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Full Length Research Paper

Bacteriological characteristics and resistance profiles of *Vibrio cholerae* O1 strains isolated in the Republic of Benin in 2020

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This study aims to carry out a bacteriological characterization and determine the resistance profile of *Vibrio cholerae* O1 strains isolated during the epidemiological season of 2020 in Benin. To achieve this goal, 43 diarrheal stool samples were analyzed. The samples were taken during the epidemic period of 2020. Bacteriological analyses consisted of enrichment of the samples in buffered peptone water followed by culture on SBCT agar. Then the characteristic colonies were subjected to microscopy, biochemical identification (oxidase, seeding and reading of TSI agar and API 20 E gallery), serotyping, and antibiotic sensitivity tests using the diffusion technique in agar medium according Kirby-Bauer method. The median age of the patients included in this study was 25 years (IQR: 15-40) with predominantly female patients. Individuals aged 11 to 25 were the most represented. Of the 43 stool samples analyzed, 22 were culture positive for *V. cholerae* and belonged to serogroup O1. The clinical manifestations observed in patients with cholera were watery diarrhea, vomiting and severe dehydration before admission to hospital. It should be noted that all of *V. cholerae* O1 strains isolated were multidrug resistant with a strong resistance to erythromycin (81.13%), ampicillin (79.96%), chloramphenicol (79.06%), and cotrimoxazole (78.12%).

Key words: Bacteriological analyses, *Vibrio cholerae* O1, antimicrobial resistance, Benin.

INTRODUCTION

Cholera is a very virulent and fatal diarrheal disease caused by ingestion of water or food contaminated with the bacteria *Vibrio cholerae* (Levade et al., 2017). Vibrio strains can grow and remain for a long time in coastal waters polluted by human feces (Gidado et al., 2018). Seven cholera pandemics have been recorded worldwide

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and continue to cause local and regional outbreaks on the African continent (Danso et al., 2020). Cholera is a major public health problem in many sub-Saharan African countries. According to World Health Organization (WHO), in 2014, 190,549 cases and 2,231 deaths were reported. Out of 2231 deaths, 1882 (84.3%) were from Africa (Bwire et al., 2017). Cholera remains endemic in many countries, in Southeast Asia, Africa, Central and South America. Transmission is closely linked to a lack of access to safe drinking water and sanitation facilities (Abana et al., 2019). Strains of vibrios produce an enterotoxin which causes profuse, painless, watery diarrhea associated with vomiting, leading to severe and fatal dehydration if treatment is not administered promptly (Ngandjio et al., 2009). The main treatment for cholera is based on rehydration. Antimicrobials are a useful therapy for decreasing the duration of diarrhea and bacterial stool and therefore reducing the volume of fluid replacement needed for treatment (Ohene et al., 2016). Widely used antimicrobials include doxycycline or tetracycline for adults, cotrimoxazole (trimethoprim = sulfamethoxazole) for children, and furazolidone for pregnant women (Ngandjio et al., 2009). Since the first pandemic that reached West Africa, some outbreaks have been frequently reported across the region (Moore et al., 2018). However, only a few small-scale studies have been performed to investigate the dynamics of recent cholera outbreaks in West Africa. Overall, the cholera epidemics studied in the region exhibited different characteristics in different countries (Moore et al., 2017). In some countries such as Benin, Ghana, Nigeria and Togo, cases of cholera have been reported every year, although their incidence is relatively low (Landoh et al., 2013; Sule et al., 2017; Moore et al., 2018; Danso et al., 2020). However, in Benin there is a glaring absence of epidemiological data on annual cholera epidemics. It is to remedy this situation and to provide recent epidemiological information on cholera that this study was initiated. It aims to carry out a bacteriological characterization and to determine the resistance profile of the strains of *V. cholerae* O1 isolated in 2020.

MATERIALS AND METHODS

Study framework

This study was conducted in the Republic of Benin. It is a coastal country in West Africa, with an area of 114,763 km^2. The annual average temperatures vary from 26 to 28°C. The climate is subequatorial in the south, with a humid tropical transition in the center and dry tropical in the north (Houehanou et al., 2015). The population was estimated at 9,983,884 inhabitants in 2013 (INSAE, 2016) of which 51.2% were women. The population is elderly with a majority of individuals over 15 years of age. The health system is structured in three levels (central, intermediate and peripheral) in a pyramidal manner (Houehanou et al., 2015). Specifically, the bacteriological diagnosis of the samples was carried out in the bacteriology section of the National Public Health Laboratory, Ministry of Public Health, Benin.

Sampling

Stool samples (43) were systematically taken during the study period (May 8 to October 20, 2020). These samples came from all suspected cases of cholera, notified by health centers and departmental health services. Thus, stool samples from each of these cases were taken into sterile jars and sent to the laboratory immediately for analysis. Samples not sent directly to the laboratory for transport were put on Cary Blair medium and sent to the laboratory within 24 h. Each sample was identified and accompanied by a completed notification form. The information on the sheet concerns the socio-demographic and epidemiological information of the patient.

Sample analysis

Isolation and identification of strains of V. cholerae

Isolation of *V. cholerae* began by enriching samples in buffer peptone water (BPW) (pH 8.4) at 37°C for 6 h, and the resulting solution was streaked onto agar with sucrose bile citrate thiosulfate (SBCT). After 24 h of incubation at 37°C, yellow colonies (sucrose fermenting, 2-3 mm in diameter) suspected of being *V. cholerae* were purified on Müeller Hinton agar for 24 h at 37°C. The purified colonies were characterized by Gram stain (Gram negative comma shaped rods), positive oxidase reaction and reaction in triple sugar iron (TSI) agar. Confirmation of the identity of the putative isolates of *V. cholerae* was achieved by seeding and reading the API 20E gallery.

Serogrouping

The previously identified serogroups of *V. cholerae* strains were serologically confirmed by a slide agglutination test using polyvalent antisera specific for *V. cholerae* O1 and O139, followed by a serotype specific monoclonal antibody.

Antimicrobial susceptibility test

All confirmed isolates of *V. cholerae* O1 were tested for antimicrobial susceptibility on Mëuller Hinton agars using the Kirby-Bauer method of diffusion of the following antibiotic discs: tetracycline (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), ampicillin (10 µg), cotrimoxazole (23.75 µg/1.25 µg), amikacin (30 µg), gentamicin (10 µg), erythromycin (15 µg), streptomycin (10 µg), cefuraxime (30 µg), nalidixic acid (30 µg), doxycycline (30 µg), azetromycin (30 µg), flucloxacillin (5 µg), cefotaxime (30 µg), ceftazidime (30 µg) and chloramphenicol (30 µg). The reference strain *Escherichia coli* ATCC 25922 was used as control. The diameters of the inhibition zones measured were interpreted according to CLSI guidelines (CLSI, 2007).

RESULTS

The sex ratio in the sampled patients was 1.39 in favor of the female sex. The median age of this study population was 25 years (IQR: 15-40). Individuals aged 11 to 15 and 21 to 25 were the most represented with a common percentage of 13.94%, followed by individuals aged 16 to 20, 26 to 30 and 36 to 40 with the same percentage of 9.30% (Table 1). Of the 43 stool samples analyzed, 22 were culture positive for *V. cholerae* and serogrouping

Table 1. Distribution of patients sampled by age and sex.

Age class (years)	Sex		Total (%)
	M (%)	F (%)	
0-5	0 (0.00)	3 (6.98)	3 (6.98)
5-10	2 (4.65)	1 (2.33)	3 (6.98)
10-15	2 (4.65)	4 (9.30)	6 (13.94)
15-20	2 (4.65)	2 (4.65)	4 (9.30)
20-25	3 (6.98)	3 (6.98)	6 (13.94)
25-30	3 (6.98)	1 (2.33)	4 (9.30)
30-35	1 (2.33)	2 (4.65)	3 (6.98)
35-40	1 (2.33)	3 (6.98)	4 (9.30)
40-45	2 (4.65)	1 (2.33)	3 (6.98)
45-50	0 (0.00)	2 (4.65)	2 (4.65)
50-55	0 (0.00)	1 (2.33)	1 (2.33)
55-60	0 (0.00)	2 (4.654)	2 (4.65)
60-65	1 (2.33)	0 (0.00)	1 (2.33)
65-70	1 (2.33)	0 (0.00)	1 (2.33)
Total	18 (41.86)	25 (58.14)	43 (100.00)

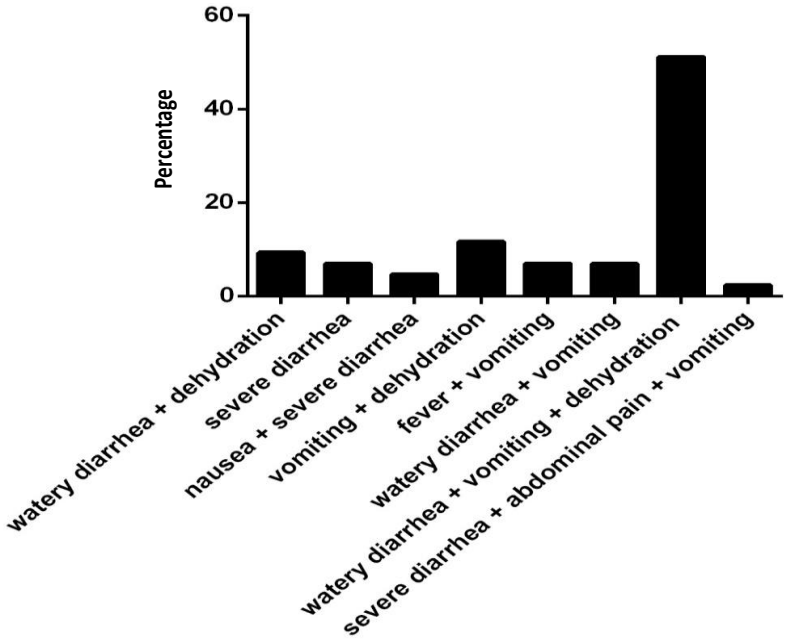

Figure 1. Percentages of different symptoms observed in patients.

resulted in *V. cholerae* O1, that is to say, a prevalence of 51%.

The examination of the notification forms having followed the samples, allowed us to identify 8 symptoms from which the patients suffered before the samples. These symptoms include: watery diarrhea + dehydration; severe diarrhea; nausea + severe diarrhea; vomiting + dehydration; fever + vomiting; watery diarrhea + vomiting; watery diarrhea + vomiting + dehydration and severe diarrhea + abdominal pain + vomiting. The most represented symptoms were watery diarrhea + vomiting + dehydration with 51.16% (n = 22), followed by fever + vomiting (11.63%; n = 05) and watery diarrhea + dehydration (09.30%; n = 04) (Figure 1).

Of the 22 strains of *V. cholerae*, 12 were from samples from female patients and 10 were from samples from male patients: 48% of the samples from female patients and 55.56% of samples from male patients, respectively.

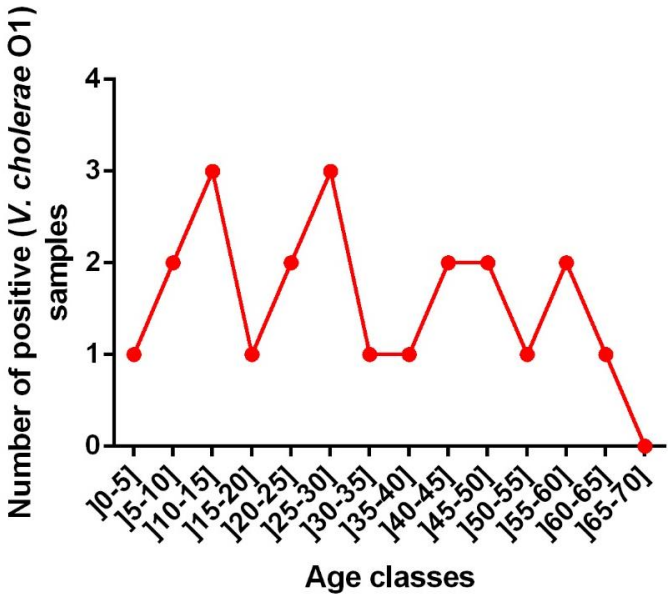

Figure 2. Distribution of positive *V. cholerae* results by age class.

Table 2. Distribution of the analysis results according to the symptoms observed in the patients sampled.

Symptom	Analysis results		Total (%)
	- (%)	+ (%)	
Watery diarrhea + dehydration	04 (19.05)	0	04 (09.30)
Severe diarrhea	03 (14.29)	0	03 (06.98)
Nausea + severe diarrhea	02 (09.52)	0	02 (04.65)
Vomiting + dehydration	05 (23.81)	0	05 (11.63)
Fever + vomiting	03 (14.29)	0	03 (06.98)
Watery diarrhea + vomiting	03 (14.29)	0	03 (06.98)
Watery diarrhea + vomiting + dehydration	0	22 (100.00)	22 (51.16)
Severe diarrhea + abdominal pain + vomiting	01 (4.76)	0	01 (02.33)
Total	21 (48.84)	22 (51.16)	43 (100.00)

Analysis of the data showed an absence of statistical significance ($p > 0.05$) between the positive proportions of the samples in the culture of *V. cholerae* and the sex of the patients from whom these samples were taken.

Of the 22 culture-positive samples for *V. cholerae*, samples from patients aged 11 to 15 and 26 to 30 years old represented the highest proportion with 13.64% each (n = 3) (Figure 2).

Of the 8 symptoms or groups of symptoms observed in patients during sampling, only watery diarrhea + vomiting + dehydration was observed in patients whose specimens tested positive for *V. cholerae* culture (Table 2). Among the culture negative samples of *V. cholerae*, the most prominent symptoms were vomiting + dehydration (23.82%), followed by watery diarrhea + dehydration (19.05%) and severe diarrhea (09.52%). But there was no statistically significant difference ($p > 0.05$) between symptoms and positive culture.

Of the 16 antibiotic discs tested on each of the 22 strains of *V. cholerae*, all strains were susceptible to amikacin, azithromycin, gentamicin, ciprofloxacin, cefuraxime, doxyclyne, cefotaxime and ceftazidine. However, a very strong resistance was noted of the strains isolated to erythromycin (n = 20), to ampicillin (n = 19), to chloramphenicol (n = 18), to cotrimoxazole (n = 17), flucloxacillin (n = 16) and nalidixic acid (n = 16) (Figure 3).

