Molecular Identification of Antibiotic Resistant ESBL, MBT and AMPC Producing Enterobacteriaceae in Vagetables

Çiğdem SÖKMEN[1], Haydar ÖZPINAR[1]

Abstract

Extended spectrum beta-lactamases (ESBLs), AmpC and metallo-beta-lactamases (MBLs) are extremely important mechanisms of antibiotic resistance, in particular, in Gram negative Enterobacteriaceae. These specific enzymes make any bacteria resistant to a wide range of beta-lactam antibiotics. The objective of this study was to investigate the occurrence of ESBL, AmpC and MBL-producing Gram negative *Enterobacteriaceae* in the fresh vegetables. A total of 108 samples of fresh vegetables sold in the public bazaars of Istanbul, Turkey was randomly collected in between July and October 2014. After pre-enrichment and inoculation on beta-lactamase selective media, presumptive isolates were characterized by Vitek® MS (bioMérieux). Phenotypic Screening and MIC determination of β-lactamases were performed by disc-approximation testing and Micronaut-S beta-lactamase VII kit (Merlin) and Software (Sifin) according to the CLSI Guidelines. The results revealed that 25,55% of 69 isolates were contaminated with ESBL-producing *Enterobacteriaceae* and the most prevalent ESBL phenotypes were found as *Klebsiella pneumoniae* (n=10; 55,5%), *E. coli* (n=7; 39%), *C. freundii* (n=1; 6%) respectively. Finally, according to the results of the antibiogram verification; strain of *Stenotrophomonas maltophilia* (n = 1) and strains of *Acinetobacter baumannii complex* (n = 3) were determined to produce metallo-beta-lactamase. The simultaneous production of both ESBL and AmpC was determined in 1 Eschericia coli. This study presented that the fresh vegetables harbored ESBL, AmpC and MBL-producing Gram negative Enterobacteriaceae, leading to a health risk for the consumers. The vegetables may, therefore, play a significant role for the food-related spread of resistant bacteria and their beta-lactamase coding genetic materials. Further molecular studies should be extended to understand the foodborne epidemiology of this emerging biohazard for the public health.

Keywords: *Enterobacteriaceae, ESBL, MBL, AmpC, Vegetable*

[1] *Food Safety and Nutrition Department of Istanbul Aydin University, Istanbul/Turkey, e-mail: haydarozpinar@aydin.edu.tr*

1.Introduction

These drugs-related developments have increased since the initiation of the use of antibiotics and the probable problems that might arise because of this, have began to be the matters in question even though the resistance arose against using these substances initially. Bacterial strains show resistance by releasing extended spectrum beta lactamase (ESBL) enzyme as a result of mutations arose with exposure to extended spectrum of beta lactam antibiotics(45).The bacteria that also have ability to produce ESBL, can be able to create multi resistance against antimicrobial agents, with chromosomal or plasmid mediated mechanisms (44).

Vegetables are contaminated with soil microorganisms so much since they are grown under and surface of the ground (14). The fecal origined enterobactericeae types,such as *Citrobacter spp, Enterobacter ssp., Escherichia coli,* and *Klebsiella spp.*,might mostly exist in water ,soil and plants (2, 36, 22, 30).Even tough these strains exist in soil as commensal harmlessly like some types of *E.coli*,the other ones exist in humans and in animals as pathogenic. Since *Enterobactericeae* types spread in a wide range, it is inevitable for them to contaminate food chain as well (12). It was reported that the *Enterobactericeae* types, which are contaminated by foods, generate ESBL, which developed resistance against antibiotics (4, 8, 11, 15, 16, 17, 28).

In addition to the fact that antimicrobial agents can be transmitted to fruit and vegetables in many ways, it can be said that the most significant transmission has been realized by mixing antibiotics used in stockbreeding for the purpose of treatment or growth, in soil by fertilizer or by mixing them directly into the soil. The bacteria existing at fruits and vegetables can gain resistance against antibiotics in case antibiotics mix into soil, and they can live in soil for many years (13, 29).

