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# A study of *Edwardsiella tarda* colonizing live Asian clam, *Corbicula fluminea*, from Pasir Mas, Kelantan, Malaysia with the emphasis on its antibiogram, heavy metal tolerance and genetic diversity

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**LEE, S. W., W. WENDY, M. C. ZALINA, A. MD RUHUL, H. SUKREE: A study of *Edwardsiella tarda* colonizing live Asian clam, *Corbicula fluminea*, from Pasir Mas, Kelantan, Malaysia with the emphasis on its antibiogram, heavy metal tolerance and genetic diversity. Vet. arhiv 83, 323-331, 2013.**

**ABSTRACT**

This paper deals with the antibiogram, heavy metal tolerance and genetic properties of *Edwardsiella tarda* colonizing live Asian Clam, *Corbicula fluminea* from Pasir Mas, Kelantan, Malaysia. Asian Clam is a popular snack for Malaysians. An antibiotic susceptibility test was carried out using the disc diffusion method against 19 types of antibiotics, namely: oxolinic acid, ampicillin, erythromycin, lincomycin, oleandomycin, amoxicillin, colistin sulphate, sulfamethoxazole, chloramphenicol, doxycycline, florfenicol, flumequine, kanamycin, nalidixic acid, novobiocin, tetracycline, fosfomycin, spiramycin and compound sulphonamides, whereas heavy metal tolerance of bacteria to $Hg^{2+}$, $Cd^{2+}$, $Cr^{6+}$ and $Cu^{2+}$ was tested using the two fold agar dilution method. The RAPD PCR profile of the bacteria was characterized using three different types of universal primers. It was found that there were most cases of antibiotic sensitivity (56.1%), followed by antibiotic resistance (38.6%) and antibiotic intermediate sensitivity (5.3%). Only kanamycin was found to be able to inhibit all the bacterial isolates present, whereas other bacterial isolates were resistant to fosfomycin, lincomycin, novobiocin and sulphamethoxazole. Additionally, all bacterial isolates were also resistant to all the heavy metals tested. The bacterial isolates present showed a percentage of genetic similarity ranging from 37.5% to 100%. In conclusion, the findings of the present study revealed that the sampled *C. fluminea* were highly exposed to antibiotic and heavy metal residues.

**Key words:** antibiogram, heavy metal, *Edwardsiella tarda*, *Corbicula fluminea*

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## Introduction

*Edwardsiella tarda* is a bacterium which has been reported to have caused several diseases in humans, such as meningitis, cholecystitis, endocarditis, osteomyelitis, soft tissue infections, bacteraemia and septicaemia (NELSON et al., 2009). Myonecrosis is another human disease caused by *E. tarda* (SLAVEN et al., 2001). Although many studies have claimed that human infections caused by *E. tarda* are rare, this bacterium has been frequently isolated from samples of human faeces, blood, urine, bile, peritoneal fluid and wounds. However, gastroenteritis due to *E. tarda* is the most frequently reported in infected humans (NELSON et al., 2009). Consuming contaminated seafood is identified as the source of the gastroenteritis infection.

Being a filter feeder, Asian clam tends to concentrate microbes present in the surrounding waters, which can cause severe illness in humans (COOK et al., 2002). Gastro-intestinal infections and related symptoms such as diarrhoea, fever, nausea, abdominal cramping, dehydration, headache, and vomiting are always blamed on the consumption of raw, semi-cooked or coliform bacteria contaminated shellfish (HOBBS and ROBERTS, 1993). However, there is no scientific report on the bacterial colonization of this clam. Hence, this paper is aimed at investigating the characteristics of *Edwardsiella tarda*, the bacteria which colonizes the Asian clam with the emphasis on the antibiogram, heavy metal tolerance and genetic property. The scientific information could be useful in understanding the nature of the food borne pathogen, *E. tarda,* concerning the risks to public health.

## Materials and methods

*Bacterial isolation.* A total of 50 pieces of live Asian clam (*Corbicula fluminea*), weighing 3 to 5 g each was purchased from the wet market at Pasir Mas, Kelantan, Malaysia. Ten grams of the flesh samples were homogenized with 100 mL of sterile physiological saline, followed by serial dilution and plating onto Xylose Lysine Dextrose (XLD) (Merck, Germany) selective agar. All the inoculated media were incubated at room temperature for 24 to 48 h. Bacterial colonies that grew on the selective media, with morphology clear in colour and a black spot in the centre, were randomly selected for identification test using conventional biochemical tests (HOLT et al. 1994) and confirmed by a commercial identification kit (BBL, USA).

