

Study of the multi-resistance of enterobacteria and nonfermentative gram-negative *Bacilli* isolated from the Bandama Rivers (Cote d'Ivoire)

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Abstract

The purpose of the Bandama river study was to contribute to the surveillance of multidrug resistant pathogens isolated from the river. It was used to isolate strains of Enterobacteria and multi-resistant non-fermentative gram-negative bacilli and to establish a database of these microorganisms. Twelve stations on the Bandama river were sampled during 2019, specially once every six months. The resistance profiles of enterobacteria and non-fermentative gram-negative bacilli to the marker antibiotics were determined for the isolated germs. A total of 69 bacterial species were isolated from the waters of the Bandama River, including 33 enterobacteria (47.8%) and 36 non-fermentative gram-negative bacilli (52.2%). The sensitivities tests performed revealed that 48.13% of Enterobacteria were resistant to both at least three antibiotic families (Beta-lactam antibiotics, Fluoroquinolones, Aminocyclitol antibiotics) and 63.16% of the non-fermentative gram-negative bacilli were resistant to both at least three antibiotic families (Beta-lactam antibiotics, Fluoroquinolones and other antibiotic families). The presence of these multi-resistant bacteria constitutes a real public health problem because of the risk of dissemination of these strains involved in human and animal infections. It is feared that the emergence of multidrug resistance to antibiotics could lead to the limitation or absence of solutions for effective treatment.

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Introduction

For more than 70 years the development and use of antibiotics have led to a major decline in terms of mortality and morbidity associated with bacterial infections throughout the world. Unfortunately, during the last decades, the use of these molecules is at the origin of an antibiotic resistance concerning more and more species due to a growing number of antibiotics (Ouédraogo, 2016). The global spread of strains of multi-resistant bacteria such as Enterobacteriaceae and non-fermentative gram-negative bacilli continues to grow (Vandenesch *et al.*, 2003). This bacterial multi-resistance to antibiotics can go as far as therapeutic impasse, considerably limiting the possibilities of using antibiotics in chemical practice. It is also a major public health problem worldwide, especially, since few new antibiotics are on the market (Vandenesch *et al.*, 2003). Indeed, this spread of antibiotic resistance could jeopardize long-term medical progress. Resistant bacteria are thought to have caused more than 650,000 infections in Europe in 2015. These resistant germs make infections more difficult to treat with an estimated increase in mortality of 33000 deaths in Europe (Cassini *et al.*, 2019).

In addition, hospital, industrial, agricultural and domestic waste and discharges from veterinary activities into the environment systematically contaminate natural aquatic environments (Sidrach Cardona *et al.*, 2014). Several studies have demonstrated the presence of strains of multi-resistant Enterobacteriaceae and non-fermentative gram-negative bacilli in the environment, both in developing countries and in developed countries. Indeed, the study of the resistance profiles of bacteria isolated from aquatic environments underlines the resistance of *E. coli* to amoxicillin, azithromycin, penicillin, erythromycin (Sidrach Cardona *et al.*, 2014; Sidrach Cardona and Bécares, 2013). In Côte d'Ivoire, research on multi-resistant bacteria in hospital effluents from the city of Abidjan showed

that 63.3% of isolated Enterobacteriaceae strains were resistant to beta-lactams, aminoglycosides and Fluoroquinolones (Guessens *et al.*, 2013). Therefore, the misuse of antibiotics has a direct impact on environmental microorganisms. The presence of these molecules results in an increase in the selective pressure on the bacteria, favoring their resistance (Hijosa-valsero *et al.*, 2011).

Indeed, surface waters are receptacles for industrial, hospital, agricultural and domestic wastewater. Thus, polluted surface water leads to the contamination of drinking water, to the direct contamination of the people who consume it.

As it is also used for watering crops, humans can be contaminated indirectly by ingesting contaminated plants (Yala *et al.*, 2017).

In Côte d'Ivoire, there are data on multi-resistant Enterobacteriaceae and non-fermentative gram-negative bacilli isolated from hospital effluents. On the other hand, there is little data relating to multiresistance in rivers. Moreover, it was admitted that in most cities of Côte d'Ivoire, wastewater, waste from factories and hospitals are directly discharged into the aquatic ecosystem without treatment.

