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Full Length Research Paper

Bacterial communities associated with the surfaces of the fresh fruits sold around Dhaka Medical College and Hospital and their anti-microbial profiles

R. B. Kabir, R. Zaman, N. E. J. Tania, Md. Asaduzzaman, A. Haque, F. B. Habib, N. N. Tanni, M. Nesa, A. Chowdhury, Md. F. Rahman, A. Sarker, K. Halder, N. Sharmin, M. Chowdhury, S. S. Nahar, M. Rahman, S. B. Shahid and S. M. Shamsuzzaman*

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Fresh fruits are popular sources of healthy diets with low energy density. Since they are consumed raw, it may act as a source of foodborne disease and a reservoir for antibiotic-resistant organisms. This study aimed to determine microbial prevalence among the fruits sold around hospital along with antimicrobial profiles. Thirty-five different types of fruits were bought from around Dhaka Medical College and Hospital (DMCH) and analyzed for the presence of bacteria. Antibiotic sensitivity, detection of ESBL, AmpC β-lactamase, and MBL positive strains were done by standard methods followed by PCR to detect ESBL, AmpC β-lactamase and MBL genes. Twenty-seven different organisms were isolated: *Klebsiella spp.* (33.33%), *Citrobacter spp.* (29.64%), *Enterobacter spp*. (22.22%), *Escherichia coli* (11.11%) and *Staphylococcus aureus* (3.70%). Among them, 48.15% were resistant to different antibiotics. Only one organism (*Citrobacter spp*.) produced ESBL phenotypically (7.69%). Two (15.38%) were positive for AmpC β-lactamase and one of these (*Enterobacter spp*.) possessed SHV and CTX-M15A genes by PCR. Imipenem resistance was 84.62% of the antibiotic-resistant organisms and 10 (90.91%) were phenotypically MBL positive. By PCR, one *Enterobacter spp*. had MBL encoding gene OXA-48. Fresh fruits, contaminated with pathogens, might be a source of resistant organisms' transmission and contribute to public health issues.

Key words: Antibiogram, bacteria, Bangladesh, fresh fruits, fruit venders around hospital.

INTRODUCTION

Fresh fruits are good sources of vitamins, minerals, phytonutrients and dietary fiber. It is also considered as a measure to decrease heart diseases and some cancers (Xyli et al., 2019). Moreover, fruits are regarded microbiologically safer than other unprocessed foods. Thus, to promote fruits and fresh products intake, the Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO)

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recommended a minimum consumption of 400 g of vegetables and fruits per day (Hölzel et al., 2018). But, fruit surfaces are not free from microorganisms, and generally consumed raw which can impose foodborne diseases (Rincón and Neelam, 2021). As a result, fresh fruits can act as a means of human exposure to antibiotic-resistant bacteria containing antibiotic resistance genes (Liu and Song, 2019). These antibiotic resistant bacteria containing antibiotic resistance genes on the fruit or vegetable surfaces give rise to risks regarding environmental health ecologically (Sun et al., 2021).

Since β-lactams, specially extended spectrum cephalosporins and carbapenems are the drugs of choice to treat resistant gram negative bacteria, β-lactamases, such as penicillinase, extended-spectrum β-lactamases (ESBLs), cephalosporinases (AmpC) and carbapenemases have emerged worldwide (Ye et al., 2018). This leads to increase use of the last line antibiotic, the pivotal alternative, colistin, which ultimately gives rise to colistin-resistant bacteria, in particular, through horizontal transfer of mcr gene (Bakthavatchalam et al., 2018).

Biswas et al. (2020) demonstrates diverse gram negative bacteria on the surfaces of fresh fruits and vegetables, among which *Vibrio spp., Lactobacillus spp., Pseudomonas spp.* and *Salmonella spp*. are dominant. The transfer of these organisms to fresh fruits and vegetables may occur at multi-stage from production to home kitchen- during production through the use of animal manure and contaminated irrigation water, during the post-harvest stage, transport, conservation and processing by handlers (Richter et al., 2019). It certainly poses a potential public health threat since they are able to exchange resistance genes with intestinal bacteria during their colonization and passage through the intestines leading to further dissemination in the environment (van Hoek et al., 2015).

Previously, chromosomally encoded MBL enzymes or chromosomally mutated genes responsible for colistin resistance was not transferable, hence were clinically negligible (Anyanwu et al., 2020; Elshamy and Aboshanab, 2020). Afterwards, various plasmid-encoded carbapenemase genes, such as Imipenem-resistant *Pseudomonas*-type carbapenemases (IMP), Verona integron-encoded MBL (VIM), New Delhi MBL (NDM) and mobilized colistin gene (mcr) have been described which are capable of horizontal transfer (Liu et al., 2016; Elshamy and Aboshanab, 2020). Recently, ESBL, cephalosporinase, carbapenemase and mcr gene-producing gram-negative bacteria isolated from fresh vegetables and fruits have been reported in several studies (Ye et al., 2018; Liu and Song, 2019; Yang et al., 2019; Manageiro et al., 2020) which is very alarming.

Therefore, in this study, we determined the extent of bacterial contamination and an anti- microbial profile of the organisms isolated from the fruit surfaces, sold and consumed in and around DMCH.

MATERIALS AND METHODS

This cross sectional study was conducted at the department of Microbiology, Dhaka Medical College, Dhaka from April 2021 to September 2021 in 5 phases at different time. Thirty five different raw fruits, namely, five apples, five mangoes, five guava, five sets of blackberry, five bunches of grapes, five oranges, four pineapples, and one sugarcane juice, were bought from the fruit venders around Dhaka Medical College and Hospital, Dhaka. Samples were transported to the laboratory carefully in sterile polythene bags for bacteriological analysis (Leff and Fierer, 2013). Fruits were washed with 20 ml sterile distilled water and fluid was transferred into separate beaker. A ten-fold serial dilution of the samples was performed in the Trypticase Soya broth (TSB).

Determination of total viable count (TVC) by bacterial enumeration

Bacterial enumeration was used to determine the number of colony forming units (CFUs) (Angela et al., 2010).

Isolation and identification of bacteria

After incubating the diluted samples in TSB for 24 h, they were subcultured in blood agar and MacConkey agar media followed by incubation at 37°C for 48 h. A single colony was further subcultured until pure culture was obtained. Identification of bacteria was performed on the basis of colony morphology, Gram's staining reaction and biochemical tests (catalase, coagulase, sugar utilization, gas and H_2S production in Triple Sugar Iron agar media, citrate utilization test in Simmon's Citrate agar, motility, indole and urease production in Motility Indole Urea agar media) (Cheesbrough, 2009).

Antimicrobial susceptibility test

Susceptibility to antimicrobial agents of all isolated organisms was determined by modified Kirby-Bauer technique using Mueller-Hinton agar media. Zones of inhibition were interpreted according to CLSI guidelines (CLSI, 2021). FDA guideline was followed for tigecycline ("Antimicrobial Susceptibility test Interpretive Criteria", 2022).

Detection of ESBL producers by double disc synergy test

30µg ceftazidime disc and amoxiclav (20 µg + 10 µg) disc were placed 20 to 25 mm apart (center to center) in the Mueller- Hinton agar plate and was observed for clear extension of the edge of zone of inhibition of cephalosporin disc towards amoxyclav disc after incubating at 37°C for 24 h (Iqbal et al., 2017).

Phenotypic detection of carbapenemase producers

Organisms were considered carbapenemase producers if positive for any of the following methods.

Double disc synergy (DDS) test

After inoculating the test inoculums (compared with McFarland standard) in Mueller- Hinton agar plates, imipenem disc was placed on the inoculated plate along with a blank disc containing 20 µl of Tris- EDTA and 20 µl of 1:320 diluted 2- mercaptopropionic

Table 1. Distribution of organisms associated with different fruit surfaces (N=27).

Organism	Apple (N=4)	Mango (N=5)	Guava (N=5)	Blackberry (N=5)	Grapes (N=2)	Orange (N=4)	Pineapple (N=1)	Sugar cane juice (N=1)	Total (N=27) [n (%)]
Klebsiella spp.	1	1+1*	2+1*	"	"	1	1	1	9 (33.33)
Escherichia coli	1	"	"	"	1	1	"	"	3 (11.11)
Enterobacter spp.	1	2	1	2	"	"	"	"	6 (22.22)
Citrobacter spp	1	1*	1*	2	1	2	"	"	8 (29.64)
Staphylococcus aureus	"	"	"	1	"	"	"	"	1 (3.70)

*Denotes presence of both organisms in the same sample. N= Total number of organisms n= number of specific organism.
Source: Author

acid, placed 10 mm apart. A clear extension of the edge of the inhibition zone of imipenem disc towards Tris- EDTA- MPA disc was observed after incubating at 37°C for 24 h (Kim et al., 2007).

Combined disc (CD) assay

Two imipenem discs (one supplemented with 5 µl of 0.5 M EDTA solution containing approximately 930 µg EDTA) were placed on an inoculated Mueller- Hinton agar plate following incubation at 37°C for 24 h. An increased zone of diameter of ≥6 mm around the disc containing imipenem supplemented with EDTA compared to the disc containing imipenem only was interpreted as MBL producer (Qu et al., 2009).

Modified Hodge test (MHT)

A lawn culture of 1:10 dilutions of 0.5 McFarland's standard *Escherichia coli* ATCC 25922 broth was done on a Mueller-Hinton agar plate. A 10 µg imipenem disc was placed in the center of the plate. Then 0.5 McFarland's standard were made by three imipenem resistant gram negative organisms and streaked from the edge of the disc to the periphery of the plate in three different directions. After overnight incubation, the presence of clover leaf shaped zone of inhibition was interpreted as MHT positive (Amjad et al., 2011).

Detection of anti-microbial resistance genes by PCR

DNA extraction

300 µl distilled water was mixed with bacterial pellets and vortexed until mixed well. Then DNA was extracted in block heater (DAIHA Scientific, Seoul, Korea) at 100°C for 10 min for boiling followed by cooling on ice pack and centrifugation at 4°C at 13500 g for 10 min. The extracted DNAs were kept at -20°C for future use (Farzana et al., 2022).

Amplification through thermal cycler

PCR was performed in a DNA thermal cycler (Eppendorf AG, Mastercycler gradient, Hamburg, Germany) after mixing of mastermix and primers with DNA template. Each PCR run comprised of preheat at 94°C for 10 min followed by 36 cycles of denaturation at 94°C for 1 min, annealing at specified temperatures for 45 s, extension at 72°C for 1 min with final extension at 72°C for 10 min. Gel electrophoresis was done in 1.5% agarose (Bethesda Research Laboratories). DNA bands were detected by staining with ethidium bromide (0.5 µl/ ml) for 30 min at room temperature and visualized with UV transilluninator (Gel Doc, Major Science, Taiwan).

Data analysis

The result of the study was recorded systematically. Data analysis was done by using 'Microsoft Office Excel 2010' program and according to the objectives of the study. The test of significant was calculated by using X^2 test. P value < 0.05 was taken as minimal level of significance.