DISCUSSION

The suspected cholera patients included in the study are

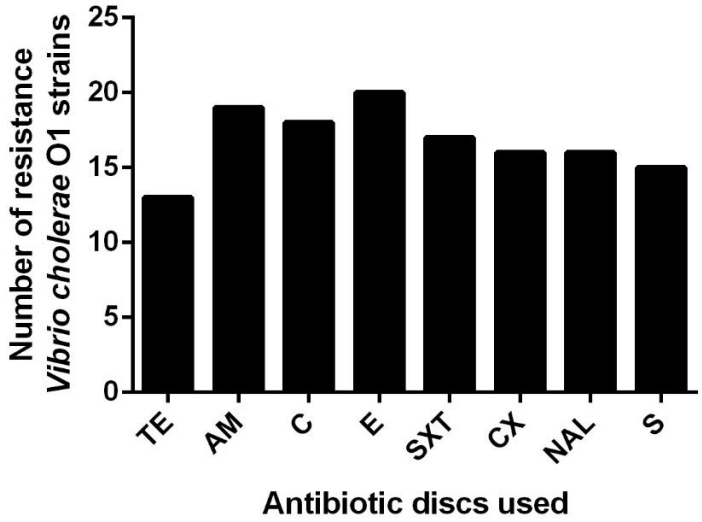

Figure 3. Resistance of *V. cholerae* strains to the antibiotics used. TE: Tétracycline; AM: Ampicillin; C: Chloramphenicol; E: Erythromycin; SXT: Cotrimoxazole; CX: Flucloxacillin; NAL: Nalidixic Acid; S: Streptomycin.

predominantly female, with a median age of 25 years. The most represented age groups were [10-15] and [20-25]. However, statistical analysis of the data has shown that the occurrence of cholera is not related to age or gender. Blacklock et al. (2015) showed that there were more women than men admitted to the Referral Hospital during the cholera epidemic in 2012 in Sierra Leone. As for Danso et al., (2020), they showed that the patients involved in the cholera epidemics in southern Ghana from 2012 to 2015 were mostly men (54.9%), which justify moreover the conclusion according to which the occurrence of cholera in an individual did not depend on sex. However, the fact that in the present study women are more represented than men, could be explained by the exclusive involvement of women in the performance of household chores and the associated risks of cholera transmission. Of the 43 samples analyzed, 22 were declared positive in the culture of *V. cholera* and confirmed to be all strains of *V. cholera* O1, that is, a prevalence of 51%. This prevalence is lower than that found by Koley et al. (2014) 65.6% and that found by Chhotray et al., (2002) in India. All strains of *V. cholerae* isolated in this study are serogroup O1. Both *V. cholerae* serogroups O1 and O139 are involved in cholera epidemics in Africa and globally (Chhotray et al., 2002; Rashed et al., 2012; Lessler et al., 2018). Several studies have shown that in West Africa the *V. cholerae* serogroup most involved in annual cholera epidemics was serogroup O1 (Dalsgaard et al., 1996; Landoh et al., 2013; Moore et al., 2018; Danso et al., 2020). This justifies the results we have obtained. All patients diagnosed positive for *V. cholerae* O1 in the present study were doing watery diarrhea, vomiting and dehydration. The main symptoms of cholera are profuse diarrhea and vomiting, after an incubation period of about 2 h to 5 days (Mengel et al., 2014). Many authors have shown in their studies that the clinical manifestations of patients diagnosed with cholera are diarrhea and vomiting and that on admission to hospital they are severely dehydrated (Ndour et al., 2006; Sule et al., 2017; Elimian et al., 2019). All 22 strains of *V. cholerae* O1 isolated were multidrug resistant with high resistance of the strains to erythromycin, ampicillin, chloramphenicol, cotrimoxazole (trimethoprim/sulfamethoxazole), flucloxacillin and nalidixic acid. Rashed et al. (2012) also showed that the strains of *V. cholerae* O1 responsible for the 2008 to 2010 cholera epidemic in Bangladesh had high resistance to erythromycin and trimethoprim/ sulfamethoxazole. As for the resistance of the strains to ampicillin and nalidixic acid, Eibach et al., (2016) made the same observations on the strains of *V. cholerae* O1 isolated during the cholera epidemics that occurred in Ghana in 2014. However, the results of the present study showed that all the strains of *V. cholerae* O1 obtained were sensitive to gentamicin, ciprofloxacin, doxyclyne and cefotaxime. The sensitivity of the strains of *V. cholerae* responsible for cholera epidemics to gentamicin, ciprofloxacin and docycline have been reported by several authors (Akoachere et al., 2013; Abana et al., 2019; Danso et al., 2020). Cotrimoxazole (trimethoprim = sulfamethoxazole), formerly first-line treatment in children, was ineffective in 78.12% of isolates. Tetracycline, recommended by the WHO for the treatment of cholera in adults, was found to be effective against 51.8% of the

strains tested and ineffective against 48.2% of the latter. This situation then poses a serious problem of therapeutic failure in the treatment of cholera with antibiotics. However, it should be noted that in the treatment of cholera, rehydration remains the main treatment.

Conclusion

This study confirms that the annual cholera epidemics in Benin are the cause of the same *V. cholerae* serogroup, which is serogroup O1 and has shown that the disease can affect all ages, and 90% of them manifest as watery diarrhea, vomiting, followed by severe dehydration. The results showed an increasing trend of multi-resistant *V. cholerae* O1 strains in Benin. Ciprofloxacin, tetracycline and doxycycline remain effective against clinical strains. But a strong resistance of these same strains to erythromycin, tetracycline, ampicillin and trimethoprim/sulfamethoxazole were noted. The results indicate an urgent need for the appropriate use of antibiotics, hygiene and sanitation of coastal villages in Benin in general to prevent the cholera epidemic.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Antibacterial activity of sweet orange (*Citrus sinensis*) juice extract on selected bacteria

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Plants have potentials to be developed into many new drugs yet to be discovered because of the countless chemical compositions in them. The investigation is targeted at the antibacterial activity of sweet orange juice extract on some bacteria using ethanol and ethyl ethanoate solvent to extract juice. Ditch method was used for the sensitivity testing against *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Neisseria gonorrheae* with a dilution factor of 10^{-10} for inoculation from pure culture of each selected bacteria. Disc method was used to test streptomycin, ciprofloxacin, gentamycin and penicillin G against test organisms as positive controls. There was no significant difference in the effect of different concentrations of the same extract on test organisms. However, there was a significant difference in the ethyl ethanoate and alcohol extracts. The ethyl ethanoate extract showed minimum inhibitory concentration at 300 mg/ml on *E. coli* (31.5 ± 0.5 mm); *N. gonorrheae* (21 ± 0.0 mm) at 200 mg/ml; *S. aureus* (22 ± 0.0 mm) and *K. pneumoniae* (37 ± 3.0 mm) at 100 mg/ml; while ethanol extract at 100 mg/ml on *E. coli* (23.5 ± 1.5 mm) and *K. pneumoniae* (25 ± 5.0 mm); *N. gonorrheae* (13.5 ± 1.0 mm) and *S. aureus* (12.5 ± 2.5 mm) at 300 mg/ml and 200 mg/ml respectively. The zones of inhibition exhibited by streptomycin ranges from *N. gonorrheae* (14-24 mm) *E. coli*; ciprofloxacin varies from 15- 21 mm on *K. pneumonia*e and *S. aureus* respectively. Gentamycin ranges from 14-20 mm on *N. gonorrheae* and *S. aureus* respectively; and penicillin G on *N. gonorrheae* (14 mm) and *S. aureus* (28 mm). It can be concluded that sweet orange juice of ethyl ethanoate extract was more effective than the ethanol extract and the positive control.

Key words: Antibacterial activities, ethanolic extract, ethanolic extract, sweet orange and microorganisms.

INTRODUCTION

Medicinal plants can be developed into many new drugs yet to be discovered because of the extraordinarily large chemical constituents found in them. The use of herbal medicine in Africa and Asia had been traced back to the time immemorial. The part of plants used as drug vary from the roots, barks, stems, leaves and seed as extracts and concoctions (Hassan et al., 2013). Many plants were used as antimicrobial agents because of various

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chemical constituents found in them. However, recently attention had been drawn towards extracts and biologically active compound from popular plant species. Plants have ability to synthesize aromatic substances such as phenolic, (for example phenolic acids, flavonoids, quinones, coumarins, lignans, stilbenes, and tannins), nitrogen compounds (alkaloids, amines), vitamins, terpenoids (including carotenoids) and some other endogenous metabolites (Alo et al., 2012). These substances serve as plant defense mechanisms against predators like microorganism, insects and herbivores (Badar et al., 2008).

Orange is one of the most important commercial fruit cultivated on all continents of the world. The importance of the orange is attributed to its diversified use and cultivation worldwide and which probably stands first among the cultivated fruits. *Citrus sinensis* (sweet orange) is widely cultivated in Nigeria and many other tropical and subtropical regions (Piccinelli et al., 2008). Sweet orange commonly called orange is a member of the family *Rutaceae* and a main source of vitamins, especially vitamin C; but also has sufficient amount of folic acid, calcium, potassium, thiamine, niacin and magnesium (Angew, 2007). Sweet orange is the major source of vital phytochemical nutrients and for a long time have been valued for their wholesome nutrition and antioxidant properties. It has been scientifically established beyond reasonable doubt that oranges are very rich in vitamins and minerals that are beneficiary to humans as nutrient and immune booster. According to Doughari and Manzara (2008), sweet orange juice can be used in the development of safe antibiotics for the treatment of bacterial infections. It was recently appreciated that other biologically active and non-nutrient compounds present in sweet orange juice such as antioxidants, as well as soluble and insoluble dietary fibers are reported to reduce the risk of cancers; while many chronic diseases such as arthritis, obesity and coronary heart diseases have been treated with sweet orange juice (Crowell, 1999).

Rehman et al. (2007) reported that essential oil of the citrus juice exhibits antifungal, antibacterial, antiviral and anti-parasitic properties. Recently, many microorganisms have developed resistance against many conventional antibiotics; because of acquisition and expression of resistant genes in them (Bakhru, 2001). Furthermore; conventional antibiotics had been associated with adverse health effects such as hypersensitivity, allergic reactions and immune suppressions (Ahmed and Beg, 2001). Hence, time had come to develop new antibiotics that are safe for the treatment of infectious disease. According to Bhardwaj and Laura (2009), fruits and plants possess secondary metabolites that can inhibit and kill most pathogens. The difference in the antibacterial activity of the various extracts showed that different extracts have varying antibacterial agents with different modes of action and bacteria susceptibility or that not all phytochemicals responsible for antibacterial activity are soluble in a single solvent (Kumar et al., 2011; Badar et al., 2008). Fruits are considered to have great potential therapeutic treatment for various microbial diseases and it is therefore necessary to carry out a study to validate the antibacterial activity of sweet orange on selected bacteria.

MATERIALS AND METHODS

Biological sample

Sample collection

Ten fresh sweet oranges (*C. sinensis*) free from insect infestation and other kinds of damage were plucked at early morning (7 am) from the Lagos State Polytechnic campus at Ikorodu area of Lagos State, Nigeria (Latitude 6.5945°N Longitude 3.3370°E).

Microorganisms

Pure cultures (clinical isolate) of test organisms were obtained from Nigeria Medical Research, Yaba (NIMER). The test organisms were purified by sub culturing and preserved on nutrient agar at 4°C before used. They included their reference numbers: *Staphylococcus aureus* (ATCC 25923) (gram positive bacteria), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883) and *Neisseria gonorrheae* (ATCC 49226) (gram negative bacteria).

Phytochemical screening of C. sinensis juice

The phytochemical analysis was carried out using the method described by Odebiyi and Sofowora (1978). The orange juices were screened for the presence of tannins, saponins, flavonoids, steroids, amino acid, terpenoids, carbohydrate, alkaloids as well as oil and fat.

Extraction of sweet orange juice

The sweet oranges were washed several times using clean water, peeled and sliced into halves and the juice were squeezed or squashed into the beaker. Orange juice (500 ml) was measured into two flasks each and 1000 ml of solvents (100% Ethyl ethanoate and 70% Ethanol) were added to the orange juice to make two different mixtures. The mixtures were left for two days to enable the solvents extract the active ingredients in the orange juice. The mixtures were filtered through Whatman filter paper (number 4) so as to obtain clean and clear filtrate from the residues of the extracts. The filtrates were concentrated in a vacuum using a rotary evaporator model (Buchi rotavapor R – 114) which ensures evaporation of the bulky solutions (filtrates) to the smaller volume concentrates (semi-solid) at temperature between 40°60°C. The resultant concentrates (extracts) were filter - sterilized using millipore filter 0.45 µm and they were ready for the antibacterial activities.

Preparation of extract concentration and the isolate

Six labeled beakers were separated into two groups. The first three breakers were used for the preparation of extract from ethyl ethanoate and the other three were used for the preparation of

Table 1. Phytochemical Constituents of *C. sinensis* juice.

Active ingrédients	Quantitative analysis	Inférence
Tannins	++	Moderate amount
Saponins	+++	High amounts
Flavonoids	++	Moderate amount
Steroids	+++	High amounts
Amino acid	+	Slightly detected
Carbohydrate	+	Slightly detected
Terpenoids	+++	High amounts
Alkaloids	+++	High amounts
Oil and fats	+	Slightly detected

extract from ethanol. In the first three beakers, 100, 200 and 300 mg/ml of extract from ethyl ethanoate was added to each beaker respectively. The same procedures were followed for preparing extract concentration from ethanol; however, the two tubes containing only the ethyl ethanoate and ethanol served as negative controls. A dilution factor of 10^{-10} of each selected organism (pure culture) was prepared and 1mL each out of these was taken for inoculation and labelled accordingly.

Antibacterial activity of the sweet orange juice extract

The extract concentrates of the sweet orange juice were screened for antibacterial activity by using a ditch (well or cup) method. Mueller-Hinton Agar was prepared based on the manufacturer prescription. Aliquot of 1 ml each of the test organism suspensions was inoculated with micro pipette onto the agar surface of each plate of the test organisms and with the aid of the hockey stick (spreader), the bacterial suspension was aseptically spread on the agar surface. The plates were allowed to absorb the organism suspensions at room temperature. A sterile cork-borer of 5mm diameter was used to punch on each agar surface in the plates to make three wells (ditches); subsequently each well was filled with 1mL of the orange juice extract of 100, 200 and 300 mg/ml respectively; and control wells containing the same volume (1 ml) of ethyl ethanoate and ethanol were made for each plate as negative control. However, positive control contained four plates of prepared Mueller-Hinton Agar based on the manufacturer prescription and the same procedure for the inoculation of test organisms was followed as early described in the ditch method and onto which discs impregnated with the following antibiotics; streptomycin, penicillin G, ciprofloxacin, and gentamycin were placed aseptically. The plates were incubated at 35°C for 24 h. Thereafter; the antibacterial activity was evaluated by measuring the diameter of the inhibition zone around the well and disc as case may be. The zone of inhibition around each well and disc was measured using a transparent metric ruler in millimeters (mm). All the tests (ditch method) were performed in duplicate and the average diameter of the two tests was calculated to give mean value and standard deviation of zone of inhibition on each organism and concentration.

Statistical analysis

$$\frac{f_1 + f_2}{2} = \text{mean value (mm)}$$

f_1 = first plate (diameter of zone of inhibition) in mm
f_2 = second plate (diameter of zone of inhibition) in mm

$$\sigma = \sqrt{\frac{\sum(x_i - \mu)^2}{N}}$$

σ = plates standard deviation
∑ = summation
N = the size of the diameter of zone of inhibition
x_i = each value of diameter of zone of inhibition
μ = mean value of the plates (diameter of zone of inhibition) in mm

The result from the above formulae (data) were then expressed as mean ± SEM (standard error mean) of duplicates and subjected to one-way analysis of variance (ANOVA), using the Statistical Analysis System (SAS 9.4 Version).

RESULTS AND DISCUSSION

Table 1 illustrates phytochemical constituents of sweet orange juice extract and the followings were present: tannins, saponin, flavonoid, terpenoid, steroid, amino acid, carbohydrate, alkaloid and oil and fat. These results were similar with the finding of Baba et al. (2018), except that amino acid was absent; while steroid and oil and fat were not analysed at all. Pytochemical analysis of the juice extracts showed that plant constituents such as alkaloids, saponins, terpenoid, tannins and flavonoid were present and that saponin in sweet orange juice was responsible for the antibacterial properties of the juice. According to Kumar et al. (2011) *Citrus sinensis* juice has large amount of saponin with haemolytic activity and cholesterol binding properties.

