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Isolation, molecular characterization and antimicrobial resistance of enterobacteriaceae isolated from fish and seafood

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ABSTRACT

This current study attempted to examine the occurrence rates of Enterobacteriaceae in seafood samples collected from different fishmongers in the province of Constantine (Northeast Algeria) over a one-year period. Total and fecal coliforms were enumerated and selected Enterobacteriaceae colonies were identified. *Escherichia coli* (*E. coli*) and *Salmonella* isolates were serotyped, after which their antimicrobial susceptibility patterns were determined. Extended-spectrum beta-lactamases (ESBL) encoding genes, virulence genes (*stx1*, *stx2* and *eae*) and plasmid-harboring were searched among *E. coli* isolates and their clonality was also assessed by pulsed-field gel electrophoresis (PFGE). Results showed that fecal coliform contamination levels are beyond the recommended limits. All strains of *Salmonella* (n = 2) isolated were of serovar infantis. One *E. coli* isolate from sardines belonged to serotype O127 and two others (one from sardines and one from shrimps) were identified as serotype O125. One *E. coli* isolate from sardines was characterized as enterohemorrhagic (EHEC) and six others as shiga toxin-producing (STEC) (three from sardines and three from shrimps). All tested strains were multidrug resistant, with five *E. coli* strains showing an ESBL phenotype and harboring the $bla_{CTX\text{-}M\text{-}15}$ gene (four from sardines and one from shrimps). Plasmids of differents sizes were extracted from seven *E. coli* isolates (five from sardines and two from shrimps) with many strains carrying more than one plasmid. *E. coli* isolates belonged to two phylogenetic groups (B1 and, mainly, E) and scattered on eight distinct clones as defined by PFGE. Some strains are grouped within the same cluster regardless of their origin and characteristics, indicating a common source of contamination. These findings demonstrate that, in the study region, sardines and shrimp may play an important role in the dissemination of virulent and multi-drug resistant enterobacteria strains.

1. Introduction

Fish and seafood are important sources of health-promoting compounds such as high-quality proteins, vitamins (A, D, E and B12), selenium and iodine, as well as polyunsaturated fatty acids (eicosapentaenoic and docosahexaenoic), which are beneficial for cardiac arrhythmias, cancer and hypertension, and play a crucial role in the maintenance of neural functions and the prevention of certain psychiatric disorders (Ortega, 2006; Swanson, Block, & Mousa, 2012).

However, the fact that fish and seafood are highly vulnerable and perishable products may make them harmful to consumers in some circumstances. The related health hazards can be classified as chemical, physical and biological (FDA, 2011). Pathogens of different bacterial taxa found in seafood and related products can be divided into three categories: bacteria often present in the

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habitat of the consumed species, such as *Vibrio* spp., non-proteolytic *Clostridium botulinum (C. botulinum)*, *Plesiomonas shigelloides* and *Aeromonas* spp.; bacteria present in the environment in general, such as *Listeria monocytogenes*, proteolytic *C. botulinum*, *Clostridium perfringens* (*C. perfringens*) and *Bacillus* spp.; bacteria present in the environment in general, such as *Listeria monocytogenes*, proteolytic *C. botulinum*, *C. perfringens* and *Bacillus* spp.; and finally bacteria which have their natural habitat in man or animals, including *Salmonella* spp., *Shigella* spp., *Escherichia coli*, *Campylobacter jejuni* and *Staphylococcus aureus* (Huss, Ababouch, & Gram, 2003).

In most cases, the bacterial hazards associated with seafood consumption do not differ significantly from those related to other foods (Lee, Rangdale, Croci, Hervio-Heath, & Lozach, 2008). Ingesting seafood contaminated with pathogenic microorganisms and/or their toxic by-products can lead to food-borne illnesses ranging from mild gastroenteritis to life-threatening syndromes, in the form of infection, intoxication, or both (Iwamoto, Ayers, Mahon, & Swerdlow, 2010).

To control these bacteria and prevent them from reaching the consumer, Good Manufacturing Processes (GMP) must be followed and effective safety and quality assurance systems should be applied based on Hazard Analysis Critical Control Point (HACCP) principles (Andres Vasconcellos, 2004).

Given that Algeria has a coastline of over 1280 km, and fish and seafood are considered essential elements of the diet, as in other Mediterranean countries, the main purpose of the present work was to investigate the presence and levels of Enterobacteriaceae contamination sardines (*Sardina pilchardus*) and red shrimp (*Aristeus antennatus*) commercialized in Constantine, Algeria.

2. Materials and methods

2.1. Seafood sampling

A total of 10 sampling campaigns were conducted during 2015 at 10 fishmongers in Constantine. Each fishmonger was visited once a month and the standard materials and sampling procedures (ISO 7218:2007) were used carefully during collection of 100 samples of sardines and 50 red shrimp samples. They were placed in sterile containers and convoyed on ice packs to the laboratory, where they were processed that same day. Information related to sample sources (fishmongers and fishing ports) was also recorded.