RESULTS

Fruits were collected on five occasions and 35 cultures were done. Among those 35 fruits 25 (71.43%) yielded growth of 27 different gram positive and gram negative organisms, two fruits showed growth of double organisms. Ranges of microbial count in apple was 1×10^2 to 6×10^2 CFU/ml, guava was 3×10^2 to 3×10^3 CFU/ml, blackberry was 0.1×10 to 3×10^2 CFU/ml, orange was 0.3×10^2 to 2.4×10^2 CFU/ml, mango was 1.2×10^2 to 8×10^2 CFU/ml, grape was 1×10^2 to 1.4×10^3 CFU/ml and pineapple was 1.6×10^2 CFU/ml, and sugar cane juice was 2×10^2 CFU/ml.

Among 27 different organisms, nine (33.33%) were *Klebsiella spp.,* eight (29.64%) were *Citrobacter spp.*, six (22.22%) were *Enterobacter spp., E. coli* were three (11.11%) and lastly *Staphylococcus aureus* were one (3.70%) (Table 1).

Fourteen (51.85%) organisms were sensitive to all drugs, and 13 (48.15%) organisms were found to have resistance to different antibiotics.

Four out of six *Enterobacter spp.* and two of the nine *Klebsiella spp.* were multidrug resistant. Within the resistant organisms, only one (7.69%) was extended spectrum-β-lactemase (ESBL) producer (*Citrobacter spp.*) and two (15.38%) were AmpC-β-lactemase producers (*Klebsiella spp.* and *Enterobacter spp.*) phenotypically. One of the AmpC-β-lactemase producers (*Enterobacter spp.*) was positive for both SHV and CTX-M15A gene (Table 2).

Table 2. Antimicrobial resistance pattern of isolated organisms from different samples (N=13).

Antibiotics	Resistant organisms, n(%)
Imipenem	11 (84.62)
Meropenem	1 (7.69)
Amoxicillin/ clavulanic acid	3 (23.08)
Doxycycline	5 (38.46)
Ciprofloxacin	1 (7.69)
Ceftriaxone	3 (23.08)
Ceftazidime	2 (15.39)
Tigecycline	1 (7.69)
Colistin	3 (23.08)
Aztreonam	1 (7.69)
Cefepime	1 (7.69)
Cefuroxime	2 (15.39)
Sulphamethoxyxazole-trimethoprim	2 (15.39)

Source: Author

Eleven (84.62%) out of 13 antibiotic resistant organisms were imipenem resistant and all of them were phenotypically detected as carbapenemase producers by CD test, DDS test or MHT. Ten (90‧ 91%) of them were phenotypically positive for MBLs (Table 3), considering one that was only positive for MHT. Among the ten MBL positive samples, only one (Enterobacter spp.) was positive for OXA-48 gene. No MBL positive organisms were positive for VIM, NDM-1, NDM-2, IMP, OXA-181, OXA-10, KPC genes. The only organism which was resistant to tigecycline, was not positive for tetA gene.

DISCUSSION

Fruits are consumed in raw state which facilitates the transfer of microorganisms on the fruit surfaces to human along with antimicrobial resistance genes (Chelaghma et al., 2021). Bacterial enumeration identified in this study highlighted the fact that fresh fruits contaminated with pathogenic organisms can act as a transmission vehicle for human diseases (Biswas et al., 2020). It can cause a lot of sufferings for the patients, like a prolonged hospital stay, which ultimately inflects the treatment cost of the patients.

The microbial load of the fruits used in this study varied with the types of food, but their presence and anti-microbial patterns highlighted the fact that, fruits could be contaminated with pathogenic bacteria and thus may act as a vehicle for transmitting diseases.

In a previous study, among 105 fruit samples, 126 bacterial isolates were identified (different species of Enterobacteriaceae and *Staphylococcus spp.*) (Al-Kharousi et al., 2016). In another study, among 25 fruit samples, 106 bacterial isolates were identified (*Escherichia coli, Salmonella spp.*, *Vibrio spp.*, *Bacillus spp.* and *Staphylococcus spp.*) (Sarker et al., 2018). In this study, 27 isolates of six different species among 35 samples were identified. These contaminations may occur from the soil through manure fertilization, or by human through direct contamination (Hölzel et al., 2018). Some studies also confirmed the effect of wastewater for irrigation playing a pivotal role in contaminating fresh farm products such as fresh vegetables and fruits (Adegoke et al., 2018). The difference between the findings may be due to the different sample size, geographic area, seasonal variation or different practices in production and transportation of fruits.

In recent years, the use of antibiotics has increased globally and antibiotic resistance genes have been described in all environment including natural and clinical habitats (Bahram et al., 2018; Chng et al., 2020). The antibiotic resistance genes find their way into the soil by animal or poultry manure and sewage sludge, that is organic manure, and then from the soil to the fruit surfaces by soil-plant system via aerosol from the environment (Zhu et al., 2017; Zhang et al., 2019; Wu et al., 2022).

They spread from fruits and vegetables to human via the food chain (Chen et al., 2019). In this study, almost half of the isolates were resistant to one or more antimicrobial groups. Among them 84.62% were resistant to imipenem, followed by 38.46% to doxycycline. 23.08 and 15.39% were resistant to third generation cephalosporin (ceftriaxone and ceftazidime). At present colistin is considered as the antibiotic of last resort and it is used when a bacteria shows resistance to most of the available antibiotics including carbapenems. In the present study, 23.08% gram negative bacilli were resistant to colistin which is a concern. In a study conducted in Bangladesh Agricultural University,

Table 3. Phenotypic detection of carbapenemase producers among imipenem resistant isolates by DDS, CD and MHT (N=11).

Phenotypical methods	Imipenem resistant organisms, n (%)
DDS	1 (9.09)
MHT	1 (9.09)
CD+DDS	3(27.27)
CD+MHT	4 (36.37)
CD+DDS+MHT	2 (18.18)

Source: Author

Mymensingh, bacterial isolates of guava such as, *Escherichia coli*, *Vibrio spp*., and *Staphylococcus spp.* were found resistant to ampicillin and cephalexin and *Salmonella spp.* were also resistant to chloramphenicol, ampicillin and cephalexin (Sarker et al., 2018). In 2018, a study on fresh vegetables revealed 83.3% prevalence of ESBL-producing strains (Iseppi et al., 2018). Whereas, in the present study, only one of the resistant isolates (*Citrobacter spp*) was positive for ESBL phenotypically. Furthermore, in this study, one AmpC β-lactamase producer harbored both SHV and CTX-M15A gene. According to a recent study, *Escherichia coli* is a predominant reservoir of CTX-M15A gene (Safain et al., 2020). In a recent study, ESBL and AmpC β-lactamase producing *Klebsiella pneumoniae* isolated from fresh fruit were reported (Mesbah Zekar et al., 2020). These findings speculate the fact that ESBL and AmpC β-lactamase gene producing organisms can cause community acquired and hospital acquired infections.

Furthermore, they are serious threat to public health, as these genes have the potential to be transmitted by horizontal gene transfer (Groussin et al., 2021). Gram negative bacteria isolated from clinical samples from patients of Dhaka Medical College Hospital of Bangladesh showed increased proportion of ESBL producers up to about 2015, after that increasing proportion of MBL producers was observed.

The reason behind it might be due to the fact that ESBL producers are treated by carbapenems and the bacteria have started producing more carbapenemase enzymes due to increased used of carbapenems. Moreover, imported fresh food products seem to be a possible reservoir of carbapenemase- producing gram negative organisms (Solaiman et al., 2021). In a study conducted in Algeria, it reported OXA-48 producing *Klebsiella pneumoniae* (Touati et al., 2017). In this study, one *Enterobacter spp.* was found harboring OXA-48.

Wide spread use of antibiotics might contribute in the emergence of multidrug resistant bacteria. Moreover, application of animal manure to the agricultural field using untreated wastewater for irrigation, unhygienic handling at post-harvest stage and during transportation and processing by handlers- all contribute to the spread of drug-resistant bacteria to fresh food products (Richter et al., 2019).

Conclusion

A global risk of transmission of multidrug resistant organisms via fresh fruits needs global attention to minimize future public health issues. The findings of this study clearly showed that there was a wide array of micro-organisms in different fruit samples collected from around a tertiary level hospital in Bangladesh; *Klebsiella spp.* was the most prevalent one. Almost half of the organisms showed resistance to multiple antibiotics and antibiotic resistance genes were found in *Enterobacter spp*. It is a matter of great concern for the consumers. To reduce the risk for the consumers, improvement of agricultural practice, which includes better water quality for irrigation, safe use of fertilizer, hygienic transportation, should be ensured for the fresh food products including fruits. Moreover, ethical use of antibiotics should be emphasized in all sectors to control antibiotic resistance.

CONFLICT OF INTERESTS

The authors declared no conflicts of interests.

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Evaluation of diagnostic tests for plague in Madagascar

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Madagascar is the country that reports the most cases of plague in the world. This is an evaluation study of plague diagnosis over a 2-year period from January 2017 to December 2018. For all suspected plague cases, peripheral RDT (performed locally), central RDT (performed in the laboratory) and PCR were performed. Parameters of the diagnostic tests used, year of study, performance and study of concordance between tests were studied. 2981 cases were collected, 21.6% were confirmed, 28.4% probable and 50% suspected. The sensitivities of peripheral RDT, central RDT and PCR were 96.55, 100 and 97.41%, respectively; the specificities were 41.43, 51.17 and 89.24%. Cohen's kappa was 0.12 between peripheral RDT and culture; 0.17 between culture and central RDT and 0.64 between CRP and culture. For pneumonic plague (PP) patient samples, sensitivities were 80.00, 66.66 and 93.33%, Cohen's kappa was 0.017 between peripheral RDT and culture; 0.013 between central RDT and culture and 0.371 between PCR and culture; sensitivities of peripheral RDT and central RDT were 75.00 and 62.50% for 2017, respectively. The null hypothesis between diagnostic tests was rejected, discordance between tests was found. Sensitivity is lowered during lung sampling and during 2017.

Key words: Plague, polymerase chain reaction, technology assessment.

