Malaysian Journal of Microbiology, Vol 17(2) 2021, pp. 155-164
DOI: http://dx.doi.org/10.21161/mjm.200925

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Evaluation of antibiotic resistance of Enterobacteriaceae isolated from edible snails

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Received 1 July 2020; Received in revised form 2 September 2020; Accepted 24 December 2020

ABSTRACT

Aims: Food safety and drug resistance in bacteria are both important issues globally. Consumption of escargot represents possible food safety problem especially when contaminated with an indicator and multi-drug resistant bacteria. Hence, this study aimed to identify and evaluate susceptibility of Enterobacteriaceae isolated from edible snails *Archachatina marginata* to antibiotics.
Methodology and results: A total of 60 edible snails, *A. marginata* were purchased from local markets in three states of Nigeria. The edible snails were starved for three days and Enterobacteriaceae were isolated using microbiological procedures. Bacteria was identified by sequencing its partial 16S rRNA, while susceptibility of the bacteria to antibiotic was determined by disc diffusion method. Enterobacteriaceae obtained were *Klebsiella* (18), *Escherichia* (16), *Citrobacter* (10), *Salmonella* (7) and *Enterobacter* (5) species. Out of the 56 isolates obtained, 21 (37.5%) were resistant to amoxicillin and amoxicillin/clavulanic acid, 9 (16.07%) were resistant to tetracycline and 4 (7.14%) were resistant to co-trimoxazole.
Conclusion, significance and impact of study: The number of isolates which show resistant to different antibiotic classes was small. However, coliform bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Citrobacter freundii*, and *Enterobacter cloacae*) isolated from edible snails represent a huge food safety risk to the consumers of edible snails. Hence, high hygienic practices are required for the consumers of edible snails to prevent infection with pathogenic bacteria.

Keywords: Edible snail, enterobacteriaceae, susceptibility

INTRODUCTION

The Enterobacteriaceae are the largest family among Gram-negative bacteria and are commonly found in the gastrointestinal tract of human and animals as commensals playing both positive and negative roles in the host (Linton and Hinton, 1988; Lauzon *et al*., 2003; Machado *et al*., 2013). They have been the most cause associated to healthcare and community infections globally especially in drug resistance (Pitout and Laupland, 2008). Members such as *Salmonella*, *Escherichia*, *Klebsiella*, *Shigella*, and *Yersinia* have been involved in food poisoning, diarrhea, pneumonia and other forms of extra-intestinal diseases (Kapperud, 1991; Rangel *et al*., 2005; Choi *et al*., 2008; Kunwar *et al*., 2013; Painter *et al*., 2013; Sangeetha *et al*., 2014; Ed-Dra *et al*., 2017). The presence of this family, mainly coliforms, indicates evidence of faecal contamination either from human or animal sources and this plays a significant role in decision making within an industrial and pharmaceutical settings where food safety standards are upheld (Tortorello, 2003).

Microbial drug resistance is currently the most important world focus as a result of its increased impact in both community and healthcare institutions. The impacts include prolonged hospitalization, recurrent infections, economic burdens, and most importantly increased mortality rate (Kong *et al*., 2016; Munita and Arius, 2016; Ezeamagu *et al*., 2018). The dynamics of bacterial drug resistance pose serious challenges on patients' management and chemotherapy, which often result in treatment failure. Bacterial drug resistance is no longer new and dated back in 19th century before the first generation beta-lactam antibiotics were introduced in clinical practice (Fevre *et al*., 2005; Saga and Yamaguchi, 2009). To date, the trend of bacterial resistance has been on the increase as more antibiotics are being introduced in clinical practice (WHO, 2014; Ezeamagu *et al*., 2018). There is a strong relationship between drug resistance and antibiotic use as several reports have linked these

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ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 17(2) 2021, pp. 155-164
DOI: http://dx.doi.org/10.21161/mjm.200925

two components (Tumbarello *et al*., 2006; Wener *et al*., 2010; Fair and Tor, 2014; Harris, 2015).

