Antimicrobial resistance and toxigenic profiles of bacteria isolated from tropical shrimps (*Farfantepenaeus notialis* and *Penaeus monodon*) in Cameroun

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Research note

Keywords: Spoilage, shrimps, Bacillus, resistance genes, enterotoxins

Posted Date: July 16th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-22912/v2

Version of Record: A version of this preprint was published at BMC Research Notes on July 29th, 2020. See the published version at https://doi.org/10.1186/s13104-020-05184-1.

Abstract

Objective: Post-harvest shrimp losses are a big problem due to the proliferation of spoilage bacteria. Presence and multiplication of these bacteria promotes the emergence of food-borne illnesses. This study was carried out to characterize specifics spoilage bacteria from tropical brackish water shrimp and black tiger shrimp shrimps.

Result: 22 Bacillus spp ; 09 SCNs staphylococcus and 04 enterobacteria such as 01 Pantoea genus ; 01 Serratia plymutica and 02 Serratia rubidaea have been identified. Resistance and virulence genes were searched. All isolates expressed resistance to at least three of antibiotics tested. 03 strains of enterobacteria were sensible to cetfazidim and Amoxicillin-Clavulanic Acid . Bacillus showed complete susceptibility to Cefixim, ertapenem and cetfazidim. Staphylococococci were susceptible to clyndamycine. Pantoea spp was resistant to all antibiotics but exhibited intermediate susceptibility to Amoxycyclin-Clavulanic Acid. 04 strains of Staphycolocci were positive to mecA genes. None enterobacteria were positives to tetracycline resistance genes. All the strains of Bacillus contained enterotoxin genes, high prevalence of 21 strain to hemolytic enterotoxins and 17 strains to cell-lyse factor production like sphingomyelinase activities. The majority of Bacillus strains present in the study poses a potential risk of food poisoning due to the prevalence of toxin genes found.

Introduction

Shrimps are great component of global seafood production. In general, they contain good quantities of digestible proteins, essential aminoacids, bioactive peptides, long-chain polyunsaturated fatty acids (Venugopal, 2009). In Cameroun, tropical brackish water shrimp (*Farfantepenaeus notialis*) and black tiger shrimp (*Penaeus monodon*) are species widely consumed (MINEPIA, 2014). Shrimps are generally safe for consumption but their exposure to handling practices may occasionally entail health risks (Pang 2002; Yang et al., 2017, Don et al., 2018). Many retailers inappropriately stored shrimps in addition to poorly handling practices causing postharvest degradation (Nga Ombede et al., 2018). Specific spoilage organisms will change characteristics of food as consequence of contamination (Leisner, 2000). Many studies showed emergence of foodborne diseases due to spoilage bacteria (Chaves et al., 2011). Gram-positive bacteria are slightly dominant in tropical warm water (Al Bulushi et al., 2010). This suggests their great implications in spoilage, especially at ambient temperature (Dabadé et al., 2015). Foodborne illness could result from the fact that those bacteria harbor enterotoxins and resistance genes (Chaves et al., 2011). The aim of this study was then to explore antibiotics resistance and virulence factor of spoilage bacteria isolated from shrimps stored in ambient temperature (25°C) in our markets.

Material And Methods

Collection and storage of samples

Experiments were carried out with 5 kilograms of tropical brackish water shrimp (*Farfantepenaeus notialis*) and the same quantity of black tiger shrimp (*Penaeus monodon*). The sampling took place from November to December 2018. Freshly harvested shrimp were purchased from 'Youpwe fisheries market' in Douala, Cameroon. Those samples were transported to the laboratory in iced box. They were then packed into four groups of 250 g portions per species in sterile plastic bags and kept at -20°C until lab processing. Every two days, one plastic bag from each species was randomly taken for enumeration and isolation of total spoilage bacteria. Before this, shrimps were removed from fridge and left at 25°C for 24h in the laboratory. This was done at room temperature because shrimps are sold in the markets without cold conditions and allowed spoilage odor to be produced.

Identification of spoilage bacteria

25 grams of shrimp per specie were aseptically added to 225 mL of peptone water and homogenized (ISO, 2013). Seven fold dilutions of each homogenate were prepared and 0.1 ml of 10^{-7} dilution was used for enumeration and isolation on nutrient agar. Replicate plates were incubated in aerobiosis and in anaerobiosis at 37°C. Thereafter counting, three characteristic isolates per plate were purified on Mueller Hinton. Pure isolates were stored at -80°C in 20% glycerol. Identification of bacterial isolates was then carried out using biochemical tests.