This is the case of the Ebrié lagoon where slaughterhouse effluents and domestic waste are discharged (Ouattara, 2016). These surface waters are used for watering crops, laundry, bathing and are an important source of drinking water supply for the populations. Thus, they constitute a means of dissemination of pathogenic and multi-resistant germs in the population.

Like the Ebrié lagoon, the waters of the Bandama watershed which extend over 11 regions and 41 departments, including the department of Korhogo, are not spared by this phenomenon of contamination of untreated wastewater. Hence the interest of this study whose theme is study of

the multi-resistance of enterobacteriaceae and non-fermentative gram-negative bacilli isolated from the waters of the Bandama river (Ivory Coast). The general objective of this study is to help monitor multidrug-resistant pathogens from the waters of the Bandama River. Specifically, it involved: isolating and identifying strains of enterobacteriaceae and non-fermentative gram bacilli, assess their level of resistance to marker antibiotics.

Materials and methods

Sampling

The sampling was carried out during the period from August 28 to October 12, 2019. A total of 24 samples were taken from the 12 Sampling Points, due to one sample per campaign, such as two campaigns carried out. The 12 stations concerned are Grand-lahou, Tiassalé, N'ziannouan, Lamto, Zambakro (Yamoussoukro), Bozi, Bouafilé, Zuénoula, Marabadiassa, Niakaramadougou, Sinématiali, Ferkessedougou (Fig. 1).

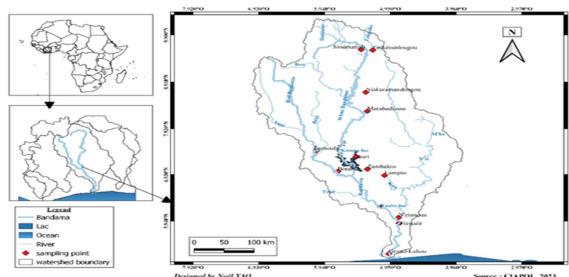


Fig. 1. Map of Bandama watershed and sampling points

The vials containing the samples were stored in a cooler containing dry ice. Then forward to the environmental laboratory. This laboratory is located at the oceanological research center in Treichville fishing port opposite the port police.

Enumeration and identification of strains of enterobacteriaceae and non-fermentative negative bacilli

Isolation and enumeration of Enterobacteriaceae and non fermentive gram-negative bacilli

resistant to antibiotics were carried out on Macconkey medium supplemented with 4 mg per liter of third cephalosporin antibiotic Ceftazidime (CAZ).

After isolation, strains of Enterobacteriaceae and resistant non-fermentive gram-negative bacilli were purified by transplanting on nutrient agar. The identification of the different strains was carried out with the Api 20^E miniature gallery. The reactions produced during the incubation period were reflected by spontaneous color changes or revealed by the addition of reagent. These reactions were read using the reading table, and identification was obtained using the API 20^E WEB software.

Realization of the antibiogram of the strains

Determination of the resistance profil to antibiotics of the strains

To determine antibiotic resistance, a standardized antibiogram in agar medium requiring several steps was carried out on all the strains isolated from water samples from the Bandama River according to the recommendations of the antibiogram committee of the French Society of Microbiology (CA-SFM, 2019).

Antibiotic Susceptibility Testing was done in the National Reference Center for Antibiotics of the Institut Pasteur of Côte d'Ivoire. The antimicrobial susceptibility of the extended spectrum enterobacteria β -lactamase isolates was determined by the Bauer-Kirby disk diffusion test using antibiotic disks (Bio-Rad, France) (Bauer and Kirby, 1966). "The double synergy test was used for detection of ESBL-producing strains. The disks of ceftazidime (30 μ g) and ceftriaxone (30 μ g) were placed around an amoxicillin/clavulanic acid disk (10/20 μ g) on Mueller Hinton agar (BioMérieux, France). The distance between the discs, center to center was 20 mm. This test was performed when the strain was categorized resistant to third generation cephalosporins.

Of these, fourteen antimicrobial agents from five antibiotic families (β -lactams, quinolones, aminoglycosides, polymixin and sulfamid) were tested. Clinical Laboratory Standards Institute (CLSI) guidelines were followed for inoculum standardization, medium and incubation conditions, and internal quality control organisms (*E. coli* ATCC 25922). Isolates were screened for the ESBL-producing phenotype by the standard double-disc synergy test, as described previously" (Jarlier *et al.*, 1988). Antimicrobial discs (concentration of antibacterial in μg) used were amoxicillin/clavulanic acid (10/20), ceftazidime (30), ceftriaxone (30), imipenem (10), nalidixic acid (30), ciprofloxacin (5), pefloxacin (5), amikacin (30), gentamycin (15) and tobramycin (10), chloramphenicol (30), fosfomycine (200), Triméthoprime/sulfaméthoxazole (1,25/23,75) and Nitrofurantoin (100). All the antibiotics were procured from Bio-rad (France).