Several mechanisms that allow bacterial survive from antibiotic treatment leading to resistance such as efflux pump, bypass of metabolic pathways inhibited by the antibiotics, modification of target site, and more importantly destruction of the antibiotics by microbial enzymes have been identified (Munita and Arias, 2016). Extended-spectrum beta-lactamases (ESBLs) are the enzymes that are extensively used by Gram-negative bacteria, especially the Enterobacteriaceae and caused resistance to most of antibiotics class from carbapenems (Coque *et al*., 2008; Pitout and Laupland, 2008; Abdelhady *et al*., 2016). Temoneira (TEM), sulfidrhyl-variable (SHV) and cefotaximase (CTX-M) genes usually reside on mobile genetic elements (plasmids), which migrate from strain to strain and between other bacterial species. Over 150 ESBLs have been reported worldwide; nevertheless, class A beta-lactamases are most extensively studied (Rupp and Fey, 2003).

Snail meat, also referred to as escargot, provides protein dietary need in various parts of the world especially in low income earners like Ghana (Ngenwi *et al*., 2010). The common edible species are *Helix pomatia*, *Helix aspersa*, *Archachatina marginata* (African giant snail) and *Achatina fulica*. The nutritional value of edible snail with respect to protein (15%), iron (3.5 mg/100 g) and low fat contents between 1.3-2.4%, essential fatty (linoleic acids and linolenic) acids has been documented (Saldanha *et al*., 2001; Su *et al*., 2004; Adegoke *et al*., 2010). There were reports that protein contents in edible snails was comparable to domestic livestock (Imevbore and Ademosun, 1988; Adegoke *et al*., 2010). The U.S. has imported snails for worth more than $4.5 million from 24 countries (Adenegan and Bolaji-Olutunji, 2012). Hence, it is economically viable for wealth generation. Since the nutritional and economic values of snail is a welcome development, especially in Africa, where the per capita animal protein consumption lies within the range of 6.8-21.8 g per capita per day (Egbunike, 1997; Jummai and Okoli, 2013; Radzki *et al*., 2017), however, transmission of infectious diseases from the consumption of edible snails has been reported (Morgan *et al*., 2002; Kirkan *et al*., 2006). Pathogens of public health importance have been isolated from edible snails such as *Staphylococcus*, *Bacillus*, *Aspergillus*, *Micrococcus* species (Adegoke *et al*., 2010; Van Horn *et al*., 2012).

Data in the literature suggest that there is a significant geographical variation in the prevalence of ESBL-producing Enterobacteriaceae globally (CDC, 2013; WHO, 2014; Harris, 2015) and transmission of infectious diseases has been reported amongst people who consumed snail meat (Morgan *et al*., 2002; Kirkan *et al*., 2006). Thus, it is imperative to identify important reservoirs of ESBL-producing Enterobacteriaceae, of which African giant edible snail (*A. marginata*) was considered in this current study. Hence, the objective of this study was to identify Enterobacteriaceae, assess its resistance determinants (ESBL and plasmid) and determine their susceptibility to different antibiotic classes.

MATERIALS AND METHODS

Sources and isolation procedures

A total of 60 *A. marginata* (20 samples each) were purchased from local markets in three states (Lagos, Ogun and River) between February and June, 2016. They were all kept in air proof bags and immediately sent to the microbiology laboratory, Babcock University for processing. The edible snails were starved for three days and washed with 70% ethanol prior to bacterial isolation from the edible snails. The snails were aseptically cut open and intestinal contents were transferred into tryptone soy broth (Oxoid, UK) and incubated overnight at 37 °C for 24 h. MacConkey, Salmonella-Shigella and eosin-methylene blue agars (Oxoid, UK) were used to selectively isolate members of Enterobacteriaceae. Besides, Gram staining was also performed according to microbiological standard procedures. A total of 56 non-duplicate isolates were selected for further screening.