*Antibiotic susceptibility test.* An antibiotic susceptibility test was conducted according to the Kirby-Bauer disk diffusion method using Mueller-Hinton agar (Oxoid, England) (BAUER et al., 1966). Pure cultures of the bacterial isolates (n = 110) were cultured in tryptic soy broth (TSB) (Oxoid, England) for 24 h at room temperature. The bacterial cells were then centrifuged at 14,500 rpm for 5 min using a minispin (Eppendorf, Germany). Concentrations of the bacterial cells were adjusted to $10^6$ colony forming unit

(CFU) using saline water and verified with a Biophotometer (BioRad, USA) before being placed onto the prepared Mueller Hinton agar (Oxoid, England). Antibiotic discs used for testing included: oxolinic acid (OA; 2 µg), ampicillin (AMP; 10 µg), erythromycin (E; 15 µg), lincomycin (MY; 15 µg), oleandomycin (OL; 15 µg), amoxicillin (AML; 25 µg), colistin sulphate (CT; 25 µg), sulfamethoxazole (RL; 25 µg), chloramphenicol (C; 30 µg), doxycycline (DO; 30 µg), florfenicol (FFC; 30 µg), flumequine (UB; 30 µg), kanamycin (K; 30 µg), nalidixic acid (NA; 30 µg), novobiocin (NV; 30 µg), tetracycline (TE; 30 µg), fosfomycin (FOS; 50 µg), spiramycin (S; 100 µg), and compound sulphonamides (S3; 300 µg) (Oxoid, England). Results were interpreted as sensitive (S), intermediate sensitive (I) and resistant (R) in accordance with the standard measurement of inhibition zones in millimetres (mm).

*Multiple antibiotic resistance (MAR) index determination.* The multiple antibiotic resistance (MAR) index of the present isolates against the tested antibiotics was calculated based on the following formula (LEE and WENDY, 2012): MAR index (multiple antibiotic resistance) = X / (Y x Z), X = total antibiotic resistance case; Y = total antibiotics used in the study; Z = total of isolates. A MAR index value equal to or less than 0.2 indicated that the tested antibiotics were seldom or never used for the Asian clam in terms of treatment, whereas a MAR index value higher than 0.2 implied that the Asian clam were highly exposed to the tested antibiotics.

*Heavy metal resistance test.* Bacterial tolerance to four elements of heavy metal, i.e., mercury ($Hg^{2+}$), cadmium ($Cd^{2+}$), chromium ($Cr^{6+}$) and copper ($Cu^{2+}$), was determined by the agar dilution method (MIRANDA and CASTILLO, 1998). Overnight, the bacterial suspension was spread onto plates of tryptic soy agar (TSA) medium, incorporated with different concentrations of $HgCl_2$, CdCl, $K_2Cr_2O_7$ and $CuSO_4$ (Fluka, Spain). Following two-fold dilutions, concentrations of both $Cd^{2+}$ and $Cr^{6+}$ ranged from 25 to 400 µg/mL, while concentrations of $Hg^{2+}$ and $Cu^{2+}$ ranged from 2.5 to 40 µg/mL and 150 to 2400 µg/mL, respectively. For the purpose of defining heavy metal resistance, the isolates were considered as resistant if growth was obtained at concentrations of 10 µg/ mL ($Hg^{2+}$), 100 µg/ mL ($Cd^{2+}$ and $Cr^{6+}$) and 600 µg/mL ($Cu^{2+}$) (ALLEN et al., 1977). The operational definition of tolerance used in this study was based on the positive bacterial growth when the concentration of heavy metals was above the concentration stated for resistance.

*Extraction of bacterial genomic DNA.* DNA extraction of the present isolates was done using the boiling technique (SAMBROOK and RUSSELL, 2001). Bacterial colonies of overnight culture on TSA were picked and suspended in Tris-EDTA (TE) buffer in a micro centrifuge tube (Eppendorf, Germany). The samples were heated at 95 °C using a water bath for 5 min, followed by immediate storage at -20 °C. The sample was then thawed at room temperature before it was centrifuged at 13,000 rpm for 10 min. The

supernatant was collected and transferred into new micro centrifuge tube whereas the pelleted cells were discarded.