A few number of studies have been available in the world regarding antibiotic resistance of vegetables. It was seen when the studies performed in Europe reviewed that *Enterobacter* types isolated from vegetables and fruits carry beta-lactam group antibiotics-resistant genes (25, 24, 3). Over 2,800 people have died in Europe every year due to the infections occured with this type of bacteria and since their treatments take along time, it has been estimated that extra care and treatment expenses were nearly 18 million Euro (46, 47).

Through this study, it was aimed the determination of resistance that would be created in the bacteria belonging to the *Enterobacteriaceae* family, which existed in the vegetables supplied from the district bazaars in İstanbul and the surrounding area, through beta-lactam group of antibiotics (Cefotaxime, Ceftazidim, Cefpodoxim) ,which have been used abroad and in our country.

2. MATERIAL and METHOD

2.1. Material

A total of 108 pieces of sample including 8 pieces of vine leaves, 8 pieces of dills, 9 pieces of spinach, 8 pieces of lettuce, 8 pieces of black cabbage, 7 pieces of white cabbage, 8 pieces of mushroom, 8 pieces of parsley, 8 pieces of mint,11 pieces of leaf beet, 8 pieces of leek, 8 pieces of rucola, 8 pieces of purslane supplied from the district bazaars in İstanbul and the surrounding area between the dates of July-November 2014 and they were put in the sterile samples carrying containers and taken quickly to the laboratory in the same day.

2.2. Method

Pre enrichment

A total of 25 grams of sample from sample materials brought for analysis in sample carrying box was weighed on the precision scales (AND GF-6100, Japan) by core method and inserted into pochettes through E.E buyyon (E.E. Broth; LABM LAB091, Lancashire, United Kingdom)sterilized tape measure, which was prepared in accordance with instruction manual for 225 ml and cooled after the sterilization process. It was incubated during 18 to 24 hours at 37 °C after being homogenized for 2 min. in the homogenizator (EasyMix, AES Chemunex, BruzFrance).

Inoculation to selective medium

Plantation was performed by spreading method of ESBL chromogen ready agar (ChromoagarTM ESBL, Paris, France), which has reached to the room temperature and operationalized with the help of 10µ sterilized loop taken from enrichment liquid. It was incubated for 18 hours at 37 °C and resulting green colored colonies with 1 to 2 mm in diameter implied to suspect ESBL positive *Klebsiella pneumoniae* and resulting pink red colonies implied to *E. coli*. Plantation was performed again with the intent of full purification by spreading method of ESBL chromogen agar taken from colonies, considered to be more purified than each petri, by special sterilized loop and It was incubated again for 18 hours at 37 °C. It was incubated once more for 18 to 48 hours at 37 °C by being passed from purified colonies to TSA(LABM /UK) ready medium by sterilized loop. Then oxidase test (Bactident Oxidase test kit) was applied to the isolates and the isolates with oxidase negative results were kept in refrigerator conditions by the time their antibiotic susceptibility and identification tests were performed.

Disk Diffusion and Disk Diffusion Confirmation Test

The single colonies created in ESBL medium were dispersed in sterile salt water solution (0.85% NaCl2). This prepared suspension was set using (108 kob/ml) densitometer (BD Phoenix Phoenix spec, USA) in accordance with standard of 0,5 McFarland (bioMerrieux, Marcy l'Etoile, France). The sample was spread on Mueller Hinton Agar (Merck-1.05437) using a cotton swab to meet 0,5 McFarland (BBL McFarland Turbidity Standard) and was kept for 3 minutes. Following this process, ready-to-use discs (Mast Group ESBL Kit CPD10, United Kingdom) including Cefpodoxime,

Cefotaxime and Ceftazidime (+/-Clavulanik acid) were placed with sterile forsep. During this process, the discs were positioned carefully complying with the instructions of CLSI (2013) in such a way that the zone regions that might occur will not overlap to each other. In order to prevent the created zones from overlapping, it was paid attention to keep 25 mm distance between the disk centers and at least 15 mm from the edge of the petri. By being reversed, the plaques were incubated for 24 h at 37 ° and the diameters of their zones occured after this process were recorded after being measured. The discs incubated for 12 h again and after completion of the incubation, the differences that occured between the zones with clavulanic acid and those without clavulanic acid were compared. After then, they were reviewed in terms of the presence of ESBL according to the instructions of kit.