Molecular Identification

Based on the preliminary tests, 22 isolates were selected for species barcoding. Genomic DNA was extracted according to the protocol described in Quick-DNA™ miniprep plus kit (Zymo research, Biolab, USA). The quality of DNA was verified by 1.5% agarose gel electrophoresis prior to PCR amplification. The hypervariable region of 16S rRNA of bacteria were amplified using PCR with primers 27F, 5'-AGAGTTTGATCMTGGCTCAG-3' and 1525R, 5'-AAGGAGGTGWTCCARCCGCA-3' synthesized by Inquaba Biotech, South Africa. An aliquot of 5.0 µg of extracted DNA was added to PCR mix containing 25 µL OneTaq Quick-Load 2X Master Mix Buffer (New England BioLabs), 0.2 mM forward and reverse primers and sterile distilled water to make up to 50 µL reaction volume. PCR was carried out with positive and negative control containing *Escherichia coli* (ATCC 25922) and all of the reagents without DNA template respectively. DNA amplification was carried out using GeneAmp PCR 9700 system (Applied Biosystems) with the following thermal programme set up: Initial denaturation at 94 °C for 3 min, denaturation at 94 °C for 30 sec, annealing at 47 °C for 30 sec, extension at 68 °C for 30 sec and final extension at 68 °C for 5 min for 30 cycles. After that, 10 µL of amplicon was resolved by 1.5% agarose gel electrophoresis.

The amplicons were then purified and sequenced using Sanger sequencing method by Inquaba Biotech, South Africa on commercial basis. Unidirectional sequence reads were performed by standard procedures and the contigs were assembled using BioEdit (version 7.2.5.0) sequence program (Hall *et al*., 1999). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor, 1969) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 17(2) 2021, pp. 155-164
DOI: http://dx.doi.org/10.21161/mjm.200925

Evolutionary analyses were conducted in MEGA6 (Tamura *et al*., 2013). All the sequences were deposited in GenBank under accession numbers: MH843162 to MH843183.

Extended β-lactamase detection

Brilliance^{TM} ESBL chromogenic agar plate (Oxoid, UK) was employed for detection of extended beta-lactamases. An overnight culture of the test organisms was prepared and standardized to 0.5 McFarland turbidity standard. Then, the ESBL chromogenic agar plates were inoculated with the respective test organisms and incubated at 37 °C for 24 h. The result was interpreted following the guidelines provided by the manufacturer.

Susceptibility test

Susceptibility test was carried out using commercial multi antibiotic disc manufactured by Abtek Biologicals Ltd (Abtek®): Amoxicillin (10 µg), cotrimoxazole (10 µg), gentamicin (10 µg), ofloxacin (5 µg), amoxicillin/clavulanate (10 µg), tetracycline (30 µg), nalidixic acid (10 µg) and nitrofurantoin (10 µg). Briefly, a single colony of pure isolate was inoculated into a test tube containing 1 mL of nutrient broth (Oxoid, UK) and incubated overnight at 37 °C. The overnight broth was then standardized to match 0.5 McFarland standard. A sterile swab stick was dipped in the standardized suspension and streaked over the surface of prepared Mueller Hilton agar plates (Oxoid LTD, Basingstoke, Hamshire, England). The antibiotic were placed on the agar surface maintaining a distance of 30 mm edge to edge. The plates were incubated at 37 °C for 24 h and the clear zone of inhibition was measured with a ruler to the nearest diameter. Results were interpreted in accordance with Clinical and Laboratory Standards Institute guidelines (CLSI, 2015).

Plasmid extraction

Plasmid extraction was achieved using ZR Plasmid Miniprep Kit (Zymo Research, USA) following the protocol provided by the manufacturer. Briefly, 1 mL of an overnight culture of the bacterial cells was transferred into 1.5 mL Eppendorf tubes and centrifuged at full speed (12,000 r.p.m) in a mini-centrifuge (Eppendorf, Germany) for 15-20 sec. The supernatant was then discarded and P1 buffer (200 µL) was added to re-suspend the pellet by vortexing. Thereafter, 200 µL of P2 buffer was mixed by inverting 2-4 times. After 1 min, 400 µL of P3 buffer was added and the mixture was then mixed gently. The lysate was allowed to incubate at room temperature for 1-2 min and was centrifuged for an additional 2 min. The supernatant was then transferred to a Zymo-Spin IN Column placed in a collection tube. The Zymo-Spin IN Collection tube assembly was then centrifuged for 30 sec and the flow-through was discarded. Exactly 200 µL of Endo-Wash buffer and 400 µL of Plasmid-Wash buffer was successively added to the column ensuring centrifugation for 30 sec and 1 min respectively. The column was transferred to a clean 1.5 mL Eppendorf tube where 30 µL of DNA Elution buffer was then added to the column and centrifuged for 30 sec to elute the plasmid DNA. Electrophoresis was carried out using 0.8% agarose and stained with ethidium bromide 0.5 g/mL for 45 min to observe the plasmid.

