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Characterization of extended-spectrum β -lactamases (ESBLs) producers in *Klebsiella pneumoniae* by genotypic and phenotypic method

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Abstract

This study aims to determine the presence of extended-spectrum (ESBL) in *Klebsiella pneumoniae* isolated from raw vegetables by genotypic and phenotypic method. Fifty-three *K. pneumoniae* isolates that were obtained by plating method were confirmed by PCR. Isolates obtained were screened for their resistance to selected antibiotics. Phenotypic tests for ESBL detection is basically to confirm production of ESBL, in this study two types of antibiotics used which were amoxycillin/clavulanic Acid (AMC, 30 µg) and ceftazidime (CAZ, 30 µg), The resistance were 5/53 (9.4%) and 1/53 (1.9%), respectively. However, it was interesting to observe that none of the *K. pneumoniae* isolates demonstrated the presence of any of the *bla* genes by using genotypic method except *bla*$_{TEM}$ gene has been detected in two isolates out of 53 isolates of *K. pneumoniae* in this research.

Introduction

Klebsiella is a lactose fermenter and nonmotile bacilli that have luxurious capsule. *Klebsiella pneumoniae* (*K. pneumoniae*) can cause bacterimia and urinary track infections particularly in hospitalized patients and immune compromised individuals as well. *K. pneumoniae* is also associated with the contamination of raw vegetables, since it is can be found in soil. Contamination in raw vegetables by this bacteria can be occur during growth stage of vegetables. In current situation, fresh produce is heavily being contaminated by bacteria and it is an emerging global problem. This problem arises due to use of animal manure, as well as untreated sewage water containing pathogens lead to the outbreaks of human illness.

Even though, *K. pneumoniae* is naturally resistant to ampicillin, amoxicillin, carbenicillin and ticarcillin, and not to extended-spectrum β-lactam antibiotics. However, many of the factors can contribute to the resistant of bacteria towards antibiotics. Adaptation of bacteria to stress environment can result in a bacteria adapt, and lead to survival and growth, becomes more virulent. This finally led to production of extended-spectrum β-lactamases (ESBLs) producers in *K. pneumoniae*.

Extended-spectrum beta-lactamases (ESBLs) have been detected in most of pathogenic Gram-negative bacteria, but they are most common in nosocomial isolates of *Klebsiella pneumoniae* (Philippon *et al*., 1989; Medeiros, 1993), giving a proportion of 75% of ESBL-producing strains (Sirot, 1995). Since then several types of ESBLs have been described (Jacoby and Medeiros, 1991). Hospital outbreaks of ESBL-producing *K. pneumoniae* been reported all around the world and recently ESBL-producing *K. pneumoniae* isolates were reported from different countries in the world (Pagani *et al*., 1994; Galas *et al*., 1999; Kariuki *et al*., 2001; Pai *et al*., 2001; Bedenic *et al.*, 2001).

Extended spectrum β-lactamase (ESBLs) are widely resistance to penicillin, cephalosporin and monobactam but not to carbapenem (Mehrgan et al., 2008). ESBLs are plasmid mediated and mostly are members of TEM-1, TEM-2 and SHV-1 family's enzymes, which produced by *Enterobacteriaceae* and *K. oxytoca* (Astal *et al.*, 2004; Vandana *et al.*, 2009; Shah *et al*., 2004; Bhattacharya *et al.*, 2006).

Commonly, resistance of *K. pneumoniae* to extended-spectrum β-lactams antibiotics is mediated by β-lactamases. *K. pneumoniae* is a majorly known

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as a host of plasmid-kocated extended-spectrum beta-lactamases (ESBL). ESBLs are clavulanate-susceptible enzymes capable of hydrolizing oxyimino-cephalosporins and monobactams, but not cephamycins and carbapenems ESBL-producing bacteria being major-therapeutic dilemma, due to availability of limited choices of antibiotics. ESBL-producing isolates are often being as a causative agent for nosocomial outbreaks (Messai *et al*., 2008).

There is limited data from Malaysia available regarding detection of bla genes in food borne isolates. In many parts of the world, 10-40% of *K. pneumoniae* isolates produced ESBLs, has been reported in Latin America (45.4%), followed by the Western Pacific (24.6%), Europe (22.6%), the United States (7.6%), and Canada (4.9%) (Tzouvelekis *et al*., 2000; Stevenson 2003). The aim of this study is to characterize extended-spectrum β-lactamases producers in *K. pneumoniae* by genotypic and phenotypic method.