INTRODUCTION

Plague is a bacterial disease caused by a small gram-negative bacillus: Yersinia pestis. It is very serious in humans as it is often fatal without prompt and appropriate treatment. Its case fatality rate is 30 to 60% for the bubonic form and almost always fatal in the pulmonary form if left untreated (OMS, 2022). Late diagnosis is one of the main causes of the spread of the disease, as it limits the effectiveness of control measures. Worldwide, between 2010 and 2015, 3248 cases of plague were recorded, 584 of which were fatal (OMS, 2022). Between 1990 and 2020, nearly 50,000 human cases of plague were reported to the World Health Organization (WHO) by 26 countries in Africa, Asia and the Americas. Plague is a disease that is still prevalent today and is one of the

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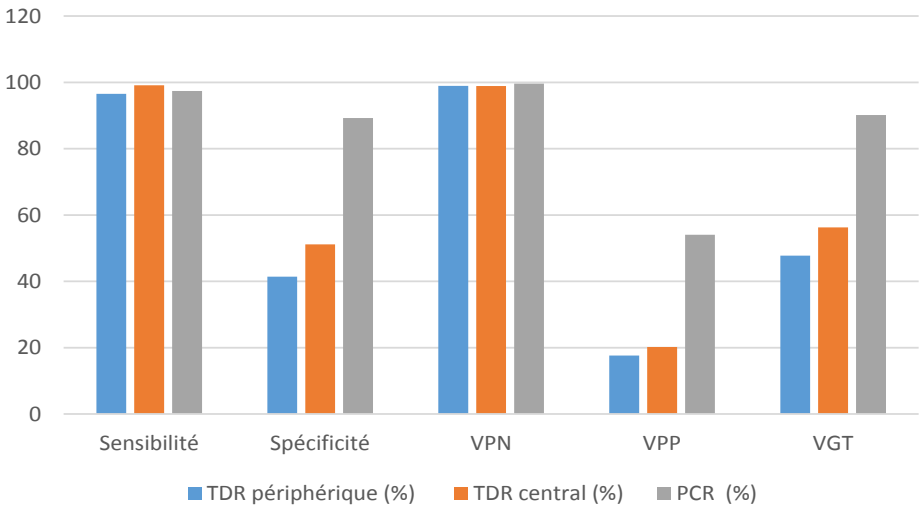

Figure 1. Global evaluation of the performance of diagnostic tests for plague in Madagascar (n = 1009).
Source: Authors

re-emerging diseases (Institut Pasteur, 2021). It remains a public health problem in many countries. In the DRC, between 2020 and 2021, 578 cases and 44 deaths related to plague were reported in the entire province of Ituri (Nations Unies, 2021). In Madagascar, cases of plague are reported every year with a seasonal upsurge between August and April. Pneumonic plague is one of the most severe forms of plague and in this country, a total of 30 cases of pneumonic plague were reported on 03 September 2021, of which 12 were confirmed and 7 were deaths (CFR=23%) (Morvan, 2021). To confirm its diagnosis, the main diagnostic methods are: Microscopy of a smear after Gram or Giemsa or Wayson staining, the rapid diagnostic test (RDT) by detection of the F1 antigen specific to *Y. pestis*, the ELISA test by detection of the anti-F1 antibody, culture and detection by molecular technique or polymerase chain reaction (PCR) (Aubry and Gaüzère,2021). Diagnostic tests must be evaluated against the gold standard for the isolation of the bacteria by bacterial culture. For years, microscopy or RDT has been evaluated to confirm the diagnosis of the plague. This study is the first to evaluate the performance of both RDT and PCR. The objective of this study is to define the null hypothesis of RDT and PCR versus bacterial culture for the diagnosis of plague in Madagascar.

METHODS

This is a retrospective analytical plague diagnostic evaluation study spanning a 2 year period from January 2017 to December 2018. The study was conducted within the Directorate of Epidemiological Health Surveillance and Response (DVSSER) by exploiting the plague database containing epidemiological and biological data. The diagnostic tests used for plague confirmation were peripheral RDT (performed locally), central RDT (performed in the laboratory), PCR and bacterial culture. The latter 3 were performed in the central plague laboratory hosted by the Pasteur Institute of Madagascar (IPM). The study included suspected plague cases for which all tests were performed and excluded cases with incomplete data. This was an exhaustive study and the parameters studied were the different diagnostic tests used (peripheral RDT, central RDT, PCR, Culture), the clinical forms (PP, BP), the evaluation of the performance of the diagnostic tests and the study of the concordance between the tests. Peripheral RDTs are RDTs that are performed at the bed of the patient, while central RDTs are RDTs performed in the laboratory. Data were processed and analyzed on Epi info 2012 version 3.5.4 software with a significance level below 0.05.

RESULTS

During the two years of the study, 2981 cases of plague were notified, 644 cases were confirmed (21.6%), 845 cases were classified as probable (28.4%) and 1492 cases were classified as suspect (50%). Of the 2981 cases, 2915 cases received peripheral RDT, 2556 cases received central RDT, 2826 PCR cases, and 1751 bacterial culture cases. One thousand nine (1009) cases had complete results on peripheral RDT (635 positive cases: 62.93%), central RDT (547 positive cases: 54.21%), PCR (209 positive cases: 20.71%) and bacterial culture (116 positive cases: 11.5%). The peripheral RDT, the central RDT and the PCR were evaluated relative to the bacterial culture which is the gold standard for the laboratory diagnosis of plague (Figure 1).

The study of the concordance of the plague diagnostic tests with the gold standard, the bacterial culture was carried out. Between bacterial culture and peripheral TDR, Cohen's kappa = 0.12, between bacterial culture and central TDR, Cohen's kappa = 0.17, between

Table 1. Study of the concordance between diagnostic tests for plague with the gold standard.

	Peripheral RDT			Central RDT			PCR		
	Bacterial culture			Bacterial culture			Bacterial culture		
	Positive (n)	Negative (n)	Total (n)	Positive (n)	Negative (n)	Total (n)	Positive (n)	Negative (n)	Total (n)
Positive	112	523	635	111	436	547	113	96	209
Negative	4	370	374	5	457	462	3	797	800
Total	116	893	1009	116	893	1009	116	893	1009
Cohen's Kappa		0.12			0.17			0.17	

Source: Authors

Table 2. Performance (%) of plague diagnostic tests according to the type of sample.

	Peripheral RDT		Central RDT		PCR	
	Bubo	Sputum	Bubo	Sputum	Bubo	Sputum
Sensitivity	99.00	80	100	66.66	98.01	93.33
Specificity	40.38	41.86	59.23	47.86	79.23	93.36
Predicted positive value (VPP)	39.21	3.15	48.79	2.94	64.7	25.22
Predicted negative value (VPN)	99.05	98.88	100	98.37	99.03	99.83

Source: Authors

Table 3. Study of the concordance of diagnostic tests for plague according to PP patient samples.

	Peripheral RDT			Central RDT			PCR		
	Bacterial culture			Bacterial culture			Bacterial culture		
	P	N	T	P	N	T	P	N	T
P	12	368	380	10	330	340	14	42	56
N	3	265	268	5	303	308	1	591	592
T	15	633	648	15	633	648	15	633	648
Cohen's kappa		0.017			0.013			0.371	

P= positive, N= negative, T= total.
Source: Authors

bacterial culture and PCR, Cohen's kappa = 0.64 (Table 1).

The sensitivities of the peripheral RDT, of the central RDT, of the PCR according to the types of samples have been shown in Table 2.

Regarding the concordance of diagnostic tests for plague, for lung samples, Cohen's kappa = 0.017 for bacterial culture and peripheral RDT, 0.013 for bacterial culture and central RDT and 0.371 for bacterial culture and PCR (Table 3). For the bubonic samples, we found respectively a Cohen's kappa 0.448; 0.448 and 0.667 (Table 3).

Concerning the concordance of the diagnostic tests for plague, for the BP patient samples, we found respectively a Cohen's kappa 0.448; 0.448 and 0.667 (Table 4).

DISCUSSION

Madagascar is one of the 46 member states of the African region of the World Health Organization (WHO) which monitors the disease through integrated disease surveillance and response. This system aims to monitor diseases with potential epidemics including 28 diseases and plague is one of these diseases. Globally, 10 of the 33 countries with a plague outbreak have reported human cases in the past 5 years. The regions that have reported these cases are limited to sub-Saharan Africa, Asia, and North and South America. Madagascar remains, with the Democratic Republic of Congo (DRC), the country which reports the most cases of plague in the world. In 2008, 2683 cases were notified and 5 countries

Table 4. Study of the concordance of diagnostic tests for plague according to BP patient samples.

	Peripheral RDT			Central RDT			PCR		
	Bacterial culture			Bacterial culture			Bacterial culture		
	P	N	T	P	N	T	P	N	T
P	100	155	255	101	106	207	99	54	153
N	1	105	106	0	154	154	2	206	208
T	101	260	361	101	260	361	101	260	361
Cohen's kappa		0.448			0.448			0.667	

P= positive, N= negative, T= total.
Source: Authors

(Madagascar, Uganda, Peru, DRC and United Republic of Tanzania) alone reported 98% of global cases. Ten years later, the total number of cases in the world is 10 times less and they are reported in only 5 countries (Bertherat, 2019).

During this study, 2981 cases of human plague were notified, 21.60% were confirmed (bacterial culture positive or RDT positive with PCR positive), 28.34% were classified as probable (RDT positive or PCR positive) and 50.05% were classified as suspect (clinical picture suggestive of plague with a favorable epidemiological link). This prevalence is similar to the study carried out previously in Madagascar from 1998 to 2016 including 13234 cases reported to the National Plague Program (PNLP) (Andrianaivoarimanana et al., 2019) and to that of Uganda from 2008 to 2016 with 255 cases of human plague reported. For this country, the diagnostic methods for plague were serology and bacterial culture (Forrester et al., 2017). In Madagascar, the plague is an endemic disease, with a seasonal upsurge between August and April (Rajerison et al., 2020). When comparing the results of plague diagnostic tests with the gold standard, the positivity rate is not similar; it is 62.93% for peripheral RDT, 54.21% for central RDT, 20.71% for PCR against 11.50% for the gold standard which is bacterial culture. We can therefore say that there are many false positives, especially for RDT. Similarly, the percentage of true negatives is underestimated. Regarding the overall performance of plague diagnostic tests in Madagascar, sensitivities were excellent at 96.55, 100 and 97.41%. On the other hand, specificities were low for RDT, 41.43% for peripheral RDT, 51.17% for central RDT and 89.24% (high) for PCR. Their PPV was low and their NPV was high. These sensitivities of RDTs are similar to those of previous study (Chanteau et al., 2003; Andrianaivoarimanana et al., 2019). On the other hand, the specificity is different from that observed in previous studies which found a specificity of RDTs at 100% (Chanteau et al., 2003). Concerning PCR, the sensitivity is similar to that found by some authors in the literature with sensitivity of between 80 and 95% (Matero et al., 2009; Loiez et al., 2003). The sensitivity can be altered (35 to 50%) for samples collected and transported under non-optimal conditions (Rahalison et al., 2000). This result is different from that observed by some authors (Matero et al., 2009; Loiez et al., 2003) who also found a PCR specificity of 100%. In this study, the pla gene was also used with other genes, including the caf1 genes for real-time PCR and the caf1 and inv genes for conventional PCR. PCR performance depends on the amplification target used and the quality of the sample, and can be further improved by changing the amplification targets. In general, high sensitivity of diagnostic tests was related to limited specificity, these results are similar to previous studies (Lutkenhoner and Basel, 2013). An ideal diagnostic test has high sensitivity coupled with high specificity. However, it is extremely difficult to meet these criteria. It is often necessary to find a reasonable balance between sensitivity and specificity. If highly pathogenic bacteria such as *Yersinia pestis* are detected in the field, sensitivity appears to be a priority, with positive samples usually being confirmed by more sophisticated laboratory methods. When rapid test kits are applied, the likelihood of false negative results should be taken into account. The low sensitivity of the tests used in the field makes it necessary to retry even negative samples in the laboratory with all the negative financial consequences. At the level of health systems, when consulting a patient with suspected plague, it is preferable that a test is available on site with a rapid result such as the RDT and that this RDT has a high sensitivity allowing immediate treatment of the patient after. This RDT will be confirmed by a more specific test such as PCR and this will gradually eliminate cases of false positive RDTs. For the diagnostic tests of the plague in Madagascar, the sensitivities of the RDTs were excellent; on the other hand their specificities were low at 41.43% for the RDT carried out at the level of health systems and at 51.17% for the RDT carried out at the level of health systems of the central plague laboratory. This means that there are many healthy people who have received treatment for plague without being sick, unnecessary treatment. On an individual level, these people will be frustrated, stressed and may experience

side effects from prescribed medications. Collectively, the effect can be positive in sensitizing the community on brush clearing, pest control, and deforestation and bushfire avoidance. The total overall value for RDTs was low, 47.77% for peripheral RDT and 56.29% for central RDT; on the other hand, it is 90.18% for PCR. Therefore, it will be necessary to search the market for RDTs which will have the best performance, especially in terms of specificity.