*Quantification of DNA sample.* The DNA extraction of isolates present was screened for its quality and quantity using a Biophotometer (BioRad, USA) at an absorbance of 260 nm and 280nm. According to SAMBROOK and RUSSELL (2001), the extracted DNA was suitable for PCR reaction when the reading of the ratio of A260 : A280 of the DNA fell between 1.8 to 2.0. The quantity of the DNA of the isolates was then determined using the following formula:

$$\text{DNA quantity } (\mu g/mL) = \frac{A260 \times 50\ \mu g/mL \times \text{total volume } (\mu L)}{\text{Volume of sample } (\mu L)}$$

*RAPD-PCR assay.* PCR reactions were performed in a 25 µl PCR mixture containing 10 mM Tris-HCl, 50 mM KCl, 0.1% Triton® X-100, 2.5 mM $MgCl_2$, 0.5 µM universal primers, 0.2 mM nucleotide mix and 1.25 U of *Taq* DNA polymerase (Promega Madison, USA). Three primers used were 785 - 5'CCGCAGCCAA-3', 786 - 5' GCGATCCCCA-3' and 795 - 5'GAGACGCACA-3'. Amplifications for the primers were carried out by programming the thermal cycler (BioRad, USA) to the following profile: 30 cycles of 94 ºC for 1 min, 36 ºC for 1 min, 72 ºC for 1.5 min, and then kept at 72 ºC for 10 min.

*Gel electrophoresis and RAPD fingerprint.* The RAPD-PCR products, together with 1000 bp and 100 bp DNA markers (Fermentas, USA), were electrophoresed on 1% agarose gel containing ethidium bromide (5 µg/ µL), submerged in 1x Tris borate EDTA (TBE) buffer. The gels for electrophoresis used in this study were prepared by boiling 1% agarose powder in 1x Tris borate EDTA (TBE) buffer solution, together with 2 µL of ethidium bromide, and poured into a mould after being cooled to 50 °C. Before starting electrophoresis using an electrophoresis machine (BioRad, USA), the gel was submerged in a TBE buffer containing ethidium bromide at 5 µL/mL concentration. Using a 10 µL micro pipette, the RAPD-PCR products, which had already been mixed with a loading dye (Promega Madison, USA) were loaded into wells of the prepared gel. Electrophoresis was run at 110 V for 90 min. Subsequently, the RAPD-PCR fingerprints of the samples were visualized and captured using a UV transilluminator (BioRad, USA).

*RAPD analysis and genetic relationship.* A data matrix was generated based on the RAPD fingerprints of the isolates by giving scores of 0 or 1 for the absence or presence of bands, respectively, at each position for all isolates. The obtained data matrix was analysed by Numerical Taxanomy and the Multivariate Analysis System (NTSYSpc) version 2.1 (ROHLF, 2000) based on the unweighted pair-group method with arithmetic means (UPGMA) (SNEATH and SOKAL, 1973). The percentage of genetic similarity between the isolates was calculated according to the DICE (1945) formulation, as follows:

$$\text{Percentage of similarity, } F = \frac{2Nxy \times 100\ \%}{Nx + Ny}$$

where, Nxy = number of shared bands; Nx = total number of bands in lane X; Ny = total number of bands in lane Y.

**Results**

A total of 110 isolates of *Edwardsiella tarda* were successfully isolated from live Asian clam (*C. fluminea)*. All the bacterial isolates present were Gram negative. They showed positive responses in the tests of motility, indole production, citrate, maltose, mannose and glycerol utilization. However, all the bacterial isolates failed to utilize several sugars, such as arabinose, sucrose, rhamnose, xylose, mannitol and sorbitol. Antibiotic susceptibility results showed a total of 56.1% antibiotic sensitive cases, 5.3% intermediate sensitive cases and 38.6% resistant cases (Fig. 1), meanwhile the MAR value was 0.39. All the bacterial isolates were found to be resistant to fosfomycin, lincomycin, novobiocin and sulphamethoxazole (Fig. 2). On the other hand, kanamycin was the only antibiotic found to be effective in controlling all the bacterial isolates present. More than 80% of the bacterial isolates were found to be sensitive to chloramphenicol, doxycycline, erythromycin, florfenicol, flumequine, oxolinic acid, spiramycin and tetracycline. In the heavy metal tolerance test, all the bacterial isolates present were found to be resistant to all heavy metals tested. RAPD analysis further revealed the percentage of genetic similarity of *E. tarda* in a range of 37.5% to 100%