RESULTS AND DISCUSSION

Species distribution

A total of 56 non-duplicate isolates of Enterobacteriaceae were obtained from 16 (28.57%), 19 (33.93%) and 21 (37.50%) in Lagos, Rivers and Ogun State respectively (Table 1). Five members of Enterobacteriaceae family were obtained: *Escherichia*, *Klebsiella*, *Citrobacter*, *Enterobacter* and *Salmonella* species; and 4 non-Enterobacteriaceae consisting of *Ralstonia* spp. (2), *Acinetobacter* spp. (1) and *Massilia* spp. (1) (Table 2).

In Table 2, the least percentage similarity of species within the family of Enterobacteriaceae to the close relatives in Genbank was 93%, indicating similar species. A review by Janda and Abbott in 2007 suggest that 16S rRNA gene sequencing provides genus identification in most cases (>90%), but less so with regard to species (65 to 83%). A more stringent boundary for species delineation was proposed to increase the accuracy of identification (Stackebrandt and Ebers, 2006). Pairwise nucleotide similarity values for the species in this study were within the acceptable range for species identification. Hence, sequencing using 16S rRNA accurately identified the bacteria under investigation. The use of 16S rRNA over other house-keeping genes in bacterial identification is probably due to its conserved nature in virtually all bacteria and hence, a more accurate measure of evolution.

Table 1: Distribution of Enterobacteriaceae isolated from edible snails across the three states.

Bacteria isolated	Sample location		
	Lagos state	Rivers state	Ogun state
Citrobacter spp.	4	2	4
Enterobacter spp.	1	3	1
E. coli	6	4	6
Klebsiella spp.	3	7	8
Salmonella spp.	2	3	2
Total	16 (28.57%)	19 (33.93%)	21 (37.50%)

The phylogenetic relationship of the five species fell into four clusters (Figure 1). Isolates obtained from Rivers State (*Klebsiella pneumoniae* PORC2SS, PORC4, *C. freundii* PORC9, *Enterobacter cloacae* PORC18 and *Citrobacter youngae* PORC7) were distributed within three clusters. The same trend was observed in isolates isolated from Ogun State. Four

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 17(2) 2021, pp. 155-164
DOI: http://dx.doi.org/10.21161/mjm.200925

Table 2: The bacteria identification, accession number and its close relatives in NCBI.

Isolates	Accession number	Location	Similarity to close relatives in NCBI (%)	Close relatives in NCBI
Klebsiella pneumoniae	MH843168	Ogun	98	*Klebsiella pneumoniae* strain IGM6-8
Escherichia coli strain	MH843169	Ogun	96	*Escherichia coli* strain 128-a pink
Escherichia coli O157:H7	MH843170	Ogun	99	*Escherichia coli* O157:H7 strain
Escherichia coli strain	MH843171	Ogun	93	*Escherichia coli* strain N15-1
Salmonella enterica subsp. *enterica* strain	MH843172	Ogun	97	*Salmonella enterica* subsp. *enterica* strain KY656602.1
Klebsiella pneumoniae strain	MH843173	Ogun	99	*Klebsiella pneumoniae* strain LSRBMoFPIKRGCFTRI49
Enterobacter cloacae strain	MH843174	Ogun	99	*Enterobacter cloacae* strain I 19
Klebsiella aerogenes strain	MH843176	Ogun	99	*Klebsiella aerogenes* strain Ka
Klebsiella pneumoniae strain	MH843177	Rivers state	98	*Klebsiella pneumoniae* strain IGM5-10
Citrobacter freundii strain	MH843178	Rivers state	99	*Citrobacter freundii* strain TY172-18
Klebsiella pneumoniae strain	MH843179	Rivers state	98	*Klebsiella pneumoniae* strain IGM6-8
Citrobacter youngae strain	MH843180	Rivers state	94	*Citrobacter youngae* strain L6
Enterobacter cloacae strain	MH843181	Rivers state	96	*Enterobacter cloacae* strain K-17
Escherichia coli O157:H7 strain	MH843182	Rivers state	96	*Escherichia coli* O157:H7 strain
Citrobacter europaeus	MH843162	Lagos	98	*Citrobacter europaeus* strain OFM49
Escherichia coli O157:H7 strain	MH843163	Lagos	99	*Escherichia coli* O157:H7 strain
Escherichia albetii strain	MH843164	Lagos	99	*Escherichia coli* strain N15-1
Escherichia coli strain	MH843165	Lagos	99	*Escherichia coli* strain Y15-3
Ralstonia pickettii strain*	MH843166	Lagos	99	*Ralstonia pickettii* strain P2W4
Ralstonia pickettii strain*	MH843167	Ogun	99	*Ralstonia pickettii* strain P2W4
Massilia sp. strain*	MH843183	Rivers state	91	*Massilia arvi* strain 14.1
Acinetobacter baumannii strain*	MH843175	Ogun	88	*Acinetobacter baumannii* strain AUBAB06

