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Occurrence of *bla*CTXM-2, *bla*SHV, *bla*TEM genes in ESBL-producing bacteria from retail sausages in Kampar, Malaysia.

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Abstract

Overuse of antibiotics in the prevention and treatment of diseases among humans and livestock has led to the emergence of multi-antibiotics resistant bacteria worldwide. This includes bacteria which produce the enzymes extended-spectrum β-lactamases (ESBLs). This study reports the presence of β-lactamases-producing bacteria (35.7%) and ESBL-producing bacteria (21.4%) from retail sausages in Kampar, Malaysia and the detection of bla_{CTXM-2} (60%), bla_{TEM} (40%) and bla_{SHV} (20%) genes, but not bla_{CTXM-1} and bla_{CTXM-9} genes in these isolates. The bacteria harboring bla_{CTXM-2} and bla_{TEM} genes were identified as *Pseudomonas pneumotropica*, whereas the bacteria harboring only bla_{SHV} gene was identified as *Klebsiella pneumoniae* subsp. *pneumoniae*. This is the first report of bla_{CTXM-2} in food source in Malaysia.

Keywords: antibiotic resistant ESBL-producing bacteria, sausages, *bla*CTXM-2, *bla*SHV, *bla*TEM

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Introduction

The overuse of antibiotics in human medicine, animals and agriculture has led to selective pressures in the adverse environment and the emergence of antibiotics resistant genes among bacteria. This has led to a great deal of attention being paid to the emergence of antibiotic resistant bacteria in both human and animal populations. As antibiotic resistant bacteria can lead to the risks of spread of resistant strains among humans and animals, this impacts morbidity and mortality from chronic diseases and increases the cost of therapy [1].

One of the current important antibiotic resistance mechanisms is the plasmid-mediated production of β-lactamases. This enzyme is able to inactivate the β-lactam antibiotics by hydrolyzing the β-lactam ring [2]. However, the prolonged exposure of bacteria to a variety of β-lactams antibiotics has led to continuous mutation and production of β-lactamases and expanding their activity towards the newly developed β-lactam antibiotics [3]. These enzymes are known as Extended-Spectrum β-Lactamases (ESBLs) and are most frequently reported as TEM, SHV and CTX-M types [1]. ESBLs confer broad resistance to penicillins, monobactams (aztreonam) and up to third and fourth generations of cephalosporins (with the exception of cephamycins and carbapenems) [4]. However, they are inhibited by sulbactams, tazobactams and clavulanic acid, a feature that is used as a standard for β-lactamases classification and for diagnostic ESBL detection purposes [2].

Recently, the recovery of ESBL-producing bacteria from different types of food products and animals was reported in some studies [5-7]. These reports have raised questions about the possible interplay of animal- and food-related reservoirs in the spread of ESBL- producing microorganisms. Hence, this study was conducted to isolate the ESBL-producing bacteria from retail sausages of poultry source, in addition to identify the antibiotic resistance and gene determinants.

Materials and Methods

Sample collection, isolation and identification

All the sausages were collected from a hypermarket and cafeteria of the university in Kampar, Perak, Malaysia. Each of the sausages was cut into small pieces to approximately two grams and cultured into five replicates of Luria-Bertani (LB) enrichment broths (Laboratories CONDA, Spain) respectively on the day of arrival.

The culture broths were then incubated at 37^{o}C for 16- to 18-hours and a loopful of the enrichment broth was streaked onto MacConkey (MAC) agar (Oxoid, England) plates for the isolation of Gram-negative bacteria. The

agar plates were then incubated aerobically at 37°C for 24-hours. Any of the gram-negative bacteria isolated from retail sausages were then subjected to a range of general biochemical tests and identified using the API 20E bacterial identification kit (bioMerieux®, France) in accordance to the manufacturer's specifications.

