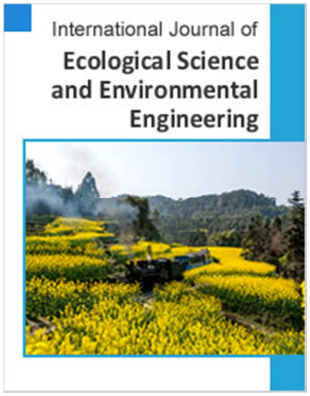
International Journal of Ecological Science and Environmental Engineering
2018; 5(2): 52-57
http://www.aascit.org/journal/ijesee
ISSN: 2375-3854

American Association for Science and Technology



Keywords
Escherichia coli,
Staphylococcus aureus,
Drinking Water,
Antibiotic Sensitivity,
Lalo,
Benin

Received: February 6, 2018
Accepted: March 1, 2018
Published: April 27, 2018

Sensitivity of Strains of *Escherichia coli* and *Staphylococcus aureus* Identified in Drinking Water to Commonly-Used Antibiotics in Lalo, Benin

Hermione Amoukpo[1, *], Nouratou Salifou[2], Tamègnon Victorien Dougnon[1, 3], Zoulkifl Salou Bachirou[1], Horace Degnonvi[1], Michel Boko[1], Roch Christian Johnson[1]

[1]Interfaculty Center of Training and Research in Environment for the Sustainable Development (CIFRED), University of Abomey-Calavi (UAC), Abomey-Calavi, Benin
[2]Faculty of Science and Technology (FAST), University of Abomey -Calavi (UAC), Abomey-Calavi, Benin
[3]Polytechnic School of Abomey-Calavi (EPAC), University of Abomey-Calavi (UAC), Abomey-Calavi, Benin

Email address

amohermy@yahoo.fr (H. Amoukpo), nourath.salifou@gmail.com (N. Salifou), victorien88@hotmail.com (T. V. Dougnon), zoulkifls@gmail.com (Z. S. Bachirou), degnonvihorace@gmail.com (H. Degnonvi), bokomichel@gmail.com (M. Boko), rochchristianjohnson@gmail.com (R. C. Johnson)

[*]Corresponding author

Citation

Hermione Amoukpo, Nouratou Salifou, Tamègnon Victorien Dougnon, Zoulkifl Salou Bachirou, Horace Degnonvi, Michel Boko, Roch Christian Johnson. Sensitivity of Strains of *Escherichia coli* and *Staphylococcus aureus* Identified in Drinking Water to Commonly-Used Antibiotics in Lalo, Benin. *International Journal of Ecological Science and Environmental Engineering*. Vol. 5, No. 2, 2018, pp. 52-57.

Abstract

In Benin, despite the high coverage of improved water sources, the majority of the population does not have access to a drinking water source at home. In the absence of a home piping system, transport and storage favor microbiological contamination of the water. Unfortunately, bacteria that indicate fecal contamination are often resistant to the majority of antibiotics. This study aims to evaluate the effectiveness of commonly-used antibiotics on the germs responsible for the microbiological contamination of drinking water in the district of Ahomadégbé, in the municipality of Lalo, Benin. Thirty-five (35) drinking water samples were collected and the efficacies of commonly-used antibiotics were tested on *Escherichia coli* and *Staphylococcus aureus* strains isolated from these samples. *Escherichia coli* strains showed complete sensitivity to Ciprofloxacin, Doxycycline, Gentamicin, Imipenem and Pristinamycin. In contrast, isolated *Staphylococcus aureus*strains were resistant to Cephalosporins, Cyclins and Macrolides, but were sensitive to Pristinamycin (Streptogramins). Strains of *Escherichia coli* and *Staphylococcus aureus* are resistant to most common antibiotics. To limit the progression of resistance to antibiotics that can still act on these strains, it is important to educate people regarding good hygiene practices, which are the most effective ways to reduce the risk of infection.