*, Non-enterobacteriaceae.

strains obtained from Ilishan in Ogun State: *E. coli* (OGUB17 and OGUB8), *Salmonella enterica* (OGUB17S) and *E. cloacae* (OGUB13) were 99% identical to two strains (*E. cloacae* PORC18 and *C. youngae* PORC7) found in Rivers State. However, they were evolutionally distinct from *E. coli* LAGA17 and *E. coli* PORC6 strains isolated from Lagos and Rivers State respectively. The phylogenetic relationship indicated that the species obtained from Ogun state were more closely related to each other compared to species in other states. It is intriguing to note that the strains obtained from close proximity areas (Lagos and Ogun state) of 76.6 km apart were evolutionally distant, whereas relatedness was observed in the strains in two distant locations (Lagos-Port Harcourt, 478 km and Ogun-Port Harcourt, 465 km). It seems therefore, that evolutionary relationship is a function of distance. However, more studies with large population are required to unravel this puzzle. In addition, analysing a set of conserved genes among the isolates will shed light on the evolutionary and functional relationships which is beyond the scope of this study. Nevertheless, the phylogenetic analysis revealed a transmission pattern between two different States (Ogun-Port Harcourt and Lagos-Port Harcourt routes) and vice versa. As snail meat provides protein dietary need for people, especially in low income earners, there is a likely possibility of inter-state trade occurring across the two routes above. Lagos and Port Harcourt are the two leading cities in terms of commerce and therefore may be the nerve centres for snail business in Nigeria.

The transmission pattern is important in disease surveillance and epidemiology especially as it concerns the general public about the recovery of Enterobacteriaceae within the intestinal tract of snails.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 17(2) 2021, pp. 155-164
DOI: http://dx.doi.org/10.21161/mjm.200925