Typing through VITEK®MS

Bacteria typing process was performed through mass spectrophotometer (Vitek ® MS, bioMerieux, France) ,using the colonies occured in TSA medium. The spreading operations were applied to the slide of the device and after then it was placed to the device and identification of the bacteria was performed.

Antibiogram Affirmation

Through disc difusion test, the antibiogram affirmation and MIC value detection with respect to the isolates yielding positive results were performed. This operation was carried out by following Micronaut-S beta-lactamase VII Paneli (Merlin Diagnostika, Germany) instruction, which can detect extended spectrum beta-lactamases (ESBL), cephalosporins (AmpC) which can inactivate aminopenisilin, *K. pneumoniae* cephalosporins (KPC), and type D carbapenamas (Ertapenem and OXA-48 beta-laktamas). MultiScan spectrometer device (Thermoscientific, Finland) was used for reading operation and the findings were automatically analysed by MCN6 software (Sifin, Germany).

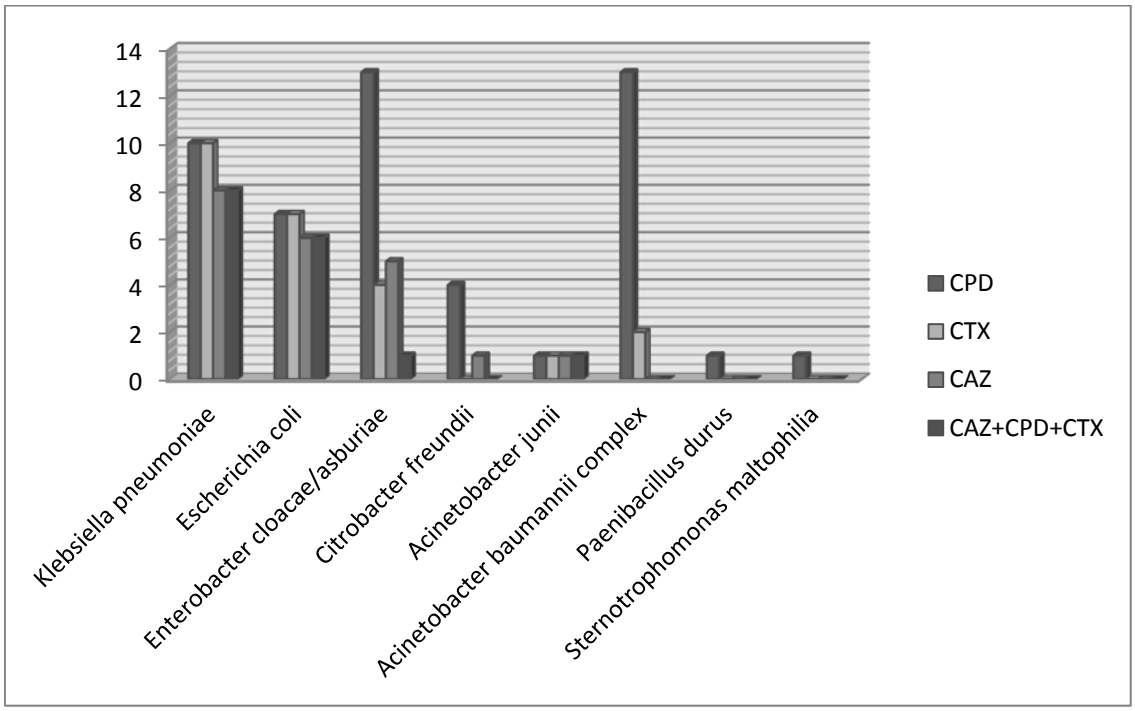
The Microbial suspension with 50 µl 0.5 McFarland standard was processed of vortice after being pipetted in Mueller Hinton Buyyon (Merck, Germany),which was prepared as 11 ml. After then, 100 µl was taken from this prepared suspension and added in each eye of the plates, where antibacterial substances had already kept as hydrated and vacuum dryed then it was incubated for 18 hours at 37º C. The reading operation was carried out with Thermofischer Multiskan FC spectrometer. The analysis of MIC data was automatically performed with MCN6 software (Sifin, Germany).