Materials and Methods

Phenotypic detection of extended-spectrum beta-lactamases (ESBLs)

All 53 isolates of *K. pneumoniae* were tested for susceptibility to various antibiotics using the disk diffusion method according to guidelines set by the National Committee for Clinical Laboratory standard M100-S15 (2005). The isolates were grown in TSB and were incubated at 37°C for 24 hours. The cultures were swabbed evenly using sterile non-toxic swab on Mueller-Hinton agar plates (Merck, Germany), which were then left to dry for 2 to 5 min before placing the antimicrobial sensitivity discs onto the agar using Disk Diffusion Dispenser (Oxoid Ltd., Hamshire, England). The culture of *E. coli* ATCC 25922 was included as a control test in the susceptibility test.

Amoxycillin/clavulanic acid (AMC, 30 µg), and ceftazidime (CAZ, 30 µg) were used as phenotypic test to confirm ESBL-production. The antibiotics cartridges with commercially prepared antibiotics discs were purchased from Oxoid (Hamphire, United Kingdom) and BBL (Becton-Dickinson Microbiology Systems, Maryland, USA). Each antibiotic test was run in duplicate on freshly prepared Mueller Hinton agar plates. All plates were incubated at 37°C for 24 hours. After incubation, the size of the inhibition zones was recorded and the levels of susceptibility (sensitivity, intermediates and resistant) were determined according to the National Commitee for Clinical Laboratory Standards (NCCLS) (2005).

Preparation of DNA template

Template DNA of *K. pneumoniae* was prepared from freshly cultured isolates by suspending 3-5 colonies from plate to 500 µl of TE buffer and boiled for 10 minutes. After boiling, the samples were cooled at -20°C for 5 min before being centrifuged at 10,000 rpm for 10 min.

Genotypic detection of bla genes by polymerase chain reaction (PCR)

The supernatant obtained was used as the DNA template in the PCR analysis. Molecular detection of bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{OXA-1}, and bla_{OXA-2} were performed by using polymerase chain reactions (PCRs). The primers used for respective detection of these genes are shown in Table 1.

Table 1. Primers and conditions of polymerase chain reaction used in this study

Primer	Nucleotide sequences	Expected size	PCR conditions
TEM-F	ATG AGT ATT CAA CAT TTC CG	867bp	95°C, 5 min; 30 cycles of 95°C, 1 min,
TEM-R	CTG ACA GTT ACC AAT GCT TA		50°C, 1 min, 72°C, 1min
SHV-F	GGG TTA TTC TTA TTT GTC GC	928bp	94°C, 5 min; 35 cycles of 94°C, 1 min,
SHV-R	TTA GCG TTG CCA GTG CTC		58°C, 1 min, 72°C, 1min
CTX-M-F	GAC GAT GTC ACT GGC TGA GC	759bp	94°C, 5 min; 35 cycles of 94°C, 45 s,
CTX-M-R	AGC CGC CGA CGC TAA TAC A		58°C, 45 s, 72 °C, 1min
OXA-1-F	ACA CAA TAC ATA TCA ACT TCG C	813bp	94°C, 5 min; 35 cycles of 94°C, 1 min,
OXA-1-R	AGT GTG TTT AGA ATG GTG ATC		58°C, 1 min, 72 °C, 1min
OXA-2-F	TTC AAG CCA AAG GCA CGA TAG	814bp	94°C, 5 min; 35 cycles of 94°C, 45 s,
OXA-2-R	TCC GAG TTG ACT GCC GGG TTG		61°C, 45 s, 72 °C, 1min

PCR amplification was performed in 25 µl reaction mixture containing 5 µl of 5x PCR buffer, 1.5 mM $MgCl_2$, 400 µM dNTP, 1 unit *Taq* DNA polymerase, 1 µM of each primer (Promega, Research Instruments, USA) and 2 µl of the DNA template solution.

The cycling conditions for detection of bla_{TEM} were as follows: initial denaturation at 95°C for 5 min; 30 cycles of 95°C for one min; 501 min ; 72 °C for 1 min; and final elongation at 72°C for 10 min. However, the cycling conditions for bla_{SHV}, bla_{CTX-M}, bla_{OXA-1}, and bla_{OXA-2} were as follows: initial denaturation at 94°C for min; 35 cycles of 94°C for 1 min; 1 min at 58°C for bla_{SHV} and bla_{OXA-1}; 45 seconds at 58°C for bla_{CTX-M}; 45 seconds at 61°C for bla_{OXA-2}, and one min at 72°C, with a single final extension step of 10 min at 72°C. for the visualization of PCR products, 10 µl of PCR products were run on 1% agarose gel at 100V for 30 min. The gel stained with ethidium bromide and viewed under ultra violet light. A DNA molecular ladder (100bp ladder) (Vivantis Technologies) was included in each gel.