Table 2 shows that at 95% confidence level, there was a significant difference in the antibacterial activities (zones of inhibition) of extracts (ethyl ethanoate and ethanol respectively) on *E. coli, K. pneumoniae, N. gonorrheae* and *S. aureus*. Furthermore, according to the Duncan Post Hoc analysis of the ANOVA; there was no significant difference between the means of the zones of inhibition of the extracts (ethyl ethanoate and ethanol respectively) on *N. gonorrheae* and *S. aureus* and also there was no significant difference between the means of the zones of inhibition of the extracts (ethyl ethanoate and ethanol respectively) on *E. coli* and *K. pneumoniae*.

Table 2. Comparison of zone of inhibition of ethyl ethanoate and ethanol extract.

Organisms / Extract type	Concentration of extracts in (mg/ml)/ Means of zone of inhibition (mm)			Minimum inhibitory concentration (MIC)(mg/ml)
	100	200	300	
Escherichia coli				
Ethyl ethanoate extract	29.5 ± 0.5	29 ± 1.0	31.5 ± 0.5	300
Ethanol extract	23.5 ± 1.5	22.5 ± 0.5	22 ± 1.0	100
Klebsiella pneumoniae				
Ethyl ethanoate extract	37 ± 3.0	36 ± 1.0	36 ± 2.0	100
Ethanol extract	25 ± 5.0	21.5 ± 3.5	19.5 ± 0.5	100
Neisseria gonorrheae				
Ethyl ethanoate extract	17.5 ± 0.5	21 ± 0.0	21 ± 1.0	200 and 300
Ethanol extract	0.0 ± 0.0	6 ± 6.0	13.5 ± 1.0	300
Staphylococcus aureus				
Ethyl ethanoate extract	19 ± 2.0	21.5 ± 0.5	22 ± 0.0	300
Ethanol extract	10 ± 0.0	12.5 ± 2.5	5.5 ± 5.5	200

ANOVA of antibacterial activities of orange juice on different bacteria at different concentrations

Source	Type III sum of squares	Df	Mean square	F	P value
Model	12487.698[a]	7	1783.96	162.133	0
Effect on organism	1148.95	3	382.983	34.807	0
Conc on organism	6.812	2	3.406	0.31	0.738
Type of the extract	810.844	1	810.844	73.693	0
Error	187.052	17	11.003		
Total	12674.8	24			

Neisseria gonorrhea[a] and *Staphylococcus aureus*[a]; *Escherichia coli*[b] and *Klebsiella pneumoniae*[b].

This means that the zones of inhibition of extracts on *E. coli* and *K. pneumoniae* were higher compared to the zones of inhibition of extracts on *N. gonorrheae* and *S. aureus*.

There was no significant difference in the effect of different concentrations of the same extract on *E. coli, K. pneumoniae, N. gonorrheae* and *S. aureus*. This means that the change in the concentration of the same extract does not affect or improve the potency of the antibacterial activities of the extract but there was a significant difference in the type of extract (ethyl ethanoate and ethanol extracts) that is to say ethyl ethanoate extract was more effective than the ethanol extract. Thus ethyl ethanoate extract showed a remarkable inhibition against *K. pneumoniae* (37±3.0 mm) and *E. coli* (29.5±0.5 mm) compared to ethanol extract on the same test organisms; which showed lower zones of inhibition. Gram negative bacteria have been reported to be more resistant to antibacterial agents due to the possession of an outer-membrane permeability barrier that prevents the antimicrobial agents to reach inner part of the bacterial cell. The antibacterial activity against *E. coli* (gram negative) and *S. aureus* (gram positive) bacteria used in this study is an indication of its broad spectrum activity. This observation is in agreement with the report of Doughari and Manzara (2008) and Kumar et al. (2011). Ethyl ethanoate at various concentrations (mg/ml) demonstrated the highest antibacterial activity against *K. pneumoniae* (37 ± 3.0 mm), *E. coli* (29.5 ± 0.5 mm), *S. aureus* (22 ± 0.0 mm) and *N. gonorrheae* with the minimum zone of inhibition (21 ± 0.0 mm) at 100 mg/ml, 100 mg/ml, 300 mg/ml and 200 mg/ml respectively. This result concurs with the Kumar et al. (2011) findings.

Kumar et al. (2011) reported a maximum zone of inhibition (16 mm) against *E. coli* with ethyl ethanoate extract of the sweet orange juice. The variation in the antibacterial activity of the various extracts showed that different extracts have varying antibacterial agents with different modes of action and bacteria susceptibility or that not all phytochemicals responsible for antibacterial activity are soluble in a single solvent (Kumar et al., 2011 and Badar et al., 2008). Ethyl ethanoate extract was found to be a good solvent for the extraction of antibacterial agent in this study as it had shown the

Table 3. Zone of inhibition of conventional antibiotics using disc method.

Antibiotics	Disc code	Organisms per Zone of Inhibition (mm) on each Antibiotics				Interpretation
		Neisseria gonorrheae	***Staphylococcus aureus***	***Klebsiella pneumoniae***	***Escherichia coli***	**Resistant / Susceptible**
Streptomycin	S – 10	14	23	20	24	Susceptible
Ciprofloxacin	CIP – 5	18	21	15	17	Susceptible
Gentamycin	GM	14	20	17	15	Susceptible
Penicillin G	P	18	28	15	14	Susceptible

highest yield in the antibacterial activity of the sweet orange juice. However, none of the conventional antibiotics (positive control) as illustrated in Table 3 could match the inhibition zone of extract from ethyl ethanoate on the test organisms. According to Hassan et al. (2013), the ethanol extracts of *Ocimum gratissimum (E. coli* 17 mm; *S. aureus* 19 mm*)* and *Vernonia amygdalina* (*E. coli* 12mm; *S. aureus* 5mm) were the most effective on majority of test organisms among the water extracts of *Ocimum gratissimum* (*E. coli* nil, *S. aureus* 13 mm) and *Vernonia amygdalina* (*E. coli* nil, *S. aureus* nil) and the drugs; teteracycine (*E. coli* 16 mm, *S. aureus* 17mm) and flagy (*E. coli* nil, *S. aureus* 11 mm) used in their study. It can be concluded that *K*. *pneumoniae* and *E. coli* were more susceptible to the extracts (ethyl ethanoate and ethanol) compared to the zones of inhibition shown by *N*. *gonorrheae* and S*. aureus*. This finding concurs with Kumar et al. (2011) who asserted the highest zone of inhibition (16 mm) against *E. coli* with ethyl ethanoate extract of sweet orange juice.

Conclusion

The results obtained from this research proved that ethyl ethanoate and ethanol extracts of sweet orange juice have varying degree of antibacterial activity against the test organisms. This suggested that extracts of sweet orange juice can be useful in developing a new drug, which can be used in treating bacterial infections caused by the test organisms in this study.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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A prospective study of bacterial isolates profile in infected open fractures

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The most common complication in open fractures is infection, which often escalates to sepsis, osteomyelitis, and amputations. The use of prophylactic antibiotics is one of the most effective strategies to prevent infection. The prevailing bacterial isolate patterns must guide the choice of antibiotics for both prophylactic and empiric therapy. This study aims to describe the bacterial isolate profiles in infected open fractures. A prospective cross-sectional study was carried out at Kenyatta National Hospital, Kenya, between October 2019 and January 2020. 66 infected open fractures were identified and pus swabs/infected tissue specimens taken for bacterial cultures. Other data were collected from patient interviews and their hospital records. Results revealed that the culture growth rate was 79%. Gram-negative isolates accounted for 73% while Gram-positive isolates were 27%. The most pre-dominant bacterial isolate was *Pseudomonas aeruginosa* (34%), followed by *Staphylococcus aureus* (27%), *Escherichia coli* (20%), *Proteus mirabilis* (16%) and *Klebsiella pneumoniae* (3%). There were more gram-negative than gram-positive bacterial isolates. The pre-dominant bacterial isolate was *P. aeruginosa* followed by *S. aureus*. The higher proportion of gram negative isolates is in variance with what is widely documented in the literature. The selection of antibiotics for both prophylaxis and empiric therapy should be tailored to the local patterns of bacterial isolates.

Key words: Bacteria isolates profile infected open fractures.

INTRODUCTION

Globally, the estimated incidence of long bone fractures is 11.5 per 100,000 people per year, occurs more in men than women, and has a bimodal age distribution, with the tibia being the most commonly affected bone (Courtbrown and Caesar, 2006). The incidence of these open fractures is high in our local setting due to unsafe modes of transport, particularly the upsurge in the use of motorcycles for public transport (Gachathi, 2016; Waithiru, 2015).

Surgical Site Infection (SSI) is one of the most common complications of open fractures. Infected open fracture wounds have historically been dreaded because of the debilitating effect on the patient. Up to the beginning of the 20th century, open fractures were often treated by

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prophylactic amputations to avoid the sequelae of infection, sepsis, and death (Pappe and Webb, 2008). Most studies report infection rates between 10 - 50% (Asif, 2011; Ioannis et al., 2014; Marcelo et al., 2017; Ondari et al., 2016). Earlier studies done at Kenyatta National Hospital have revealed higher rates than those observed globally. Asif (2011) reported an SSI rate of 50% in all open fractures, while Ondari et al. (2016) found a 28% infection rate in Gustilo II open fractures.

Many guidelines have been developed to reduce the rate of these infections (ACS/TQIP, 2019; British Orthopedic Association Standards for Trauma (BOAST), 2019; Hoff et al., 2011). The cardinal principles include antibiotic prophylaxis, debridement/ irrigation, fracture stabilization, and soft tissue coverage. Early administration of antibiotics and urgent surgical debridement are the most critical strategies in infection prevention. Determining the profile of microbial isolates from infected wounds and their sensitivity/resistance patterns is crucial in developing antibiotic protocols for prophylaxis and empirical therapy.

About 70% of all open fractures get contaminated at the time of injury, mainly by organisms from the patient's skin and the surrounding environment (Gustilo and Anderson, 1976). Further contamination occurs in the course of management at the hospital. A study done to determine if organisms isolated from wounds before initial debridement are similar to those causing infection after debridement found that the isolates were not similar and that infections were mainly caused by nosocomial microorganisms (Sitati et al., 2017). The profile of microbial isolates in infected open fractures may vary from one health institution to another and from time to time. Overall, the commonest isolate is *Staphilococus. aureus*. (Gusrilo and Anderson, 1976; Ashwin and Thomas, 2018).

This study was conducted at the Kenyatta National Hospital (KNH) Orthopedic Department. KNH is the largest national teaching and referral hospital in the East African region with over 1800 bed capacity. It is situated along Hospital Road, Upper Hill area in Nairobi about 5km from the city center. It receives the highest number of trauma patients from its vast catchment area. The high number of trauma cases makes it possible to achieve a good sample size within a reasonable period with results that would better represent the situation in the surrounding referring facilities. The objective of the study was to determine the profile of bacterial isolates in infected open fractures.

MATERIALS AND METHODS

This study was conducted in the Orthopedics wards at Kenyatta National Hospital between October 2019 and January 2020. It was a prospective descriptive cross-sectional study of patients aged between 18 and 75 years with infected open appendicular fractures following initial debridement. The ASEPSIS Score was used to determine infection -scores of 21 and above (Wilson et al., 1986). We excluded patients on chemotherapy and long-term steroids as well as those with diabetes mellitus.

Wound assessment for infection was done on the 3rd day following initial debridement. Non- infected wounds were re-assessed five days later. The sample size was 66 patients, which had been calculated using the Fisher's formula; base on the prevalence of Sitati et al. (2016) in the same hospital, which revealed an infection rate of 58.9%. The first participant was randomly selected. Consecutive sampling was then applied where all subsequent patients with infected open fractures following initial debridement and fitting the criteria were included until the sample size was achieved. Superficial infection was defined as involvement of only skin and subcutaneous tissue with no fluctuation in deep tissue or deep tissue dehiscence beyond the fascia. Deep infection was defined as fluctuation on palpation or purulent discharge from deep tissue layers below the fascia.

Pus swabs were taken from infected wounds using the Levine Method (Gardner et al., 2006). Infected tissue specimens were taken from patients with deep infections during their repeat debridement in theatre as well as during bed-side wound dressing. The Specimens were submitted to the microbiology laboratory within one hour of collection for microscopy, culture and sensitivity. They were cultured within one hour after delivery to the laboratory. Sheep Blood Agar was used for culture, incubated at 35 to 38 degrees Celsius for 18 h followed by further 18 h of sensitivity testing if growth was obtained. Bacterial identification was based on both gram-stain features and colony morphology.

Patients were interviewed and their hospital records checked to obtain demographic data, mechanism of injury, fracture characteristics and wound grading. A questionnaire was utilized to collect the data, which was entered into SPSS version 22 for analysis. Descriptive analysis was done for demographic and other baseline characteristics, while associations were analyzed using chi-square tests. The study was approved by the hospital/university ethics board and all patients gave informed consent.

RESULTS

Baseline characteristics

A total of 66 patients with infected open fracture wounds were recruited into the study. There were 57 (86) males and 9 (14%) females, a ratio of 6.3:1. The minimum age was 19 years while the maximum was 59 years; a range of 40 years. The median age was 36 and the mean age was 36.38 years.

Most of the injuries (59%) resulted from motorcycle accidents followed by motor vehicle accidents at 24%. The commonest site of open fractures was Tibia/fibula shaft followed by tibia plafond and foot/ankle at 30, 20 and 14% respectively. Most of the infected open fractures were Gustilo grade II (48%) followed by Gustilo IIIA (33%), and only 2 patients had Gustilo I fractures. 35% of the patients had their initial wound debridement done within 24 h, while 47 and 18% had their initial debridement done between 24 – 48 h and after 48 h respectively.

Culture growth

Figure 1 show that the Proportion of specimens with

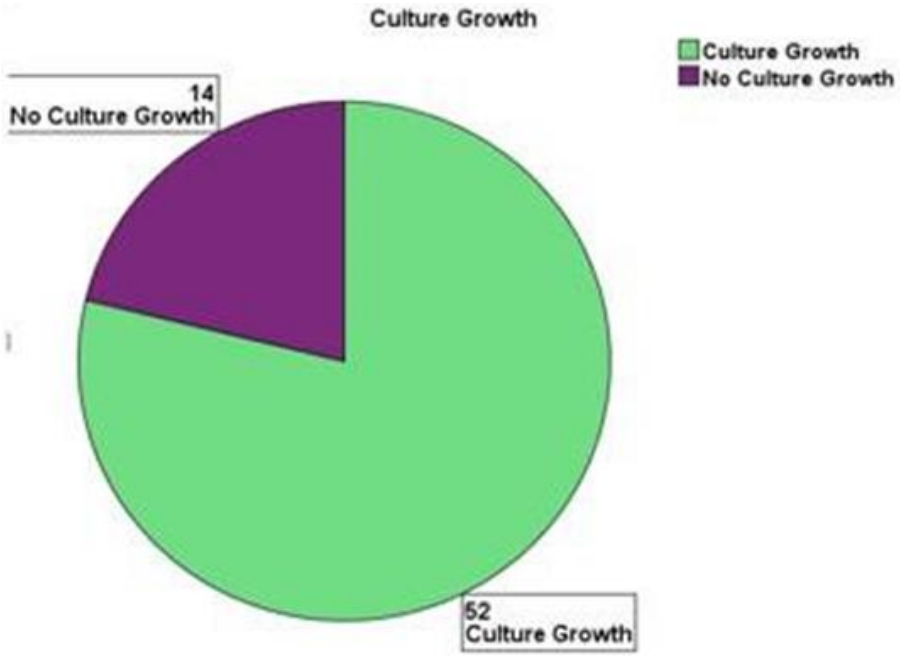

Figure 1. Proportion of specimens with culture growth.