Detection of resistance profile

An overnight bacterial pre-culture was diluted to obtain a turbidity of 0.5 McFarland. Kirby Bauer techniques were used to perform susceptibility testing (Antibiogram Committee of the French Society of Microbiology, CA-SFM, 2012). Broth cultures were aseptically swabbed on Mueller Hinton agar. Antibiotic disks were placed on the agar plates and incubated 24 hours at 37°C. Inhibition zone diameters were measured after 24hours. Susceptibility or resistance was determined according to CA-SFM (2012). Antibiotic disks (HIMEDIA, India) used were amoxicillin (30 µg), amoxicillin-clavulanic acid (30 µg), cefixim (5 µg), ceftazidim (30 µg), ceftriazone (30 µg), ciprofoxacin (5 µg), clindamycin (10 µg) colistin (25 µg), ertapenem (10 µg), erythromycin (15 µg) fosfomycin (50 µg), imipenem (10 µg), nalidixic acid (30 µg), oxacillin (1 µg), penicillin (10 µg), pristinamycin (15 µg) and vancomycin (30 µg).

Molecular identification of antibiotic resistance genes

DNA was extracted from the overnight colony obtained by spreading on Muller Hinton agar. DNA was extracted from isolates using Qiagen blue extraction kit. PCR mixture of 23 µl contained 10 Nmole of genomic DNA, 0,5 µl of each primer, 0.5 µl dNTP, 2.5 µl Buffer with MgCl2, 0,125 µl Taq DNA Polymerase, and 16 µl distilled water. For enterobacteria and staphylococci isolates, PCR detection was performed for the following different resistance genes : macrolide-resistance genes, vancomycin resistance genes, methicillin resistance genes and tetracycline resistance genes. PCR conditions included initial

denaturation for 5 minutes at 94 °C, followed by 40 cycles of denaturation for 45s at 94 °C, annealing for 55s at 45°C, extension for 90 s at 72 °C and final extension for 10 min at 72 °C. PCR conditions for mecA were in accordance with those described by Igbinosa and Beshiru (2019). For *Bacillus*, virulence and enterotoxins genes were investigated. Genes encoding hemolysin (hbl-D/A), non-hemolytic enterotoxin (nheB), *B. cereus* enterotoxin T (bceT) and enterotoxin FM (entFM) were screened. Table S1 show the primers used in the study. Virulence factors targeting genes coding for two phospholipases associated with cell lysis, sphingomyelinase (sph) and phosphatidylinositol-specific phospholipase C (piplc) were also investigated in accordance with Matarante et al. (2004) and Mohammadou et al. (2014). PCR products were analyzed on 1.5% (w/v) agarose gel stained (120 volts at 35 min) with ethidium bromide (0.5 µg/ml) and visualized by UV. Table S2 shows the primers of virulence factor genes detection.

Results

Identification of spoilage bacteria

Shrimps contained more than 10^7germs/g after 24 hours in ambient storage. Figure S1 shows the microbial population present in tropical shrimps. Results showed the main presence of Gram-positive bacteria such as *Bacillus spp* and Coagulase Negative Staphylococci (CNS). Some isolates of enterobacteria (*Serratia spp* and *Pantoea spp*) were also identified.

Resistance profile

All the isolates of Coagulase Negative *Staphylococci* were susceptible to clindamycin. They showed total resistance to oxacillin and penicillin. The isolates were resistant to ciprofloxacin, colistin and vancomycin (55.56%), to nalidixic acid (88.89%). 15 isolates showed resistance to ceftazidim, ceftriaxon, cefixim and amoxicillin. Table 1 presents the antimicrobial susceptibility profile of *Staphylococci*.