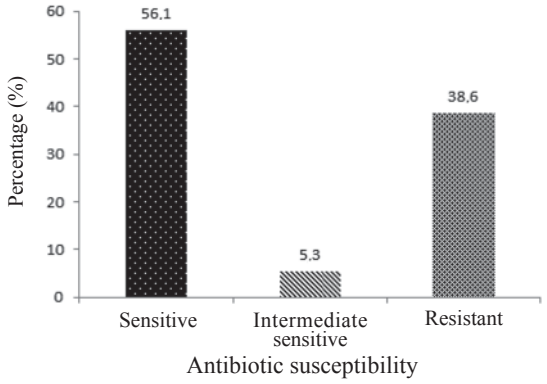


Fig. 1. Percentage of sensitivity of *Edwardsiella tarda* isolated from live Asian clam of Pasir Mas, Kelantan, Malaysia

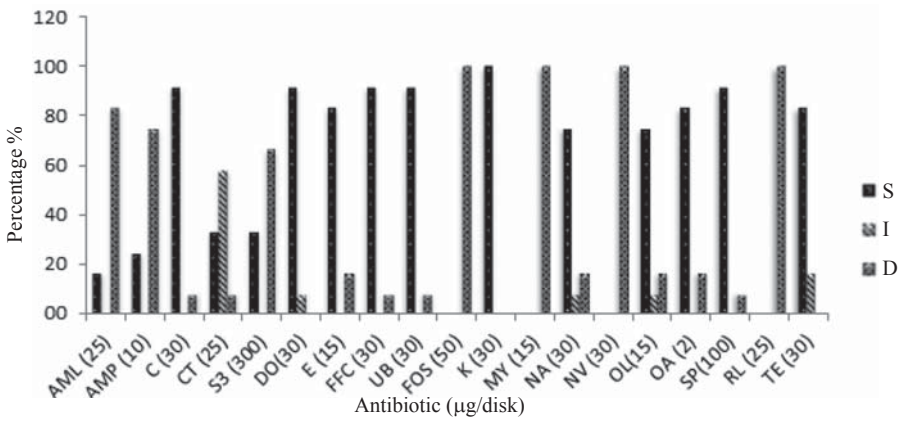


Fig. 2. Percentage of sensitivity of *Edwardsiella tarda* isolated from live Asian clam of Pasir Mas, Kelantan, Malaysia against 19 antibiotics

**Discussion**

This study revealed the phenotypic and genotypic characteristics of *E. tarda* colonizing the Asian clam of Pasir Mas, Kelantan, Malaysia. Findings from the study indicated the potential risk of gastrointestinal infection due to the consumption of Asian clam colonized by *E. tarda*. All isolated bacterial strains were pathogenic, as they performed beta hemolysis in human red blood cells.

Interestingly, kanamycin was the only antibiotic that could inhibit the growth of *E. tarda* isolates present. This suggests that kanamycin may be prescribed for gastrointestinal infection due to consumption of Asian clam contaminated by *E. tarda*. Other antibiotics, such as: chloramphenicol, doxycycline, erythromycin, florfenicol, flumequine, oxolinic acid, spiramycin and tetracycline, may also be used in treatment since a high percentage of antibiotic sensitive cases were observed among these antibiotics. Besides, fosfomycin, lincomycin, novobiocin and sulphamethoxazole may be used as a medium supplement to isolate pathogenic *E. tarda,* as these antibiotics failed to inhibit the growth of all isolated bacteria.