1. Background

Today, 71% of the world's population has access to a drinking water service that is available at all times and free of contamination [1]. Of those who do not have such access, 1.3 billion people have an improved water point less than 30 minutes away from their home and 263 million people have access to limited services, requiring a round trip more than 30 minutes in length to recover water [1]. In developing countries, when a home piping system is absent, the chore of obtaining water imposes a daily back-and-forth for women, between their home and the nearest source [2]. This supply system imposes a phase of transport and a phase of storage of water at home [2, 3]. Several studies have shown that the transport and storage of water at home promote the microbiological contamination of water [2-10]. These different studies indicate that the lack of hygiene during transport and storage of drinking water is at the root of its microbiological contamination, which acts directly on human health by causing diarrheal diseases or cases of intoxication.

Every year, there are approximately 1.7 billion cases of child diarrhea worldwide and 525,000 children under the age of five die from diarrheal diseases due to poor sanitation and hygiene, and unsafe water [11]. In Benin, water-related diseases such as gastrointestinal diseases and diarrhea account for at least 49% of the country's epidemiological situation [12]. These diseases remain very common in rural areas and constitute a heavy economic burden for households [3].

Cases of diarrhea can last for several days, dehydrating the body and depriving it of the mineral salts needed for survival [11], and water plays a decisive role in the appearance of bacterial diarrhea [13]. In developing countries, bacterial diarrhea remains a serious public health problem, especially since the resistance of these strains is progressing, leading to increased treatment failures [13]. Some bacterial strains have acquired multidrug resistance against antibiotics [13], the result of a very old adaptation process that has been accelerated by the excessive use of antibiotics in the twentieth century, both in terms of human health and animal health [14]. In recent years, the extraordinary efficacy of antibiotics has been called into question by the evolution of bacteria towards greater and greater resistance, resulting in bacteria being isolated against which the best antibiotics have no effect [14]. This phenomenon constitutes a major threat for medicine, putting its practices and advances in question [14]. Regarding drinking water, fecal contamination indicator bacteria such as *Escherichia coli* and enterococci are now resistant to antibiotics; thus, several infections can no longer be effectively treated [15]. The most commonly used antibiotics, namely sulfonamides, ampicillin and cyclins, are increasingly inactive [13], leading to the testing of other molecules, such as fluoroquinolones, which are more expensive and therefore less accessible to populations for which bacterial diarrhea is a concern [13].

In Benin, the management of diarrheal diseases is carried out through the implementation of inadequate and unsettled antibiotic therapy. Populations also have access to this antibiotic therapy because of self-medication, through the free sale of antibiotics in pharmacies and on the black market. All these factors lead to the resistance of germs to commonly-used antibiotics. This study aims to evaluate the effectiveness of commonly-used antibiotics against the germs responsible for the microbiological contamination of drinking water in the district of Ahomadégbé, in the municipality of Lalo, Benin.

2. Methods

2.1. Study Site

This study was conducted in the district of Ahomadégbé, in the municipality of Lalo, Benin (Figure 1). The municipality of Lalo is an administrative subdivision of the Couffo department and includes eleven (11) districts. The district of Ahomadégbé is subdivided into four villages, with a total population estimated at 5,403 inhabitants [16].