Table 1: Antimicrobial susceptibility of *Staphylococci*

Antibiotics	S if ≥	R if <	S %	I %	R %
Ciprofloxacin	25	22	**3** (33.33%)	**1** (11.11%)	**5** 5.56%)
Pristinamycin	22	19	**5** (55.56%)	**1** (11.11%)	**3** (33.33%)
Penicillin	29	18	**0**	**0**	**9** (100%)
Fosfomycin	14	14	**6** (66.67%)	**0**	**3** (33.33%)
Erythromycin	22	17	**5** (55.56%)	**1** (11.11%)	**3** (33.33%)
Clindamycin	15	15	**9** (100%)	**0**	**0**
Nalidixic Acid	20	15	**1** (11.11%)	**0**	**8** (88.89%)
Vancomycin	17	-	**4** (44.44%)	**0**	**5** (55.56%)
Oxacillin	21	21	**0**	**0**	**9** (100%)
Colistin	15	15	**4** (44.44%)	**0**	**5** (55.56%)

S= sensible; R= resistant; I= intermediate

Enterobacteria showed total resistance to ceftriazon, cefixim, amoxicillin and ertapenem. Only isolates of *Pantoea spp* showed intermediate resistance to amoxicillin - clavulanic acid and total resistance to all other antibiotics tested. *Bacillus spp* were susceptible to imipenem. 59.09% of *Bacillus spp* exhibited intermediate resistance to ciprofloxacin and

amoxicillin-clavulanic acid. Table 2 shows antimicrobial susceptibility profile of enterobacteria and *Bacillus spp*.

Table 2: Antimicrobial susceptibility of enterobacteria and Bacillus species

Antibiotics			Enterobacteria			*Bacillus spp*		
	S if≥	R if <	S	I	R	S %	I %	R %
Amoxicillin - clavulanic Acid	23	16	3	1	-	**8** (36.36%)	**13** (59.09%)	**1** (4.55%)
Vancomycin	17	-	2	-	2	**7** (31.82%)	**-**	**15** (68.18%)
Ceftazidim	21	19	-	1	3	**-**	**-**	**22** (100%)
Impenem	21	18	3	-	1	**22** (100%)	**-**	**-**
Ciprofloxacin	25	22	1	2	1	**6** (27.27%)	**6** (27.27%)	**10** (45.45%)
Ceftriazone	26	23			4	**0**	**1** (4,55%)	**21** (95,45%)
Cefixim	25	22			4	**0**	**0**	**22** (100%)
Amoxicillin	23	16			4	**0**	**1** (4,55%)	**21** (95,45%)
Ertapenem	28	26			4	**0**	**0**	**22** (100%)

S= sensible; R= resistant; I= intermediate

Resistance and enterotoxins-virulence genes

Bacillus isolates isolated harbor at least two enterotoxins genes. Only one isolate was positive to hemolysin gene. Three isolates were negative to sph and piplo virulence genes. All *Bacillus* isolates (08) harbor *Bacillus cereus* enterotoxin T and also the sph gene. 04 isolates of *Staphylococci* exhibited the presence of MecA resistant gene. All enterobacteria isolates were tested negative to tetracycline resistance gene. Figures 1 shows the results of PCR product migration.

Discussion

Identification of spoilage bacteria

This study was carried out to explore resistance and virulence genes of spoilage bacteria isolated from shrimps in Cameroun. Results of this study are similar to those of Dabadé et al. (2015) who mainly found positive Gram bacteria, especially lactic bacteria and enterobacteria in black tropical brackish water shrimp. Those Authors revealed that potentially spoilage bacteria in tropical shrimps in Benin may be H_2S-producing dominant group of bacteria. Finding in this study suggests that *Bacillus* and *Staphylococci* species are responsible for spoilage detected in tropical shrimp. The results of this study are different from those of Srinivasan and Saranraj (2017) who found *Vibrio cholerae, Pseudomonas fluorescens, Salmonella* Typhi*, Staphylococcus aureus* and *Escherichia coli*. These microorganisms are common in polluted water, so it is possible to find them in the seafood. The results obtained are comparable to those of Al Bulushi et al. (2010) who who also isolated *Staphylococci* in their study. They also related that the frequency of *Staphylococci* in marine environments depend on geographic location. *Bacillus* species in coastal fisheries are also common, despite the relative abundance of *Staphylococcus spp* (Al bulushi et al., 2010). Abundance of *Bacillus* in coastal waters and marine fisheries depends on temperature, depth and decline distance from the coast and, suggests that *Bacillus* species in coastal water originate from runoff (Rüger et al., 2000). Mohammadou et al. (2014) reported *Bacillus* species such as *Bacillus cereus, Bacillus thuringiensis* and *Bacillus anthracis* were well-known as food poisoning in spoilage food. Gram-positive bacteria were more abundant than Gram-negative in tropical water. According to Al bulushi et al. (2010), their influence in spoilage remain little probed. Findings of this study also showed low frequency of enterobacteria such as *Serratia* and *Pantoea* isolates who grow in a broad range of temperatures and substrates according to Garrity et al. (2005). Many species related with food spoilage, have been described as opportunistic human pathogens (Mardanehand and Mohammad, 2013; Mahlen, 2011; Déletoile et al., 2009).

