtained from Thai yardlong beans at a market in Mueang exhibited more resistance (63.64%) than sensitivity (36.36%). ~~The~~ MDR analysis showed that ~~patterns of~~ this isolate was~~ere~~ resistant to ~~present in~~ four classes of antibiotics and were resistant to seven out of 11 antibiotics in total. Another isolate (E50501) of ESBL-producing *E. coli* from Thai basil in Tha Sala also had an MDR profile including~~n~~ four antibiotic classes and was resistant to five out of 11 antibiotics. However, this isolate had slightly more antibiotic sensitivity (54.55%) than resistance (45.45%). The proportional sensitivity was similar to that in MDR isolate (C30501). An MDR *E. coli* isolate derived from an MDR *K. pneumoniae* (A80301) was also isolated from Thai yardlong beans in Mueang district and was resistant to four classes of antibiotics (6/11) and exhibited greater resistance (54.55%) than sensitivity (45.45%). Although the *K. pneumoniae* isolates (B90101, C70101, and G70301) were

Commented [DEDP26]: It is sufficient to refer to non- β-lactam

Commented [DEDP27]: Resistance to which antibiotics

obtained from several vegetables (Thai eggplant, winged bean, and cucumber, respectively) in different Mueang markets, all exhibited MDR to three classes of antibiotics (3/11), though these isolates were more susceptible (72.73%) to eight other antibiotics in comparison with their resistance (27.27%). Consequently, these MDR patterns were more frequently found in *E. coli* than in *K. pneumoniae* isolates and were more frequently detected in samples from Mueang markets, which had the highest proportion of contamination in Thai yardlong beans (Table 6). Several of the above isolates showed MDR activity, but their sensitivities to other antibiotics ensure these are still available for infection treatment.

We then characterized seven ESBL-encoding genes (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX\text{-}M1}$, *bla*$_{CTX\text{-}M9}$, *bla*$_{GES}$, *blaVEB,* and *bla*$_{PER}$) by PCR and confirmed the identity of these genes by DNA sequencing. This demonstrated that the *bla*$_{SHV}$-ESBL gene (57.14%) (8/14) was the most prevalent ESBL-encoding gene in the *E. coli* and *K. pneumoniae* (Table 7), with four out of 14 (28.57%) isolates from each species. Notably, there was no detection of other ESBL-coding genes in *K. pneumoniae* isolates. Furthermore, there was limited detection of MDR genes in the *E. coli* isolates, with two (40%) and one (20%) being positive for *bla*$_{TEM}$ and *bla*$_{CTX\text{-}M1}$/*bla*$_{CTX\text{-}M9}$/*bla*$_{GES}$ genes, respectively. There was no detection of *bla*$_{VEB}$ or *bla*$_{PER}$ genes in any of the *E. coli* or *K. pneumoniae* isolates.

Multiple ESBL-encoding genes were found in the *E. coli* isolates (2/5). A60301 and C30501 were isolated from Thai yardlong beans and winged beans from Mueang and Tha Sala markets, respectively, including two sets of three ESBL-coding genes: *bla*$_{TEM}$/*bla*$_{CTX\text{-}M1}$/*bla*$_{GES}$ and *bla*$_{TEM}$/*bla*$_{SHV}$/*bla*$_{CTX\text{-}M9}$. Each ESBL genes amplified from *E. coli* isolates was detected by agarose gel electrophoresis (Figure 1).

Dendrogram relationship of isolated strains of *E. coli* and K. pneumoniae

Phylogenetic trees of five *E. coli* isolates were generated by GelClust (Figure 2A). This demonstrated that the A60301 isolate was more closely related to C30501 than to other *E. coli* isolates, with an identity of approximately 73%. The G30501 isolate is probably another strain that is related to the E20301 and E50501 isolates (with 77% identity). The E20301 and E50501 strains were more closely related to each other than to other isolates (~84% identity). The phylogenetics of the five *E. coli* isolates could thus be used~~implied~~ for individual strain identification. Each isolate of *E. coli* obtained from the same vegetable or market was ~~of~~ different strain, but as E20301 and E50501 came from the same vegetables, they were likely to be closely related.