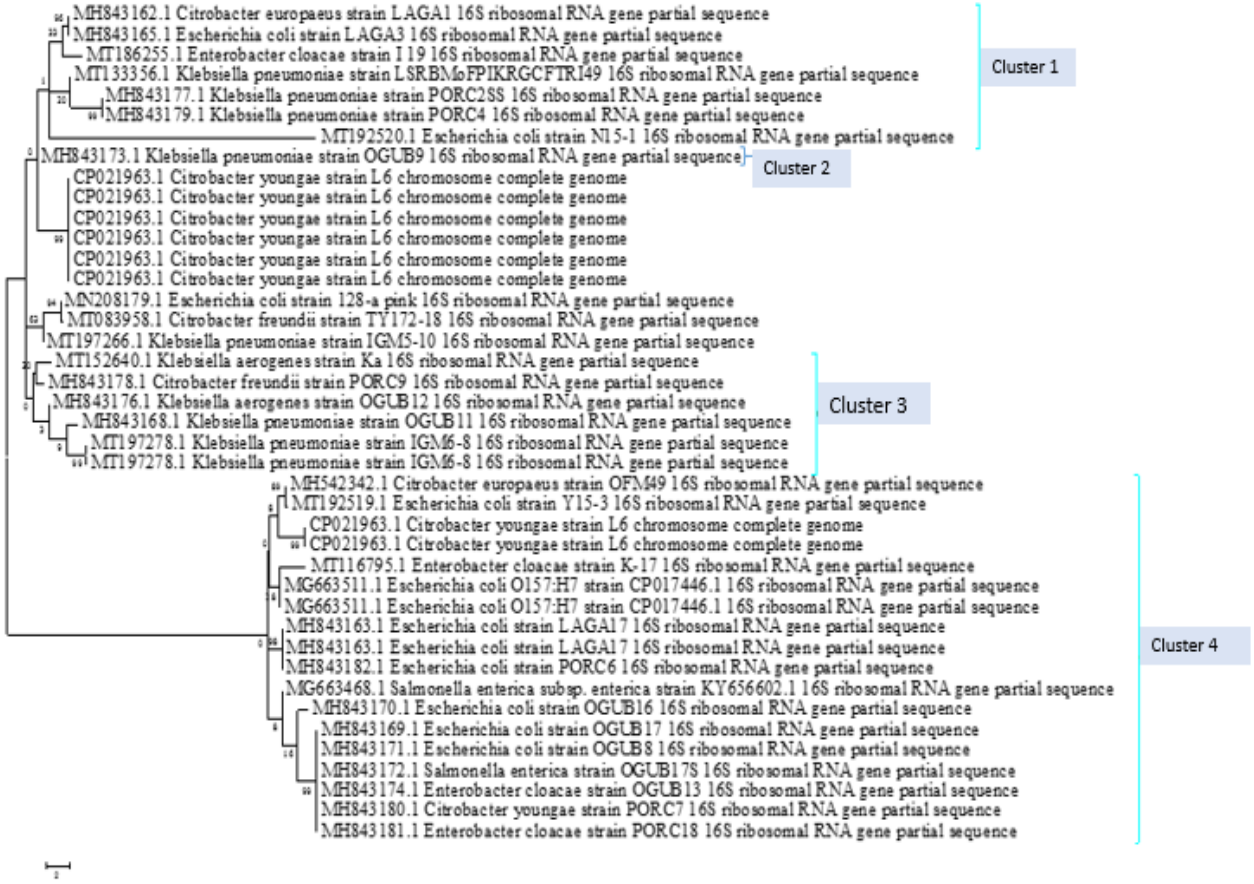

Figure 1: Phylogenetic tree illustrating the relationship among the Enterobacteriaceae identified and their close relatives in NCBI. The evolutionary history was inferred using the Neighbor-Joining method and distances were computed using the Jukes-Cantor method. Cluster 1: *C. youngae* strain LAGA1, *E. coli* strain LAGA3, *K. pneumoniae* strain PORC2SS and *K. pneumoniae* strain PORC4; Cluster 2: *K. pneumoniae* strain OGUB9; Cluster 3: *C. freundii* strain PORC9, *K. aerogenes* strain OGUB12 and *K. pneumoniae* strain OGUB11; Cluster 4: *E. coli* strain LAGA17, *E. coli* strain PORC6, *E. coli* strain OGUB16, *E. coli* strain OGUB17, *E. coli* strain OGUB8, *S. enterica* OGUB17S, *E. cloacae* strain OGUB13, *C. youngae* strain PORC7 and *E. cloacae* strain PORC18.

Consequently, snails serve as reservoirs for pathogenic bacteria, which could constitute health hazard to humans. The bacteria reported in this work were comparable to species obtained by other authors (Agbonlahor *et al*., 1994; Charrier *et al*., 2006; Adegoke *et al*., 2010; Nwiyi and Amaechi, 2011; Chukwudi and John, 2013). Of particular interest was the isolation of coliforms (*Escherichia*, *Citrobacter*, *Enterobacter* and *Klebsiella* species) which serve as indicator (evidence of faecal contamination) in water, food and pharmaceutical industries. The presence of these pathogenic organisms represents a significant health risk for humans who consuming snail meat. For instance, *E. coli* O157:H7 is the cause of many health issues in both humans and animals. Although most infections of *E. coli* O157:H7 in humans have been traced to exposure to transmission routes such as food or water, person-to-person and direct contact with animals or animal manure (Swerdlow *et al*., 1992; Kiranmayi *et al*., 2010). However, the isolation of this bacterium in this study is an important food safety issue that need serious public attention. This is because the consumption of contaminated food especially meat dishes have been linked to illness (Wells *et al*., 1991). Human infection caused by *E. coli* O157:H7 can cause various clinical manifestations ranging from asymptomatic cases to death. This strain infects the alimentary tract and induces abdominal cramps with hemorrhagic diarrhea and enterohemorrhagic disease that can cause systemic illness by hemolytic uremic syndrome (HUS), which manifests as hemolytic anemia, thrombocytopenia, and acute renal failure. Hence, it is an important food pathogen that causes diarrhea, hemorrhagic colitis, and HUS in humans (Armstrong *et al*., 1996). It has been reported that HUS can develop in both acute, potentially life-threatening illness and lifelong, chronic illness (Pollari *et al*., 2017; Atnafie *et al*., 2017; Erickson *et al*., 2019).