Antimicrobial susceptibility profile

The presence of *Pantoea spp* and *Serratia spp* in food is often neglected, when compared to classic multidrug-resistant Gram-negative pathogens (Halpern et al., 2011, Melo et al., 2018, Cunningham and Leber. 2018) likewise in this study. They are opportunistic pathogens among the most common causes of nosocomial diseases and transmissible by food ingestion. Resistance diversity of *Bacillus* isolates in the study is not similar to the works of Mohammadou et al. (2014). Those authors revealed relative susceptibility of *Bacillus* isolates to antibiotics unlike our results. Chaves et al. (2011) also found susceptibilities of *Bacillus* isolated from Brazilian food to gentamicin and tetracycline. Drug resistance of isolates in this study may be attributed to the spores of *Bacillus* species. Mohammadou et al. (2014) found that *B. subtilus* was able to produce bacteriocins such as subtilin in Mbuja.

Resistance and enterotoxins-virulence genes

Tetracycline resistance genes can be explored to current and emerging multidrug-resistant pathogens including carbapenem-resistant Enterobacteriaceae. None enterobacteria presented tetracycline resistance genes, although they were all resistant to ertapenem. MecA genes as presented in this study about *Staphylococci* are similar to those of Ali (2014) and Igbinosa and Beshiru (2019). MecA gene is present in all *Staphylococci* isolates and is known to encode penicillin binding protein 2a (PBP2a). Beta-lactam resistance is mostly attributed to mutations in mecA gene, but other genetic elements may also be considered for the explanation of the mechanism of resistance. Half of *Bacillus* isolates were positive to HBL genes. These results are similar to those of Chaves et al. (2011). The same authors demonstrated prevalence of hemolytic toxins in *Bacillus* isolates from fermented foods. Many authors correlate prevalence of enterotoxins HBL-NHE (Boonchai et al., 2008). According to Trans et al. (2010), wall peptidase (sph genes and PIPLC genes) are involved in adhesion, biofilm formation and virulence. Prevalence of entFM genes in more than half of *Bacillus* isolates is located on the chromosome and appears to be common to *Bacillus thuringiensis* and *B. cereus*. Detection of EntFM is similar to the results of Mohammadou et al. (2014). Presence of Bcet gene is evidence of presence of *B. cereus* among isolates. Agata et al. (1995) also demonstrated that prevalence of this gene carried out other isolates than *B. cereus*. The majority of the isolates isolated pose a potential risk of food poisoning due to their antibiotic resistance profile and the prevalence of toxin genes found in *Bacillus* isolates. The surveillance of the quality control of those seafoods should be enhanced in the country, from the water to the final sellers. This is important and urgent to protect consumers' health.

Limitations: To better understand the links between spoilage and the bacteria involved, it would be useful to identify isolates using advanced methods as 16S rRNA gene sequencing or species-specific PCR. The strains will be kept at -20°C for sequencing in Helsinki (Finland).

Abbreviations

CNS : Coagulase Negative *Staphylococci*

Declarations

Ethics approval and consent to participate: Not Applicable.

Consent for publication: Not Applicable.

Availability of data and materials: All data generated or analyzed during this study are included in this published article and supplementary information files.

Competing interests: Authors declare no competing interest.

Funding: This study has been self-funded by the authors.

Authors' contributions: Authors' contributions: SNNO, FT and VD drafted the protocol. HK, ED, RPJMN, JPG, CT, AY performed the analysis. HK, ED and VD carried out the statistical analysis. SNNO, VD, ED wrote the draft of publication. AY and JD supervised the work and reviewed the manuscript. All the authors read the final version of the manuscript.

Acknowledgements: The authors are grateful to Prof Pascal HOUNGNANDAN, National University of Agriculture, Ketou (Benin) who allowed this collaboration between the both teams from Benin and Cameroon.