We also created a phylogenetic tree for all isolated *K. pneumoniae* strains, which had a wider range of genetic heterogeneities than was present in the *E. coli* isolates (Figure 2B). The cluster analysis and related dendrogram showed that five molecular genetic clusters were represented individually in each strain of *K. pneumoniae*. The H80301 strain was similar to I70201 (85% identity), although these were the least related to any of the other stains (Figure 2B). G70301 was related with 67% identity to A80301/E20502 and had 55% identity with H30301. The A80301 and E20502 isolates were shown to be of the same strain. Moreover, H30301 showed 60%

Commented [DEDP28]: italics

identity with C70101. The final clusters delineated for the relationship of C70101 and A70201/B90101 isolates had 58% identity. A70201 and B90101 were of different strains with 67% identity. The *K. pneumoniae* strains showed a high diversity from all types of vegetables and markets, with each isolate coming from an independent source.

Discussion

Previously, many studies have reported the prevalence of ESBL-producing Enterobacteriaceae bacteria; in particular, *E. coli* and *K. pneumoniae* have been found in human and animal foods and in food-producing animals (3, 13, 17-25). The isolation of both ESBL-producing species from vegetable samples has also been described in many studies (3, 6, 9, 10, 21, 25-28). Although the prevalence and role of ESBL producers in vegetables, such as cabbages, cucumbers, tomatoes, corianders, and lettuce that are cultivated ubiquitously worldwide, have been examined, there have been few studies on vegetables (yardlong beans, winged beans, basil, eggplant, and young cashew leaves) specifically found in either Thailand or tropical countries and that are frequently consumed in Southern Thailand. ~~In this study, we have determined that the highest prevalence of isolates of ESBL-producing *E. coli* and *K. pneumoniae* producers is in Thai yardlong beans and Thai basil samples, representing 8.3%–8.8% of all samples.~~ This is the first report describing the number of ESBL producers that have been found contaminating Thai basil. In this study, we have determined that the highest prevalence of isolates of ESBL-producing *E. coli* and *K. pneumoniae* producers is in Thai yardlong beans and Thai basil samples, representing 8.3%–8.8% of all samples. Nevertheless, the prevalence of ESBL-producing *E. coli* and *K. pneumoniae* was lower when compared with that of other studies (different types and sample sizes of vegetables), with frequencies of 10.1%, 13.3%, 16.4%, and 43.3% (6, 10, 21, 27). Therefore, this may imply that the variation in the prevalence of ESBL-producing strains depends on the sample size collection and types of vegetables tested.

We have determined that the *E. coli* and *K. pneumoniae* strains isolated here contained a range of antibiotic resistance. These isolates were resistant to broad-spectrum AMP (85.71%) and third-generation cephalosporin antibiotics, including CTX, CRO, CAZ, and CPD (40%–70%). Our results are in accordance with a previous review by Falagas and Karageorgopoulos that showed that *E. coli* and *K. pneumoniae* bacteria were high producers of ESBL enzyme, which could hydrolyze many common antibiotics, i.e., penicillin (the same antibiotic class with AMP), cephalosporins, and ATM (carbapenem), leading to the antibiotic resistance in these bacteria (2). Furthermore, Zurfluh et al. reported that 26 isolates of ESBL-producing *E. coli* and *K. pneumoniae* derived from imported vegetables in Dominican Republic, India, Thailand, and Vietnam were resistant to AMP (100%) and to narrow-spectrum cephalosporins: cephalothin (INN) and CTX (88.3%) (27). Kim et al. determined the antibiotic susceptibility of ESBL-producing *E. coli* and K. pneumoniae, isolated from sprout samples in South Korea, and showed these were resistant to CTX (100%)(6). Another study showed that all of ESBL-producing *E. coli* isolated from raw vegetables were completely resistant to AMP, CTX, and other antibiotics (piperacillin, cefazoline, and nalidixic acid (28). *E. coli* isolated from fresh vegetables showed

Commented [DEDP29]: More suited to the introduction

Commented [DEDP30]: What was the percentages in the study by Falagas and Karageorgopoulos. Were these isolates from vegetables, if so which types?