Variance analysis

The statistical analyzes and the tables were carried out respectively using the software graphpad prism version 8.0 and Excel 2016. The analysis of variance was carried out with the software graphpad prism version 8.0 to study the degree of difference between the different parameters studied.

In the event of a significant difference between the parameters studied, the classification of the means (homogeneous group) is carried out with the Tukey test. The significance level (α) is 0.05. Statistical differences with a probability value less than 0.05 are considered significant. When the probability is greater than 0.05 the statistical differences are not significant.

Results

A total of 69 bacterial species were isolated from water samples from the Bandama River, including 33 enterobacteria (47.8%) and 36 non-fermentative gram-negative bacilli (52.2%).

Table 1. Frequency (N) of occurrence of isolated and identified Enterobacteriaceae

| Enterobacteriaceae | Frequency (N) | Percentage (%) |
|------------------------------|---------------|----------------|
| <i>Klebsiella pneumoniae</i> | 18 | 54.55 |
| <i>Escherichia coli</i> | 7 | 21.21 |
| <i>Pantoé ssp.</i> | 8 | 24.24 |
| Total | 33 | 100 |

Table 2. Frequency (N) of occurrence of isolated and identified gram-negative bacilli

| Non-Enterobacteria | Number (N) | Percentage (%) |
|----------------------------------|------------|----------------|
| <i>Aeromonas hydrophila</i> | 12 | 33.3 |
| <i>Pseudomonas aeruginosa</i> | 11 | 30,6 |
| <i>Aeromonas salmonicida</i> | 05 | 13,9 |
| <i>Chromobacterium violaceum</i> | 08 | 22,2 |
| Total | 36 | 100 |

Table 3. Resistance profile of Enterobacteriaceae isolated from Bandama River waters

| Antibiotics and their families | <i>K. pneumoniae</i> (N=18) | <i>E. coli</i> (N= 7) | <i>Pantoe ssp.</i> (N=8) |
|--------------------------------------|-----------------------------|-----------------------|--------------------------|
| Beta-lactams | | | |
| Amoxicillin + clavulanic acid (AMC) | 18 (100) | 7 (100) | 7 (87,5) |
| Ceftazidime (CAZ) | 18 (100) | 7 (100) | 8(100) |
| Ceftriaxone (CRO) | 18 (100) | 7 (100) | 8(100) |
| Imipenem (IPM) | 0 (0) | 0 (0) | 0 (0) |
| Fluorouinolones | | | |
| Acide nalidixique (NA) | 13 (72,22) | 7 (100) | 4 (50) |
| Ciprofloxacin (CIP) | 6 (33,33) | 7 (100) | 6 (75) |
| Pefloxacin (PEF) | 14 (77,78) | 5 (71,43) | 8 (100) |
| Aminosides | | | |
| Gentamicine (CN) | 3 (16,67) | 0 (0) | 1 (12,5) |
| Tobramycin (TOB) | 4 (22,22) | 0 (0) | 0 (0) |
| Amikacin (AK) | 4 (22,22) | - | - |
| Phenicols | | | |
| Chloramphenicol | 2 (11,11) | 0 (0) | 0 (0) |
| Other families | | | |
| Fosfomycine (FF) | 11 (61,11) | 0 (0) | 4 (50) |
| Trimethoprime/sulfamethoxazole (CLT) | 10 (55,55) | 1 (14,26) | 2 (100) |
| Nitrofurantoin (F) | 1 (5,55) | 0 (0) | 0 (0) |

