

Characteristics of pathogenic bacteria isolates associated with the gut and gill of molluscan shellfish, Eastern Cape, South Africa

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Abstract

Introduction: Infectious diseases attributable to the consumption of raw and lightly cooked molluscan shellfish are caused by bacterial agents that are associated either with human waste disposed into the ocean or those of marine origin. This has led to the increase in prevalence of bacterial agents and microbial resistance to antibiotics.

Method: Samples of *Perna perna* L. were dissected and homogenized for bacterial isolation. Six types of bacteria were isolated, identified and evaluated for their antimicrobial susceptibility profiling

Results: The microbial counts obtained revealed significant differences in the pathogenic bacteria populations found in the gut and gill of *Perna perna*. The morphological characteristics of the isolates were found to be of different shapes. All the isolates were catalase positive and oxidase negative except *Vibrio* spp. which was positive to the latter. Chloramphenicol and ciprofloxacin were the most effective antibiotics against all the isolates, each exhibiting 100% efficacy. The highest resistance to ampicillin was observed in *Salmonella* spp., *Shigella* spp., *Listeria monocytogenes* and *Staphylococcus xylosum*.

Conclusions: The results of this study revealed that marine habitats are reservoirs of bacterial pathogens and there is increased resistance to antibiotics, thereby constituting risks to public health. Therefore, appropriate surveillance measures to safeguard the health of shellfish consumers' should be established.

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Introduction

Marine ecosystems are increasingly contaminated through multifarious man-made activities such as the discharge of sewage and industrial effluents into the ocean. This has resulted into drastic reduction of shallow waters fish populations and invertebrates, ecological imbalance resulting into large scale disappearance of numerous flora and fauna, increased pollution of marine with chemicals and toxins which affect the microbial populations [1]. The microbiota of bivalve mollusca have been thoroughly investigated since mussels may concentrate pathogenic microorganisms in their tissues and as such cause diseases to humans through their consumption [1]. However, the South African Molluscan Shellfish Monitoring and Control Programme is working tremendously with the shellfish farmers, laboratories, National Regulator for Compulsory Specification (NRCS), Department of Health (DoH) and Municipalities to enforces microbiological monitoring in commercial shellfish farms in order to minimize the risk of disease and poisoning through consumption of molluscan shellfish [2].

The outbreaks of infections have been attributed to bacterial and viral agents that are associated either with human wastes disposed to the ocean or bacterial pathogens indigenous to coastal marine environments [3]. Besides accumulating naturally occurring microorganisms and other pathogenic microorganisms, these shellfish are also prone to contamination by toxin-producing dinoflagellates, faecal pathogens especially the species of *Salmonella*, *Shigella*, *Campylobacter*, *Listeria*, *Escherichia coli*, *Plesiomonas shigelloides* and enteric viruses from sewage-polluted waters [3]. Thus, the international Commission on Microbial Specification for Foods have rated raw molluscan shellfish as the second highest hazard of all foods [4].

Perna perna (L.) is one of the economically and commercially important marine bivalves found along the south and east coast of South Africa [5]. The species naturally inhabits rocky shores along

the coast and adjacent to estuaries that are increasingly contaminated with anthropogenic chemicals. It is harvested as a food source for its nutritional values. Along with the benefits derived from the consumption of *Perna perna* however, comes the potential risk of contamination. The harmful effect of the presence of the pathogenic microorganisms is augmented by the fact that *Perna perna* are consumed raw or under-cooked which may not completely ensure the inactivation of microbes or toxins, thereby creating a critical concern in terms of food safety [6]. *Perna perna* (L.) was chosen for this study because of its abundance and wide distribution along the east coast of South Africa, also it is commonly exploited for subsistence consumption [7].

Scientists have reported that the frequency of antibiotic resistance in bacteria from seafoods has significantly increased. The current treatment of infections caused by these bacteria should be re-examined as the severity of these infections makes antibiotic resistance in bacteria a critical public health concern [8, 9]. The worldwide emergence of multi-drug resistant bacterial strains has rendered many of the current drugs used for treatment useless, causing treatment failures [10].

In South Africa, there is a dearth of information available on the bacterial pathogens isolated from *Perna perna* (L.) and their antibiotic susceptibility profiles. Therefore, the present study was undertaken to examine the presence of microorganism in the gut and gill of *Perna perna* as well as determining their susceptibility to important antimicrobial agents.