A gold standard or reference test is the best diagnostic test already available for the pathology of interest and which can be used in a "reasonable" situation, that is to say applicable in patients in the clinic or in research (Versi, 1992). Here, the bacterial culture is the gold standard recommended by the WHO; it allows the isolation of *Yersinia pestis*. Agreement between judgments (diagnostic tests) is defined as the conformity of two or more pieces of information that relate to the same subject. The rate of agreement is therefore estimated by the Kappa coefficient proposed by Cohen (1960). It is an index that varies between 0 and 1, which reflects a level of agreement or concordance as much as its value is close to 1. In this study, between peripheral RDT and bacterial culture, Cohen's kappa was 0.12; between central RDT and bacterial culture it was 0.17 and between PCR and bacterial culture it was 0.64. It can be said that there is a poor degree of agreement between peripheral RDT and bacterial culture and also between central RDT and bacterial culture; in contrast, there is a good degree of agreement between PCR and bacterial culture. These RDTs are therefore not recommended as diagnostic tests for plague because of this discrepancy. It is best to search the market for RDTs that will match the best bacterial culture. It is best to search the TDR market that will have the best matches with the bacterial culture.

The sensitivities of the diagnostic tests were low for peripheral RDT and central RDT in PP patient samples at 80.00 and 66.66%, respectively. The sensitivities were high during the PP patient samples, 99.00 and 100% and during the PCR regardless of the type of sample, 93.33% during the pulmonary samples and 98.01% during the BP. Low sensitivity to PP could be related to the very short reaction and relative insufficient affinity and avidity of the antibodies used. An additional reason could be the low volume of the lung sample. During PB and PP patient samples, the specificities of the peripheral RDT for these two types of sampling were 40.38 and 41.86%, respectively; for the central RDT, they were 59.23 and 47.86% and 79.23 and 93.36% for the PCR. The specificity of RDTs regardless of the type of sample is limited; it was higher for BP than for PP. This study is similar to that observed previously between 2002 and 2007 which found a specificity of 60.5% against 44.7% (Andrianaivoarimanana et al., 2019). For PCR, the specificity is higher than that of RDTs although it is not excellent, unlike RDT; it is higher in PP than in BP.

During PP patient samples, the sensitivities and specificities of RDTs were low; therefore, true positive cases were diagnosed with false negative. This is serious because these are cases of PP with a high risk of lethality in the absence of prompt treatment within 48 to 72 h. For those around the undiagnosed, untreated patient, no preventive measures have been taken and since the transmission of PP is aerial, it will be transmitted to the family, to those around them and with the risk of an epidemic PP difficult to manage after. Since the sensitivity of RDTs in PP patient samples is limited, their specificity is also limited. The use of these RDTs when PP is suspected requires a question; it will be necessary to search the market for RDTs that will have the best sensitivities and specificity. Otherwise, for the suspicion of this clinical form of plague, PCR should be used first if it has better sensitivity and specificity. In this study, the latter were respectively 92.30 and 93.36%. An improvement in the molecular technique to increase their performance could be done and in this case PCR would be used on the first line in a case of PP. According to the literature, the performance of PCR depends on the amplification target used; perhaps changing the targets could help improve their performance (Matero et al., 2009). During this study, for PP patient samples, the degrees of agreement between the diagnostic tests and the gold standard were very poor with a Cohen's kappa of 0.017 and 0.0013 for the RDTs and the bacterial culture and of 0.371 for PCR and bacterial culture. The RDTs used are therefore not recommended in the first line in cases of suspected PP. Other suppliers of RDTs with a good match with the bacterial culture for PP patient samples should be sought in the market. It is also important to improve the quality of the sample as this contributes to the improvement of the tests. Training of health workers on the quality of the sample is essential. They need to know the ideal type of sample, how to properly sample, and how to store and ship samples. Health workers also need to be trained in the use and interpretation of RDT. On the other hand, during the PB patient samples, the concordances between the diagnostic tests and the gold standard were acceptable. The degrees of agreement between RDTs and bacterial culture were moderate with a Cohen’s kappa similar to 0.448; agreement was good between PCR and bacterial culture with a Cohen’s kappa of 0.667. It is therefore always recommended to use RDT if BP is suspected.

This study remains limited due to the retrospective nature of the study; therefore not all suspected cases of plague during this study period received 2 RDTs, PCR and bacterial culture at the same time. The evaluation of the performance of each of these tests was therefore not likely to be exhaustive on all suspected cases of plague. Some cases have been tested with RDTs and bacterial culture, some do not have bacterial culture results and some do not have PCR results.

Conclusion

Plague is a disease that still exists in Madagascar and in rare countries in the world. Test performance was performed on 1009 cases that had complete results and were assessed against bacterial culture. A poor degree of agreement was found between the RDT and the gold standard. On the other hand, there is a good degree of agreement between the PCR and the gold standard. In PP patient samples, this agreement was very poor to poor for diagnostic tests for plague. The sensitivity of RDT was low for PP patient samples. The null hypothesis was rejected, a discrepancy in diagnostic tests was observed, it will be necessary to search the market for RDT that will have the best sensitivities and specificity, especially in the event of suspected PP. Training of health workers in plague endemic areas on the quality of samples, performing RDT and their interpretations is necessary. The performance of PCR can be further improved by changing the amplification targets.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Molecular characterization of multiple antibiotic-resistant *Pseudomonas aeruginosa* isolated from selected hospital fomites and hands of health care workers in Ondo, Nigeria

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The study reported the molecular characterization, antibiotic susceptibility profile and the nature of resistance genes in the multiple antibiotic resistant Pseudomonas aeruginosa isolated from selected hospital fomites and hands of health care workers in Ondo, Nigeria. Various fomites and hands of health care workers were swabbed for the detection of Pseudomonas aeruginosa. Each sample was cultured separately on MacConkey and Centrimide agar plates and incubated for 18-24h at 37oC. Pure isolates were obtained using Analytical Profile Index (API) 20E kit. Kirby Bauer's disc diffusion technique was used to decide the susceptibility of the pure isolates to known antibiotics. Resistant genes to 3 or more antibiotics were determined by Polymerase Chain Reaction (PCR) using appropriate primers. Two hundred Gram-negative bacterial isolates were recovered from 480 swab-stick samples analyzed out of which 54 were Pseudomonas aeruginosa. All the P. aeruginosa isolates showed total resistance to augmentin, cefixime and cefuroxime meanwhile 30 were resistant to nitrofuratoin, gentamycin 11, ceftazidime 7, ofloxacin 2 and ciprofloxacin 1. 31 (57.4%) were resistant to three or more classes of antibiotics. Out of the 12 representative isolates, 6 harboured blaCTX-M (585 bp) gene and were not susceptible to beta lactam antibiotics while 4 of the 7 Aminoglycoside (gentamycin) resistant isolates harboured aac-3-iv (286 bp) gene. In conclusion, different hospital fomites might be possible sources of nosocomial infections.

Key words: *P. aeruginosa*, antibiotic-resistance, hospital workers, Ondo, Nigeria.

INTRODUCTION

Nosocomial infections are hospital acquired infections and its severity is due to the level contamination of the hospital environment and the intrinsic characteristics of the organism. They are due to infections with organisms such as *Klebsiella* spp., *Shigella* spp., *Escherichia* coli, *Acinetobacter* spp., *Streptococcus* spp., *Staphylococcus aureus*, *Enterococcus* spp., *Proteus* spp., *Salmonella* spp. and *Pseudomonas* spp (Moti et al., 2018).

A major cause of public health concern is the worldwide dissemination of multi- or extensively drug resistant 'high

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risk clones' of *P. aeruginosa*, which need urgent attention (Lopez-Causape et al., 2018; Horcajada et al., 2019). The routes of transmission of the pathogen include environmental and patient-to-patient contamination, hands of healthcare workers (after contact with contaminated fomites, and infected patient amongst others. It can also survive on dry fomites from 6 h to 6 month due to its rugged adaptability and survival ability (Pachori et al., 2019; Centre for Disease Control, 2020).

An emerging global health threat are infections caused by *P. aeruginosa*, which are life-threatening because of their mechanisms for survival, adaptation and resistance to multiple antibiotics classes (Moradali et al., 2017). Also, the organism's pathogenicity is related to the complexity of its genome and various virulence factors (Maurice et al., 2018).

Its survival mechanisms include quorum sensing, viable but not culturable state, biofilm formation and antibiotic resistance mechanism (Dey et al., 2019; Verderosa et al., 2019).

The ability of *P. aeruginosa* to colonize a lot of habitats, persistence and prevalence in health care settings and antimicrobial resistance is due to its ability to use diverse responsive mechanisms such as reduced permeability, degrading enzymes, active efflux and modification of the antimicrobial targets (Moradali et al., 2017). It is also innately resistant to a lot of anti-microbials because of its ability to prevent membrane penetration by antimicrobial molecules or to release them if penetration occurs. Some active antimicrobials include: some b-lactams (e.g. ceftolozane-tazobactam, piperacillin tazobactam, ceftazidime-avibactam, ceftazidime, imipenem, meropenem, cefepime and doripenem), fluoroquinolones (e.g. levofloxacin and ciprofloxacin), aminoglycosides (e.g. tobramycin, amikacin and gentamicin) and so on (European Centre for Disease Prevention and Control, 2018).

MATERIALS AND METHODS

Selection and collection of samples

Swab stick samples of fomites and hands of health care workers were collected from Mother and Child Hospital, Ondo and State Specialist Hospital, Ondo, Ondo State, Nigeria after approval of ethical clearance with a reference number (MCHO/06/15/002) by the research committee of the institutions.

Identification and characterization of bacterial isolates

Fomites samples were collected using sterile swap sticks and were immediately transported to the Laboratory for identification. Samples collected were streaked on Cetrimide agar for the isolation of *Pseudomonas aeruginosa*

Presumptive identification of isolates was done using colony morphology and Gram staining reaction. Pure isolates were furthered identified by biochemical tests, such as catalase, citrate, Methyl Red-Voges Proskaeur, motility and sugar fermentation test (Olutiola et al., 2000).