Table 1. Cross tabulation of delayed debridement and type of bacteria cultured.

Delayed Debridement × Gram Stain Cross-tabulation			Gram Stain		Total
			Gram Positive	Gram Negative	
Delayed Debridement	Within 24 h	Count	7	16	23
		Expected Count	6.3	16.7	23.0
	Beyond 24 h	Count	11	32	43
		Expected Count	11.7	31.3	43.0
Total		Count	18	48	66
		Expected Count	18.0	48.0	66.0

The actual counts are similar to the expected counts when no association exists.

culture growth and there was culture growth in 52 specimens (79%) and no culture growth in 14 specimens (21%).

There was a 79% (n = 52) culture growth rate out of the 66 specimens. 81% (n = 42) had single bacterial isolates while 21% (n = 10) had 2 isolates each, thus the total number of isolates studied were 62.

Bacterial isolates

There were 45 (73%) gram negative and 17 (27%) gram positive bacterial isolates. Gram positive bacteria comprised of *S. aureus* while the Gram negatives included *Pseudomonas aeruginosa*, *Escherichia coli*, *Proteus Mirabilis and Klebsiella pneumoniae*.

Effect of delayed initial debridement on type of bacteria cultured

Table 1 shows cross tabulation of delayed debridement and type of bacteria cultured. Here the actual counts are similar to the expected counts when no association exists. Also, Table 2 reveals Chi-square tests for association between delayed debridement and type of bacteria cultured. Both tables indicate that there was no statistically significant association between delayed initial debridement and the type of bacteria cultured (P=0.673).

Proportion of specific bacterial isolates

Specific bacterial isolates proportion is described in

Table 2. Chi-square tests for association between delayed debridement and type of bacteria cultured.

Chi-Square Tests	Value	df	Asym Sig.1 sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	0.178[a]	1	0.673		
Continuity Correction[b]	0.017	1	0.895		
Likelihood Ratio	0.176	1	0.675		
Fisher's Exact Test				0.774	0.442
Linear-by-Linear Association	0.175	1	0.675		
No. of Valid Cases	66				

Chi square value is 0.178, with P value of 0.673. The level of statistical significance was set at 0.05.

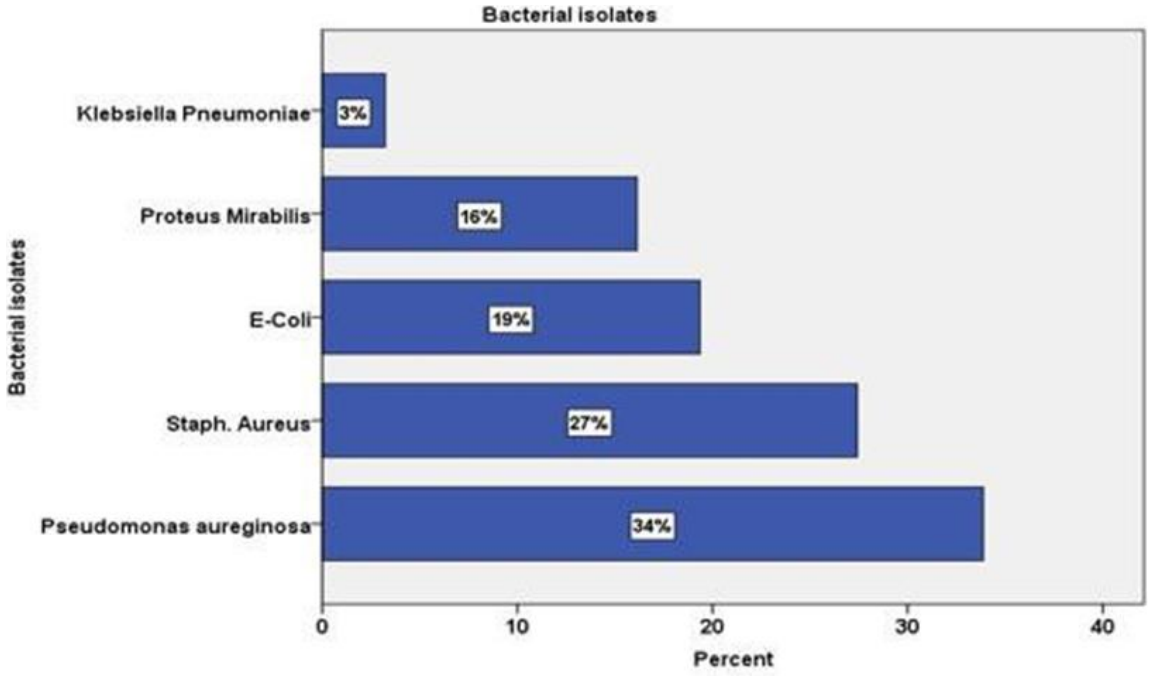

Figure 2. Bacterial isolates cultured.

Figure 2. The most pre-dominant bacterial isolate was *P aeruginosa* (34%), followed by *S. aureus* (27%), *E. coli* (20%), *P. mirabilis* (16%) and *K. pneumoniae* (3%).

DISCUSSION

The baseline characteristics concerning gender and age were similar or close to what was observed in other local studies (Gachathi, 2016; Waithiru, 2015; Ondari et al., 2016). The youthful male preponderance is consistent with the expected gender roles in the local society where males are mostly the bread winners, thus more likely to be involved in risky activities in the transport sector and other industries.

Most of the injuries resulted from motorcycle accidents (59%), followed by motor vehicle accidents (24%). Similar findings were noted by Gachathi in Eldoret: 67% of all injuries resulted from road traffic accidents, among which 65 were from motorcycle accidents while 33% resulted from motor vehicle accidents (Gachathi 2016). Most of the open fractures were Gustilo II (48%) and Gustilo IIIA (33%). The small number of infected Gustilo I fractures (3%) reflects the known minimal infection rate in this group (Gustilo and Anderson, 1976).

35% of the patients had their initial wound debridement done within 24 h as recommended by current literature. Many studies have shown no increase in infection rates when debridement is not done within the historical 6 h period (Reuss and Cole, 2007; Harley et al., 2002). The current consensus is to debride wounds within 24 h on a semi-emergency theatre list, mostly at day time when optimum operating room equipment and personnel are accessible, except for wounds with vascular injury, gross

contamination or compartment syndrome whose intervention should be emergent (BOAST, 2017; ACS/TQIP, 2019). There were patients who had their initial debridement done between 24 – 48 h (47%) and after 48 h (18%). The main reasons for the delays were; late presentation mainly from referring hospitals, lack of blood and blood products, patients being too sick for operations and needing further stabilization by specialists in other departments, and theatre space being taken over by more dire emergencies.

The high culture growth rate achieved (79 %) was attributed to the Levine technique for pus swab specimen collection. In a study comparing three different methods, Levine technique had the highest accuracy with a sensitivity of 90%. The mean concordance between swab specimens obtained using Levine's technique and tissue specimens was 78% (Gardner et al., 2006).

The overall proportion of gram negative bacterial isolates (73%) was higher than that of gram positive isolates (27%). The most pre-dominant bacterial isolate overall *was P. aeruginosa* (34%), followed by *S. aureus* (27%), *E. coli* (20%), *P. mirabilis* (16%) and *K. pneumoniae* (3%). These findings were similar to a few studies found in the literature, which found an overall higher proportion of gram negative bacteria. Among the gram positive bacteria, these three studies found *S. aureus* to be the most predominant (Ako-Nai et al., 2009; Al-Saadi et al., 2018; Nobert et al., 2016). Overall, the most common bacteria isolated from open fractures, as demonstrated in most studies is *S. aureus* (Ashwin and Thomas, 2018; Gustilo and Anderson, 1976).

Local studies at our hospital by Ondari et al. (2016) and Sitati et al. (2017) showed a higher overall proportion of gram positive than gram negative isolates, with *S. aureus* being the most predominant. Our results were different from these two studies. The two studies were quite similar to our study with respect to the setting, population and methodology, with the main difference being the study period. This shows how the pattern of bacterial isolates in a given health institution may vary from time to time, and it is also possible that there was an outbreak of pseudomonas infection in the wards during the period of this study. With delays in initial debridement, it is also possible that the wounds were already contaminated with pseudomonas bacteria prior to initial debridement. However, there was no statistical correlation between delayed initial debridement and the gram-stain characteristics of isolated bacteria (P value = 0.673). The mixing of patients who are awaiting debridement with long-stay patients may also contribute to this possible contamination.

Conclusion

There was a higher proportion of gram negative (73%) than gram positive (27%) bacterial isolates in infected open fracture wounds in our setting. The most common isolates were *P. aeruginosa*, followed by *S. aureus, E. coli, P. mirabilis and K. pneumoniae*. The higher proportion of *Pseudomonas* infection is different from what is documented in the literature and thus points to possible cross-contamination in the wards.

RECOMMENDATIONS

1) Patients awaiting initial debridement should be admitted to a separate trauma ward to minimize the chances of cross-contamination.
2) Surveillance for bacterial isolate profiles in infected wounds should be enhanced to inform a regularly updated prescription protocol for prophylactic/ empiric antibiotic.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Letter to Editor

Multidimensional aspects of surgical site infections due to non-tuberculous mycobacteria

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We read the recent publication on “Surgical site infections” (SSI) by Braga et al. (2021) with great interest. Moreover this paper has sensitized the surgeons, practitioners, microbiologists and quality managers on the need to look for non-tuberculous mycobacteria (NTM) as a cause for SSIs. Though NTM is one another cause for SSIs, and scientific articles and case reports including the present report have supported the views, NTM do not receive due attention in regular clinical practice. Here, we would like to highlight on other dimensions of this entity related to patients, professionals, laboratory, quality, medical malpractice and educational.

Non-recognition of NTM in patients with SSI contributes to morbidity, exposes them for multiple medications, enhances hospital stay, escalates healthcare cost, and thus causes strained Doctor-Patient relationship (Chipidza et al., 2015). Professionals handling SSI in the interest of patients, by and large switch over to a combination of higher antimicrobials without searching for the causative agents for SSI, which predispose to the development of drug resistance and delay in recovery. Apart from that, this attitude pushes the SSI to chronicity, contributes to treatment failure and causes anxiety among healthcare providers involved in patient care. Over all NTM receives suboptimal attention in clinical practice and discussion.

Despite the reports on NTM as a cause for SSI from different parts of the world including the present one (Braga et al., 2021), the Clinical Microbiologists and Laboratory personnel handling the pus samples of SSI do not process them for NTM unless asked for. Hence, it is suggested that pus samples of SSI shall be subjected to Ziehl Neelson staining routinely in the interest of patient care and if needed for culture. In addition, the sterilization techniques adopted for surgical materials shall be checked periodically from the point of patient safety.

With regard to quality enhancement, the internal and external audit team shall design protocols and standard operating procedures (SOPs) to include routine screening of SSI samples for NTM. These shall be reviewed in hospital infections control committee meetings, as it is preventable. Moreover, notification of NTM and discussion on this may likely assist early diagnosis and institute appropriate medications.

Current status of non-consideration of NTM as a cause for SSI may lead to delay in diagnosis, dissatisfaction among patients and deviation in therapy (Legeais, 2019). As these predispose to malpractice claims (Choudhary et al., 2020), current health science education shall be tuned to focus on NTM similar to the teaching, learning and assessment (TLA) adopted for *Mycobacterium tuberculosis* and lepra bacilli so as to acquire required skills. It is also suggested that NTM as a cause of SSI shall be remembered by the practitioners, recalled during clinical rounds, recognized by the available clinical and laboratory means; and explained to the patients and caregivers for medication adherence (Legeais, 2019) which shall strengthen provider patient partnership.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Virulence and multi-resistance of gram-negative bacilli strains isolated from some artisanal fermented dairy products sold in secondary schools in Benin

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This work aims at assessing toxin production capability and antibiotic resistance profiles of thermo-tolerant Gram-negative bacilli strains isolated from three types of fermented dairy products (yoghurt, dèguè millet and dèguè couscous). Samples collected in Abomey-Calavi and Cotonou were analyzed for microbial, biochemical and molecular parameters. Samples were contaminated with thermo-tolerant Gram-negative bacilli strains at 13.88%. The high contamination rate was recorded with the samples of dèguè couscous and the lowest contaminated samples were dèguè millet. Morning samples were more contaminated. *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Escherichia coli* and *Enterobacter cloacae* were the most identified bacteria. The most isolated species in the dry season was *E. coli.* In the rainy season, *K. pneumoniae* and *E. cloacae* were the most isolated species. *K. pneumoniae* was the most formative of biofilm (38.9%). About 12% of the isolated strains were extended-spectrum beta-lactamases (ESBL) producers. The higher resistance rate was observed with amoxicillin and doxycylin (92.3%). Imipenem was the most efficient molecule on the isolated strains with 00% of resistance. The blaTEM gene was detected in 51.43% of the isolated strains followed by blaSHV (37.14%), blaCTX-M (8.57%) and blaOXA-1 (2.86%). It is necessary to train sales people on good hygiene practices for food during their production and their distribution.

Key words: Fermented milk products, thermo-tolerant gram-negative bacilli, toxins, antibiotics resistance, Benin.

INTRODUCTION

Milk is a complex ecosystem for various microorganisms including bacteria. The widely consumed milk products are cheese, yoghourt and curdle milk (Pal and Awel, 2014). Dairy products undergo lactic fermentation to

ensure food safety through acidification and production of bacteriocins that antagonize the growth of pathogenic bacteria, and improve the final quality of dairy products by producing aromatic compounds. There is an increase demand of natural food without any artificial additive and pathogenic microorganisms. However, several germs such as yeasts and bacteria are responsible for altering the marketable and hygienic quality of these dairy products (Abdel-Aziz et al., 2016).

Food borne pathogens cause outbreak of food borne diseases through pathogenic microorganisms or their toxins (Smith and Fratamico, 2018). Thus, in developing countries, foodborne infections remain a major public health problem since they can affect a large part of population (Djogbe et al., 2019). Milk and their derived products are reported to be important sources of food borne pathogen such as bacteria (Oliver et al., 2005; Zagare et al., 2012). In Benin, traditional techniques for improving preservation and production of yoghurt and dèguè are expensive and less accessible to processors (Sessou et al., 2013; Tohoyessou et al., 2020).

Milk and its derivate products contamination by pathogenic bacteria is largely due to processing, handling, and unhygienic conditions. Garas et al. (2017) describe the presence of microorganisms that can cause food poisoning are viruses and bacteria. These include thermo-tolerant Gram-negative bacilli such as *Clostridium botulinum*, *Clostridium perfringens*, *Campylobacter*, *Salmonella* and *Escherichia coli* (Mon et al., 2015). Nowadays, food poisoning and safety is very important subject all over the world.