Table 4. Resistance profile of gram-negative bacilli isolated from Bandama River waters

| Antibiotics and their families | <i>Aeromonas hydrophila</i> (N= 12) | <i>Aeromonas salmonicida</i> (N= 5) | <i>Chromobacterium violaceum</i> (N=8) | <i>Pseudomonas aeruginosa</i> (N=11) |
|-------------------------------------|----------------------------------------|----------------------------------------|-------------------------------------------|-----------------------------------------|
| Betalactamines | | | | |
| Amoxicillin+clavulanic acid (AMC) | 12 (100) | 0 (0) | 8 (100) | 0 (0) |
| Ceftazidime (CAZ) | 12 (100) | 5 (100) | 8 (100) | 11 (100) |
| Ceftriaxone (CRO) | 0 (0) | 5 (100) | 8 (100) | 11 (100) |
| Imipenem (IPM) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Fluoroquinolones | | | | |
| Nalidixic acid (NA) | 12 (100) | 5 (100) | 8 (100) | 11(100) |
| Ciprofloxacin (CIP) | 6 (50) | 5 (100) | 8 (100) | 0 (0) |
| Pefloxacin (PEF) | 6(50) | 5 (100) | 8(100) | 0 (0) |
| Aminosides | | | | |
| Gentamicin (CN) | 0 (0) | 0 (0) | - | 0 (0) |
| Tobramycin (TOB) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Amikacin (AK) | 0 (0) | - | - | 0 (0) |
| Phenolics | | | | |
| Chloramphenicol | 0 (0) | 0 (0) | 8 (100) | - |
| Other families | | | | |
| Fosfomycin (FF) | 12 (100) | 5(100) | 8 (100) | 11 (100) |
| Trimethoprim/sulfamethoxazole (CLT) | 12 (100) | 5 (100) | - | 11(100) |
| Nitrofurantoin (F) | 0 (0) | 0 (0) | 8 (100) | 0 (0) |

Enterobacteriaceae species were composed of 18 *Klebsiella pneumoniae* (54.6%), followed by 8 *Pantoea* spp (24.2%) and 7 *Escherichia coli* (21.2%). On the other hand, non-fermentative gram-negative bacilli comprised 12 *Aeromonas hydrophila* (33.3%), 11 *Pseudomonas aeruginosa* (30.6%), 8 *Chromobacterium violaceum* (22.2%) and 5 *Aeromonas salmonicida* (13.9%) (Table 1 and 2).

Sensitivity tests carried out on isolated bacteria revealed high levels of resistance to the same families of antibiotics both in enterobacteria and in non-fermentative gram-negative bacilli. Most of the enterobacteriaceae were resistant to 5 families of antibiotics at the same time, in particular the family of Betalactams, Fluoroquinolones, Aminosides, phenolics, other families (Fosfomycin, Trimethoprim/sulfamethoxazole).

Concerning non-fermentative gram-negative bacilli, resistance was observed with the families of beta-lactams, fluoroquinolones and other families (Table 3 and 4).

Discussion

According to Blaak *et al.* (2015), the ubiquity of multi-resistant bacteria in the environment has

been demonstrated worldwide. Enterobacteria and multi-resistant non-fermentative gram-negative bacilli were detected in various samples of municipal effluents, university hospitals (Yopougon and Treichville) (Ouattara *et al.* 2014; Guessennd *et al.* 2013).

In Côte d'Ivoire, the hospital effluent evacuation network consists either of a pipe leading directly to the receiving environment (rivers, lagoons, etc.), as in certain university hospitals (UCH), or of a connection to the municipal sanitation network (Ouattara, 2016).

The connection of the hospital effluent evacuation network to the municipal wastewater evacuation network could partly justify the presence of multi-resistant bacteria in the waters of the Bandama river. The results of the identification and the antibiogram confirm the presence of multi-resistant bacteria belonging to different bacterial families in these waters (Yala *et al.* 2017). In this study, the isolation of multi-resistant Enterobacteriaceae reveals a predominance of *Klebsiella pneumoniae*. The predominance of this species had already been highlighted during a previous study by Guessennd *et al.* (2013) on hospital effluents and wastewater from the city of Abidjan.

The resistance profiles carried out on the isolated Enterobacteriaceae revealed that all the strains were sensitive to Imipenem and 48.13% of the Enterobacteriaceae are resistant to at least three families of antibiotics (Beta-lactams, Fluoroquinolones, Aminocyclitol derivatives).

It is noted that the beta lactams were less active whereas the most active family is that of phenolics (chloramphenicol). Indeed, a resistance rate of 100% to Amoxicillin + clavulanic acid (AMC), to Ceftazidime (CAZ) and to Ceftriaxone has been observed. However, the resistance rate was 11.11% for Chloramphenicol. Our results are in agreement with those obtained by (Guessennd *et al.* 2013) on hospital effluents and wastewater from the city of Abidjan.