Antibiotic susceptibility test

The *P. aeruginosa* isolates that were susceptible to commonly used antibiotics were identified using disc diffusion method and the susceptibility test was interpreted following Clinical and Laboratory Standard Institute (CLSI, 2013) guidelines. Discs immersed into concentrations of different antibiotics (gentamycin, augmentin, ceftazidime, nitrofurantoin, ofloxacin, ciprofloxacin, cefixime and cefuroxime (Oxoid Ltd, UK) were carefully inserted on the inoculated Mueller –Hinton agar plate with the aid of sterile forceps and incubated for 18 to 24 h at 37^{o}C. The dimensions of inhibition were taken with a transparent calibrated ruler.

Amplication and detection of PCR products

Twelve representative multiple antibiotic resistant *P. aeruginosa* isolates were further examined to detect resistance genes Cefotaximase- Munich (*blaCTX-M)* and aminoglycoside 3-N-acetyltransferase *(aac3*-IV) using Polymerase Chain Reaction (PCR). Isolates were selected on the basis of their reaction to antibiotics (Colom et al., 2003; Van et al., 2008).

RESULTS

Occurrence of *Pseudomonas aeruginosa* cultured from hospital fomites and hands of health workers in Ondo

Table 1 shows the occurrence of *P. aeruginosa* cultured from hospital fomites and hands of medical personnel. Of the 54 *P. aeruginosa* isolates cultured, 19 (35%) were cultured from bed, trolley 15 (28%), door handles 5 (9%), wash hand basin 8 (15%), mattress 5 (9%), bed sheet 1 (2%) and health care worker 1 (2%). However, *P. aeruginosa* was not recovered from other hospital fomites such as incubator, drip stand, cupboard and kidney dish.

Antibiotic susceptibility profile of *Pseudomonas aeruginosa* cultured from hospital fomites and hands of health care workers in Ondo

The antibiotic susceptibility profile of *P. aeruginosa* to the various antibiotics tested (augmentin, ceftazidime, cefixime, cefuroxime, ciprofloxacin, gentamycin, nitrofuratoin and ofloxacin) is shown in Table 2.

Multiple antibiotic resistance patterns of *Pseudomonas aeruginosa* cultured from hospital fomites in Ondo

Multiple antibiotic resistance patterns of *P. aeruginosa* are represented in Table 3. Multiple antibiotic resistances were defined as resistance to at least 3 or more different classes of antibiotics. The classes of antibiotics used include Beta-lactams (augmentin, cefixime, ceftazidime and cefuroxime), Fluoroquinolones (ciprofloxacin and

Table 1. Occurrence of *P. aeruginosa* from Hospital Fomites and Hand of Health Care Worker in Ondo.

Sample	Number of Isolates (n)	Percentage of Isolates (n)%
Bed	19	35
Trolley	15	28
Wash hand basin	8	15
Mattress	5	9
Door handle	5	9
Bed sheet	1	2
Hand of health care worker	1	2
Incubator	-	-
Drip stand	-	-
Cupboard	-	-
Kidney dish	-	-
Total	54	100

Source: Authors

Table 2. Antibiotic susceptibility profile of *P. aeruginosa* cultured from Hospital Fomites and Hand of Health Care worker in Ondo.

Antibiotic (µg)	Abbreviation	No. of Isolates	Number of isolate occurrence (%)		
			Susceptibility	Intermediate	Resistant
Augmentin (30 µg)	AUG	31	0	0	31 (100)
Ceftazidime (30 µg)	CAZ	31	22 (71)	2 (6)	7 (23)
Cefixime (5 µg)	CXM	31	0	0	31 (100)
Cefuroxime (30 µg)	CRX	31	0	0	31 (100)
Ciprofloxacin (5 µg)	CPR	31	30 (97)	0	1 (3)
Gentamycin (10 µg)	GEN	31	20 (65)	0	11 (35)
Nitrofurantoin (300 µg)	NIT	31	1 (3)	0	30 (97)
Ofloxacin (5 µg)	OFL	31	29 (94)	0	2 (6)

Source: Authors

Table 3. Multiple antibiotic resistance pattern of *P. aeruginosa* cultured from hospital fomites and hand of health care worker in Ondo.

Isolate	No. of antibiotic class	Multiple antibiotic resistance pattern	Frequency
P. aeruginosa	5	AUG, CAZ, CRX, CXM, CPR, GEN, NIT, OFL	1
	5	AUG, CRX, CXM, GEN, NIT, OFL	1
	4	AUG, CRX, CXM, GEN, NIT,	2
	4	AUG, CAZ, CRX, CXM, GEN, NIT	6
	3	AUG, CAZ, CRX, CXM, GEN,	1
	3	AUG, CRX, CXM, NIT	20
		Total	31(100%)

Source: Authors

ofloxacin), Aminoglycosides (gentamycin) and Nitrofurans (nitrofurantoin). Thirty-one (57.4%) of the fifty-four *P. aeruginosa* isolates obtained in this study showed multiple resistance to at least three different classes of antibiotics. All the thirty one *P. aeruginosa* exhibited multiple antibiotic resistances, ranging from three to five

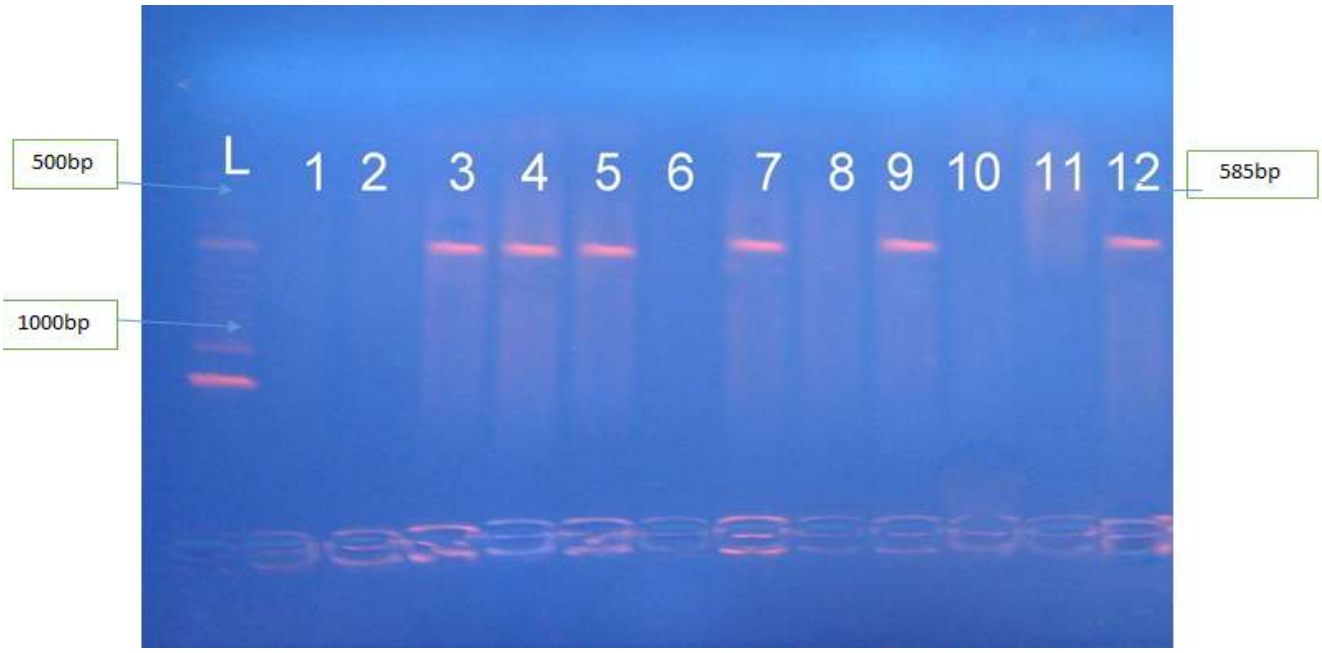

Plate 1. Agarose gel electrophoresis of the amplification product coding blaCTX-M (585 bp) gene in selected multiple antibiotic resistant *P. aeruginosa.* Lane L= DNA marker (100 bp), 3 = SPB1, 4 = SPB4, 5 = MmT17b, 7 = MND2, 9 = MCD5, 12=SPT10bp. Source: Authors

different classes. The *P. aeruginosa* isolates exhibited 6 different patterns with “GEN, CXM, AUG” appearing the most frequent.

Molecular Detection of *bla*CTX Resistance gene in *Pseudomonas aeruginosa*

Plate 1 shows the agarose gel electrophoresis of *bla* CTX-M (585 bp) gene in the 12 multiple antibiotic resistant (MAR) *P. aeruginosa* selected. Six of the beta-lactam antibiotics resistant isolates are depicted by Lanes 3, 4, 5, 7 and 9.

Molecular detection of *aac-3-iv* resistance gene in *Pseudomonas aeruginosa*

Plate 2 presents the MAR *P. aeruginosa* that harbor *aac-3-iv* (286 bp) gene. Four of the 7 representative isolates that were resistant to gentamycin antibiotics are depicted by Lanes 3, 4, 5 and 7 harboured *aac-3-iv* resistance gene of molecular weight of 286 bp.

DISCUSSION

The data obtained from this study revealed that some of the hospital fomites with which body have contact were contaminated with potential pathogens. The recovery of *P. aeruginosa* is an indication of gross contamination which calls for great concern considering the health risks of those that often have body contact with the fomites especially the immunocompromised patients. This is in line with some researchers (Hayden et al., 2006; Carling et al., 2008) who reported various disease causing organisms or nonpathogenic organisms can contaminate different surfaces and medical equipment often used in hospitals.

Thirty one (57.4%) of the 54 isolates of *P. aeruginosa* was multiple antibiotics resistant. Resistance was seen in three or more different classes of antibiotics. The trend of decreased antibiotic resistance observed in the study agrees with Lewis et al. (2012); Messadi et al. (2008) and Joseph et al. (2013) who earlier reported decreased resistance of *P. aeruginosa* to Ceftazidime and Ciprofloxacin but different from Senthamarai (2014), Mohanasundaram (2011) and Ibukun et al. (2007) where high resistance against Ceftazidime in their studies was reported.