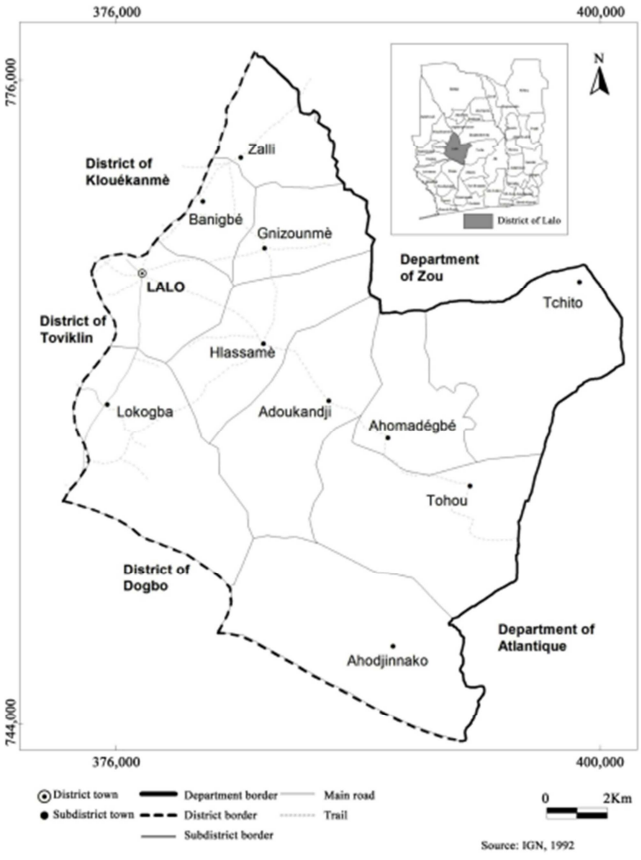


Figure 1. *Location map of the municipality of Lalo, Benin.*

2.2. Description of the Study

This is a study designed to evaluate the effectiveness of commonly-used antibiotics against the germs responsible for microbiological contamination of the drinking water consumed by populations of the district of Ahomadégbé.

2.3. Study of the Sensitivity of Germs to Antibiotics

2.3.1. Sampling

Thirty-five (35) drinking water samples were collected in sterile 500-ml bottles, which were filled three quarters-full.

2.3.2. Transport to the Laboratory

The bottles were carefully labeled and numbered. Transport from the sampling point to the Laboratory of Research in Applied Biology (LARBA) of the Polytechnic School of Abomey-Calavi (EPAC), University of Abomey-Calavi, was conducted using a cooler at 4°C. Once in the laboratory, the samples were kept in the refrigerator.

2.3.3. Laboratory Analyses

The effectiveness of commonly-used antibiotics was evaluated by isolating certain germs and studying their sensitivities to antibiotics. Given the strong presence of *Escherichia coli* revealed by an earlier study concerning the microbiological quality of drinking water in the district of Ahomadégbé [10] and the adverse health effects of *Staphylococcus aureus*, including the production of enterotoxins responsible for foodborne illness [17], antibiotic sensitivities were studied in *Escherichia coli* and *Staphylococcus aureus*.

(i). Isolation

(a) Escherichia coli

*Escherichia coli*was isolated using Eosin Blue Methylene (EMB) medium, after 48 hours of incubation at 44°C. *Escherichia coli* colonies are dark blue and show a metallic reflection when examined under reflected light. These colonies were isolated and purified three times on Muller-Hinton agar, followed by a mobility test, Gram staining (Gram negative bacilli), and tests of oxidase (negative oxidase), urea (negative urea) and indole (positive indole).

(b) Staphylococcus aureus

Staphylococci were isolated from Chapman medium incubated at 37°C for 48 hours. Colonies of staphylococci are small and yellow in color. To confirm the presence of *Staphylococcus aureus*, the following method was used:

3 ml of Brain Heart Broth (BCC) were removed from sterile hemolysis tubes;

suspicious colonies of staphylococci were removed from each tube and crushed;

after incubation at 37°C for 24 hours, 0.1 ml of each suspension was taken and 0.3 ml of rabbit plasma was added;

thenew suspension was incubated at 37°C for 19 hours.

A mass settling at the bottom of the tube indicates a positive result.

Small, yellow, coagulase-positive colonies were isolated and purified three times on Chapman medium, followed by gram staining (Gram-positive Cocci).

(ii). Sensitivity of Germs to Antibiotics

Sensitivity of *Escherichia coli* and *Staphylococcus aureus* to antibiotics was tested on Muller-Hinton agar medium. The diffusion method from a 24-hours culture on Muller-Hinton agar was used.

In 3 ml of saline (0.9% NaCl) from sterile hemolysis tubes, a pure colony of *Escherichia coli* was suspended. Muller-Hinton agar was inoculated by flooding, after which excess suspension was removed and the agar surface was dried for 5 minutes. Antibiotic disks were deposited on the agar surface and then samples were incubated for 24 hours.