Indeed, these actors have shown that no strains of Enterobacteriaceae were resistant to imipenem, however, these were resistant to at least three families of antibiotics (Beta-lactams, Fluoroquinolone and Aminocyclitol derivatives).

This observation reflects the presence of multi-resistant bacteria in surface waters, particularly in the waters of the Bandama River. This multiresistance could be explained by bacterial selection pressure due to the presence of antibiotics and other molecules in untreated hospital, agricultural and domestic effluents, observed in the Bandama River. Moreover, the exchange of genetic material could in any case explain this resistance observed in Enterobacteriaceae isolated from the waters of the river, during their transit in the digestive tract of humans and animals treated with antibiotics. Enterobacteriaceae can acquire some resistance to antibiotics (Servais and Passerat 2009). Consequently, the bacteria present in the waters of natural environments can acquire a form of antibiotic resistance there by exchanging genetic material with them (Servais and Passerat, 2009).

Of the 5 (five) strains of non-fermentative gram-negative bacilli, 63.13% of the strains were resistant to at least three families of antibiotics (Beta-lactams, Fluoroquinolones and other families). Beta-lactams were less active and Aminocyclitol derivatives were the most active. Indeed, a resistance rate of 100%, to Amoxicillin + clavulanic acids (AMC), to Ceftazidime (CAZ) and to Ceftriaxone (CRO) has been observed.

However, the resistance rate was 0% to Gentamicin (CN), Tobramycin (TOB), and Amikacin (AK). These results of the evaluation of the level of resistance of non-fermentative gram-negative bacilli show the presence of BMR in the waters of the Bandama River. This resistant character observed in these four bacterial genera would be natural or acquired. Certainly, several studies have clearly shown the natural residence in *Pseudomonas aeruginosa*. (Baba Ahmed-Kazi Tani and Arlet, 2014) which allows it to resist several beta-lactams by production of an inducible class C chromosomal beta-lactamase (Baba Ahmed-Kazi and Arlet, 2014). Indeed, this would not be inhibited by clavulanic acid and would hydrolyze C₃G such as Ceftazidime and Cefepime (Mérens *et al.* 2011).

In addition, broad-spectrum beta-lactamases constitute a group of enzymes whose particularity is to confer resistance to first, second and third generation cephalosporins. In addition, acquired resistance in *Pseudomonas aeruginosa* would certainly result from a genetic modification that would be due either to a mutation or to the acquisition of a resistance gene (Mérens *et al.* 2011). Undeniably, the fact that the outer membrane wall of gram-negative bacilli is less permeable would promote multiresistance (Kulkarni *et al.* 2015).

The resistance of the genus *Aeromonas* could be explained by different resistance mechanisms: enzyme secretion, impermeability of the outer membrane and efflux pump (Li *et al.* 2015; Li

and Nikaido, 2009). This resistance observed in *Aeromonas* could also be due to its origin in water contaminated by hospital effluents and wastewater. A study conducted in Ireland showed that river water sampled next to a hospital effluent discharge point contained more strains of *Aeromonas* producing Broad-spectrum Beta-lactamases and resistant to antibiotics (Drieux-Rouzet and Jarlier, 2014).

In this study, the inactivity of Beta-lactams observed in the species (Enterobacteriaceae and non-fermentative gram-negative bacilli) would be linked to the fact that Beta-lactams are considered to be the most frequent family used in the world. These antibiotics are involved in the treatment of approximately 55% of bacterial infections (Yala *et al.* 2017).

Conclusion

This study showed the presence of multi-resistant bacteria (Enterobacteriaceae and non-fermentative gram bacilli) in the waters of the Bandama River. Particularly high rates of resistance to antibiotics, often major, have been observed and in general Enterobacteriaceae and non-fermentative gram bacilli are resistant not only to several families of antibiotics, but also to several antibiotics of the same family. This observation once again raises my question of the impact of discharging certain untreated effluents such as hospital and municipal effluents directly into the environment. It is important to point out that the control of multi-resistant bacteria from surface water (river, lagoon, river, etc.) used by certain companies for the purpose of transformation into drinking water or directly by the population for the purpose of consumption, watering and entertainment, remains a major public health issue since these strains are the cause of many infections.

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