Antibiotic resistance developed by pathogenic organisms is a global menace and has escalated over the years by the emergence of multi-drug resistant strains among these pathogens (Aslam et al., 2018). Development of resistance to antimicrobial agents by pathogens is a fitness trait acquired to survive in whatever environment they find themselves (Koskella et al., 2011). *P. aeruginosa* is known to exploit high level of intrinsic and acquired resistance mechanism to bombard quite a lot of antibiotics (Wilkie et al., 2021). A study suggests that the resistance of *P. aeruginosa* increases

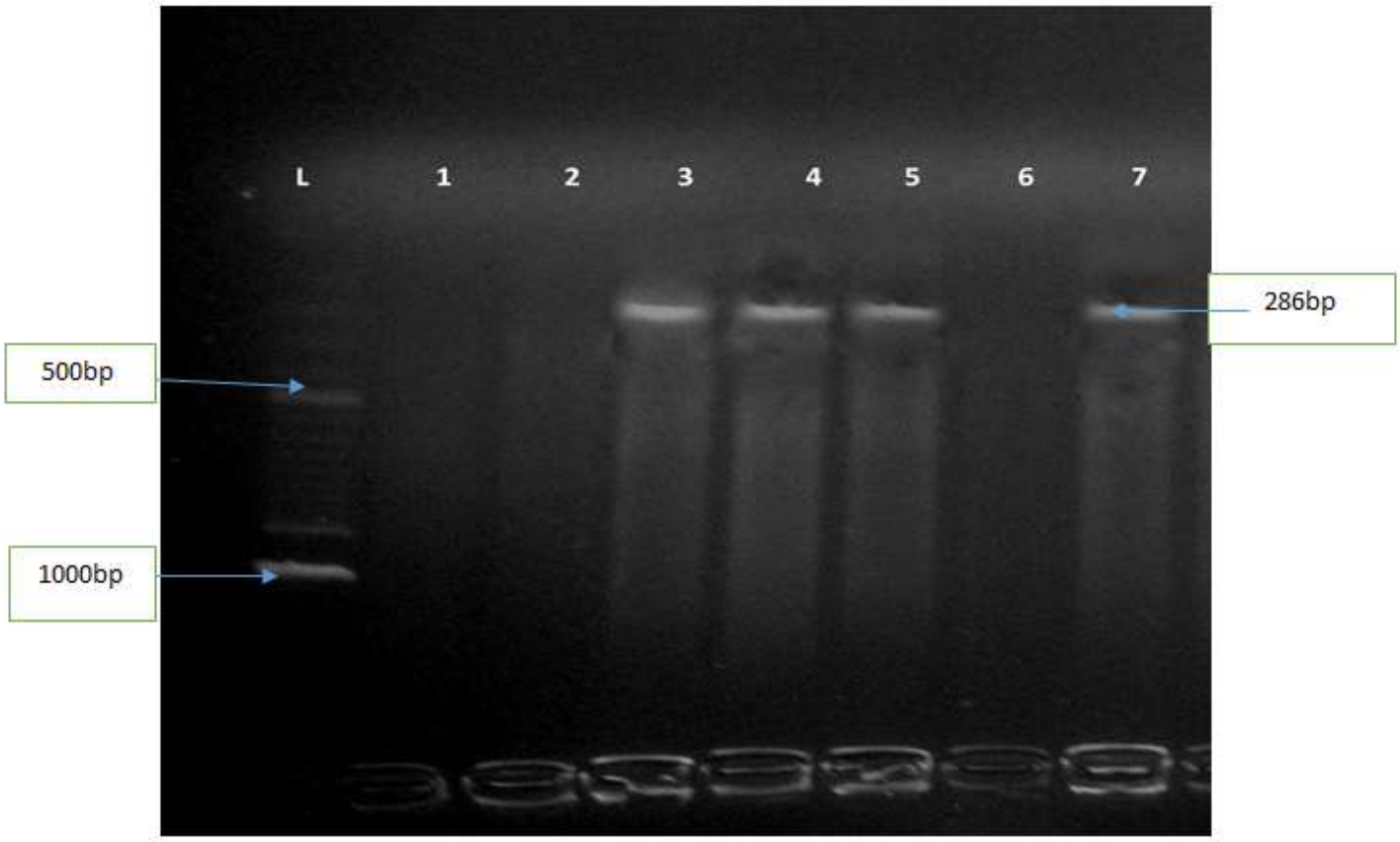

Plate 2. Agarose gel electrophoresis of the amplification product coding *acc-3-iv* (286 bp) gene in selected multiple antibiotic resistant *P. aeruginosa*. Lane L= DNA marker (100 bp), 3 = SPB1, 4 = SPB4, 5 = MmT17b, 7 =MND2.
Source: Authors

due to the uncontrolled usage and disposing of antibiotics in the environment. Treatment may fail to recover by constant contact of resistance isolates (Nasreen et al., 2015).

This study also revealed the detection of *bla*CTX-M (585 bp) and *aac-3-iv* (286 bp) resistance genes in *P. aeruginosa* isolates cultured from door handles of children and neo-natal wards in Mother and Child hospital and from beddings and trollies of post-natal ward in State Specialist hospital. The detection of *bla*CTX-M (585 bp) and *aac-3-iv (*286 bp) resistance genes, account for the resistance observed against beta-lactam group of antibiotics and gentamycin used, respectively. This agrees with Polotto et al. (2012) where *bla*CTX was detected in *P. aeruginosa* in his study.

Studies have revealed that unlike some exceptions, the CTX-M enzymes have nearly displaced other extended-spectrum Beta lactamase (ESBLs) enzymes in Enterobacteriaceae, including TEM and SHV ESBL variants (Cantón, 2008; Hawkey and Jones, 2009; Rodriguez-Villalobos et al., 2011). This displacement might have occurred not only as a consequence of the extraordinary dissemination of the corresponding *bla*CTX-M genes in highly mobilizable genetic platforms, including plasmids and transposons, but also because of these platforms within successful clones (Cantón and Coque, 2006; Rogers et al., 2011; Woodford et al., 2011). Another reason for this increase is the co-resistant phenomenon in CTX-M producing organisms, particularly to aminoglycosides and fluoroquinolones, which might facilitate co-selection processes (Morosini et al., 2006; Cantón and Ruiz-Garbajosa, 2011).

Apart from this general overview, within the CTX-M enzymes, the CTX-M-15 and CTX-M-14 are by far the most important ones, virtually invading all human and animal compartments as well as the environment all over the world (Cantón, 2008; Hawkey and Jones, 2009; Dolejska et al., 2011; Hiroi et al., 2012). Nevertheless, temporal emergence and penetration of these enzymes in different epidemiological scenarios might also explain the current epidemiology of CTX-M enzymes. Antibiotic consumption and dissimilar risk factors in different geographic areas and groups of patients and particularities of different compartments might have also contributed to the current CTX-M scenario (Carattoli, 2008; Rodríguez-Baño and Navarro, 2008; Rodríguez-

Baño and Pascual, 2008; Oteo et al., 2010a; Naseer and Sundsfjord, 2011).

Conclusion

This study showed that the different hospital fomites in the study location may be possible sources of nosocomial infections. It also revealed the presence of resistance genes (*bla*CTX-M, 585 bp and *aac-3-iv,* 286 bp) in the multiple antibiotic resistant *P. aeruginosa* isolates which accounted for the multiple antibiotic resistance observed. The susceptibility pattern of *P. aeruginosa* to ciprofloxacin (97%), ofloxacin (94%) and ceftazidime (71%) in this study showed the effectiveness of these drugs in the treatment of infections caused by *P. aeruginosa.*

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Diversity and microbiological quality of fruit juices produced in southern Benin

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The fruit juice production sector is growing rapidly in Benin's major urban centers and appears to have enormous hygienic insufficiencies. This study aimed to identify and evaluate the microbiological quality of the different fruit juices produced in southern Benin. The methodological approach to the first objective consisted of a semi-structured survey coupled with direct observations of fifty enterprises produced fruit juices in the Municipalities of Cotonou and Abomey-Calavi. The microbiological quality evaluation of pineapple juice (*Ananas cosmosus*) consisted in the enumeration of microorganisms that alter both hygienic and market ability qualities of foodstuffs, using standard methods. This study revealed a strong diversity of fruit juices. There are thirteen (13) different mono-fruity juices, fifteen (15) poly-fruity juices with pineapple and nine (09) poly-fruity juices without pineapple. The pineapple mono-fruity juice is produced by almost all the enterprises (98%). Poly-fruity juices with pineapple are the most popular among consumers. From the microbiological point of view, a strong variation of the microbial load was noticed from one enterprise to another. With the exception of a few enterprises, about 18% of pineapple juices are microbiologically non-compliant. These pineapple juices contain mycotoxigenic yeasts and moulds, lactic acid bacteria, thermotolerant coliforms and *Staphylococcus aureus*. The consumption of these poor sanitary quality juices poses a real public health problem, as it exposes consumers to the severe food poisoning risks.

Key words: Fruit juice, diversity, pineapple, *Ananas cosmosus*, microbiological quality, non-compliance.

INTRODUCTION

Fruit is often recommended to prevent and reduce the risk of chronic degenerative diseases, cardiovascular diseases and diabetes (Benton and Young, 2019). They remain an important source of minerals, vitamins,

phytoconstituents and phenolic compounds (Asandé et al., 2020). Thus, many fruits contain phenolic amino acids, organic acids such as tartronic acid (Sun et al., 2018), minerals such as Ca, K, Mg, Fe and Se (Doharey et al., 2021) and vitamins such as vitamin C, riboflavin and niacin (Zaini et al., 2011). Several medicinal properties such as anxiolytic, anticonvulsant, antidepressant, anti-inflammatory, analgesic and anti-asthmatic effects have been associated with fruits (Tiwari et al., 2013). For a healthy diet and better overall health, the Food and Agriculture Organization of the United Nations recommends a daily fruits consumption of at least 400 g (FAO, 2020). However, the adoption of this recommendation, especially in most developing countries, is very difficult. Indeed, in these low-income countries the diet is essentially based on cereals and tubers (FAO, 2020).

In Benin, the abundant consumption of fresh fruit by the population faces several difficulties, namely: high prices, inadequate preservation technologies, high post-harvest losses due to poor sales, and the fragility and seasonality of fruit. Faced with this situation, fruit transformation into other by-products is essential. The processing of fruit into fruit juice is widespread in Benin. Indeed, fruit juice is a nutritious drink that not only contains vitamins, minerals, proteins and protective antioxidants, but also provides an additional source of water (Heyman and Abrams, 2017).

Noumavo et al. (2022) showed that the fruit juice production sector is growing rapidly in southern Benin. It creates huge employment and generates significant income for the actors in the sector. However, the production and conservation conditions of these juices do not guarantee the required food safety. Indeed, the production methods of these juices are often artisanal, under unsatisfactory hygienic conditions. The ingestion of food or drink contaminated by certain infectious or toxigenic agents is a public health problem throughout the world. Foodborne diseases affect one in ten people every year and 420,000 people die from them (Petruzzi et al., 2017). These foodborne infectious diseases can also have a significant economic impact because of treatment cost.

This study aims to identify and evaluate the microbiological quality of the different fruit juices produced in southern Benin.

MATERIALS AND METHODS

Geographical area

The present study was carried out in southern Benin, particularly in the municipalities of Abomey-Calavi (6°26'9112 "N; 2°21'3396 "E) and Cotonou (6°21'9216 "N; 2°25'0998 "E). These two municipalities contain the majority of fruit juice producing enterprises in southern Benin. Indeed, they are very close to Allada department, an area of large fruit production, particularly pineapple (*Ananas cosmosus*) in Benin.