Materials

Collection and samples preparation

Grab sampling of *Perna perna* (L.) was carried out in which about 200–250 live organisms were collected into sterilized containers from Algoa Bay (33°49'36"S,

25°47'42"E), Port Elizabeth, Eastern Cape Province of South Africa. They were transported in cooler boxes on ice packs to the Microbiology Department Laboratory at the University of Fort Hare, Alice, for dissection and examination. Samples were sterilized with 70% ethanol and rinsed three times in order to remove the attached bacteria. Shells were then opened using a sterile scalpel blade. The shell liquor of the mussels was discarded and the gill and gut were removed, homogenized and aseptically transferred to sterile test tubes.

Media used

All the enrichment broth and media used in this study were obtained from Oxoid Ltd (UK).

Bacteriological examination

Enrichment cultures

Twenty five grams (25 g) of the homogenized gills and guts was inoculated into 225 ml of the following selective enrichment broths for each isolates: Alkaline peptone water (Bacto peptone 1%, NaCl 1%) for *Vibrio* spp.; Tryptone soya broth with Casamino acids for *Escherichia coli*; Buffered peptone broth, Rappaport-Vassiliadis broth and Tetrathionate broth for *Salmonella* spp. pre-enrichment and selective enrichment steps respectively. *Shigella* broth to which novobiocin (0.5 µg/ml) had been added for *Shigella* spp.; *Listeria* Enrichment broth for *Listeria* spp. and Tryptic soy broth plus 10% NaCl for *Staphylococcus* spp. Serial dilutions (up to 10⁻³) were made into the appropriate broth. All broths were incubated at 37°C for 24 h [11].

Conventional culture method

Aliquots of 0.2 mL of the 10⁻³ dilution from each of the enrichment medium was poured into the appropriate agar media plates as follows: Agar plates of Thiosulphate Citrate Bile Sucrose for *Vibrio*, Xylose lysine desoxycholate agar for *Escherichia coli*, *Salmonella-Shigella* agar for *Salmonella*, MacConkey agar for *Shigella*, PALCAM agar for *Listeria*

and Baird-Parker agar for *Staphylococcus*. The plates were incubated at 37°C for 18-24 h [11]. Assay was carried out in three replicates.

Isolation and identification

Individual colonies from each agar media were randomly selected and sub-cultured. The plates were placed on a colony counter and the number of colony forming units was taken. The following tests were carried out on all the pure isolates: Gram staining, oxidase test strips (Oxoid, UK), catalase using 3% hydrogen peroxide (H₂O₂). Analytical profile index (API) 20E and API staph system] (bio-Mérieux, Marcy l'Etoile, France) were used for the identification [12].

Scanning electron microscopy examination of the isolates

For electron microscopy examination, the samples were coated with gold palladium and examined using JEOL JSM 6390 LV SEM at 15Kv (Japanese Electron Optical Lab.) Scanning Electron Microscope (SEM).

Antimicrobial susceptibility profiling

Susceptibility of the isolates against different antimicrobial agents was determined *in vitro* by employing the standardized agar disc-diffusion method according to Clinical and Laboratory Standards Institutes [13].

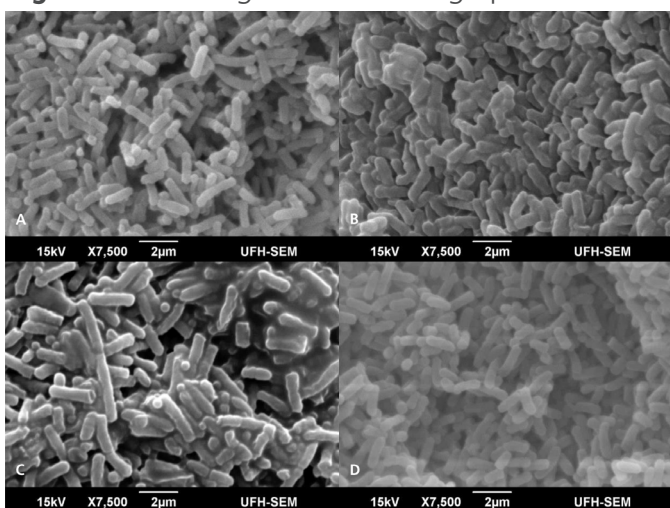
Statistical analysis

The experimental data was expressed as mean ± standard deviation (SD) of the three replicates. Statistical analysis was done by using MINITAB program (version 17 for Windows) (Minitab Inc., Pennsylvania, USA). One-way analysis of variance (ANOVA) was used to compare the data in each sample among the isolates. p < 0.05 was considered statistically significant.