2.2. Bacterial counts, isolation and identification

In triplicate, 25 g from each sample were aseptically homogenized with 225 ml of buffered peptone water in a stomacher blender for 2 min (Stomacher® 400 Circulator, Seward, UK). Total and fecal coliform counts were performed according to the ISO 21528-2:2004 methodology, whereas *Salmonella* was isolated as described under ISO 6579:2002, for which samples were pre-enriched in buffered peptone water at 37 °C for 18–24 h followed by enrichment in Rappaport Vassiliadis and Muller-Kauffman Tetrathionate-Novobiocin broths at 42 °C for 18–24 h. Enriched samples were further plated onto XLD and Hektoen agar and incubated at 37 °C for 18–24 h.

Five Enterobacteriaceae colonies per sample were randomly selected and identified using API 20E biochemical tests (BioMérieux. Marcy l'Étoile, France) and *E. coli* strains were serotyped. The commercial kit CerTest *E. coli* O157:H7 (CerTest Biotech, Zaragoza, Spain) was used to assess the presence of *E. coli* O157 and the antisera nonavalent + /trivalent II, trivalent III (Bio-Rad, France) was used to assess the presence for the others serotypes of *E. coli*.

The species identification of strains was confirmed using MALDI-TOF-mass spectrometry (Bruker Daltonics GmbH, Leipzig, Germany) which includes more than 2500 species of 433 microorganism genera according to the biotyper manufacturer ans it is ongoing continued expansion (Singhal, Kumar, Kanaujia, & Virdi, 2015). To these *E. coli* strains, we added an *E. coli* previously isolated and identified from a sample of common cuttlefish *(Sepia officinalis)* originating from La Marsa fishing port and fishmonger number 11. Phylogenetic groups of *E. coli* isolates were determined following the method of Clermont, Christenson, Denamur, and Gordon (2013), and their clonality was assessed by pulsed-field gel electrophoresis (PFGE) at the Public Health Institution of Turkey (Ankara) with *Xba*I DNA restriction as described by Durmaz et al. (2009). *Salmonella* isolates were also serotyped according to the Kauffmann-White scheme. The antisera for *Salmonella* identification were supplied by Bio-Rad, France.

2.3. Antimicrobial susceptibility testing and ESBL detection

All *E. coli* and *Salmonella* isolates were subjected to susceptibility testing against 15 antibiotics using the Kirby-Bauer antimicrobial disk diffusion procedure on Muller-Hinton agar; the results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing guidelines (EUCAST, 2015). The antimicrobial disks (BioMérieux, Marcy L'étoile, France) tested contained the following antibiotics: 25 μg ampicillin (AMP), 25 μg amoxicillin (AML), 20/10 μg amoxicillin/clavulanic acid (AMC), 30 μg cefalothin (CF), 75 μg cefoperazone (CFP), 30 μg cefoxitin (FOX), 30 μg amikacin (AN), 30 μg kanamycin (K), 10 μg gentamicin (GM), 30 μg neomycin (N), 10 μg tobramycin (TM), 30 μg tetracycline (TE), 30 μg chloramfenicol (C), 50 μg fosfomycin (FOS) and 1.25/23.75 μg trimethoprim-sulfamethoxazole (SXT). To identify ESBL producing isolates, a double-disk synergy (DDS) test was performed following the Clinical Laboratory Standards Institute guidelines (CLSI, 2012).

2.4. Plasmid detection, characterization of ESBL and virulence genes

All *E. coli* isolates were incubated at 37 °C overnight on brain heart infusion (BHI) agar plates, after which DNA was extracted as described previously (Alves-Guerreiro, 2012, pp. 34–60) and stored at −20 °C until analyses. Plasmids were extracted from *E. coli* isolates using sucrose-mediated detergent lysis as described by Saha, Saha, Niyogi, and Bal (1989). To assess the size of any exiting plasmids, reference *E. coli* strains (39R861 and CECT679) harboring plasmids of known sizes (7.4 Kb, 37.6 Kb, 66.2 Kb, and 154 Kb) were migrated concurrently with our tested strains as well as a DNA size marker.

The genes bla_{OXA}, bla_{SHV}, and $bla_{CTX\text{-}M}$ encoding the most clinically prevalent ESBL types were detected by PCR and subtyped by sequencing the PCR products (Ahmed et al., 2007). The intimin-encoding *eae* gene and *stx1*and *stx2* genes encoding Shiga toxins were detected by PCR using specific primers and procedures described previously (Blanco et al., 2006; Khandaghi, Razavilar, & Barzegari, 2011).

2.5. Statistical analysis

Total and fecal coliforms loads were transformed into $\log_{10}$ CFU/g before performing statistical analyses. Variations in bacterial levels between fishmongers were assessed using the ANOVA statistical test. Differences in *Salmonella* spp. contamination as well as responses to antibiogram and genes harbored were analyzed using Fisher's exact test. The Student's *t*-test was also used to assess contamination disparities between sardines and red shrimp. All these tests were performed using GraphPad Instat prism 6.04