The sensitivity of *Staphylococcus aureus* colonies was tested in the same way as described above. The antibiotics tested are presented in Table 1.

Table 1. *List of antibiotics used to test sensitivities of Escherichia coli and Staphylococcus aureus.*

	Family	Antibiotic	Acronymdisk	Disk load	Critical diameters (mm)	
					Sensitive (D) ≥	Resistant (d) <
List of antibiotics tested against *Escherichia coli*	Aminoglycosides	Gentamicin	GEN	500µg	22	26
	Beta-lactams (Cephalosporins)	Amoxicillin	AMX	25µg	23	16
		Amoxicillin +Clavulanic acid	AMC	30µg	23	16
		Cefotaxime	CTX	30µg	26	23
		Ceftazidime	CAZ	30µg	21	19
		Ceftriaxone	CRO	30µg	26	23
		Aztreonam	AMT	30µg	23	21
		Imipenem	IPM	10µg	24	17
	Fluroquinolones	Ciprofloxacin	CIP	5µg	25	22
	Cyclins	Doxycycline	DOX	30UI	19	17
	Streptogramins	Pristinamycin	PTN	15µg	22	*19*
List of antibiotics tested against *Staphylococcus aureus*	Beta-lactams (Cephalosporins)	Cefotaxime	CTX	30µg	26	23
	Trimethoprim-sulfa	Trimethoprim / sulfamethoxazole	SXT	15µg	16	13
	Cyclins	Doxycycline	DOX	30UI	19	17
	Lincosamides	Lincomycin	LCN	30µg	21	17
	Macrolides	Erythromycin	E	15UI	22	19
	Streptogramins	Pristinamycin	PTN	15µg	22	*19*
	Nitro-furan	Furans	FM	30µg	15	15

Source: Antibiogram Committee of the French Microbiology Society (CA_SFM) [18]

Diameters of zones of inhibition were interpreted as follows:

a. if the diameter of the inhibition zone ≥ D (see Table 1), the strain was said to be sensitive (S);
b. if the diameter of the zone of inhibition < d, the strain was said to be resistant (R);
c. if d ≤ diameter of the zone of inhibition < D, the strain was said to be intermediate (I).

The antibiogram was read by measuring the diameters of the halves surrounding the antibiotic disks and corresponding to the growth inhibition zones, and comparing them to the recommended critical limits [18].

3. Results

3.1. Sensitivity of *Escherichia coli* to Commonly-Used Antibiotics

Escherichia coli strains showed total sensitivity to Ciprofloxacin (Fluoroquinolones), Doxycycline, Gentamicin, Imipenem and Pristinamycin (Table 2). Conversely, they showed nearly complete resistance to Amoxicillin, and a significant resistance, ranging from 50% to 75%, to Amoxicillin + clavulanic Ac, Aztreonam, Cefotaxime and Ceftriaxone. The antibiogram of one colony of *Escherichia coli* is shown in Figure 2.

Table 2. *Sensitivity of Escherichia coli to commonly-used antibiotics.*

Families	Antibiotics	Price of antibiotic (F CFA)			Sensitivity
		Tablets	Injectables	Syrup	
Aminoglycosides	Gentamicin GEN 500		2430		S
	Amoxicillin AMX 25	580		1005	R
	Amoxicillin + Clavulanic acid AMC 30	8175		5900	R
	Cefotaxime CTX 30		3560		R
Beta-lactams	Ceftazidime CAZ 30		12740		I
	Ceftriaxone CRO 30	3025			R
	Aztreonam AMT 30		13930		R
	Imipenem IPM 10		9090		S
Fluoroquinolones	Ciprofloxacin CIP 5	3505	1170		S
Cyclins	Doxycycline DO 3O	1250			S
Streptogramins	Pristinamycin PTN 15	15200			S