Commented [DEDP31]: italics

cephalosporin resistance, such as to CTX and CAZ (92%) (21). In our study, the most frequent resistance was to cephalosporins, such as CTX and CAZ (100%) and AMP (60%) in *E. coli* only, which was not only consistent with the above reports for isolates from raw vegetables but also for isolates from clinical specimens (60%–62% to cephalosporins) and healthy subjects (72% to AMP) in low- and middle-income countries (1, 5, 21, 28). The resistant patterns from sources of *E. coli* may be associated with each other because of transmission of resistant genes between human and environments. Moreover, in this study, MDR patterns in *E. coli* (21.43%) as well as *K. pneumoniae* (28.57%) were present in 50% of ESBL-producing isolates that shared resistance of three to four antibiotic classes in β-lactam antibiotics (aminopenicillin, cephalosporin, and carbapenem) and non-β-lactam antibiotics (aminoglycosides and TE). Our results correlated with previously described MDR strains, particularly against β-lactam CTX and non-β-lactam CHL and TE found in iceberg lettuce (26). An MDR *E. coli* strain that was resistant to β-lactam (AMP, INN, and CTX) and non-β-lactam (trimethoprim and TE) antibiotics was isolated from imported Thai yardlong beans to Switzerland (27). Two *E. coli* strains isolated from sprouts were MDR to CTX, CAZ, cefepime, ATM, ciprofloxacin (CIP), and trimethoprim/sulfamethoxazole (6). We have also found strong resistance to multiple antibiotic classes in *E. coli*, highlighting concerns of MDR. Although the presence of MDR in *K. pneumoniae* isolates was higher than that in *E. coli* isolates, other antibiotic sensitivities remained high (except for AMP). Conversely, Bhutani et al. (2015) reported major resistance to CTX (86%) by two *K. pneumoniae* isolates from iceberg lettuce (26).

The *K. pneumoniae* strains isolated here commonly carried the blaSHV gene, which was also present in several *E. coli* strains. However, *E. coli* isolates from selected vegetables seem be strong producers of ESBL enzymes via the co-existence of several ESBL-encoding genes. The *E. coli* A60301 and C30501 strains isolated from yardlong beans and winged beans carried the blaTEM, blaCTX-M1, and blaGES and the blaTEM, blaSHV, and blaCTX-M9 genes, respectively. Their co-expression may result in stronger ESBL production and increased antibiotic resistance or MDR. Consequently, strong ESBL-producing *E. coli* strains may be harbored in Thai yardlong beans and wing beans.

Not surprisingly, the blaSHV genes were frequently found both in *E. coli* and *K. pneumoniae* because this gene is a common genetic type found in the ESBL-producing Enterobacteriaceae family (4, 27). blaSHV and blaTEM were recognized as being ESBL and were initially reported from clinical isolation as were the blaVEB, blaPER, and blaGES genes, although five types of blaCTX-M were originally mainly found in environmental bacteria (Kluyvera spp.) (4, 27). The relevant genes are thought to have been mobilized into conjugative plasmids and thus transferred to pathogenic bacteria (27). Community-onset ESBL-associated infections, including urinary and bloodstream system, are principally caused by *E. coli* producing blaCTX-M type ESBLs (27). Therefore, the relevant genes might contribute to the presence of ESBLs in the community transmitted by animal sources to humans via food chains or patient-to-patient transmission (27). Notably, in this study, the A60301 and C30501 *E. coli* isolates that co-harboured~~expressed~~ those relevant ESBL gene types may be associated as antibiotic-resistant predictors that can pass

Commented [DEDP32]: AS the level of enzyme production was not measured one cannot make thi sclaim

Commented [DEDP33]: This could be mentioned as future research needed, but cannot be deducted from the results .

between food chains (vegetable) and human transmission in community if consumers lack good hygiene.

Carbapenems are widely regarded as the antibiotics of choice for the treatment of severe infections caused by ESBL-producing Enterobacteriaceae (4). In our study, the antibiotic susceptibility of ESBL-producing *E. coli* and *K. pneumoniae* isolates was completely resistant to carbapenem (IPM) and mostly resistant (85.71%) to aminoglycosides such as AK and CN. Carbapenem sensitivities similar to these have also been noted in other studies (83% and 99.3%–100%) (21, 29).

The prevalence of *E. coli* and *K. pneumoniae* isolates was demonstrated by the range of different diversities of strains determined using phylogenetic tree analysis. ESBL-producing bacteria may, therefore, be involved in terms of its spread to community, causing infectious diseases.