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Figures

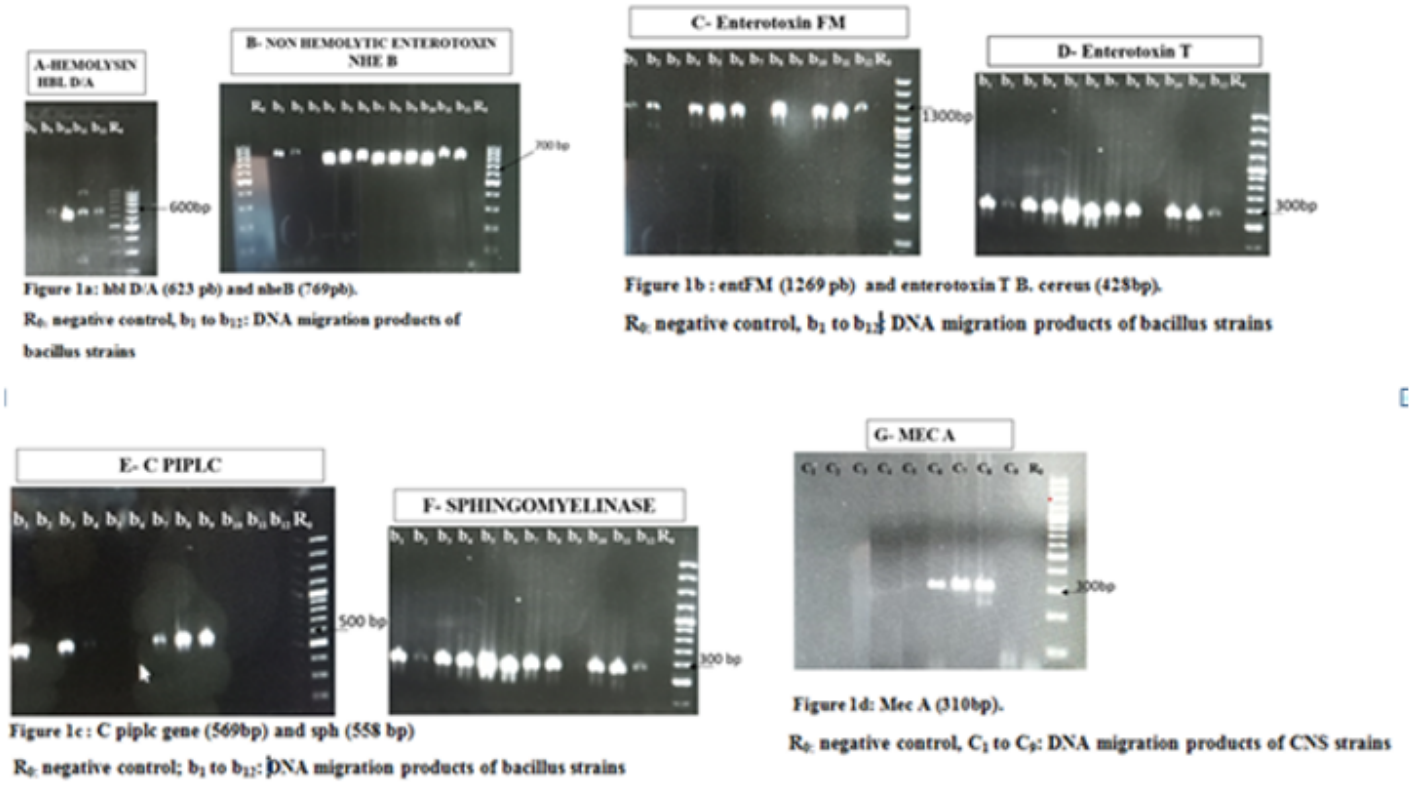

Figure 1a: hbl D/A (623 pb) and nheB (769pb).
R_0: negative control, b_1 to b_{12}: DNA migration products of bacillus strains

Figure 1b : entFM (1269 pb) and enterotoxin T B. cereus (428bp).
R_0: negative control, b_1 to b_{12}: DNA migration products of bacillus strains

Figure 1c : C piplc gene (569bp) and sph (558 bp)
R_0: negative control; b_1 to b_{12}: DNA migration products of bacillus strains

Figure 1d: Mec A (310bp).
R_0: negative control, C_1 to C_9: DNA migration products of CNS strains

Figure 1

PCR gene migration

Supplementary Files

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