The Gram-negative bacilli in particular *Enterobacteriaceae* are becoming increasingly resistant to antibiotics particularly to carbapenems (Pfeifer et al., 2010; Nordmann et al., 2011). Carbapenems remain the beta-lactam with the broadest spectrum of activity. Their excellent antibacterial activity is linked to their rapid transmembrane penetration through the external wall of Gram-negative bacilli. In addition, the have stability against most natural or acquired beta-lactamases, including chromosomal and plasmid cephalosporinases and extended spectrum beta-lactamases (Nordmann and Carrer, 2010).

Considering the numerous cases of food poisoning induced by the multi-resistant bacteria, it is necessary to investigate the resistance ability of potentially pathogenic microorganism founded in foods such as fermented milk products.

Thus, the aim of this work was to identify and characterize thermo-tolerant Gram-negative bacilli isolated from three artisanal fermented dairy products (yoghurt, dèguè millet and dèguè couscous) collected from the schools of Abomey-Calavi and Cotonou (Benin).

MATERIALS AND METHODS

Sampling and samples collection

The three dairy products (yoghurt, dèguè millet and dèguè couscous) samples were randomly collected from 15 schools of Abomey-Calavi and Cotonou (Figure 1). Those samples were preferentially taken from inside schools' vendors. For each products, two samples were taken two time daily (morning and evening) and repeated twice in a week with. Thus, 180 samples (60 yoghurt, 60-dèguè couscous and 60-dèguè millet) were collected during this study. Collected samples were carried, in ice (about 4°C), to the laboratory for further analysis.

Microbiological analyses

Each collected sample (10 g) was aseptically homogenized into sterile tryptone salt water (90 ml). From this solution, a serial decimal dilution was made to obtain dilution 10^{-1}, 10^{-2}, 10^{-3}, up to 10^{-7}. For the detection and enumeration of total and fecal coliforms, dilutions 10^{-1} to 10^{-3} were used.

Total and fecal coliforms

Total and fecal coliforms were enumerated on Violet Red Bile Glucose Agar (VRBA, OXOID CM0485) medium following a previously describe method (Milani et al., 2011). Dilutions from 10^{-1} to 10^{-3} were homogenized with 15 ml of VRBA medium and after solidify, a second stratum of about 5 ml was poured. The incubation was performed at 30°C for 24 h (total coliforms) and at 44°C for 24 h (fecal coliforms).

Thermo-tolerant Gram-negative bacilli

Tryptone Bile X Glucuronide agar and Eosin Methyl Blue Agar (OXOID, CM0069) were used for the isolation of thermo-tolerant Gram-negative bacilli. The identification of such bacilli was completed with indole test and the Api20E gallery (Riegel et al., 2006).

MALDI-TOF mass spectrometry

After isolation, the MALDI-TOF mass spectrometry was used to confirm the microbial identification. Thus, the MALDI-TOF target plate (Bruker Daltonics ™) was used to receive bacterial samples. The deposited samples was covered with 1.5 µl of the matrix solution (Sigma, Lyon, France) completed with acetonitrile 50% (500 µl), trifluoroacetic acid 10% (250 µl) and water (250 µl). The mixture was then sonicated for 10 min, centrifuged (13000 g, 5 min) and transferred to a clean polypropylene tube. The target plate and matrix were then dried at room temperature before MALDI-TOF/MS (BrukerDaltonics, Germany) identification (Pfleiderer et al., 2013). A positive control was used (*E. coli* ATCC 8739).

Biofilm formation test

The thermo-tolerant Gram-negative bacilli capacity to produce

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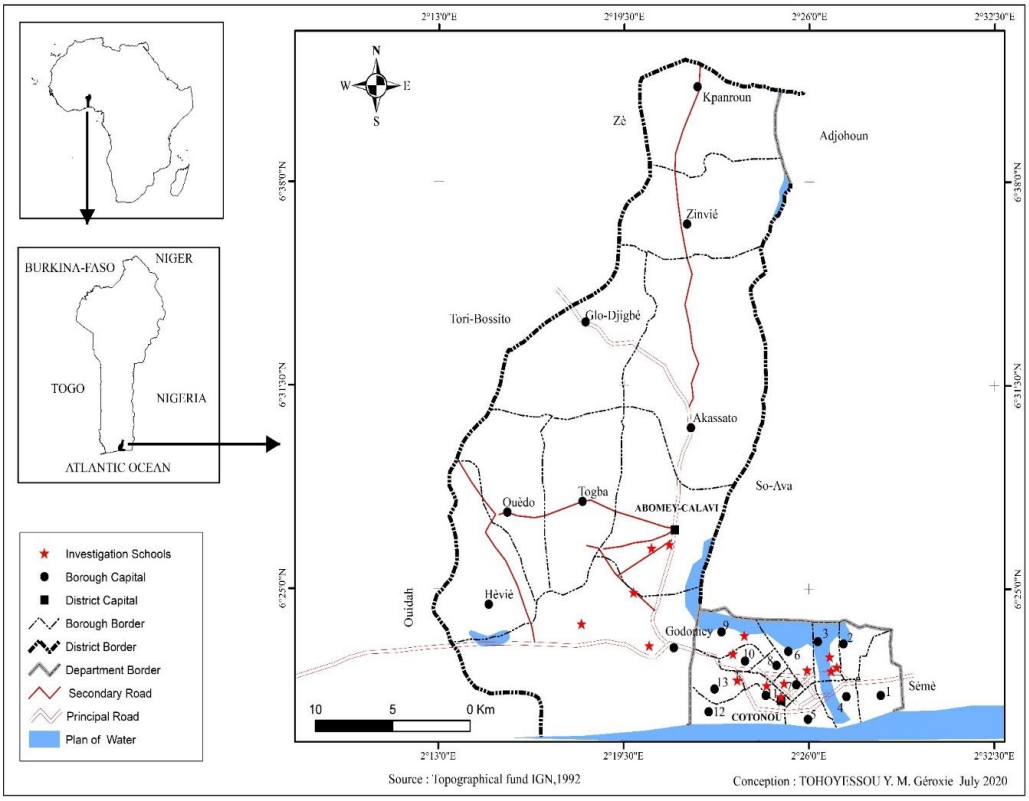

Figure 1. Localization of the investigated schools for the samples collection.

biofilm was determined according the method described by Christensen et al. (1985). Briefly, the 48 well microplate was used to assess, *in vitro*, qualitative biofilm production. Thus, on the microplates, 10 µl of 18 h old bacteria suspension was diluted with 150 µl of Brain Heath Infusion (BHI) and incubated for 24 h at 37°C. After incubation, wells were washed three times with sterile physiological water (0.2 ml) in order to eliminate the free bacteria. Biofilms formed by adhesion of sessile organisms to the microplate in each well are stained with crystal violet (0.1%) for 10 min. The excess dye was removed after thorough washing and the plates were left at room temperature for drying (Stepanović et al., 2000).

Antibiotic susceptibility of strains of thermo-tolerant Gram-negative bacilli

The susceptibility of the identified thermo-tolerant Gram-negative bacilli to 16 conventional antibiotic molecules was performed using the diffusion method (CASFM/EUCAST, 2019). The 16 antimicrobial agents (BioRad®) tested were: cefoxitin (FOX 30 µg), norfloxacin (NOR 5 µg), doxycyline (DOX 30 µg), trimethoprim sulfamethoxazole (Sxt 23.75 µg), amoxicillin-clavulamic acid (AMC 20/10 µg), amoxicillin (AMX 25 µg), ceftriaxone (CRO 30 µg), azetronam (ATM 30 µg), imipenem (IPM 10 µg), piperacilin tazoubatam (PTZ 36 µg), ticarcillin (TIC 75 µg), cefepim (FEP 30 µg), ceftazidime (CZD 10 µg), ciprofloxacin (CIP 5 µg), cefotaxime (COX 5 µg) and gentamycin (GMN 10 µg).

Penicillinase test

The penicillinase production was detected using a tube acidimetric method test on all the isolated strains as previously described (Koneman et al., 2006). Briefly, to obtain a purplish red color, 300 µl of 1% phenol red was added to the mixture. The final reaction volume is 1 ml. Two young colonies were suspended in 500 µl of physiological water (9%NaCl). The emulsion was supplemented with 150 µl of pH 8 benzyl-penicillin (600 mg of benzyl-penicillin + 400 µl of sterile distilled water). *E. coli* ATCC 25922 was used as a control. After incubation (37°C, 1 h) orange or yellow color in the tubes indicates penicillinase production.

Phenotypic detection of extended spectrum beta-lactamase

To carry out this test, a bacterial suspension (10^6 CFU/ml) was used (CASFM/EUCAST, 2019). Cefotaxim (COX 3 µg) and cefipim (FEP 30 µg) in the presence of amoxicillin + clavulanic acid (AMC) was used. AMC disc was placed in the center of Muller-Hinton agar between the two-cephalosporin discs at a distance of about 30 mm. After incubation (37°C for 18 h), the positive result was a potentiation of the corkscrew-shaped inhibition zone between the COX and AMC discs and then between the AMC and FEP discs (CASFM/EUCAST, 2019).

Hodge test

To perform this test, with *E. coli* ATCC 25922 suspension (1/10 dilution of a 0.5 McFarland suspension) and ertapenem disc (10 µg). Bacteria to be tested were seeded in line at about 20 to 25 mm of distance, from the ertapenem disc to the tip of the agar. After incubation (37°C, 24 h), the shoot of *E. coli* ATCC 25922 near the ertapenem disc around the streak of the tested strain produced a depression in the agar indicating carbapenemase activity. If the growth of *E. coli* ATCC 25922 continues to be inhibited even in the

Table 1. The PCR programs used for the amplifications.

Genes	Initial denaturation	Denaturation	Hybridization	Extension	Final extension	Number of cycles
BlaTEM	94°C for 5 min	94°C for 30 s	52°C for 30 s	72°C for 1 min	72°C for 10 min	30
BlaSHV	94°C for 10 min	94°C for 40 s	60°C for 40 s	72°C for 1 min	72°C for 7 min	30
BlaCTX-M	94°C for 10 min	94°C for 40 s	60°C for 40 s	72°C for 1 min	72°C for 7 min	35
BlaOXA-1	94°C for 10 min	94°C for 40 s	60°C for 40 s	72°C for 1 min	72°C for 7 min	30
STX-1	94°C for 2 min	94°C for 1 min	62°C for 90 s	72°C for 1 min	72°C for 5 min	30
STX-2	94°C for 2 min	94°C for 1 min	62°C for 90 s	72°C for 1 min	72°C for 5 min	30

Table 2. The sequences of primers used.

Gene	Sequences	Weight (bp)	Reference
BlaTEM	5′-TTGGGTGCACGAGTGGG TTA-3′ 5′-TAATTGTTGCCGGGAAGCTA-3′	467	Anago et al. (2015)
BlaSHV	5’ATT TGT CGC TTCTTT ACT CGC-3’ 5’ TTT ATG GCG TTACCT TTG ACC-3’	713	Dallenne et al. (2010)
BlaCTX-M	5’-ATG TGC AGYACC AGT AAR GT 3’ 5’-TGG GTRAAR TAR GTS ACC AGA 3’	688	Dallenne et al. (2010)
BlaOXA-1	5′-ATATCTCTACTGTTGCATCTCC-3′ 5′-AAACCCTTCAAACCATCC-3′	564	Dallenne et al. (2010)
Stx1	5’-TGTAACTGGAAAGGTGGAGTATACA-3’ 5’-GCTATTCTGAGTCAACGAAAAATAAC-3’	210	Meng et al. (1997)
Stx-2	5’-GTTTTTCTTCGGTATCCTATTCC-3’ 5’-GATGCATCTCTGGTCATTGTATTAC-3’	484	Meng et al. (1997)

presence of the strain tested the test result is negative (Girlich et al., 2012).

Detection of genes encoding the production of β-lactamases and of toxins for *E. coli*

All the ESBL-producer Gram-negative bacilli were used to detect genes encoding multi-resistance (TEM, CTX-M, SHV and OXA-1). In addition, the Shiga toxins genes (Stx-1 and Stx-2) were researched for E. coli isolates. The DNA template was extracted using a boiling method. Briefly, in 500 µl of sterile water a bacteria colony was incubated at 95°C for 10 min. after, the suspension was centrifuged (12000 rpm for 5 min), and 10 µl of the supernatant was used as target DNA (Rasmussen and Morrissey, 2008). DNA extracts were used for the polymerase chain reaction (PCR) reaction. The 25 µl PCR mixture containing 12.5 µl of 2xMaster Mix (BioLabs), 1.5 µl of forward primer and 1.5 µl of reverse primer, and 4 µl of DNA was used. The PCR program used for the amplifications are in the Table 1 and the primers used are in the Table 2. After 30 min electrophoresis (150 V) on 1.5% agarose gel containing ethidium bromide, 10 µl of the PCR products were visualized with a 100 bp molecular weight ladder.

Data analysis

The MS Office Excel 2010 spreadsheet was used for data

Table 3. Bacterial load of fecal coliforms and thermo-tolerant Gram-negative bacilli (TTGNB) in samples collected in Cotonou and Abomey-Calavi.

Sample	fecal coliforms (UFC/ml)	TTGNB (UFC/ml)
Yoghurt	$0.39*10^5 \pm 0.05*10^5$	$7.21*10^5 \pm 0.52*10^5$
Dèguè Millet	$11.12*10^5 \pm 0.96*10^5$	$3.09*10^5 \pm 0.17*10^5$
Dèguè Couscous	$7.75*10^5 \pm 0.16*10^5$	$10.81*10^5 \pm 0.61*10^5$

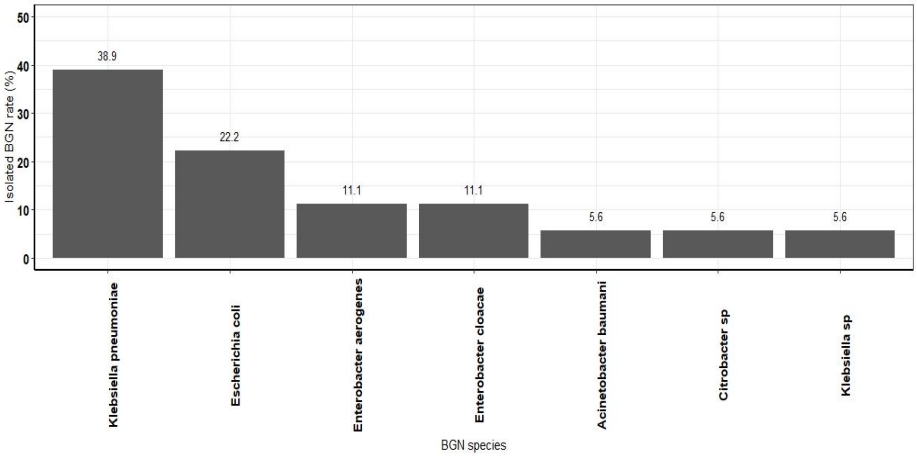

Figure 2. Rates of the different species of thermo-tolerant Gram-negative Bacilli identified.

processing. R 3.6.1 software was used for p-value test and for graphing. The test is considered statistically significant if $p < 0.05$.