Screening and confirmation of ESBL-producing bacteria
Initial screening test for ESBL-producing bacteria was performed by using Kirby-Bauer disc diffusion antibiotic susceptibility test. A single colony of pure culture from the overnight MacConkey agar plate was inoculated into 5 mL of Trpytic Soy Broth (TSB) (Merck, Germany) and incubated at 37°C overnight. The turbidity of the inoculums in the TSB was adjusted to 0.5 McFarland standard and the plates were screened with five types of antibiotics, which included aztreonam (ATM-30), cefotaxime (CTX-30) (BD BBL™, United States), cefpodoxime (CPD-10), ceftriazone (CRO-30) and ceftazidime (CAZ-30) (Oxoid, England), based on the Clinical and Laboratory Standards Institute (CLSI) standard [8]. Any isolate that showed resistance to any of the antibiotic in initial screening test was subjected to double disc synergy test for ESBL production.

In this method, a disc containing amoxicillin-clavulanic acid (AMC-30) (Oxoid, England) was placed at the center of Mueller-Hinton agar. Discs containing ceftazidime (CAZ-30) and cefotaxime (CTX-30) (BD BBL™, United States) were applied 15 mm apart from the disc at the center, which was the amoxicillin-clavulanic acid (AMC-30) in accordance to CLSI protocols. After overnight incubation at 37°C, ESBL enzyme production by the bacteria isolated would be indicated by a keyhole zone phenomenon towards the amoxicillin-clavulanic acid (AMC-30) disc. Phenotypic confirmatory test for ESBL production was carried out using combination disc method. An increase of $\geq$ 5 mm in a zone diameter for either antimicrobial agent (cefotaxime (CTX-30) or ceftazidime (CAZ-30)) tested in combination with clavulanic acid versus the zone diameter of the agent when tested alone after incubation at 37°C for 16- to 18-hours was considered positive for ESBL production [8].

Antibiotic susceptibility testing
In addition, the bacteria were also subjected to further Kirby-Bauer antibiotic susceptibility tests using additional nine types of antibiotics, which were ampicillin (AMP-10), penicillin (P-1), oxacillin (OX-1), piperacillin (PRL-75), imipenem (IMP-10), meropenem (MEM-10), norfloxacin (NOR-10) (Oxoid, England), gentamicin (CN-10), and tetracycline (TE-30) (BD BBL™, United States).

Total DNA extraction and Polymerase chain reaction
Total DNA was extracted using fast-boil method as highlighted in Kor *et al.*, 2013 [9]. Using established primers of $bla_{\text{CTXM-1}}$, $bla_{\text{CTXM-2}}$, $bla_{\text{CTXM-9}}$, bla_{TEM} and bla_{SHV} [10-11], PCR amplifications were carried out using PCR reagents from Invitrogen (USA) and established conditions as listed by the authors. The amplified products were subjected to 1.5% (w/v) agarose gel electrophoresis, stained with ethidium bromide before visualization under UV light using the UV transilluminator (Syngene, United Kingdom).

Results

A total number of 14 bacterial strains with different morphologies were isolated from eight different types of sausages. The zone of inhibition in diameter to the nearest millimeter was measured for each antibiotic tested and summarized in Table I. In this study, 11 (78.6%) out of the 14 bacterial isolates were resistant to the aztreonam, 11 (78.6%) bacterial isolates showed resistance to cefotaxime, all of the bacteria (100%) were resistant to cefpodoxime, while 10 (71.4%) of the bacteria were resistant to ceftriazone. Majority of the bacterial isolates were susceptible to ceftazidime as only six (42.9%) were resistant to ceftazidime.

Besides, high resistance was also observed towards ampicillin (64.3%), penicillin and oxacillin (78.6%), while the isolates were more susceptible towards piperacillin and the carbapenem and tetracycline group of antibiotics. All (100%) the bacteria were susceptible to norfloxacin.

As shown in Table II, five (35.7%) bacterial isolates (A1, E3, F1, F2 and G1) showed the keyhole zone phenomenon on the Mueller-Hinton agar, and are possible ESBL-producers. In phenotypic confirmatory test, only three isolates (A1, E3 and G1) showed $\geq$ 5 mm increase in the zone diameter around the disc with clavulanic acid over the disc with ceftazidime alone.