S: Sensitive, R: Resistant, I: Intermediate Sensibility
Results of LARBA, 2016

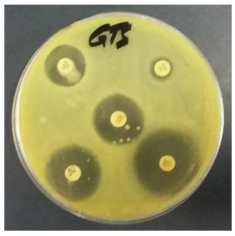


Figure 2. *Testing the sensitivity of one colony of Escherichia coli to commonly-used antibiotics; (Results of LARBA, 2016).*

3.2. Sensitivity of *Staphylococcus aureus* to Commonly-Used Antibiotics

Most strains of *Staphylococcus aureus* isolated were resistant to Cephalosporins, Cyclins and Macrolides (Table 3). We also noted sensitivity to Pristinamycin, which belongs to the Streptogramin family and was the most expensive antibiotic tested in the current study. The antibiogram of one colony of *Staphylococcus aureus* is shown in Figure 3.

Table 3. *Sensitivity of Staphylococcus aureusto commonly-used antibiotics.*

Family	Antibiotic	Price of antibiotic (F CFA)			Sensitivity
		Tablets	Injectables	Syrup	
Beta-lactams (cephalosporins)	Cefotaxime CTX 30		3560		R
Trimethoprim-sulfa	Trimethoprim / sulfamethoxazole SXT 15	2005		1260	I
Cyclins	Doxycycline DOX 30	1250			R
Lincosamides	Lincomycin LCN 30	1180			R
Macrolides	Erythromycin E 15	4135			R
Streptogramins	Pristinamycin PTN 15	15200			S
Furans/Nitrofurans	Furans FM 30	1490			I

S: Sensitive, R: Resistant, I: Intermediate Sensitivity
Results of LARBA, 2016

Figure 3. *Testing the sensitivity of one colony of Staphylococcus aureus to commonly-used antibiotics; (results of LARBA, 2016).*

4. Discussion

This study aimed to evaluate the sensitivity of germs responsible for water contamination to commonly-used antibiotics. Antibiotic sensitivity was only evaluated using strains of *Escherichia coli* and *Staphylococcus aureus*, considering that among the germs responsible for the water contamination identified, these two germs are the most dangerous for human health.

The *Escherichia coli* strains tested in this study showed total resistance to Amoxicillin, which was the cheapest antibiotic tested and is therefore the most accessible to the population. These results are comparable to those obtained by N'Diaye concerning *Escherichia coli* strains [19]. Results obtained after addition of clavulanic acid showed a significant resistance, ranging from 50% to 75%. The same results were obtained for Aztreonam, Cefotaxime and Ceftriaxone. In contrast, all *Escherichia coli* strains were sensitive to Imipenem. Similarly, the two *Escherichia coli* strains tested by N'Diaye were also sensitive to Imipenem [19]. A similar study conducted in Algeria evaluated the antibiotic resistance of *Enterobacter* species to antibiotics and revealed that all strains of *Enterobacter* species were sensitive to Imipenem and resistant to Amoxicillin/clavulanic acid [20]. In our study, all *Escherichia coli* strains showed total sensitivity to Ciprofloxacin, in the Fluoroquinolones family. These results corroborate those of Seck, which revealed 100% sensitivity to Ciprofloxacin [21]. Dosso *et al.* found that the resistance of germs to antibiotics most commonly used in current practice leads to testing other molecules, such as Fluoroquinolones, that are more expensive and therefore less accessible to populations [13]. The *Escherichia coli* strains isolated from our samples also showed total sensitivity to Gentamicin, Doxycycline and Pristinamycin. It is noted that the resistance of germs to antibiotics that are meant to fight against them increases through a long process. A study conducted at the University Hospital of Beirut in Lebanon, between 2005 and 2009, showed evolution of the antibiotic sensitivity of *Escherichia coli* strains over five years [22].