3. Results

In this study, total of 70 pieces bacteria isolated from a total of 13 kinds of vegetables including 108 pieces and the resistance of *Enterobactericeae* type bacteria against extended spectrum β-lactam group antibiotics was observed through phenotypic methods. In the subsequent oxidase test, oxidase negative results was obtained in 69 colonies.

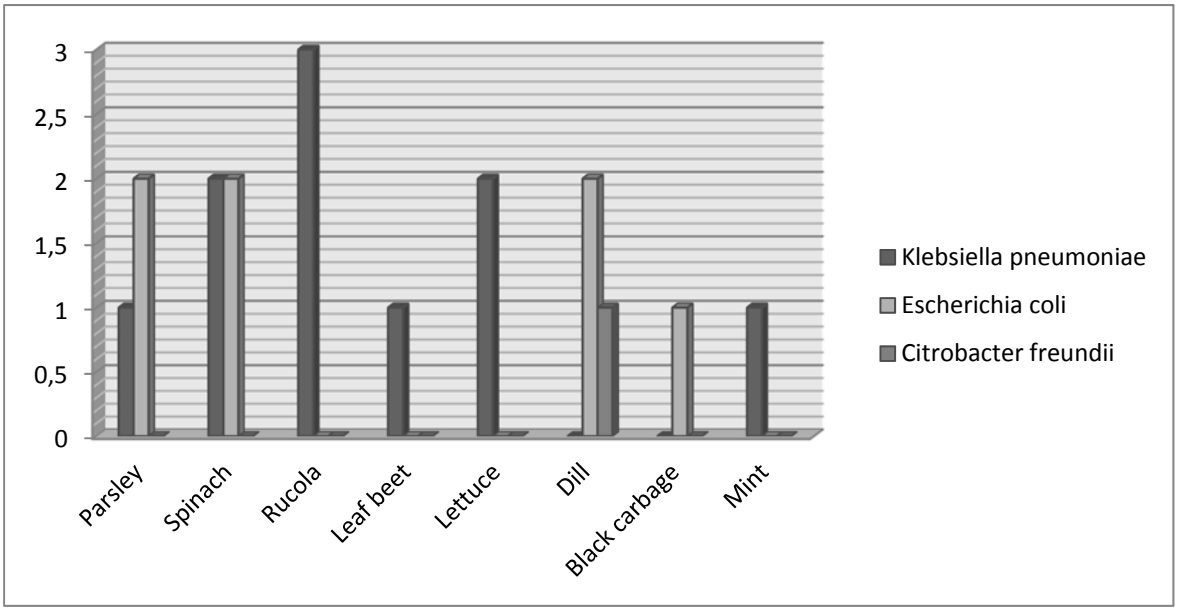
The izolated bacteria were determined as follows: *Enterobacter cloacae/asburiae*, *Acinetobacter baumanni complex*, *Klebsiella pneumoniae*, *Escherichia coli*, *Citrobacter freundii*, *Acinetobacter junii*, *Aeromonas hydrophila/caviae*, *Enterobacter aerogenes*, *Paenibacillus durus*, *Stenotrophomonas maltophilia*, *Vibrio metschnikovii*. According to the results of antibiotic disc diffusion, which was applied to 69 pieces of isolates, it was determined that 21 pieces of isolates were ceftazidime (CAZ) resistant, 24 pieces of isolates were cefotaxime (CTX) resistant, 50 pieces of isolates were cefpodoxime (CPD) resistant. It was also determined that a total of 16 isolates resisted simultaneously against three types of antibiotics (CAZ, CTX, CPD) (Chart 3.1).