None of the bacterial isolate harbored any $bla_{\text{CTXM-1}}$ and $bla_{\text{CTXM-9}}$ genes, but there was a high prevalence of $bla_{\text{CTXM-2}}$ among the bacterial isolates from sausages. Three out of the five possible ESBL-producers (F1, F2 and G1) were screened positive for the presence of $bla_{\text{CTXM-2.}}$ Both F2 and G1 also co-harbored $bla_{\text{TEM.}}$ and were identified to be *Pasteurella pneumotropica* although only the latter showed positive in phenotypic confirmatory test. Interestingly, the isolate H1 which was resistant towards nine of the antibiotics including cephalosporin and augmentin, but showed negative results for double disc synergy and phenotypic confirmatory tests, was the only isolate which was tested positive for bla_{SHV} and was identified to be *Klebsiella pneumoniae* subsp. *pneumoniae*. Both isolates A1 and E3 showed positive results for both the double disc synergy and phenotypic confirmatory tests, but they did not harbor any of the ESBL gene tested.

Table I. *Antibiotics susceptibility profile of the bacterial isolates. Aztreonam (ATM-30), cefotaxime (CTX-30), Aztreonam (ATM-30), cefotaxime (CTX-30), cefpodoxime (CPD-10), ceftriazone (CRO-30), ceftazidime (CAZ-30), ampicillin (AMP-10), penicillin (P-1), oxacillin (OX-1), piperacillin (PRL-75), imipenem (IPM-10), meropenem (MEM-10), norfloxacin (NOR-10), gentamicin (CN-10), and tetracycline (TE-30)*

Sample in assigned code	Diameter of zone of inhibition (mm)														
	ATM-30	**CTX-10**	**CPD-10**	**CRO-30**	**CAZ-30**	**AMP-10**	**P-1**	**OX-1**	**PRL-75**	**IPM-10**	**MEM-10**	**CN-10**	**NOR-10**	**TE-30**	**AMC-30**
A1	R(0)	R(19)	R (14)	R(15)	R (10)	S (25)	R (0)	R (0)	S (25)	S (25)	R (18)	R (10)	S (17)	R (9)	S (40)
B1	S(30)	S(29)	R (16)	S(29)	S (26)	S (18)	R (0)	R (0)	S (23)	S (24)	S (28)	S (15)	S (26)	S (18)	S (21)
C1	R(23)	R(19)	R (0)	R(21)	S (25)	R (0)	R (0)	R (0)	S (26)	S (34)	S (36)	S (20)	S (26)	R (11)	R (0)
C2	R(10)	R(18)	R (0)	R(20)	S (25)	R (0)	R (0)	R (0)	S (26)	S (30)	S (34)	S (24)	S (32)	S (15)	R (13)
D1	R(26)	R(22)	R (0)	R(22)	S (24)	R (0)	R (0)	R (0)	S (21)	S (26)	S (30)	S (16)	S (23)	S (19)	R (9)
E1	R(10)	R(21)	R (0)	R(22)	S (25)	R (9)	R (0)	R (0)	S (28)	S (34)	S (42)	S (25)	S (34)	S (20)	R (0)
E2	R(0)	R(0)	R (0)	R(0)	R (0)	R (8)	R (0)	R (0)	R (0)	R (10)	R (14)	R (11)	S (19)	R (10)	R (0)
E3	R(0)	R(21)	R (14)	S(26)	R (0)	R (0)	R (0)	R (0)	S (29)	S (32)	S (36)	S (30)	S (20)	S (34)	R (0)
F1	R(0)	R(19)	R (0)	R(18)	R (13)	S (40)	S (34)	S(20)	S (35)	S (36)	S (30)	S (27)	S (19)	S (23)	S (34)
F2	R(0)	R(19)	R (0)	I 20)	R (15)	S (40)	S (32)	S(19)	S (35)	S (36)	S (28)	S (28)	S (20)	S (24)	S (38)
G1	R(0)	R(19)	R (0)	R(18)	R (14)	S (40)	S (30)	S(20)	S (34)	S (36)	S (29)	S (28)	S (19)	S (24)	S (39)
H1	R(15)	R(18)	R (6)	R(18)	S (22)	R (0)	R (0)	R (0)	R (16)	S (30)	S (31)	S (16)	S (29)	S (21)	R (15)
H2	S(29)	S(28)	R (17)	S(26)	S (26)	R (0)	R (0)	R (0)	S (25)	S (25)	S (31)	S (16)	S (26)	S (19)	R (0)
H3	S(28)	S(27)	R (15)	S(25)	S (25)	R (0)	R (0)	R (0)	S (26)	S (25)	S (30)	S (15)	S (25)	S (19)	R (0)
No. of resistant isolates	11/14 (78.6%	11/14 (78.6%)	14/14 (100%)	10/14 (71.4%)	6/14 (42.9%)	9/14 (64.3%)	11/14 (78.6%)	11/14 (78.6%)	2/14 (14.3%)	1/14 (7.1%)	2/14 (14.3%)	2/14 (14.3%)	0/14 (0%)	3/14 (21.4%)	9/14 (64.3%)