MAR index found in this study revealed that the sampled Asian clams were highly exposed to the tested antibiotics. This finding was similar to previous investigations of the MAR index among cultured aquatic organisms in Malaysia, including ornamental fish, bullfrogs (*Rana catesbeiana)* Malaysian Giant Prawns (*Macrobrachium rosenbergii)*, freshwater Asian Seabass (*Lates calcarifer)* and White Leg shrimps (*Litopenaeus*

*vannamei*) (LEE et al., 2009a, 2009b, 2009c, 2009d). However, LEE et al. (2011) discovered that *E. tarda* isolated from wild freshwater fish possessed a low MAR value, i.e., 0.15. Hence, we may conclude that an incidence of a high MAR index value may due to the level of contamination of the antibiotic residues in the sampled area, as the bacteria isolated from cultured aquatic animals displayed a higher MAR index compared to the bacteria from wild aquatic animals. Obviously, the wild, sedentary and filter feeding freshwater Asian clam has a greater chance of exposure to antibiotic residues accumulated in sediment, compared to the free-swimming fish.

Resistance to all tested heavy metals reported in the present study was also similar to the cases reported by LEE et al. (2009b, 2009c, 2009d) which involved all bacterial isolates, *E. tarda* isolates from cultured bullfrogs (*Rana catesbeiana*), Malaysian Giant Prawn (*Macrobrachium rosenbergii*) and freshwater Asian Sea bass (*Lates calcarifer*). Hence, we can conclude that the high incidence of heavy metal resistance may due to the high level of heavy metal residue contamination in the sampled area, due to agricultural and industrial activities.

NUCCI et al. (2002) and ACHARYA et al. (2007) reported that RAPD-PCR failed to discriminate *E. tarda* appropriately. This was also supported by the study of LEE and NAJIAH (2008), where *E. tarda* isolated from various freshwater fish was also not discriminated by RAPD-PCR, according to their hosts and sources. Thus, *E. tarda* may possess high genetic diversity due to environmental adaptation. A similar case was also reported in the study by RENDERS et al. (1996) where the percentage of genetic similarity of *E. tarda* in the study ranged from 37.5% to 100%.

In conclusion, this study was the first in reporting the presence of *E. tarda* in Asian clam. High antibiotic and heavy metal resistance activity of the bacterial isolates present was observed, giving us insight information on the degree of exposure of the sampled Asian clam to these antibiotic and heavy metal residues .

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**LEE, S. W., W. WENDY, M. C. ZALINA, A. MD RUHUL, H. SUKREE: Proširenost bakterije *Edwardsiella tarda* u azijske školjke *Corbicula fluminea* na području Pasir Mas, Kelantan u Maleziji s naglaskom na antibiogram, podnošljivost na teške metale i genetsku raznolikost. Vet. arhiv 83, 323-331, 2013.**

**SAŽETAK**

U radu je prikazan antibiogram, podnošljivost na teške metale i genetska svojstva bakterije *Edwardsiella tarda* naseljene na živoj azijskoj školjci *Corbicula fluminea* na području Pasir Mas, Kelantan u Maleziji. Azijska se školjka često upotrebljava za zakusku prilikom razonode u Maleziji. Osjetljivost na antibiotike određena je disk difuzijskim postupkom na 19 vrsta antimikrobnih tvari: oksolinsku kiselinu, ampicilin, eritromicin, linkomicin, oleandomicin, amoksicilin, kolistin sulfat, sulfametoksazol, kloramfenikol, doksiciklin, florfenikol, flumekvin, kanamicin, nalidiksičnu kiseinu, novobiocin, tetraciklin, fosfomicin, spiramicin i sulfonamide. Podnošljivost bakterije na $Hg^{2+}$, $Cd^{2+}$, $Cr^{6+}$ i $Cu^{2+}$ određena je postupkom dvostrukog razrjeđenja agara. PCR-om nasumično umnožena polimorfna DNA bakterije bila je određena upotrebom različitih tipova univerzalnih početnica. Ustanovljeno je da je 56,1% izolata bakterije bilo osjetljivo na antimikrobne tvari, 38,6% otporno, a 5,3% umjereno osjetljivo. Samo je kanamicin bio djelotvoran na sve pretražene izolate. Neki izolati bili su otporni na fosfomicin, linkomicin, novobiocin i sulfametoksazol. Svi bakterijski izolati bili su otporni na sve pretraživane teške metale. Genetska srodnost pretraženih izolata kretala se od 37,5% do 100%. Može se zaključiti da su uzorci školjke *C. fluminea* izloženi ostacima antibiotika i teških metala.

**Ključne riječi:** antibiogram, teški metali, *Edwardsiella tarda*, *Corbicula fluminea*