Chart 3. 1 The resistance distributions of the isolated *Enterobacteriaceae* types with respect to different antibiotics



The available quantities in the vegetables, of the data determined as ESBL positive within all samples, are given at Chart 3.2.

Chart 3.2 ESBL positive types of *Enterobacteriaceae* isolated from vegetables



Besides all data, according to the results of the antibiogram affirmation process, two more different resistance excluding from ESBL were determined. These resistances are MBL and AmpC. The enzymes distributions that were created by bacteria are given in Table 3. 1.

Table 3.1. The distributions of ESBL, MBL, AmpC enzymes according to bacteria type in vegetables. (n=69)

Type of Microorganism	**GSBL**	**MBL**	**AmpC**
Klebsiella pneumoniae	10	-	-
Escherichia coli	7	-	1
Citrobacter freundii	1	-	-
Acinetobacter baumanni complex	-	3	-
Stenotrophomonas maltophilia	-	1	-
TOTAL	**18**	**4**	**1**

4. Discussion and Conclusion

Antibiotic resistance has become a significant problem growing increasingly all over the world and threatening public health by restricting the use of drugs used in the treatment of diseases. The World Health Organization (WHO) and the Food Agriculture Organization (FAO) have started many studies to raise awareness of public about this subject (64).

It was reported in the studies performed that *Enterobactericeae*, producing ESBL was detected in some animal products, such as

meat, milk, fish and chicken meat. On the other hand, there is not so much study with respect to vegetables. Whereas, the vegetables being produced in the fields carry more risk due to probable contamination arose from production areas and irrigation sources. For this reason, in this study, the vegetables was analysed in terms of ESBL.

Since the vegetables grow contacting directly with external environment (land, water), they can be contaminated by microorganisms and in addition, it was revealed in the studies that microorganism population, harmful for health, have increased in the vegetables, not watering with clean water(14). It was determined through the researches that *E. coli and Klebsiella spp*. strains were identified in food, and the same serotypes were identified in patients consuming these foods (7, 6).

The extended spectrum beta-lactamase strains released in *Enterobactericeae* strains constitutes a major problem in terms of public health. In a survey performed between the years of 2001 and 2002, in order to spesify the frequency of *Enterobacteriaceae* bacteremia creating ESBL in feces of polyclinics, it was revealed that the frequency of ESBL carriers increased from 2.1% to 7.5%(37). It was determined in another study that the frequency of *Enterobacteriaceae* bacteremia creating community originated ESBL was 4.1% (38). It was revealed in the studies that some of the factors playing role in spreading multi resistance caused by bacteria were faecal carriers, intestinal colonization, international travel and household transfer (39, 40).In addition to the ESBL analysis, apart from the disk diffusion tests and mikrodilision tests, which were carried out complying with internationally approved CLSI 2013 procedures, typing with mass spectrophotometer (Vitek ® MS) and antibiogram validation operations were performed in this study.

When the specially isolated pathogenic bacteria from vegetables was compared with world literatüre, it has been seen that they showed similarity with each other. The isolated bacteria have been determined as follows: *Enterobacter cloacae/asburiae*, *Acinetobacter baumanni complex*, *Klebsiella pneumoniae*, *Escherichia coli*, *Citrobacter freundii*, *Acinetobacter junii*, *Aeromonas hydrophila/caviae*, *Enterobacter aerogenes*, *Paenibacillus durus*, *Stenotrophomonas maltophilia*, *Vibrio metschnikovii*. The types that have been mostly seen in literature were *Enterobacter cloacae/asburiae, Klebsiella pneumoniae, Escherichia coli, Citrobacter freundii* (9, 10, 27, 34).