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 17(2) 2021, pp. 155-164
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Isolation of *S. enterica* subsp. *enterica* strains in this study is a warning signal that consumption of snail meat or products could be an avenue for infection to humans. Serrano *et al*. (2004) conducted a microbiological quality of terrestrial gastropods prepared for human consumption and observed that *Salmonella* contaminated the cooked products. Previous investigations in the analysis of uncooked snails have demonstrated that *Salmonella* is a frequent contaminant and has been isolated in Nigeria and Morocco (Andrews and Wilson, 1975; Obi and Nzeako, 1980). Non-typhoidal *Salmonellae* is responsible for gastroenteritis and usually characterized by fever, vomiting and severe diarrhea. A yearly estimate of over a million of *Salmonella* infections were reported in United States with 19,000 hospitalizations and about 400 deaths (Scallan *et al*., 2011; Bell *et al*., 2016). Salmonellosis is usually linked to consumption of contaminated foods including other food varieties and are usually dose dependent (Painter *et al*., 2013; Bell *et al*., 2016).

Food contamination with antibiotic-resistant bacteria is of serious threat to public health and the antibiotic determinants can easily be transferred to medically important bacteria through any repertoires of transfer mechanisms. The first genomic island of *Salmonella* which contain antibiotic resistant cluster was reported in *S. enterica* serovar Typhimurium DT104 (Van *et al*., 2007). This genomic island has been reported to be transferable between *S. enterica* and *E. coli* (Doublet *et al*., 2005). In developing countries where raw food hygiene is frequently poor and antimicrobial resistance epidemiology is at its developmental stage, the impact of *Salmonella* contamination and infections may be costly especially in children and immune-compromised patients. Snails naturally have high population of indigenous bacteria including coliforms as well as other poisonous substances (ICMSF, 2005). The meat can easily be contaminated by pathogens and serve as vehicle of transmission to consumers. According to Adagbada *et al*. (2011), there is a close association between snails and microorganisms due to their sources of food (sewage, manure rotten materials and poor latrine system) which increase the microbial load. Feeding on decaying or faecal matter as well as contaminated water by the snails could be the route of contamination in this study.

Status of resistance determinants and susceptibility pattern

The resistance determinants analysis showed that none of the isolates produced the ESBLs and possess plasmid (Table 3). Of the 56 organisms obtained, 21 (37.5%) were resistant to amoxicillin and amoxicillin/clavulanic acid, while 9 (16.07%) and 4 (7.14%) were resistant to tetracycline and co-trimoxazole respectively. One *E. coli* strain was resistant to ofloxacin. Resistance of isolates to beta-lactam antibiotics (amoxicillin and amoxicillin/clavulanate) were highly related to other antibiotic classes. There was no plasmid found in species investigated and the number of isolates resistant to different antibiotic classes were small.

Extended spectrum beta lactamases (ESBL) are enzymes extensively employ by Gram-negative bacteria to metabolize fluoroquinolone class of antimicrobials upon exposure or treatment. This resistance mechanism has been associated with acquisition of plasmid. Many bacteria with plasmids have potentials to spread antibiotic resistant genes, heavy metal resistant genes or any other resistance determinants through horizontal gene transfer mechanisms (Millan, 2018). In this study, we found no ESBLs and plasmid. Consequently, the bacteria may not be as virulent as those found in clinical setting which are usually difficult to treat due to acquisition of genetic elements. In addition, absent of this element may render the strains less virulent as plasmids code for virulent genes in bacteria (Elwell and Shipley, 1980; Guiney *et al*., 1994; Hammerl *et al*., 2012). The strains of bacteria in this study are, therefore, unlikely to transmit resistant or virulent genes to other neighbouring organisms within the same environment. However, although they did not possess plasmid and ESBLs, it does not negate their potentials for future acquisition of the same and mobilization of other resistance determinants such as integrons, thus, representing a public health risk.