One of the major limitations of the study is that the collection of detailed data on places of the harvest was not possible. Moreover, we included only two districts, both facts might have its limitation in collecting a true representative sample for Southern of Thailand. Nevertheless, the collection of a truly random sample by one person and investigating all samples in one laboratory might have reduced the variability observed.

Commented [DEDP34]: In the abstract it said the isolates were100% sensitive/inhibited by imipenem.

Conclusions

As per our findings, it was determined that retail vegetables at local markets in Nakhon Si Thammarat may be reservoirs for the spread of ESBL-producing *E. coli* and *K. pneumoniae*. This may lead to antibiotic resistance through food chain transmission if consumer hygiene is poor. Future studies should investigate the presence of ESBL-producing *E. coli* and *K. pneumoniae* in fresh vegetables and to explore whether the exchange of resistance genes between these ESBL-producing *E. coli* and *K. pneumoniae* and other enterobacterial species in the human gut does indeed occur.

Acknowledgments

The authors thank the Research Institute for Health Sciences Walailak University, School of Allied Health Sciences, Walailak University, and Department of Biomedical Sciences, Faculty of Medicine, Prince of Songkla University for providing the required laboratory instruments.

References

1. Nji E, Kazibwe J, Hambridge T, Joko CA, Larbi AA, Damptey LAO, et al. High prevalence of antibiotic resistance in commensal Escherichia coli from healthy human sources in community settings. Sci Rep. 2021;11(1):3372.

2. Falagas ME, Karageorgopoulos DE. Extended-spectrum beta-lactamase-producing organisms. J Hosp Infect. 2009;73(4):345-54.

3. Boonyasiri A, Tangkoskul T, Seenama C, Saiyarin J, Tiengrim S, Thamlikitkul V. Prevalence of antibiotic resistant bacteria in healthy adults, foods, food animals, and the environment in selected areas in Thailand. Pathog Glob Health. 2014;108(5):235-45.
4. Pitout JD, Laupland KB. Extended-spectrum beta-lactamase-producing Enterobacteriaceae: an emerging public-health concern. Lancet Infect Dis. 2008;8(3):159-66.
5. Teklu DS, Negeri AA, Legese MH, Bedada TL, Woldemariam HK, Tullu KD. Extended-spectrum beta-lactamase production and multi-drug resistance among Enterobacteriaceae isolated in Addis Ababa, Ethiopia. Antimicrobial Resistance & Infection Control. 2019;8(1):39.
6. Kim HS, Chon JW, Kim YJ, Kim DH, Kim MS, Seo KH. Prevalence and characterization of extended-spectrum-beta-lactamase-producing Escherichia coli and Klebsiella pneumoniae in ready-to-eat vegetables. Int J Food Microbiol. 2015;207:83-6.
7. Mritunjay SK, Kumar V. A study on prevalence of microbial contamination on the surface of raw salad vegetables. 3 Biotech. 2017;7(1):13.
8. Saksena R, Malik M, Gaind R. Bacterial contamination and prevalence of antimicrobial resistance phenotypes in raw fruits and vegetables sold in Delhi, India. J Food Saf. 2020;40(1):e12739.
9. Blaak H, van Hoek AH, Veenman C, Docters van Leeuwen AE, Lynch G, van Overbeek WM, et al. Extended spectrum ss-lactamase- and constitutively AmpC-producing Enterobacteriaceae on fresh produce and in the agricultural environment. Int J Food Microbiol. 2014;168:8-16.
10. Richter L, Du Plessis EM, Duvenage S, Korsten L. Occurrence, Identification, and Antimicrobial Resistance Profiles of Extended-Spectrum and AmpC β-Lactamase-Producing Enterobacteriaceae from Fresh Vegetables Retailed in Gauteng Province, South Africa. Foodborne Pathog Dis. 2019;16(6):421-7.
11. Bubpamala J, Khuntayaporn P, Thirapanmethee K, Montakantikul P, Santanirand P, Chomnawang MT. Phenotypic and genotypic characterizations of extended-spectrum beta-lactamase-producing Escherichia coli in Thailand. Infect Drug Resist. 2018;11:2151-7.
12. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect. 2012;18(3):268-81.
13. Abayneh M, Tesfaw G, Woldemichael K, Yohannis M, Abdissa A. Assessment of extended-spectrum beta-lactamase (ESBLs) - producing *Escherichia coli* from minced meat of cattle and swab samples and hygienic status of meat retailer shops in Jimma town, Southwest Ethiopia. BMC Infect Dis. 2019;19(1):897.
14. Versalovic J, Koeuth T, Lupski JR. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. 1991;19(24):6823-31.
15. Fox JT, Renter DG, Sanderson MW, Thomson DU, Lechtenberg KF, Nagaraja TG. Evaluation of Culture Methods To Identify Bovine Feces with High Concentrations of Escherichia coli O157. Appl Environ Microbiol. 2007;73(16):5253.