When the studies performed in Turkey and internationally rewieved, it was seen that while the resistance oriented studies performed on vegetables, in Turkey are almost any, on the other hand, according to the world literature, the existing resistance including its genes were determined through examining the resistance profiles of the microorganisms isolated from the analysed vegetables in the world. It has been revealed through the studies performed in Germany that the strains, such as *Enterobacter cloacae, Enterobacter gergoviae, Pantoea agglomerans,*

Pseudomonas aeruginosa, Pseudomonas putida, Escherichia coli ve *Enterococcus faecalis,* which were *isolated* from the raw vegetables carry the genes encoding β-lactam resistance and for this reason, they constitute a potential risk for consumers with the presence of β-laktam resistant bacteria (25,26). Since the tests of disc diffusion and antibiogram affirmation carried on 7 pieces of *E.coli* strains, resulted GSBL (+),it was revealed that this study shows parallelism with the studies performed in Germany.

In the studies carried out on organic and conventional vegetable and fruits in Netherlands and France (3, 23, 24); the types of *Rahnella aquatilis, Serratia fonticola, Pantoea agglomerans, Acinetobacter sp., Stenotrophomonas sp., Rahnella sp., Proteus sp., Pantoea sp., Klebsiella sp., Ewingella sp., Escherichia sp. and Erwinia sp Enterobacteriaceae* were isolated. It was declared that most of these bacteria were originated with soil and environment, and the potantial pathogenic types which could affect immunity were scarcely existed among these bacteria and 13% of products tested were strains producing extended spectrum beta-lactamase. In addition, when the genes of the strains isolated were examined, it was seen that the genes of *bla*$_{CTX\text{-}M\text{-}15}$, *bla*$_{CTX\text{-}M\text{-}1}$, $_{CTX\text{-}M\text{-}9}$ showed similiarity with the genes of bacteria existed in the human natural flora. It should be noted taking into account the results of this study that the origin of the bacteria existed in human flora and carrying the genes of extended spectrum beta-lactamase might be the raw vegetables taken into the body and metabolism.

In this study, 18 pieces of 69 isolated bacteria (25,55%) yielded certain results of GSBL(+). The range of these isolates were *Klebsiella pneumoniae* (n=10, 55,5 %), *Escherichia coli*(n=7, 39 %), *Citrobacter freundii*(n=1, 6 %).The bacteria producing ESBL, such as *K. pneumoniae, E. coli and C. Freundii,* were isolated from the large leafed vegetables growing close to soil. On the other hand, any bacteria producing ESBL wasn't detected at vine, which is more far from the ground than the other vegetables and at mushroom, which is closer to the ground than the other vegetables. The major contamination source of resistance genes for vegetables is animal manure. Antibiotics have been used with other veterinary medicines with the intent of preventing animal diseases and getting high efficiency, speeding up the growth of animals. Due to unconscious use of these medicines and insufficient controls on them might cause formation of residues (65). Therefore, contamination risk have been increased with direct contact of raw consumed vegetables to manure. The findings obtained in this study are important and support the opinion of that fecal contamination is possible. When the studies performed on this issue examined it was seen that *E. coli* strain could be isolated from the vegetables, such as spinach and lettuce and this strain can be passed to vegetable as a result of fecal contamination of vegetable with water and/or soil (35, 3).

It was seen through this study that as well as ESBL, metallo-beta-lactamase (MBL),which is a kind of important resistance in terms of antibiotic resistance has also been seen in vegetables. In the study, metallo-beta-lactamase enzymes originated resistance was determined in the strains of one piece of *Stenotrophomonas maltophilia* and 3 pieces of *Acinetobacter baumannii.*

It was revealed in further studies carried out regarding this topic that the bacteria of *Serratia marcescens, Klebsiella pneumoniae, Citrobacter freundii, Pseudomonas aeruginosa, Pseudomonas putida, Acinetobacter spp.* and *Alcaligens xylosoxidans* have also produced MBL(59, 60, 61). Due to the invention of transferrable MBL enzyme, resistance against carbapenems that is an important group of antibiotic have been occured, which is threatening in terms of public health.