Table 1. Identification of bacterial isolates from *Perna perna*

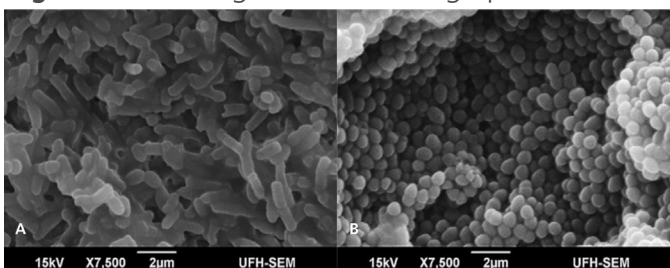
Shape	Gram reaction	Oxidase test	Catalase test	Colonial morphology	API test (%)	Identification
Curved rod	Negative	Positive	Positive	Yellow coloured colonies	95	<i>Vibrio fluvialis</i>
Rod	Negative	Negative	Positive	Pink colonies to red bile salt, precipitate surrounding the colonies	99.9	<i>Escherichia coli</i>
Rod	Negative	Negative	Positive	Colourless with black centre, Light pink colonies with distinct black centre	99.9	<i>Salmonella spp.</i>
Rod	Negative	Negative	Positive	Smooth, colourless colonies	95	<i>Shigella spp.</i>
Rod	Positive	Negative	Positive	Gray green surrounded by dark halo	99.9	<i>Listeria monocytogenes</i>
Cocci	Positive	Negative	Positive	Jet black colonies surrounded by clear zone	80	<i>Staphylococcus xylosus</i>

Figure 1: Scanning Electron Micrograph of Isolates.



A) Curved rod shaped *Vibrio cholerae*; B) Rod-shaped *Escherichia coli*; C) Rod-shaped *Salmonella spp.*; D) Rod-shaped *Shigella spp.*

Figure 2: Scanning Electron Micrograph of Isolates.



A) Rod-shaped *L. monocytogenes*; B) Grape-like cluster *S. xylosus*

Results

Isolation and identification of bacteria in *Perna perna*

In this study, six types of bacteria were isolated from *Perna perna*, identified and evaluated for their antimicrobial susceptibility profiling. The results indicated that four of the isolates were Gram-negative and two Gram-positive. Individual isolates from both the gill and gut had similar morphological shapes when observed under light and scanning electron microscopes. Four of the isolates were rod shaped, one curve shaped and one coccus shaped (**Table 1, Figure 1-2**). All were catalase positive, *Vibrio spp.* was oxidase positive while the remaining were oxidase negative. The bacterial isolates were identified with >80% acceptable probability using analytical program interface identification system and the colonial characteristics of the isolates were equally noted. The microbial counts of *V. fluvialis*, *E. coli*, *Salmonella spp.*, *Shigella spp.*, *S. xylosus* and *L. monocytogenes* isolated from the gut and gill of *Perna perna* are significantly different ($p < 0.05$) as shown in **Figure 3**.

Antibiotic susceptibility of the identified isolates

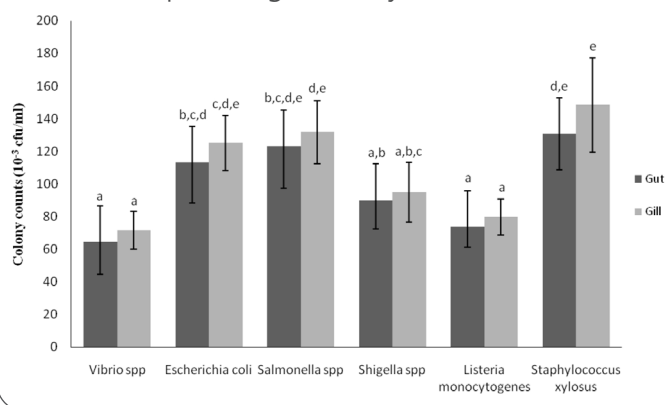
The susceptibility and resistance patterns of the isolated bacteria are shown in **Table 2**. The overall

Table 2. Susceptibility pattern of the isolates against different antibiotics.