Diversity evaluation of fruit juices

This study was conducted from November 2020 to February 2021. Fifty (50) fruit juice enterprises previously identified by Noumavo et al. (2022) were involved in the present study. These included semi-industrial (05) and artisanal (45) enterprises. Within the artisanal enterprises (A), several sub-groups can be distinguished: A1 enterprise have no mechanized equipment except for a manual capper; A2 enterprise have an extractor and/or grinder, all stainless steel or not, and then a manual capper; A3 enterprise have an extractor and/or grinder and a press, a cooker, all stainless steel, and then a manual capper; and finally A4 companies have a cooker, an extractor, a press, a pasteurizer all stainless steel, and then a manual capper. Different fruit juices census was carried out by a semi-structured survey coupled with direct observations.

Microbiological analysis of fruit juices

The microbiological investigation was carried out only on mono-fruity pineapple juice at the Laboratory of Microbiology and Food Technology (University of Abomey-Calavi, Benin). A total of 100 juice samples were collected (two samples per enterprise). The sampling, transport and storage of the samples were carried out under technical conditions that prevented any modification of their microbial flora. The preparation of decimal dilutions was done under aseptic conditions according to Speck (1976) method. Microorganism's enumeration was performed according to the method used by Ohin et al. (2018). Thus, Total Mesophilic Aerobic Microorganisms were counted on Plate Count Agar (Oxoid, England) after incubation at 30°C for 72 h. Total Coliforms and Faecal Coliforms were isolated on Violet Red Bile Lactose agar (Liofilchem Diagnostici, Italy) after incubation at 30 and 44°C, respectively for 24 h. Lactic acid bacteria were counted on MRS (de Man, Rogosa and Sharpe) agar after incubation at 37°C for 72 h. Tryptone Sulfite Neomycin agar (Biokar Diagnostics, France) was used to enumerate anaerobic sulfite-reducing bacteria (44°C for 24 h). Yeasts and molds were enumerated on Sabouraud agar (Biokar Diagnostics, France) supplemented with Chloramphenicol (25°C for 5 days). Finally, *Staphylococcus aureus* were isolated on Baird Parker agar (Biokar Diagnostics, France) supplemented with egg yolk and potassium tellurite after incubation at 37°C for 48 h.

Data management and analysis

Data from the survey forms and microbiological analysis were encoded using the Microsoft Excel 2013 spreadsheet for descriptive statistics (mean, proportion and standard deviation). The R.4.0.0 software was used to carry out the Analysis of Variance (probability level of 5%) and the Student-Newman-Keuls test.

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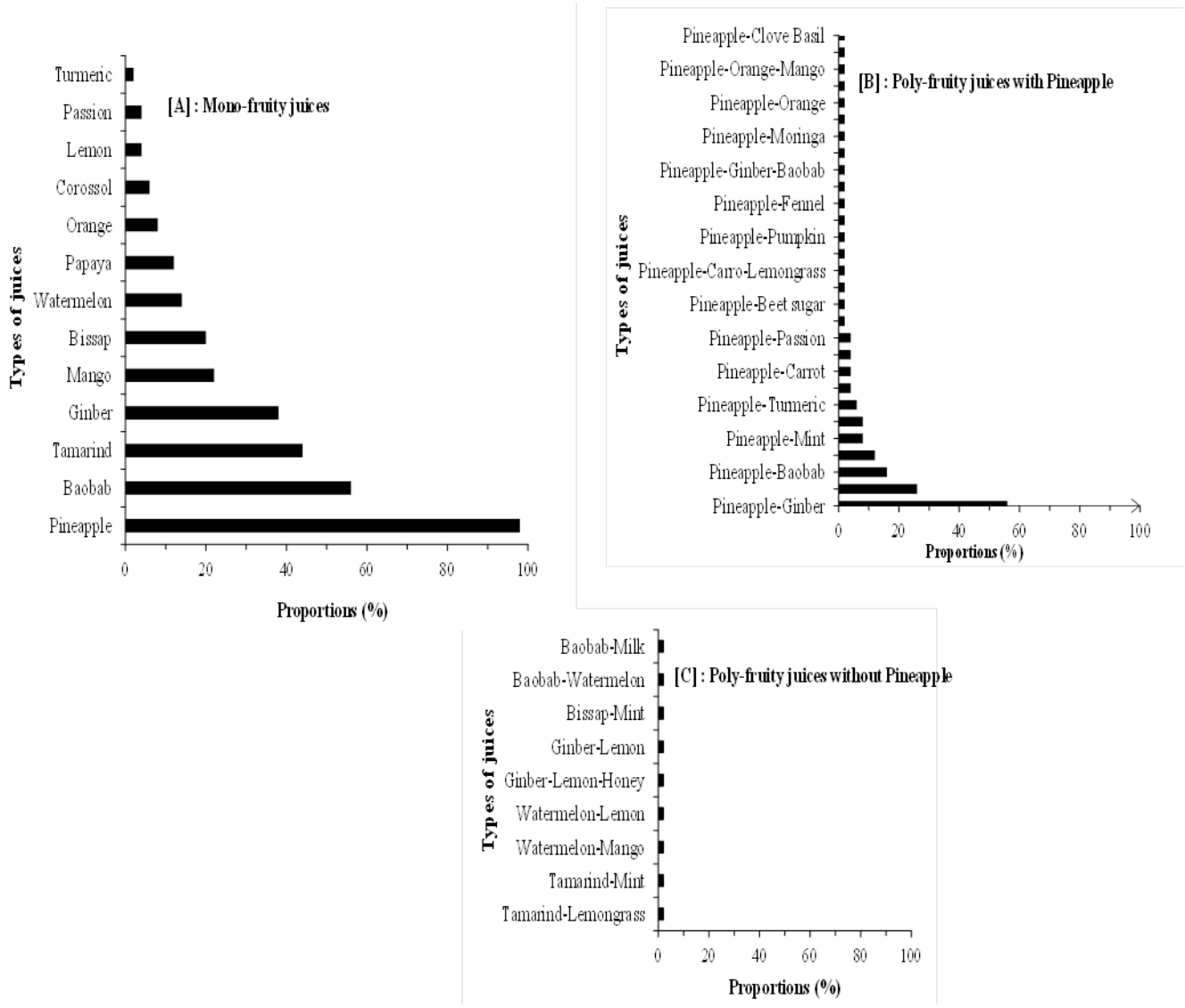

Figure 1. Diversity of fruit juices,
Source: Authors

RESULTS AND DISCUSSION

Diversity of fruit juices produced

The types of juice encountered during our surveys are verydiverse. There are thirteen (13) different mono-fruity juices, fifteen (15) poly-fruity juices with pineapple and nine (09) poly-fruity juices without pineapple. Indeed, among the most common mono-fruit juices (Figure 1A), we note pineapple (*A. cosmosus*) juice produced by almost all the enterprises surveyed (98%), baobab (*Adansonia digitata*) juice (56%), tamarind (*Tamarindus indica*) juice (44%) and ginger (*Zingiber officinale*) juice (38%).

Apart from mono-fruit juices, several types of cocktails (poly-fruit juices) have been recorded (Figure 1B). Among the cocktails with pineapple, Pineapple-Ginger is produced by just over half of the enterprises (56%). It is followed in order of proportion by Pineapple-Watermelon (26%), Pineapple-Baobab (16%) and Pineapple-Tamarind (12%) cocktails. Note that a few rare enterprises sporadically produce cocktails without pineapple (Figure 1C). Poly-fruity juices with pineapple are the most popular among consumers.

It should be noted that a diversity of juices is produced. Thus, apart from the pineapple used to sweeten the various cocktails, sour, honey or milk is also used for those who develop an allergy to pineapple. These

particularities are therefore on special order of the customer. Apart from pineapple, other raw materials are obtained in local markets such as the Dantokpa and Abomey-Calavi markets.

Microbiological quality of pineapple juice

In this study, the evaluation of the microbiological quality of the pineapple juice collected consisted in the search for certain microorganisms responsible on the one hand, for the deterioration of the hygienic quality of food (Total Coliforms, Fecal Coliforms and *S. aureus*) and on the other hand, the deterioration of the marketable quality of food (yeasts, molds and lactic acid bacteria). Figure 2 presents the different microbial groups sought and their abundance according to the industrial size of the companies producing these juices.

More specifically, Figure 2A illustrates the variation in the load of Total Mesophilic Aerobic Flora (TMAF) of pineapple juice according to the type of business. Indeed, the TMAF load is a good indicator of hygiene, which makes it possible to assess microbial pollution and the general quality of a foodstuff (Ayadi and Touahmia, 2021). With regard to the statistical analyses carried out, the load in TMAF varies very significantly from one type of enterprises to another ($p < 0.01$). The juices from the A1 and A2 artisanal type enterprises harbor a very abundant TMAF in comparison to the juices from the other types of enterprises. This result corroborates the many hygienic shortcomings noted in craft businesses, particularly those of types A1 and A2. Within type A1 and A2 companies, all stages of the production process are carried out manually except for capping, which is done using a capping machine, and grinding, which is done using an extractor and/or a crusher (A2). On the other hand, production lines are a little more mechanized in other types of craft businesses and even better in Semi-Industrial (SI) businesses. It should be noted, however, that the TMAF load of the juices from SI companies is much higher than those of the A4 type artisanal companies which contain the lowest TMAF loads. This atypical result illustrates the difficulty that some SI companies have in respecting the rules of good hygiene practice. Indeed, they are often satisfied with a simple washing with soapy water before and after production without dismantling the devices for proper cleaning. This could favor the accumulation of water and fruit residues in non-accessible areas and consequently the proliferation of spoilage and pathogenic microorganisms.

Similar results were obtained by Ogodo et al. (2016) following the analysis of the microbiological quality of fruit juices packaged and marketed in South-East Nigeria. In this study, the bacterial load of pineapple juice reached 4.4×10^5 CFU/ml. In another study by Amin et al. (2018) on mango juice the bacterial load varied from 1.10^3 to 3.10^3 CFU/ml.

The microbiological profile of pineapple juice relative to the two groups of microorganisms affecting the marketability of food (yeast-molds and lactic acid bacteria) investigated in this study is presented in Figure 2B and C. A trend of the variation of the microbial load from one type of enterprise to another is practically similar to that of the TMAF. However, the juices from A4-type artisanal businesses and SI businesses contain a very negligible load of yeasts and molds and lactic acid bacteria. According to the guidelines and standards for the interpretation of analytical results in food microbiology of the State of Quebec (Dumont, 2019), the microbial load in yeasts and molds of pasteurized fruit juices must not exceed 10^3 CFU/ml. With regard to this threshold relating to yeasts and molds, we can say that approximately 20% of the samples of pineapple juice coming from artisanal enterprises type A1 and A2 and from SI businesses as well as 12.5% of those from artisanal businesses type A3 are of unsatisfactory microbiological quality, and therefore unfit for human consumption (Table 1). All the pineapple juice samples from A4-type artisanal businesses are of satisfactory quality with regard to their yeasts and molds content. These samples will lose their organoleptic characteristics more quickly than those from A4-type artisanal businesses. In fact, molds can produce pectinases which are degrading enzymes with a high potential for modifying the taste, smell and appearance of fruit juices. The presence of these molds may be due to insufficient hygiene within the production units with a high preponderance of heat-resistant molds (Konan, 2016).