The *Staphylococcus aureus*strains tested showed resistance to Cefotaxime, Doxycycline, Lincosamide and Erythromycin. In a recent study in Togo, 60% of *Staphylococcus aureus*strains were sensitive to Cefotaxime, 22.2% to Lincomycin, 17.8% to Erythromycin and 15.6% to Doxycycline [23]. In their study in Nigeria, Nwankwo and Nasiru showed that 52.4% of *Staphylococcus aureus*strains are sensitive to Erythromycin [24]. *Staphylococcus aureus*strains in the present study showed an intermediate sensitivity to Trimethoprim/Sulfamethoxazole. Anani and *al*. found that 33.3% of *Staphylococcus aureus*strains are sensitive to Trimethoprim/Sulfamethoxazole [23]. In the current study, sensitivity to Pristinamycin was noted in all *Staphylococcus aureus*strains. These results differ from those obtained in Togo, where only 17.8% of the strains were sensitive to Pristinamycin [23].

An analysis of the prices of antibiotics shows that strains of *Escherichia coli* and *Staphylococcus aureus*are sensitive to antibiotics that are not financially accessible to the population of a rural area, such as the district of Ahomadégbé, in the municipality of Lalo.

5. Conclusion

The present study regarding the sensitivity of germs responsible for the contamination of drinking water to commonly-used antibiotics allowed us to note the resistance of *Escherichia coli* and *Staphylococcus aureus* to most antibiotics financially accessible to the population. These germs constitute a great threat to the health of consumers, especially children. To limit the spread of resistance to the few antibiotics that continue to act on strains of *Escherichia coli* and *Staphylococcus aureus*, it is urgent to fight self-medication and educate people concerning good hygiene practices, which are the most effective means of reducing the risk of infection.

Ethics Approval and Consent to Participate

The ethical protocol that authorized this study has been validated by the National Committee of Ethics for Health Research (No. 123 / MS / DC / SGM / + DFR / CNERS / SA).

Acknowledgements

We are very grateful to the populations of the district of Lalo and the health staff of the CDTUB. We are thankful to the Anesvad Foundation for partially supporting the field work.

Conflict of Interests

The authors declare that they have no competing interests

References

[1] OMS. Eau: Aide-mémoire N° 391. 2017. http://www.who.int/mediacentre/factsheets/fs391/fr/. Accessed Sept 08 2017.

[2] Lalanne F. *Etude de la qualité de l'eau le long de la chaîne d'approvisionnement au niveau des consommateurs dans 10 villages de la Province du Ganzourgou, (Région du Plateau Central, Burkina Faso)*. Fondation 2iE et UNICEF, 2012. p. 70.

[3] BMZ, IOB. *Évaluation d'impact des programmes d'approvisionnement en eau potable et d'assainissement au Bénin: Le risque d'effets éphémères*. La Haye, Pays-Bas: BMZ (Ministère fédéral allemand pour la coopération et le développement économiques) et IOB (Inspectie Ontwikkelingssamenwerking en Beleidsevaluatie (Service néerlandais de l'évaluation des politiques et des opérations)), 2011. p. 218.

[4] Dikassa L, Mock N, Magnani R, Rice J, Abdoh A, Mercer D, *etal*. Maternal behavioural risk factors for severe childhood diarrhoeal disease in Kinshasa. *International Journal of Epidemiology*. 1993; 22: 327-33. DOI: 10.1093/ije/22.2.327.

[5] Gentilini M, Viens P. *Tropical communicable diseases*. Paris: Eurotext; 1993. p. 132.

[6] Gil A, Lanata C, Kleinau E, Penny M. *Children's feces disposal practices in developing countries and interventions to prevent diarrheal diseases. A literature review*. Washington DC: Environmental Health Project. US Agency for International Development; 2004. p. 67.

[7] Judah G, Donachie P, Cobb E, Schmidt W, Holland M, Curtis V. Dirty hands: Bacteria of faecal origin on commuter's hands. *Epidemiology and Infection*. 2009; 138 (3): 409-14. DOI: 10.1017/S0950268809990641.

[8] Onabolu B, Jimoh OD, Igboro SB, Sridhar MKC, Onyilo G, Ilya R. Source to point of use drinking water changes and knowledge, attitude and practices in Katsina State, Northern Nigeria. *Physics and Chemistry of the Earth*. 2011; 36: 1189-96. DOI: 10.1016/j.pce.2011.07.038.