Table 2. *Summary of screening results for 14 bacterial isolates for detection of bla_{CTXM-1}, bla_{CTXM-2}, bla_{CTXM-9}, bla_{SHV} and bla_{TEM}.*

Bacterial strain	Double disc synergy test	PhenotypicCon-firmatory test	***bla*$_{\text{CTXM-1}}$**	***bla*$_{\text{CTXM-2}}$**	***bla*$_{\text{CTXM-9}}$**	***bla*$_{\text{SHV}}$**	***bla*$_{\text{TEM}}$**	Bacteria species based on API 20E bacterial identification kit
A1	Positive	Positive	Negative	Negative	Negative	Negative	Negative	*Serratia plymuthica*
B1	Negative	Negative	Negative	Negative	Negative	Negative	Negative	*Enterobacter cloacae* (95.1%)
C1	Negative	Negative	Negative	Negative	Negative	Negative	Negative	*Pseudomonas aeruginosa* (76.4%)
C2	Negative	Negative	Negative	Negative	Negative	Negative	Negative	*Pseudomonas fluorescens* (58.7%)
D1	Negative	Negative	Negative	Negative	Negative	Negative	Negative	*Enterobacter cancerogenus* (99.9%)
E1	Negative	Negative	Negative	Negative	Negative	Negative	Negative	*Pseudomonas luteola* (98.8%)
E2	Negative	Negative	Negative	Negative	Negative	Negative	Negative	*Pasteurella pneumotropica/ Mannheimia haemolytica* (99.9%)
E3	Positive	Positive	Negative	Negative	Negative	Negative	Negative	*Chryseobacterium meningosepticum* (87.5%)
F1	Positive	Negative	Negative	Positive	Negative	Negative	Negative	*Pasteurella pneumotropica* (56.9%)
F2	Positive	Negative	Negative	Positive	Negative	Negative	Positive	*Pasteurella pneumotropica* (49.7%)
G1	Positive	Positive	Negative	Positive	Negative	Negative	Positive	*Pasteurella pneumotropica* (49.7%)
H1	Negative	Negative	Negative	Negative	Negative	Positive	Negative	*Klebsiella pneumoniae* subsp. *pneumoniae* (97.6%)
H2	Negative	Negative	Negative	Negative	Negative	Negative	Negative	*Pantoea* spp. *3* (92.2%)
H3	Negative	Negative	Negative	Negative	Negative	Negative	Negative	*Klebsiella oxytoca* (97.4%)

Discussion

As stated in CLSI standard, the use of more than one type of antimicrobial agent for screening purpose can improve the sensitivity of ESBL production. In year 1988, double disc synergy test was described by Jarlier and others to detect the production of ESBL enzyme in *K. pneumoniae* and *E. coli* isolates. This keyhole zone phenomenon is also known as "lens of inhibition", which is an extension in the zone of inhibition surrounding the peripheral discs towards the disc placed at the centre caused by the synergy activity between the clavulanic acid and cefotaxime or ceftazidime.