Also, the consumption of these juices exposes consumers to real risks of food poisoning. Indeed, fungal contamination can lead to the formation of allergens and the production of toxigenic compounds such as mycotoxins (aflatoxins, ochratoxin A, patulin, etc). These toxigenic compounds pose a real public health problem because of their responsibility in the occurrence of acute and chronic toxicity phenomena. Their prolonged exposure through food has been associated with cancers and diseases of the kidneys, liver and immune system (Tovide et al., 2017). Unfortunately, there is no single solution in the fight against mycotoxins. It is therefore important to take all the necessary measures to reduce and control the level of mycotoxins in agri-food products (Gauthier, 2016).

The microbial loads of germs indicating fecal contamination (coliforms) and mucocutaneous contamination (*S. aureus*) are presented in Figure 2D, E and F. The analysis of variance revealed that there is a very highly significant difference in these microbial groups from one type of business to another. Samples from type A1 and A2 artisanal businesses contain more coliforms (Total and Fecal) than samples from type A3 and SI artisanal establishments. On the other hand, the samples from artisanal type A4 establishments do not contain coliforms at all. Thus, all samples (100%) of type

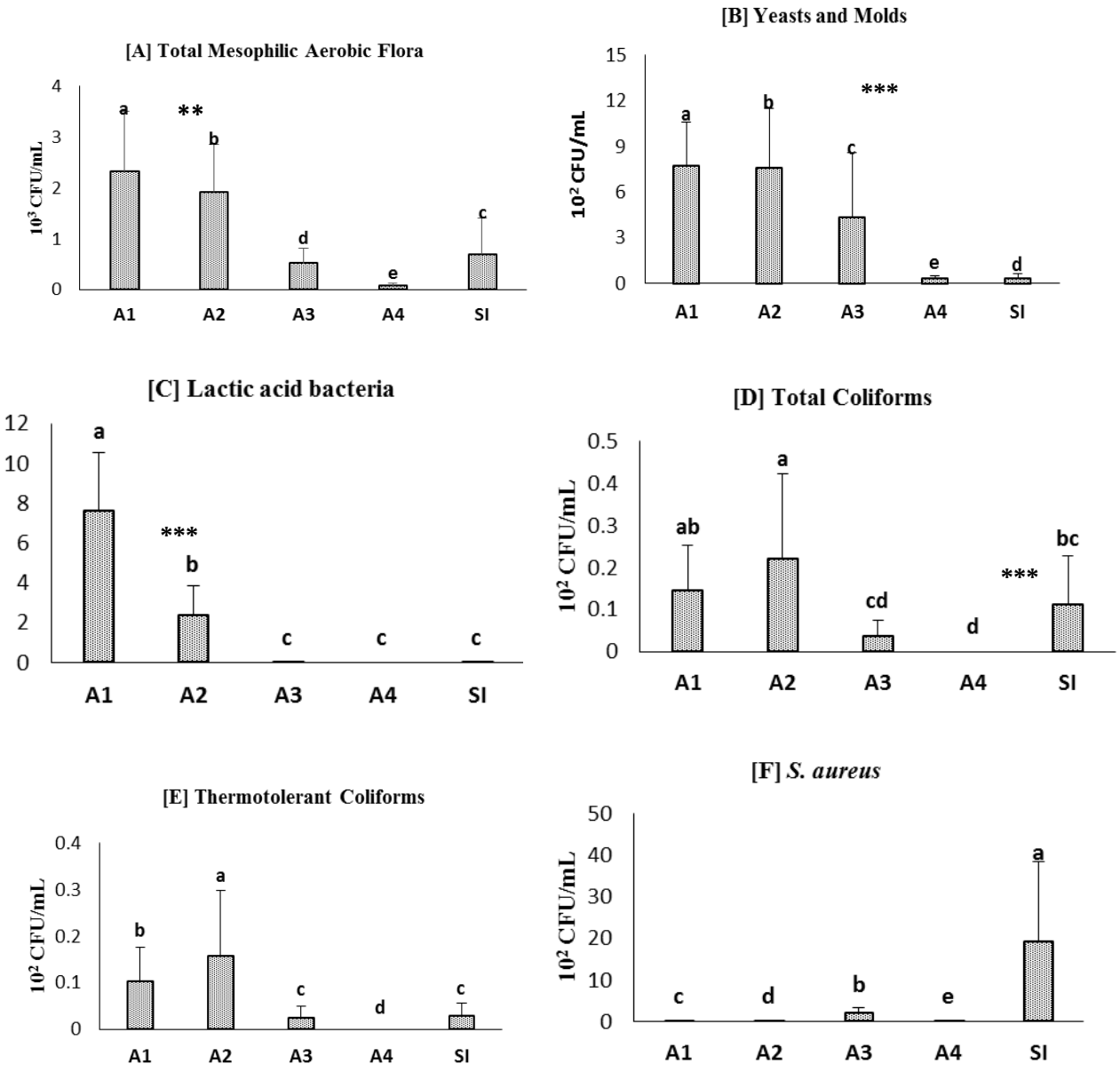

Figure 2. Diversity and microbial load variation of pineapple juices according to the industrial size of enterprises. A1: Artisanal enterprise type 1; A2: Artisanal enterprise type 2; A3: Artisanal enterprise type 3; A4: Artisanal enterprise type 4; SI: Semi-Industrial enterprise. ° = p > 0.05 (not significant), * = p < 0.05 (significant), ** = p < 0.01 (very significant), *** = p < 0.001 (very highly significant), CFU/g = Colony Forming Unit per milliliter of sample; on the same graph, the means with different letters are significantly different with probability level of 5% according to ANOVA.
Source: Authors

A4 artisanal establishments are suitable for human consumption if we stick to the guidelines mentioned earlier. About 20% of the samples from artisanal businesses A1 and SI and 10% of those from artisanal establishments A2 and A3 have a fecal coliform microbial load above the normative threshold (Table 1). Once again and in view of our results, the mechanization of pineapple juice production companies does not always guarantee good quality of the juices produced. Regardless of the industrial size of an agri-food processing establishment, the adoption of rules of good hygiene practice remains essential. Although we cannot be satisfied with our results, they are still lower than the 40% non-compliance obtained by Rahman et al. (2011) in a similar study. Asghar et al. (2018) also proved that apple, carrot, orange and sugar cane juices had a high

Table 1. Non-conformity rate of analysed pineapple juice samples.

Industrial size	Non-conformity (%)		
	Yeasts and molds	Fecal coliforms	General
A1	20	20	20
A2	22.72	9.52	22.72
A3	12.5	12.5	12,5
A4	0	0	0
SI	20	20	20

A1: Artisanal enterprise type 1; A2: Artisanal enterprise type 2; A3: Artisanal enterprise type 3; A4: Artisanal enterprise type 4; SI: Semi-Industrial enterprise.
Source: Authors

load of total and fecal coliforms. Similar results were obtained by Onuoha et al. (2018) on simple fruit juices and cocktails packaged and sold in the metropolis of Owerri (Nigeria). It should be noted that these last studies were carried out for the most part on non-pasteurized fruit juices. The presence of these germs in the pineapple juices analyzed in our study may also be due to a pasteurization defect. Indeed, pasteurization is a technique of preservation by heat treatment which makes it possible to considerably reduce the microbial load in a food. It is a simple and inexpensive technique for small agro-industries (Hounhouigan et al., 2020). However, at the level of some fruit juice production companies surveyed, there is a problem of non-compliance with pasteurization scales and lack of appropriate equipment to carry out this preservation treatment.

The presence of coliforms is on one hand an indicator of fecal contamination, by germs that are not directly pathogenic, but whose presence suggests the existence of pathogenic germs for human beings. With the exception of the genus *Klebsiella* and a few others, fecal coliforms are made up of nearly 80% *Escherichia coli*. Three major groups of *E. coli* are involved in diarrheal syndromes in humans and young children, especially in developing countries. These are enterotoxigenic *E. coli*, the cause of traveler's diarrhea and childhood diarrhea; enteropathogenic *E. coli*, agent of infantile gastroenteritis; and finally enterohaemorrhagic *E. coli*, agent of hemorrhagic colitis. *E. coli* serotypes *O157* and *O157: H7* are enterohemorrhagic *E. coli*, agents of serious food poisoning linked to the consumption of meat, unpasteurized apple juice, etc. The second serotype is also called verotoxigenic *E. coli* (VTEC) (Delarras, 2008).

A single germ indicator of mucocutaneous contamination was sought in pineapple juice. This is *S. aureus* known to be common to the bacterial flora of human skin (Alabi et al., 2021), whose microbial load in this study varied markedly ($p < 0.001$) between the juices of the different types of business. Contrary to the general trend observed with the other germs sought, juices from semi-industrial SI companies are highly contaminated with *S. aureus*. The *S. aureus* microbial load of these juices is at least 8 times higher than that of juices from other types of companies. This result remains a priori paradoxical because within semi-industrial companies, manual activities are quite limited compared to craft companies. However, it should be noted that one of the 5 SI companies investigated does not peel the pineapple fruits before grinding. Indeed, often not well-washed pineapple fruits are directly crushed with the skin using a crusher. When we refer to the method of transporting pineapple fruits from the fields or supply markets to the processing units and to the method of receiving and storing pineapple fruits, it is easy to see that the bare hand is heavily used. The fruits are even in many cases received on the ground. It should also be noted that within these companies, the washing of the packaging bottles, the filling and the capping are done by hand in conditions that are not often hygienic. To all this is added the defects of pasteurization often noted within companies in general. All these observations, although not exhaustive, could explain this high load of *S. aureus* in juices from SI enterprises.

This presence of *S. aureus* in the pineapple juice analyzed is very worrying. Indeed, food poisoning due to *S. aureus* does not result primarily from the ingestion of the bacterium itself, but rather from the toxins preformed in the contaminated food (Moloi et al., 2021). Staphylococcal food poisoning is mainly caused by a particular group of toxins called enterotoxins (Chebana et al., 2021). These enterotoxins are heat stable, highly toxic and persist in food even after adequate cooking (Ghalehnoo, 2018). Twenty-two (22) Staphylococcal Enterotoxins (ES) have been described and designated by the letters ESA to ESV in the chronological order of their discovery (Hennekinne et al., 2010). Staphylococcal Food Poisoning (SFP) is characterized by a sudden onset of symptoms (1 to 6 h after ingestion), with vomiting, abdominal pain and stomach cramps (Fetsch et al., 2014). SFP can lead to hospitalizations, especially among young, old, pregnant and immunocompromised (Murray, 2005).

Conclusion

This study of fruit juices diversity and quality in southern Benin provides important data for restructuring the sector. This study revealed a strong diversity of mono-fruity and poly-fruity juices. Mono-fruity juice of pineapple is produced by almost all the enterprises. Pineapple is incorporated in majority of poly-fruity juices. From the microbiological point of view, a strong variation of the microbial load was noticed from one enterprises to another. With the exception of a few enterprises, about 18% of pineapple juices are microbiologically non-compliant. These pineapple juices contain mycotoxigenic yeasts and moulds, lactic acid bacteria, thermotolerant coliforms and *S. aureus*. The consumption of these juices poses a real public health problem, as it exposes consumers to the severe food poisoning risks.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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