Formatted: Font: Italic

16. Ranjbar R, Tabatabaee A, Behzadi P, Kheiri R. Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR) Genotyping of Escherichia coli Strains Isolated from Different Animal Stool Specimens. Iran J Pathol. 2017;12(1):25-34.
17. Eibach D, Dekker D, Gyau Boahen K, Wiafe Akenten C, Sarpong N, Belmar Campos C, et al. Extended-spectrum beta-lactamase-producing Escherichia coli and Klebsiella pneumoniae in local and imported poultry meat in Ghana. Vet Microbiol. 2018;217:7-12.
18. Ryu SH, Lee JH, Park SH, Song MO, Park SH, Jung HW, et al. Antimicrobial resistance profiles among Escherichia coli strains isolated from commercial and cooked foods. Int J Food Microbiol. 2012;159(3):263-6.
19. Le QP, Ueda S, Nguyen TN, Dao TV, Van Hoang TA, Tran TT, et al. Characteristics of Extended-Spectrum beta-Lactamase-Producing Escherichia coli in Retail Meats and Shrimp at a Local Market in Vietnam. Foodborne Pathog Dis. 2015;12(8):719-25.
20. Montso KP, Dlamini SB, Kumar A, Ateba CN. Antimicrobial Resistance Factors of Extended-Spectrum Beta-Lactamases Producing Escherichia coli and Klebsiella pneumoniae Isolated from Cattle Farms and Raw Beef in North-West Province, South Africa. BioMed Res Int. 2019;2019:4318306.
21. Ye Q, Wu Q, Zhang S, Zhang J, Yang G, Wang J, et al. Characterization of Extended-Spectrum β-Lactamase-Producing Enterobacteriaceae From Retail Food in China. Front Microbiol. 2018;9:1709.
22. Tekiner sH, Özp1nar H. Occurrence and characteristics of extended spectrum beta-lactamases-producing Enterobacteriaceae from foods of animal origin. Brazilian J Microbiol. 2016;47:444-51.
23. Hiroi M, Yamazaki F, Harada T, Takahashi N, Iida N, Noda Y, et al. Prevalence of extended-spectrum beta-lactamase-producing Escherichia coli and Klebsiella pneumoniae in food-producing animals. J Vet Med Sci. 2012;74(2):189-95.
24. S R.
25. Kaesbohrer A, Bakran-Lebl K, Irrgang A, Fischer J, Kämpf P, Schiffmann A, et al. Diversity in prevalence and characteristics of ESBL/pAmpC producing E. coli in food in Germany. Vet Microbiol. 2019;233:52-60.
26. Bhutani N, Muraleedharan C, Talreja D, Rana SW, Walia S, Kumar A, et al. Occurrence of Multidrug Resistant Extended Spectrum Beta-Lactamase-Producing Bacteria on Iceberg Lettuce Retailed for Human Consumption. BioMed Res Int. 2015;2015:547547.
27. Zurfluh K, Nüesch-Inderbinen M, Morach M, Zihler Berner A, Hächler H, Stephan R. Extended-Spectrum-β-Lactamase-Producing Enterobacteriaceae Isolated from Vegetables Imported from the Dominican Republic, India, Thailand, and Vietnam. Appl Environ Microbiol. 2015;81(9):3115.
28. Song J, Oh S-S, Kim J, Shin J. Extended-spectrum β-lactamase-producing Escherichia coli isolated from raw vegetables in South Korea. Sci Rep. 2020;10(1):19721.

29. Devrim I, Gulfidan G, Gunay İ, Agın H, Güven B, Yılmazer MM, et al. Comparison of in vitro activity of ertapenem with other carbapenems against extended-spectrum beta-lactamase-producing Escherichia coli and Kleibsella species isolated in a tertiary children's hospital. Expert Opin Pharmacother. 2011;12(6):845-9.