However, the guideline recommendations for phenotypic confirmatory test by CLSI are restricted to a few types of bacteria, which are *K. pneumoniae*, *K. oxytoca*, *E. coli* and *P. mirabilis* [8]. In agreement with some researchers, CLSI standard guidelines should be extended to other bacteria genus and species as long as they do not produce any false- negative or positive results [12-14]. Indeed, it has been shown that any bacteria which produce KPC-beta lactamases or hyperproduction of K1-beta lactamases may cause false-positive results, whereas isolates that produced high levels of AmpC enzymes may cause false-negative results [12]. Hence, these bacteria will interfere with phenotypic tests interpretation of ESBL-producers. As observed in Table I and Table II, nine of the isolates proved to be augmentin resistant although they were resistant to at least one cephalosporin or showed positive keyhole phenomenon. These could indicate carriage of AmpC genes which leads to augmentin resistance, and the false negative results in the isolates. However, this remains to be investigated.

Both F2 and G1 which co-harbored $bla_{\text{CTXM-2}}$ and bla_{TEM} were identified to be *Pasteurella pneumotropica. P. pneumotropica* is part of the commensal oropharyngeal floras of many animals, including dogs and cats, and is usually considered a rare but opportunistic human pathogen [15]. Interestingly, this organism is not commonly reported from food source.

Although there have been reports of bla_{CTXM} from patient isolates in Malaysia [16], our study here is the first report of the high prevalence of $bla_{\text{CTXM-2}}$ gene amongst possible ESBL-producers isolated from food source in Malaysia. As highlighted by Liebana *et al*. [17], $bla_{\text{CTXM-1}}$, $bla_{\text{CTXM-14}}$, and $bla_{\text{CMY-2}}$ are usually the most frequent genes identified in *Escherichia coli* and *Salmonella* from food animals. In most countries like United Kingdom, South India and Iran, $bla_{\text{CTXM-1}}$ was the most predominant gene found in ESBL isolates [10, 18-19]. In a study by Luvsansharav *et al.* [20], $bla_{\text{CTXM-9}}$ was predominant in ESBL-producing *Enterobacteriaceae* among the fecal carriage in rural Thai communities, followed by the $bla_{\text{CTXM-1}}$. Likewise, $bla_{\text{CTXM-2}}$ has been detected in Israel, Japan, and most South American countries [21-23].

Interestingly, the isolate H1 was identified to be *Klebsiella pneumoniae* which was the only bacterium that harbored the ESBL gene categorized under *Enterobacteriaceae* family in this study. This is rather unexpected because high prevalence of *Enterobacteriaceae* are usually isolated and reported from animal food. Indeed, several types of *Enterobacteriaceae* that harbored ESBL genes were isolated from ready-to-eat-fruits in Nigeria such as *Klebsiella oxytoca* (31.7%), *Klebsiella pneumoniae* (22.0%), *Enterobacter gergoviae* (13.4%), *Pantoea agglomerans* (3.7%) and so on [24]. Besides, there was also report of a large nosocomial outbreak through the food chain in an acute care hospital in Barcelona, Spain, for the dissemination of $bla_{\text{SHV-1}}$ and $bla_{\text{CTX-M-15}}$ producing *K. pneumoniae* [25].

In this study, both *Serratia plymuthica* (isolate A1) and *Chryseobacterium meningosepticum* (isolate E3) showed positive results for both the double disc synergy and phenotypic confirmatory tests, but they did not harbor any ESBL gene tested here. This may due to the reason where both these bacteria harbored other types of ESBL genes that were not tested in the study, which included bla_{AmpC} gene and bla_{OXA} gene [26-27]. This is possible as *S. plymuthica* was shown to be resistant to oxacillin, while *C. meningosepticum* was resistant to both the ampicillin and oxacillin.

In conclusion, this is the second report of ESBL-genes from poultry food source after *bla*SHV was discovered in sushi [11]. It is paramount that control measures are to be implemented to minimize public health risk through the consumption of contaminated food. Hence, efforts should be directed to increase awareness and hygienic practices during the food processing and postharvest procedures.

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