Results and Discussions

During the past decade, ESBLs producing gram-negative bacilli especially *E. coli* and *K. pneumoniae* have emerged as serious pathogens both in hospital and community acquired infections worldwide. Occurance of ESBL among clinical isolates vary greatly worldwide and geographically and are rapidly changing over time (Babypadmini *et al.*, 2004). There is limited data available regarding ESBL producing detected from food-borne bacterial isolates.

Basically, *Klebsiella* isolates are naturally resistant to ampicillin, because of constitutively expressed chromosal class A beta-lactamase (Livermore, 1995; Haeggman *et al.*, 2004). In this research, *K. pneumoniae* isolates are expected to present an intrinsic resistance to ampicillin (Heritage *et al*., 1999), however 50.9% (27/53) of all isolates in this study showed resistance to this antibiotic. Phenotypic tests for ESBL detection is basically to confirm production of ESBL, however cannot detect the subtype of ESBL. In this study two types of antibiotics used which were amoxycillin/clavulanic Acid (AMC, 30 µg), and ceftazidime (CAZ, 30 µg), the resistance were 5/53 (9.4%) and 1/53 (1.9%) respectively shown in Table 2.

Several studies reported that the β-lactamase gene, in ESBL producing *K. pneumoniae* strains, was co-transferred with the non-β-lactam antibiotics (Sirot *et al.*, 1988; Petit *et al*., 1988; Livermore, 1995). ESBL production is encoded via genes that are commonly located on large conjugative plasmids and while these plasmids are easily transmitted among different members of the *Enterobacteriaceae*, results in strains that contain multi-resistant plasmids. Therefore, ESBL-producing isolates are resistant to a wide range of classes of antibiotics (Sirot, 1995; Bradford, 2001).

Over the past few years, the occurance of ESBL producing strains among clinical *Klebsiella* isolates has steadily increased. Since ESBL are most frequently encoded on plasmids, which encode other antibiotic resistance genes, that express ESBL are commonly resistant to other antibiotic agents (Jacoby and Sutton, 1991). These plasmids are easily transmitted between bacteria and this account for ESBL producing isolates that are resistance to a variety of antibiotics. According to Podschu and Ullmann (1998), the multidrug resistant *Klebsiella* strain is unfortunately accompanied by a relatively high stability of the plasmids.

Furthermore, the inhibitor-resistant TEM β-lactamases founded in clinical isolates of *E. coli*, but also several strains of *K. oxytoca*, *K. pneumoniae*, and *Citrobacter freundii* (Lemozy *et al*., 1995; Bret *et al*., 1996). According to literatures/previous research, *bla* genes mostly found in clinical isolates and less research available on the detection of bla genes in food-borne isolates. However, it was interesting to observe that none of the *K. pneumoniae* isolates demonstrated the presence of any of the *bla* genes except bla_{TEM} gene has been detected in two isolates out of 53 isolates of *K. pneumoniae* in this research (Figure 1).

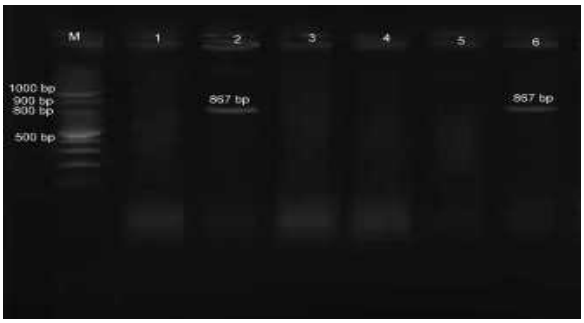

Figure 1. Agarose gel electrophoresis of the bla_{TEM} (867 bp). Lane 1= 100bp DNA marker; Lane 2= negative result for bla_{TEM} ; Lane 3 = positive result for bla_{TEM}; Lane 4 = negative result for bla_{TEM}; Lane 4= negative result for bla_{TEM}; Lane 5= negative result for bla_{TEM}; Lane 6= negative result for bla_{TEM}; Lane 7= positive result for bla_{TEM}.