RESULTS

Environment of the fermented milk products sale points

Some sales sites are unhealthy with the observation of rubbish and open gutters wastewater. Some vendors do not have adequate garbage bins for garbage collection. Dairy products are prepared at home and the rest of the activities carried out at the sale sites are done on the sale sites, using the water available at the site. Most vendors rarely change utensils rinse water. No vendors wear masks and gloves, so the hands of some vendors are in direct contact with money and the millet and couscous.

Microbiological quality of fermented dairy products

Enumeration of fecal coliforms and thermo-tolerant Gram-negative bacilli in sampled fermented milk products

The germ count results in the fermented milk products analyzed, in CFU/ml, were compiled in Table 3. Table 3 represent the microbial load of yoghurt, dèguè millet and dèguè couscous. Results indicated that the microbial loads vary according to the type of samples. For fecal coliforms, the most contaminated samples were dèguè millet samples ($11.12*10^5$ UFC/ml) and the least contaminated were yoghurt samples. For Gram-negative bacilli, couscous samples were the most contaminated and the least contaminated samples were those of dèguè millet ($3.09*10^5$ UFC/ml).

Different species of thermo-tolerant Gram-negative bacilli found in the fermented milk products

Twenty-five Gram-negative bacilli strains were isolated from the 180 samples tested. The four most represented species were strains of *Klebsiella pneumoniae* (38.9%), *E. coli* (22.2%), *Enterobacter aerogenes* (11.1%) and *E. cloacae* (11.1%). While *Acinetobacter baunami*, *Citrobacter* sp and *Klebsiella ornithinolytica* strains were the least isolated (5.6%) (Figure 2).

Distribution of thermo-tolerant Gram-negative bacilli isolates according to the type of fermented milk products

Figure 3 presents the different thermo-tolerant Gram-negative bacilli of strains of obtained as a function of the

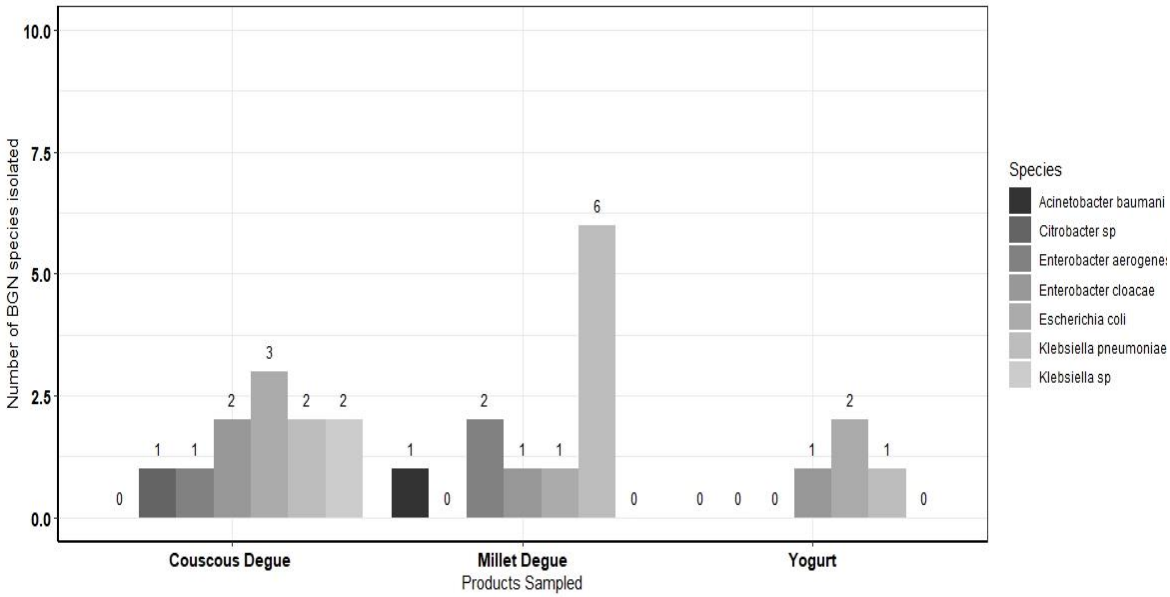

Figure 3. Distribution of thermo-tolerant Gram-negative Bacilli strains according to fermented milk products.

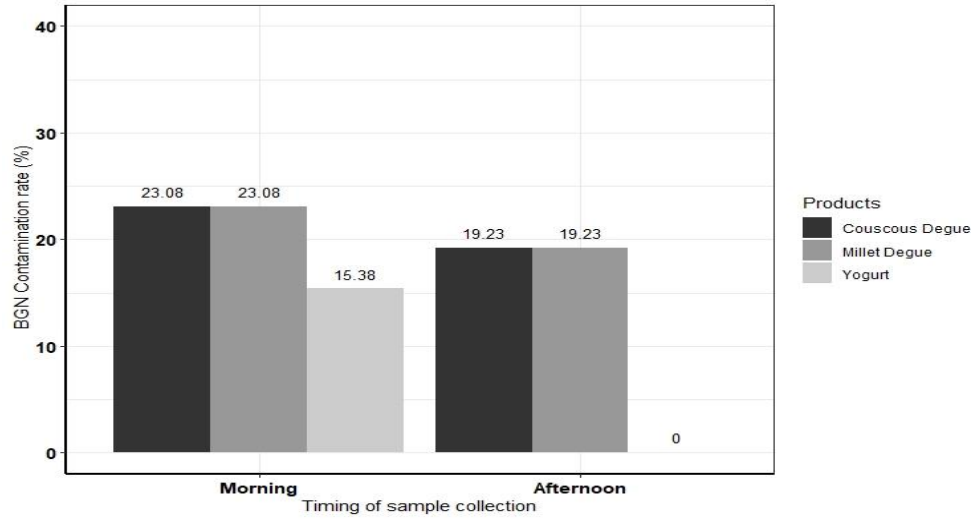

Figure 4. Distribution of thermo-tolerant Gram-negative bacilli strains according to the time of collection.

fermented milk products analyzed. A total, 7 species of thermo-tolerant Gram-negative bacilli were identified. It appears that *E. coli* strains were the most dominant in yoghourt and dèguè couscous. In the samples of dèguè millet *K. pneumoniae* was the most present. It is noted a presence of six different species in dèguè couscous, five species in dèguè millet and three species in yoghourt. *Enterobacter cloacae*, *E. coli*, and *K. pneumoniae* were present in dèguè couscous, dèguè millet and yoghourt. The distribution of species according is not significantly different (p> 0.05).

Distribution of thermo-tolerant Gram-negative bacilli according to the collection period

Samples of dèguè couscous and dèguè millet were more contaminated in the morning than in the afternoon with respective proportions of 23.08 and 19.23% (Figure 4). The yoghourt was contaminated in the morning with a proportion of 15.38% and not in the afternoon. In general, high contamination was recorded in dry season (Figure 5). The most commonly species found in the dry season was *K. pneumoniae* followed by *E. coli* and in the rainy

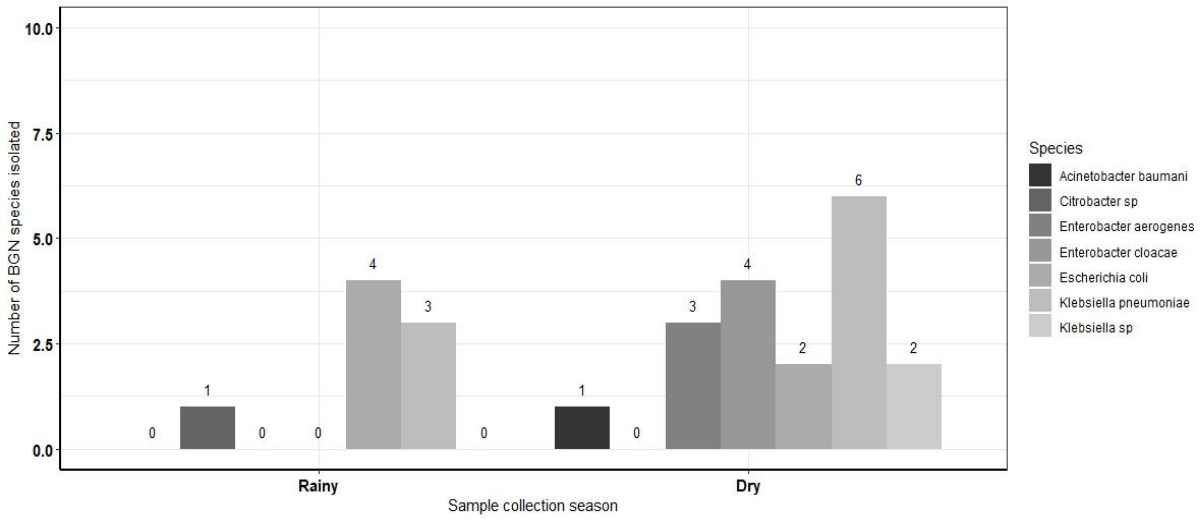

Figure 5. Distribution of thermo-tolerant Gram-negative bacilli strains according to the season of collection

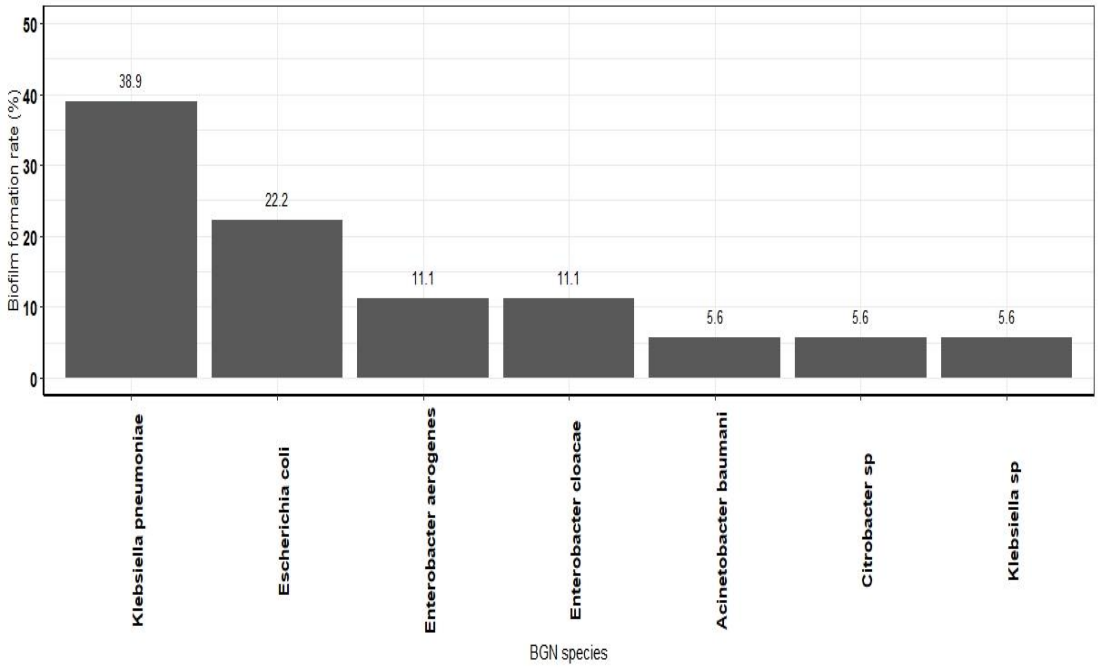

Figure 6. Biofilm production rate according to the different species of thermo-tolerant Gram-negative bacilli.

season, the species most commonly found were *E. coli* and *K. pneumoniae*.

Biofilm formation according to the isolated species of thermo-tolerant Gram-negative bacilli

The biofilm production capacity by the thermo-tolerant Gram-negative bacilli strains shows that *K. pneumoniae* was the most biofilm formative (38.9%) followed by *E. coli* (22.2%) and *Enterobacter aerogenes*, *E. cloacae* (11.1%) (Figure 6). *Acinetobacter baumani*, *Citrobacter* sp. and *Klebsiella ornithinolytica* were the lowest biofilm producers (5.6%). Meanwhile, the production of biofilm is not statistically significant different among the isolated species ($p > 0.05$). Gram-negative bacilli strains isolated from dèguè millet (34.62%) were the highest biofilm producer while those isolated from yoghourt were the

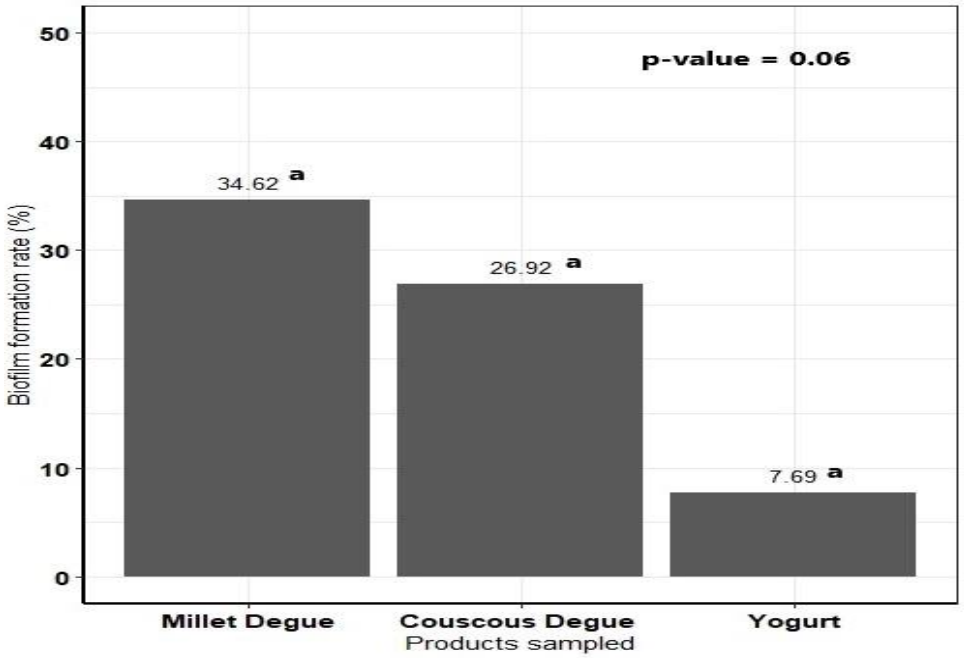

Figure 7. Biofilm production rate according to thermo-tolerant Gram-negative bacilli strains in the different samples.

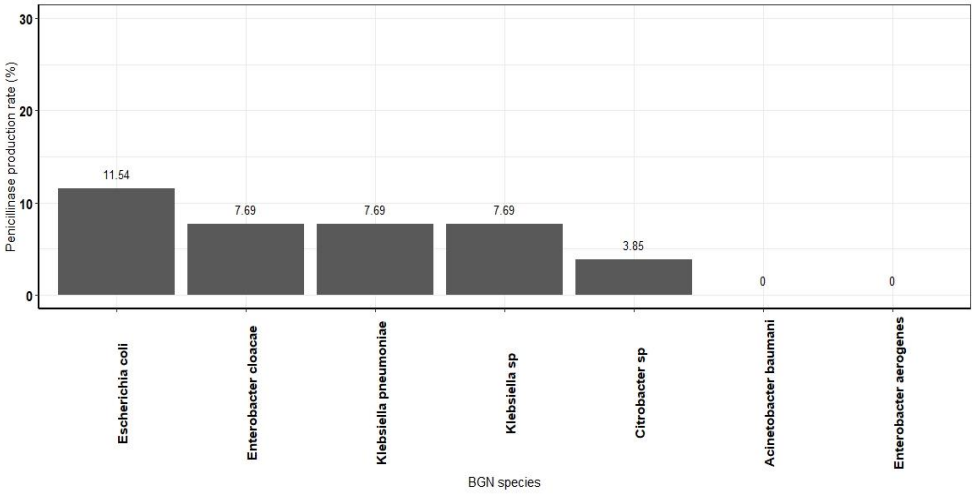

Figure 8. Penicillinase production rate according to thermo-tolerant Gram-negative bacilli strains in the different samples.

lowest (7.69%) (Figure 7). Biofilm production is statistically different from isolated species in function of samples types (p=0.06).