In other studies performed, regarding *Stenotrophomonas maltophilia* strains, the existence of MBL enzyme was determined (18, 32, 33). In the studies performed regarding salads (21), it was reported that *Stenotrophomonas maltophilia* in the ratio of 78% was isolated, but in the studies performed regarding vegetables, it was seen that MBL resistance that could occur in isolates of *S. Maltophilia* wasn't mentioned about so much. When the clinical researches were taken into account, this strain existing in the vegitables will likely gain resistance in the same way. On the other hand; MBL enzymes being produced in the strains of *A. baumannii* all over the world and in Turkey were determined as the types of Imipenemase (IMP), Verona Imipenemase (VIM) ve New Delhi Metallo beta-lactamase (NDM) -1(41, 42, 43).While the genes encoding these enzymes were reported to be existed in *Acinetobacter* strains in a limited number in Europe including the Mediterranean countries, they have been encountered in Asian countries as endemic (48).

In this study; AmpC beta lactamase resistance was determined in one piece of *E. coli* strain. When the AmpC beta-lactamase resistance examined it was seen that *Citrobacter* ve *Enterobacter* types of *Enterobacteriaceae* family could produce AmpC and in particular, *Escherichia coli*, an important fecal bacteria, producing AmpC could be isolated from the vegetables (31, 20, 3).

The multiresistance increasingly becoming a problem have been seen in *Enterobacteriaceae* strains at the present time and spreaded developing resistance mechanisms against different antibiotic groups (56, 57, 58). It was reported that the multiresistance usually arose as a result of removing plasmide and transposon, which carry genetic determinants of different resistance mechanisms (49, 50). Thus, antibiotic-resistant bacteria genes can easily be transferred to the opportunistic pathogens. This transfer was firstly presented with ampicillin resistance encoded by ESBL genes of the bacteria existing in lettuce (62) and spinach(63). One of the reasons for multi resistance is active eflux pumps and the

recent studies have focused on this mechanism (51, 52, 53).

The extended spectrum beta-lactamases (ESBL) or AmpC-type beta-lactamases encoded in plasmide are responsible from the resistance existed against the extended spectrum cephalosporins in *E.coli*s strains (55). Since the plazmides encoding ESBL in these bacteria, often carry resistance genes to the aminoglycosides, they show multi resistance (54). In their study performed in 2015 (20) Njage and Buys reported that *E. coli* strains isolated from lettuce produce both extended spectrum beta-lactamases and AmpC. It was seen in this study that AmpC-resistant *E. coli* strain encountered in antibiogram affirmation section in the last phase of the analysis showed similarity with other studies in terms of showing multi resistance against ESBL and AmpC.

In this study; the resistance of enteric bacteria isolated from vegetable samples against extended spectrum beta-lactamases metallo-beta-lactam and AmpC-type antibiotics was determined by using phenotype methods, ,but considering them unsufficient, in addition to these methods, typing with mass spectrometry and lastly, MIK values were identified by antibiogram affirmation.

In conlusion, it was seen in this study that the enteric bacteria isolated through determining enteric bacteria producing ESBL, MBL ve AmpC, developed multi resistance against antibiotics. It was revealed in the studies that extensively used antibiotics could cause multi resistance on microorganisms over the years and major problems would occur in treatment of infection occured by these organisms.

It should be kept in mind that the bacteria having the ability to develop resistance, are not only clinical or social originated, they can also originate from foods (raw consumed vegetables) and they have ability to pass to the human organism. The contamination of this type of bacteria should be prevented and minimized. In healthy life, with the intent of preventing the resistance against antibiotics in particular, in order that the vegetables that are likely potential resistance source should be kept under control, livestock sector and also all inputs on the basis of product should be controlled strictly. The amount of antibiotics used in livestock raising, from farms of which, the fertilizer used in the vegetables was supplied, accordingly, the presence of the enteric bacteria producing ESBL contained in the content of fertilizer and cleanliness of water resources supplied for vegetables are considerably important issues in terms of food safety and public health.

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