[9] Johnson RC, Segla H, Dougnon TV, Boni G, Bankole HS, Houssou C, *etal*. Situation of water, hygiene and sanitation in a peri-urban area in Benin, West Africa: The case of Sèmè-Podji. *Journal of Environmental Protection*. 2014; 05 (12): 1277-83. DOI: 10.4236/jep.2014.512121.

[10] Johnson RC, Boni G, Amoukpo H, Barogui Y, Diez G, Agossadou D, *et al*. Microbiological quality assessment of drinking water in Lalo Commune, Benin (West Africa). *Journal of Water Resource and Protection*. 2016; 08 (08): 816-22. DOI: 10.4236/jwarp.2016.88066.

[11] OMS. Maladies diarrhéiques: Aide-mémoire n°330. 2017. http://www.who.int/mediacentre/factsheets/fs330/fr/.Accessed Sept 08 2017.

[12] MEE. *Plan d'action national de gestion intégrée des ressources en eau du Bénin*. Cotonou: 2011. p. 74.

[13] Dosso M, Coulibaly M, Kadio A. Place des diarrhées bactériennes dans les pays en développement. *Journée en Hommage au Professeur A. DODIN*. 1998.

[14] Andremont A. Antibiotiques et antibiorésistance, un avatar singulier de l'histoire planétaire. 2016. http://questionsdecommunication.revues.org/10392. Accessed Sept 30 2017.

[15] Sobsey MD, Abebe L, Andremont A, Ashbolt NJ, de_Roda_Husman AM, Yew-Hoong_Gin K, *et al*. *Antimicrobial resistance: An Emerging Water, Sanitation and Hygiene Issue*. OMS, 2014. p. 16.

[16] INSAE. *Effectifs de la population des villages et quartiers de ville du Benin (RGPH-4, 2013)*. Cotonou: Institut National de la Statistique et de l'Analyse Economique, 2016. p. 85.

[17] Anses. *Staphylococcus aureus* et entérotoxines staphylococciques: Famille des Staphylococcacceae Genre Staphylococcus Bactérie. 2011. https://www.anses.fr/fr/system/files/MIC2011sa0117Fi.pdf.Accessed Oct 08 2017.

[18] CA_SFM. *Comité de l'antibiogramme de la Société Française de la Microbiologie: Recommandations 2012*. Société Française de Microbiologie; 2012. p. 59.

[19] N'Diaye A. *Etude bactériologique des eaux de boissons vendues en sachet dans quatre communes d'Abidjan* [Doctorat en pharmacie]. Bamako, Mali: Université de Bamako; 2008.

[20] Khennouchi N. *Evaluation de l'antibiorésistance du genre Enterobacter aux antibiotiques* [Thèse en microbiologie appliquée]. Algérie: Université d'Annaba; 2016.

[21] Seck A. *Données sur la résistance des souches à l'origine d'infections nosocomiales* [Doctorat en pharmacie]. Dakar, Sénégal: CHU de Dakar, Sénégal; 2001.

[22] Hamouche E, Sarkis DK. Evolution de la sensibilité aux antibiotiques de *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* et *Acinetobacter baumanii* dans un CHU de Beyrouth entre 2005 et 2009. *Pathologie Biologie*. 2012; 60: 15-20. DOI: 10.1016/j.patbio.2011.03.011.

[23] Anani K, Adjrah Y, Ameyapoh Y, Damintoti-Karou S, Agbonon A, deSouza C, *et al*. Effects of hydroethanolic extracts of *Balanitesaegyptiaca* (L.) Delile (Balanitaceae) on some resistant pathogens bacteria isolated from wounds. *Journal of Ethnopharmacology*. 2015; 164: 16-21. DOI: 10.1016/j.jep.2015.01.051.

[24] Nwankwo EO, Nasiru MS. Antibiotic sensitivity pattern of *Staphylococcus aureus* from clinical isolates in a tertiary health institution in Kano, Northwestern Nigeria. *Pan African Medical Journal*. 2011; 8 (4).