Therefore, broad antibiotic resistance extending to multiple antibiotic classes is now a frequent characteristic of ESBL-producing enterobacterial isolates. Even though, the majority of ESBL-producing organisms have been reported from hospitalized patients admitted to intensive care units (ICUs), but infections can occur through the fresh produce from farm as well due to usage of antibiotics in agriculture field. The consequences that might be happen is treatment failures in patients who received inappropriate antibiotics and outbreaks of multi drug resistant, Gram-negative pathogens, which have required expensive control efforts.

In order to avoid economic loss, precaution steps need to be taken. For example, usage of antibiotic dosage needs to decrease in agriculture. The higher dosage of antibiotic will lead the bacteria resistant to the wide range of antibiotics, and this will lead to mutation occur. Besides that, multi-drug resistance patterns of clinical *K. pneumoniae* isolates particularly for expanded-spectrum beta-lactamase is becoming an important problem, particularly taking into account the limited choice of antimicrobial agents for treatment and the risk of transfer of resistance to other enteric organisms.

In brief, most outbreaks have occurred in debilitated, hospitalised patients located in ICU's.

Table 2. Prevalence of resistance to antimicrobial agents of *Klebsiella pneumoniae* from raw vegetables and phenotypic detection of ESBL

Antibiotics	Susceptibility Range (mm)			Susceptibility		
	S	I	R	S	I	R
				No. (%)	No. (%)	No. (%)
Macrolides						
Erythromycin (15 µg)	≤13	14-22	≥23	1/53 (1.9)	48/53 (90.6)	4/53 (7.5)
Clarithromycin (15µg)	≤13	14-17	≥18	0/53 (0)	0/53 (0)	0/53 (0)
Ciprofloxacin (5 µg)	>21	16-20	≤15	46/53 (86.8)	0/53 (0)	7/53 (13.2)
Penicillin						
Carbanicillin (100 µg)	≥ 23	20-22	≤ 19	26/53 (49.1)	3/53 (5.7)	24/53 (45.3)
Ampicillin (10 µg)	≥17	14-16	≤13	10/53 (8.9)	16/53 (30.2)	27/53 (50.9)
Piperacillin (100 µg)	≥21	18-20	≤7	10/53 (8.9)	16 /53 (30.2)	27/53 (50.9)
Ticarcillin (75 µg)	≥15	12-14	≤11	35/53 (66.0)	3/53 (5.7)	15/53 (28.3)
Mezlocillin (75 µg)	≥21	18-20	≤17	27/53 (50.9)	6/53 (11.3)	20/53 (37.7)
Cephalosporins						
Ceftazidime (30 µg)	≥18	15-17	≤14	51/53 (96.2)	1/53 (1.9)	1/53 (1.9)
Cephazolin (30 µg)	≥18	15-17	≤14	51/53 (96.2)	0/53 (0)	2/53 (3.8)
Aminoglycosides						
Gentamycin (10 µg)	≥18	15-17	≤14	38/53 (71.7)	0/53 (0)	15/53 (28.3)
Tetracyclines						
Tetracycline (30 µg)	≥19	15-18	≤14	38/53 (71.7)	2/53 (3.8)	13/53 (24.5)
Sulfonamides						
Trimethoprim (5 µg)	≥10	11-15	≤16	29/53 (54.7)	2/53 (3.8)	22/53 (41.5)
Penicillin Combinations						
Amoxycillin/ Clavulanic acid (30 µg)	≥ 13	14-17	≥ 18	45/53 (84.9)	3/53 (5.7)	5/53 (9.4)

*National Committee for Clinical Laboratory standard M100-S15 (2005)

Nevertheless, outbreaks have been described in out-of-hospital locations such as nursing homes, geriatric centres and rehabilitation units (Trick, 2001; Hollander, 2001). Risk factors for acquisition of ESBL-producing Enterobacteriaceae are severity of illness will get worst and prolonged hospital stay. Besides that, usage of broad-spectrum antibiotic usage will weakened the immune system as well.

Conclusion

In conclusion, the findings of this research have the serious effect regarding the detection of ESBL in fresh produce. Precautions steps need to be taken to avoid outbreaks. Antibiotics usage needs to be controlled, so that the resistance level of *K. pneumoniae* towards several classes of antibiotics can be controlled. The data presented might be useful for the further research.

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