Penicillinase, ESBL and carbapenemase production by the thermo-tolerant Gram-negative bacilli species

The production of penicillinase by isolated thermo-tolerant Gram-negative bacilli strains was highly observed with *E. coli* (11.54%), followed by *Enterobacter cloacae, K. pneumoniae* and *Klebsiella* sp. (7.69%). *Acinetobacter baumani* and *Enterobacter aerogenes species* were not penicillinase-producers (Figure 8). Only three strains were of ESBL producers. Of the seven species isolated, only *E. coli* (7.69%) and *E. aerogenes* (3.85%) produced ESBL (Figure 9). None of the Gram-negative bacilli strains were carbapenemase-producers.

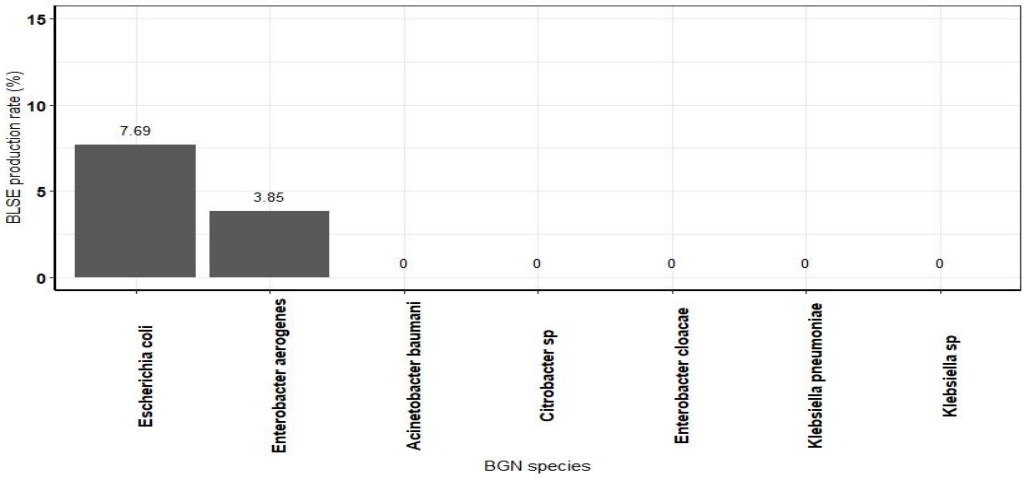

Figure 9. BLSE production by the rate according to thermo-tolerant Gram-negative bacilli strains in the different samples.

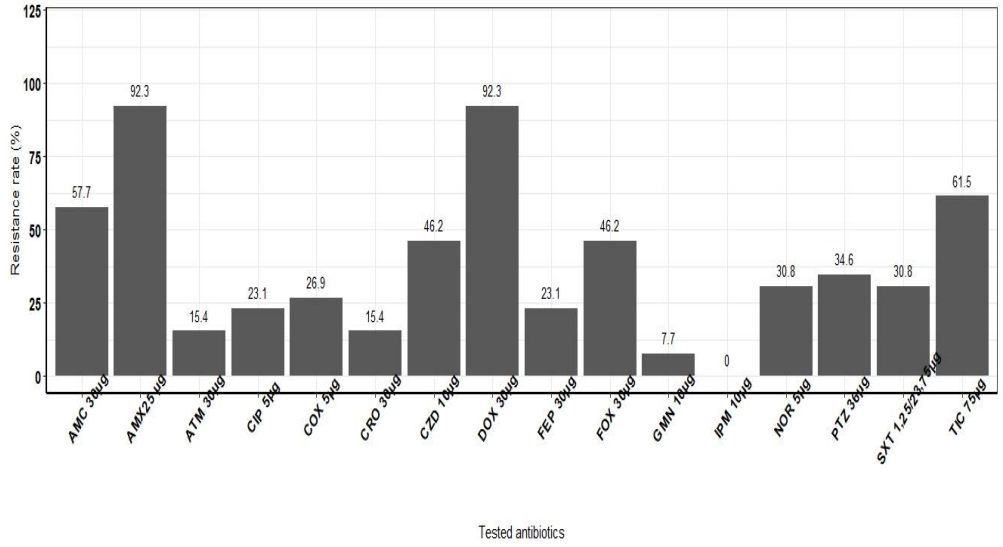

Figure 10. Resistance rate of isolated thermo-tolerant Gram-negative bacilli strains to antibiotics. Legend: Amoxicillin-clavulamic acid (AMC 20/10 µg), Amoxicillin (AMX 25 µg), Azetronam (ATM 30 µg), Ciprofloxacin (CIP 5 µg), Cefotaxim (COX 5 µg), Ceftriaxone (CRO 30 µg), Ceftazidim (CZD 10 µg), Doxycylin (DOX 30 µg), Cefepim (FEP 30 µg), Cefoxitin (FOX 30 µg), Gentamycin (GMN 10 µg), Imipénèm (IPM 10µg), Norfloxacin (NOR 5 µg), Piperacilin tazoubatam (PTZ 36 µg), trimethoprime sulfamethoxazole (Sxt 23.75 µg), Ticarcillin (TIC 75 µg).

Susceptibility to antibiotics of thermo-tolerant Gram-negative bacilli strains

Most of thermo-tolerant Gram-negative bacilli were resistant to β-lactam antibiotics. The highest resistance rate (92.3%) was recorded with amoxicillin and doxycylin followed by ticarcillin (61.5%) and amoxicillin+ clavulanic Acid (57.7%). The only sensitivity was observed with imipenem which showed no resistance (Figure 10).

Presence of genes encoding the production of β-lactamases and toxins genes among identified E. coli

The blaTEM gene was the most observed (51.43%) followed by blaSHV gene (37.14%). Genes encoding blaTEM, blaSHV and blaCTX-M were observed in the *E. coli* isolates at respective proportions of 17.14, 8.57 and 8.57%. Only one strain of *K. pneumoniae* carried the blaOXA-1 gene. blaCTX-M was only present in *E. coli*

Table 4. proportions of presence of genes coding for the production of β-lactamases.

	BlaTEM	BlaSHV	BlaCTX-M	BlaOXA-1
Enterobacter cloacae	3 (8.58%)	2 (5.71%)	0 (0%)	0 (0%)
Enterobacter aerogenes	2 (5.71%)	1 (2.86%)	0 (0%)	0 (0%)
Acinetobacter baumani	0 (0%)	1 (2.86%)	0 (0%)	0 (0%)
Citrobacter sp	1 (2.86%)	0 (0%)	0 (0%)	0 (0%)
K. ornithinolytica	1 (2.86%)	1 (2.86%)	0 (0%)	0 (0%)
K. pneumoniae	5 (14.28%)	5 (14.28%)	0 (0%)	1 (2.86%)
Escherichia coli	6 (17.14%)	3(8.57%)	3 (8.57%)	0 (0%)
Total	**18 (51.43%)**	**13 (37.14%)**	**3 (8.57%)**	**1 (2.86%)**

strains. The only isolated *Acinetobacter baumani* carried only blaSHV gene (Table 4). No strain of *E. coli* isolated carried genes coding for the production of shiga-toxins.

DISCUSSION

The observation of the sites investigated during our study, indicate insalubrity of certain sites with garbage and open gutters wastewater. Some sellers do not have adequate garbage collection bins. Fermented dairy products were displayed on makeshift tables. Inadequate garbage bins were observed with sellers for garbage collection. This increase the attraction of flies, which are not only indicators of poor hygiene, but also vectors of fecal contamination germs (Samapundo et al., 2016). Dairy products are prepared at home and the rest of the activities carried out in the context of the sale is done at the point of sale, using the water available at that site. Most female vendors rarely change rinsing utensils water. No sellers wear masks and gloves, so the hands of some vendors are in direct contact with the money and sold food. These practices increase the possibility of cross-contamination among the vended products. All this is detrimental to good practices in the preparation and sale of these desserts. Previous studies on street foods in Benin made similar observations (Moussé et al., 2016). The microbiological quality of fermented dairy products reveals the presence of fecal coliforms and thermo-tolerant Gram-negative bacilli isolates including *E. coli.* This found illustrates a failure of hygiene and implementation of Good Manufacturing Practices (GMP) recorded during the survey. Kouame-Sina et al. (2010) did similar report on the contamination occurs from hands, sanitation of vendors as well as the environment, the water used in the production. Also, according to Zelalem and Bernard (2006), high level of contamination of milk products might be explain by initial contamination originating from the udder surface, washed water, milking materials and utensils used for filtering the milk.

Thus, during our study, among the thermo-tolerant Gram-negative bacilli isolates, 38.9% were *K. pneumoniae* and 22.2% were *E. coli*. For *E. coli*, this rate is lower than the 38% reported by Bagré et al. (2014) on raw milk. The presence of different strain of *E. coli* gives a good indication of fecal pollution and contamination of milk products which lead to gastroenteritis and food poisoning in human (Galal et al., 2013). Presence of *E. coli* in milk products constitute a public health hazard. This contamination by *E. coli* could be explained also by the low hygiene level including handlers' hand, quality of water used and the used utensils during the processing and dairy products sale. The exposure of dairy products for sale in bowls and packet can be a source of contamination (Zagare et al., 2012). Similar results were early reported on fermented milks in Burkina Faso (Barro et al., 2002; Koussou et al., 2007; Savadogo et al., 2010). In our study dèguè, couscous and dèguè millet samples were more contaminated in the morning (23.08%) than in the afternoon (19.23%) with respective proportions of 23.08 and 19.23%. The yoghourt samples were only contaminated at 15.38% in the morning. This could be explained by the fact that the initial contamination will be affected by the lowering of pH and the antimicrobial activities of lactic acid bacteria present by the production of bacteriocines (Savadogo et al., 2004; Labioui et al., 2005). Moreover, their decrease may be due to inappropriate conditions for their development such as temperature (Le Conte and Navajas, 2008). The formation of biofilm by strains of thermo-tolerant Gram-negative bacilli, *K. pneumoniae* displays highest rate of 38.9% followed by the strains *E. coli* with a proportion of 22.2%. Samples of dèguè millet contained more biofilm-forming thermo-tolerant Gram-negative bacilli (34.62%) strains. The formation of biofilm by food-borne thermo-tolerant Gram-negative bacilli strains (especially dairy products) is very serious for human, especially for children. Biofilms are reported to be involved in both device associated infections and tissue infections such as pneumonia and osteomyelitis (Costerton et al., 1999). The study of the sensitivity to antibiotics of the strains of isolated thermo-tolerant Gram-negative bacilli showed the existence of variable resistance proportions according to the antibiotics families. Indeed, against the 16 antibiotics tested, the strains of thermo-tolerant Gram-negative Bacilli show a resistance rate of 92.3% to

amoxicillin and to doxycillin, a resistance rate of 61.5% to ticarcillin and 57.7% resistance to amoxicillin+ clavulanic acid. These founded are similar to those of Bagré et al. (2014) on raw and curdled milk sold in Ouagadougou who found a high rate of resistance of *E. coli* strains to amoxixcillin (78.26%) and the highest rate of resistance to amoxicillin+ clavulanic acid (100%). Our data is similar to those of Virpari et al. (2013) which observed the highest resistance rate to Ampicillin (43.75%). This high resistance level could be explained by the abusive and uncontrolled use of antibiotics both in medicine and agriculture, especially in developing countries. This abusive use could induce the acquisition of antibiotic resistance factors by microorganisms (Okeke et al., 1999; Lesch et al., 2001; Trivedi et al., 2011), only *E. coli* (7.69%) and *Enterobacter aerogenes* (3.85%) strains produced ESBL. These rates are lower than the 16% observed by Lonchel et al. (2012) in Cameroon on clinical strains. The difference observed can be explained by the origin of the strains involved. Indeed, the strains of clinical origin acquired resistance to antibiotics because they are currently in contact with molecule other than food strains. The observed resistance is due to the abusive and uncontrolled antibiotics use. On the other hand, our strains come from food materials that are supposed to not yet been confronted with the abusive antibiotics use. The only sensitivity was observed with imipenem, which showed a resistance rate of 00%. This suggests that imipenem is still a treatment of choice against the Gram-negative bacilli. During our research, *E. coli* strains carriers' blaCTX-M gene and had the highest level of blaTEM. Studies in the food sector have observed the presence of blaSHV, blaTEM and blaCTXM-M genes in *E coli* strains at high levels. These are the studies conducted by Saad et al. (2019) on chickens in Egypt and that carried out in Iran by Dallal et al. (2018). These studies have shown that the more the strains develop a multidrug resistance to antibiotics the more they are carriers of resistance genes. In our research, we observed the presence of two multi-resistant *E. coli* strains that are both biofilm-forming, ESBL-producing and carriers of blaCTX-M, blaTEM and blaSHV genes. This state of affairs is very bad for the health of consuming populations.

Conclusion

It appears from our study that artisanal fermented dairy products sold in some secondary schools of Abomey-Calavi and Cotonou are contaminated with thermo-tolerant Gram-negative bacilli. They have capabilities for biofilm formation, penicillinase production and ESBL production. Resistance strains observed in food are a reality. Awareness raising and training of sellers on good hygiene and manufacturing practices for fermented milk products are necessary to reduce bacterial contamination in order to increase the safety of these products and thus preserve the health of young consumers.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Molecular characterization of water-borne multi-drug resistant *Escherichia coli*

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Antibiotics are very important in the fight against infectious disease caused by bacteria and other microbes for decades. Today microbes have developed ways to resist antimicrobial agents targeted at them. We sought to characterize and simultaneously detect virulence genes associated water-borne antibiotic resistant *Escherichia coli*, obtained from water sampled from ground and surface water sources. The Analytical Profile Index (API) was use for the identifications of *E. coli* isolates. The Kirby-Bauer disk diffusion method was employed for susceptibility testing. A DNA•STRIP molecular assay technology designed for detection of shiga toxin genes was used for the molecular characterization. *E. coli* isolates showed a high (32.99%), resistance to penicillin, and was highly susceptible (93.8%) to nitrofurantoin. *E. coli* was confirmed Polymerase Chain Reaction (PCR). None of the confirmed multidrug resistant *E. coli* isolate had genes for stx1 and stx2. However, the *eae* intimin virulence gene was found on 6% of the multidrug resistant *E. coli* isolates. It was concluded that *E. coli* has developed a very high resistance to the various antibiotics. Second, the genotype EHEC test based on the DNA•STRIP technology used in this study has proved to be efficient and reliable in the molecular characterization of the multi-drug resistant *E. coli* isolates. The DNA•STRIP Genotype EHEC technology test is therefore recommended for pathogenic *E. coli* detection and monitoring. There is also a need to revise strategies towards the multidrug resistance programme.

Key words: DNA•STRIP Technology, *Escherichia coli,* polymerase chain reaction, drug resistance, water bourne.