The absence of resistant plasmid in the isolates did not hamper the expression of resistance to certain antibiotics. For instance, in this study, it shows the isolates were resistant to amoxicillin, amoxicillin/clavulanic acid, tetracycline and co-trimoxazole (Table 3). This is an indication that the resistance observed was likely to be intrinsically mediated by bacterial outer membrane and active efflux. In a review by Cox and Wright (2013), a number of studies indicated that certain genes and genetic loci contribute to intrinsic resistance phenotype. The observed resistance of the bacteria in this present study was in tandem with results of genomic and antibiogram studies on human microbiota and environmental bacteria which revealed high level of intrinsic resistance (D'Costa *et al*., 2006; Dantas *et al*., 2008; Sommer *et al*., 2009). In an attempt to survive in harsh environment, many antibiotic producing bacteria have chromosomally encoded resistant elements which usually are located within the antibiotic biosynthetic cluster (Cundliffe *et al*., 2001; Cox and Wright, 2013). Although, the antibiotic-producing ability of the isolates in this study is not known, however, a study implicated *E. coli* and *K. pneumoniae* as antibiotic producers (Woappi *et al*., 2013). This might have been the case for these strains in addition to the aforementioned factors.

It is common for members of the family Enterobacteriaceae within snail environment to be less resistant because antibiotic pressure is either minimal or completely absent. Reports in literatures revealed that members of this family in other environmental settings have potentials to produce ESBLs and highly resistant to different antibiotic classes (CDC, 2013). In this study, innate resistance or other resistance mechanisms might have been responsible for the observed resistance against aforementioned antibiotics. However, resistance

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 17(2) 2021, pp. 155-164
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Table 3: Resistance pattern of the isolates against selected antibiotic classes and status resistance determinants.

Antibiotics	Number of species and percentage resistance to different antibiotics (%)				
	Citrobacter spp. n=10	*Klebsiella* spp. n=18	*Salmonella* spp. n=7	*Enterobacter* spp. n=5	*E. coli* spp. n=16
AMX (10 µg)	3 (30.0)	7 (38.9)	3 (42.9)	2 (40.0)	6 (37.5)
COT (10 µg)	1 (10.0)	2 (11.1)	1(14.3)	0	0
GEN (10 µg)	0	0	0	0	0
OFL (5 µg)	0	0	0	0	1 (6.3)
AUG (30 µg)	3 (30.0)	7 (38.9)	3 (42.9)	2 (40.0)	6 (37.5)
TET (30 µg)	3 (30.0)	2 (11.1)	0	1 (20.0)	3 (18.8)
NAL (10 µg)	0	0	0	0	0
NIT (300 µg)	0	0	0	0	0
ESBL	0	0	0	0	0
Plasmid	0	0	0	0	0

n, sample size; AMX, amoxicillin; COT, cotrimoxazole; GEN, gentamicin; OFL, ofloxacin; AUG, amoxicillin/clavulanate; TET, tetracycline; NAL, nalidixic acid; NIT, nitrofurantoin; ESBL, Extended Spectrum beta-lactamase.

to the commonly used fluoroquinolone class of antimicrobials have been reported in 15-20% of patients with bacteraemia caused by *E. coli* (Sharma *et al*., 2010).

CONCLUSION

The results revealed that the members of the Enterobacteriaceae encountered in this study contained neither ESBL nor plasmids, but few were resistant to different antibiotic classes. In addition, evolutionary relationship was noted more among isolates from distant than close isolation areas. The isolation of coliform bacteria (*E. coli*, *K. pneumoniae*, *C. freundii*, and *E. cloacae*) in snails represents a huge food safety risk to consumers of escargot. Hence, snails are reservoirs of pathogenic bacteria and high hygienic practices are required for consumers of snail meat to prevent infection with these pathogens. Awareness programs are highly recommended to the general public.

ACKNOWLEDGEMENTS

Authors wish to express appreciation to Babcock University for providing financial support for execution of this project through Babcock University grant number: BU/RIIC/2016/006.

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 ISSN (print): 1823-8262, ISSN (online): 2231-7538