Antibiotic susceptibility of isolates						
Antimicrobial agent	<i>V. cholerae</i>	<i>E. coli</i>	<i>Salmonella</i>	<i>Shigella</i>	<i>L.monocytogenes</i>	<i>S. xylosus</i>
K	R	S	S	S	R	R
AMP	-	S	R	R	R	R
NA	R	I	S	S	R	R
CIP	-	S	S	S	S	S
C	S	S	S	S	S	S
TE	S	S	S	S	R	S
EFT	-	S	S	S	R	R
AK	-	S	S	S	R	R

K: Kanamycin, AMP: Ampicilin, NA: Nalidixic acid, CIP: Ciprofloxacin, C: Chloramphenicol, TE: Tetracycline, EFT: Ceftiofur, AK: Amikacin, Intermediate: I, Resistant: R, Sensitive: S, --: not tested

Figure 3: Scanning Distribution of bacteria pathogens in the gut and gill of *Perna perna* (L.) collected from Algoa Bay. Data are presented as means \pm SD of three replicates. Bar graphs with different superscript are significantly difference ($P < 0.05$).



susceptibility of the isolates to antibiotics was 64% while resistance was 34% with the rest being intermediate. Chloramphenicol, tetracycline and ciprofloxacin proved to be more effective than the other antimicrobial agents used in this study. Generally, the highest susceptibility was observed in chloramphenicol 21.4%, followed by tetracycline and ciprofloxacin with 17.9% each while the lowest was seen in ampicillin. About 80% of the isolates showed resistance to ampicillin while none was resistant to ciprofloxacin and chloramphenicol.

Discussion

Bacterial populations in the gill and gut of *Perna perna*

The bacteria growth pattern in the samples show that *Staphylococcus* spp., was predominant; followed by *Salmonella* spp., *E. coli*, *Shigella* spp., *L. monocytogenes* and *Vibrio* spp., respectively. This finding agrees with previous report that shellfish are prone to contamination by faecal pathogen from polluted waters [14].

Several *Vibrio* species, native to both marine and estuarine environments have been identified as the causative agents of human illnesses in association of shellfish consumption [3]. Also, the occurrence of *E. coli* in shellfish has been encountered in different parts of the world [15], while the presence of *Salmonella* spp. in seafood and water cause salmonellosis, characterized by fever along with gastroenteritis, abdominal cramps and diarrhea [16]. These organisms are the leading causes of food-borne illnesses worldwide [17]. *Shigella* spp. have been isolated from shellfish and its infections manifested normally in watery, loose stools to more severe symptoms, including fever, abdominal pain, tenesmus and bloody diarrhea [17]. *Shigella* is important agent of diarrheal disease as it has a low infectious dose and long survival time in clams and oysters [18]. The

low counts of *L. monocytogenes* and *Vibrio* spp. in this study confirmed the findings of Feldhusen [14] who observed that indigenous pathogenic bacteria are usually found at fairly low levels in fresh food samples. *L. monocytogenes* causes human disease called listeriosis and frequently related to seafood consumption [19]. *Staphylococcus* spp. possesses the ability to cause large scale foodborne disease outbreak through the consumption of shellfish [20].

Antibiotic susceptibility of the isolates

The increasing emergence of antibiotic-resistant pathogens associated with fish and shellfish is of major public health concern [21]. The overall susceptibility of the isolates to antibiotics was 64% while resistance was 34% with the rest being intermediate. The isolates were mostly susceptible to chloramphenicol, tetracycline and ciprofloxacin. This finding agreed with a previous study carried out by Ahmed et al. [22] where *Staphylococcus* spp. was susceptible to ciprofloxacin, chloramphenicol and tetracycline isolates from fish belly. The incidence of *Vibrio* infections due to the consumption of raw or improperly cooked seafood has shown a sustained increase since 2000 in USA [23]. The first-line drugs including tetracycline and fluoroquinolones have remained highly effective against *Vibrio* spp. [24].

Both *Shigella* spp. and *Salmonella* spp. were susceptible to all the antibiotics used in this study except ampicillin, against which resistance was recorded. The sensitivity of enteric *Salmonella* spp. to these antibiotics have been observed in different regions of India [24]. *L. monocytogenes* isolate in this study was found to be susceptible to antibiotics that can be used to treat individuals with listeriosis. In addition, a combination of ampicillin and aminoglycoside can be also used for treatment of listeriosis [25].

About 80% of our isolates showed resistance to ampicillin while none was resistant to ciprofloxacin and chloramphenicol. In recent years, resistance to ampicillin is often observed in bacterial isolates from

marine environments [26]. The present study indicates that bacteria isolated were resistant to more than one antibiotic (**Table 2**). This result shows that spread of multidrug-resistant isolates in seafood will be of great concern for public health [27].

Conclusion

The present study revealed that there are bacterial pathogens in the gut and gill of shellfish which may pose health risk to the consumers if eaten raw or slightly cooked. The presence of these bacterial pathogens is often due to sea pollution with human and animal sewage. The study also reveals that some of the bacterial isolates were multidrug-resistant.

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Conflict of interests

The authors declared no conflict of interests.

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