INTRODUCTION

Antibiotic resistance is a major challenge worldwide. It is a threat towards the attainment of the Sustainable Development Goals (SDGs) on health as well as gains made in health and development (Assembly, 2015, 2011). Today antibiotic-resistant genes imparting resistance to various antibiotics have been recognized in different water environments. This includes drinking water worldwide (Marathe et al., 2017; Odonkor and Addo, 2018). The major threat for health globally is the potential transmission of these genes from the environmental microbes unto human pathogens (Karkman et al., 2017). Prospect of drinking water passing on pathogens to

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people, thus causing disease, is well documented (Bengtsson-Palme and Larsson 2016; Pan and Chu, 2018).

The spread of multi-antibiotic resistant microbes in water environments is an important public health problem with stakeholders such as policy makers and physicians worried about their future capacity and ability to treat various infectious diseases (Fitzpatrick and Walsh, 2016; Da Silva et al., 2011; Schmidt, 2002). Ultimately, the life time of an antibiotic hinges largely on the rise and dissemination of resistant bacteria. However, early interest relating to antibiotic resistant bacteria has predominantly been focused on medical settings and nosocomial infections.

The persistence of antibiotics in waterways accounts for the increase in bacteria resistance to antibiotics as observed in water environments. This ensues through “selective pressure” and horizontal gene transfer (Alonso et al., 2001). The phenomenon of selective pressure normally occurs in the presence of antibiotics owing to the heightened persistence or development of microbial strains carrying resistance genes (Economou and Gousia, 2015; Holmes et al., 2016). These genes block the mechanism of cellular destruction caused by antibiotic compounds. Resistance genes are usually found on plasmids that are susceptible to horizontal gene transfer. This explains medium through which antibiotic resistant bacteria and associated genes are spread in the water environment (Cheng et al., 2016; Gothwal and Shashidhar, 2015; Schmidt, 2002). It is important to note that these plasmids are extrachromosomal gene structures, and have the ability to move thus, allowing for plasmid encoded genes to be transferred across cell membranes. This method of gene transfers permits profusion and allows variety of resistant microbes to increase quickly in water environments contaminated with antimicrobial compounds. These waterways may therefore become reservoirs and incubators for antibiotic resistant bacteria (Alonso et al., 2001; Odonkor and Addo, 2018).

Escherichia coli is commonly recognized as the principal channel for the spreading of antibiotic resistance genes and associated virulence vectors. This is due to their profusion within such environments (Cheng et al., 2016; Gothwal and Shashidhar, 2015; Schmidt, 2002; Tauxe et al., 1997). For example, β-Lactam antibiotics are known to account for about a half of the global antimicrobial utilization (Alpay-Karaoglu, 2007). Resistance to various β-lactam antibiotics such as penicillins and cephalosporins is usually facilitated by the β-lactamase enzymes. *E. coli* strains have also become resistant to ampicillin by plasmid-mediated class A β-lactamase enzyme such as TEM-1. They tend to mutate to extended spectrum activity (Odonkor and Addo 2011; Odonkor and Ampofo, 2013; Livermore, 1995). Epidemiological research findings show that TEM-1 is the most common plasmid mediated *B*-lactamase between medical Gram-negative bacteria (Bush and Jacoby, 1997).

However, there are other genes that are of public importance and are specific to *E. coli.* These genes include *stx1* and *stx2*, the *eae* intimin gene, and the *ipaH* (invasion plasmid antigene H) gene (Odonkor and Ampofo, 2013). They do not only confer resistance to *E. coli* but they are also known to be toxin producing. Although there are several studies assessing multi drug resistance (MDR) in *E. coli*, not much work has been done on these genes specific to *E. coli.* The aim of this study was to therefore to characterize antibiotic-drug resistant water borne *E. coli* and simultaneously detect associated virulence genes.

MATERIALS AND METHODS

E. coli isolation and identification

E. coli isolates were obtained from drinking water samples collected from ground water and surface water sources. *E. coli* organisms were identified using conventional methods (such as catalase test, indole test, nitrate reduction test, etc). This was then confirmed with Analytical Profile Index (API 20E). The *E. coli* control strain ATTC 25922 was obtained and used as the positive control.

E. coli susceptibility testing

Antibiotic susceptibility testing was done using the Kirby-Bauer disk diffusion methods as described by the Clinical and Laboratory Standards Institute (CLSI) (Lacy et al., 2004). Confirmed *E. coli* isolates obtained from overnight cultures on nutrient agar brought was brought to an equivalent of 0.5 McFarland. This was done by the suspension of isolates in sterile saline until the desire turbidity (0.5 McFarland) was achieved. The suspension was then streaked on complete surface of dishes with Mueller Hinton agar. The *E. coli* isolates obtained were tested against 14 antibiotics that were in use at the time of this study. These antibiotics and their strength are shown (Table 1).

All plates were incubated at 37°C for 24 h. This was after the antibiotic disks were placed aseptically on the streaked Mueller-Hinton agar dishes. Clinical and Laboratory Standards Institute (CLSI) protocols were used to interpret the results (Lacy et al., 2004).

Molecular characterization

A DNA strip technology test (GenoType EHEC) was used for the molecular characterization. The GenoType EHEC allows for a combined characterization and identification of the following genes: *eae* intimin gene, *ipaH* (invasion plasmid antigene H) gene, stx*1* shiga toxin gene and *stx2* shiga toxin gene. Three steps were involved in this molecular characterization method as follows: DNA extraction from *E. coli* culture, a multiplex amplification with biotinylated primers and a reverse hybridization. A template ensuring interpretation of the banding pattern was obtained and used (Prère and Fayet, 2005)

RESULTS

Results in Table 2 show the number of *E. coli* isolates

Table 1. Antibiotics and their corresponding disc concentration used.

Antibiotic	Disc concentration
Amikacin (AMK)	30 µg
Ampicillin (AMP)	10 µg
Cefotaxime (CTX)	30 µg
Cefuroxime (CXM)	30 µg
Chloramphenicol (CHL)	30 µg
Ciprofloxacin (CIP)	5 µg
Co-trimoxazole (COT)	25 µg
Erythromycin (ERY)	15 µg
Gentamicin (GEN)	10µg
Nalidixic acid (NAL)	10 µg
Nitrofurantoin (NIT)	300 µg
Penicillin (PEN)	10 units
Pipemidic acid (PA)	20 µg
Tetracycline (TET)	30 µg

Table 2. Isolates of *E. coli* obtained from the water sources.

Water source	Number of *E. coli* isolates obtained	Total (%)
Surface water	62	63.90
Ground water	35	36.10
Total	97	100

Table 3. Antibiogram of water-borne *E. coli* isolates.

Antibiotic	Susceptibility			
	Disc concentration	Resistant number (%)	Intermediate number (%)	Sensitive number (%)
Amikacin (AMK)	30 µg	7 (7.22)	1 (1.03)	89 (91.75)
Ampicillin (AMP)	10 µg	11 (11.32)	41 (42.27)	45 (46.39)
Cefotaxime (CTX)	30 µg	4 (4.12)	4 (4.12)	89 (91.75)
Cefuroxime (CXM)	30 µg	28 (28.87)	18 (18.65)	51 (52.58)
Chloramphenicol (CHL)	30 µg	18 (18.56)	12 (12.37)	67 (69.07)
Ciprofloxacin (CIP)	5 µg	8 (8.25)	17 (17.53)	72 (74.22)
Co-trimoxazole (COT)	25 µg	10 (10.31)	6 (6.19)	81 (83.50)
Erythromycin (ERY)	15 µg	23 (23.71)	24 (24.74)	50 (51.55)
Gentamicin (GEN)	10µg	5 (5.15)	4 (4.12)	88 (90.72)
Nalidixic acid (NAL)	10 µg	4 (4.12)	6 (6.19)	87 (89.69)
Nitrofurantoin (NIT)	300 µg	4 (4.12)	2 (2.060	91 (93.81)
Penicillin (PEN)	10 units	32 (32.99)	51 (52.58)	14 (14.43)
Pipemidic acid (PA)	20 µg	13 (13.40	20 (20.62)	64 (65.98)
Tetracycline (TET)	30 µg	21 (21.45)	47 (48.45)	29 (29.90)

obtained from the various water sources**.** It can be seen from the table that 62 of the *E. coli* isolates accounting for 63.90% of the entire *E. coli* isolates obtained were from surface water sources and the rest (36.10%) were from ground water sources.

Results in Table 3 present the antibiogram patterns of the *E. coli* isolates tested against the 14 antibiotics used. Results show that the *E. coli* strains were most resistant to penicillin (32) representing 32.99%. On the other hand, the isolates were most susceptible to susceptible to

Table 4. Antibiotic resistance profile *of E. coli* isolates from the water sources.

No. of antibiotics	Isolates showing resistance	
	No.	(%)
One	10	17.24
Two	18	31.03
Three	13	22.41
Four	8	13.79
Five	3	5.17
Six	1	1.72
Seven	2	3.45
Eight	1	1.72
Nine	1	1.72
Ten	1	1.72

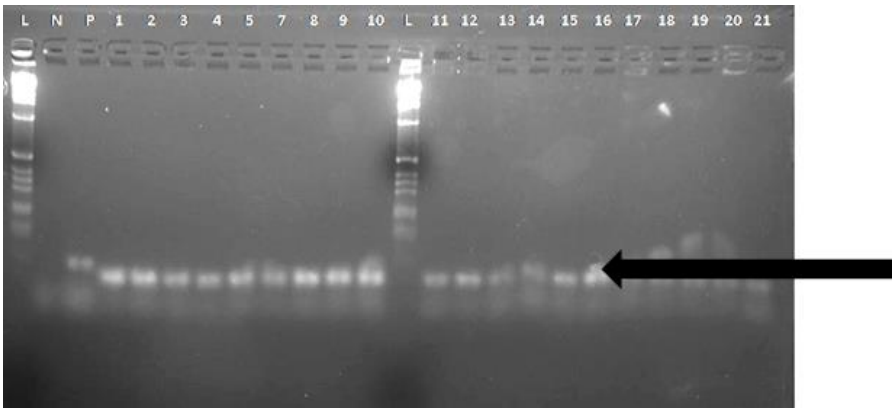

Figure 1. Diagram with arrow showing an amplicon on the gel.

nitrofurantoin (93.8%).

Results shown in Table 4 provide a summary of resistances. It can be observed from the table that 10 of the *E. coli* isolates were resistant to just one antibiotic, while one *E. coli* isolate was resistant to 10 out of the 14 antibiotics tested.

However, of great interest and importance as shown in Table 4 is that 48 out of the 97 (49.48%) *E. coli* isolates tested were resistant to 2 or more antibiotics. Thus, falls under the multi drug resistance classification (Hill et al., 2005).

Figure 1 shows a diagram of amplification of bands on the agarose gel. This is shown for the first few multi-drug resistant *E. coli* isolates. This is an important step, prior to hybridization. The success of DNA extraction and successful amplification is indicative of the presence of bands.

Results in Table 5 summarize the molecular characterization performed on the 48 multi drug resistant *E. coli* isolates. The PCR confirmed all isolates as *E. coli.* None of the confirmed multidrug resistant *E. coli* isolate had genes for stx1 and stx2. However, the *eae* intimin virulence gene was found on 6% of the multidrug resistant *E. coli* isolates.

DISCUSSION

Generally, a drug resistance refers to a phenomenon where there is a microbial resistance to at least two of the following classes of antibiotics: quinolones, amino-glycosides and lactams (Hill et al., 2005).

In this study, penicillin resistance was found to be very high. This has serious implications for public health, as the chances of these antibiotics curing infections caused by *E. coli* are significantly hampered. On the other hand, it is worth nothing that nearly all the 97 strains of *E. coli* (93.8%) found to be susceptible to nitrofurantoin, thus presenting a good choice for treatments. However, the rate at which these microbes are increasingly resistant to various antibiotics with an estimated over 10 million mortality attributable to antimicrobial-resistant infections annually by 2050 (O'Neill, 2018), the susceptibility of *E. coli* to just a single antibiotic as observed in this study

Table 5. Molecular characterization results.

Molecular parameter	Positive		Negative	
	Number	%	Number	%
cc	48	100	0	0
uc	48	100	0	0
E. coli	48	100	0	0
ipaH	0	0	48	100
stx1	0	0	48	100
stx2	0	0	48	100
eae	3	6	45	94

may not be considered a significant threat.

E. coli are generally apathogenic and belong to the enterobacteria group of microbes. They will not normally cause infections or disease. However, some strains such as Shiga toxin-producing *E. coli* (STEC), which are verotoxin-producing are known to possess pathogenic properties in humans as well as animals (Jaeger and Acheson, 2000).

Pathogenicity of a given bacteria strain such as *E. coli* is to a large extent influenced by virulence factors. In the case of *E. coli*, these factors include, capsule, adhesins, toxins and invasins, which are often present in a large genetic block on chromosome and may be horizontally transmitted between various strains. In this study, we found that 6% of the multiple resistant *E. coli* carried the *eae* virulence gene. Notwithstanding, a low percentage (6%) of virulence *eae* genes found on the stains of *E. coli*, the real danger is the potential to transfer these genes within the *E. coli* group and even with other members of the enterobacteria. Furthermore, presence of eae gene in water sources is of a grave public health concern, since spread of these virulence factors aside transmitting through food and water can also transfer from person to person (Karch et al., 2000).

Several disease outbreaks have been linked to the *eae* genes. The *E. coli* strains carrying eae gene have previously been isolated from adults who presented with the hemolytic-uremic syndrome. Similarly, it was also isolated in 101 children also presenting with hemolytic-uremic syndrome. However, in this case, the *E. coli* strains lacked the intestinal adherence factor intimin, which is known to be encoded by the *eae* gene (Bielaszewska et al., 2006). Gerber et al. (2002) reported that 97% of *E. coli* that were shiga toxin producing were isolated from children in Australia and Germany, were found to carry the *eae* gene. It worth noting that in this particular case the estimated mean incubation period was 8 days, longer than the 3 to 4-day incubation period previously reported for shiga-toxin-producing *E. coli* O157: H7.13 (Mead and Griffin, 1998).

Also, in this study, we found that all multiple resistant *E. coli* isolates were non stx1 and stx2 genes producing. *E. coli* carrying shiga toxin (stx1 and stx2) genes are known to possess pathogenic properties in humans as well as animals, thus they cause infections (Jaeger and Acheson, 2000).

E. coli are also known to be significant reservoirs of several genes that code for antibiotic resistance (Bucknell et al., 1997). Thus, major risk for public health lies in the potential for the transfer of resistant genes from environmental bacteria to human pathogens. The capacity of the resistant bacteria and associated genes to move across ecosystems is well documented (Von Wintersdorff et al., 2013).

Conclusion

This study found that all *E. coli* was highly resistant to penicillin and highly susceptible to nitrofurantoin. Furthermore, none of the isolates was gene producing for the virulence factor Stx1 and *stx2*. However, 3 of the multidrug resistant *E. coli* isolates representing 6% were found to be *eae* virulence factor producing. Thus, the principal risk for public health is that resistance genes may be transmitted from environmental bacteria to human pathogens. We recommend the DNA•STRIP Genotype EHEC technology test is therefore recommended for pathogenic *E. coli* detection and monitoring. There is also a need to revise strategies towards the multidrug resistance programme.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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