OPEN ACCESS

African Journal of Microbiology Research

September2021
ISSN 1996-0808
DOI: 10.5897/AJMR
www.academicjournals.org

ACADEMIC JOURNALS
expand your knowledge

About AJMR

The African Journal of Microbiology Research (AJMR) is a peer reviewed open access journal. The journal commenced publication in May 2007. The journal covers all areas of microbiology such as environmental microbiology, clinical microbiology, immunology, virology, bacteriology, phycology, molecular and cellular biology, molecular microbiology, food microbiology, mycology and parasitology, microbial ecology, probiotics and prebiotics and industrial microbiology.

Indexing

CAB Abstracts, CABI's Global Health Database, Chemical Abstracts (CAS Source Index) Dimensions Database, Google Scholar, Matrix of Information for The Analysis of Journals (MIAR), Microsoft Academic, Research Gate

Open Access Policy

Open Access is a publication model that enables the dissemination of research articles to the global community without restriction through the internet. All articles published under open access can be accessed by anyone with internet connection.

The African Journal of Microbiology Research is an Open Access journal. Abstracts and full texts of all articles published in this journal are freely accessible to everyone immediately after publication without any form of restriction.

Article License

All articles published by African Journal of Microbiology Research are licensed under the Creative Commons Attribution 4.0 International License. This permits anyone to copy, redistribute, remix, transmit and adapt the work provided the original work and source is appropriately cited. Citation should include the article DOI. The article license is displayed on the abstract page the following statement:

This article is published under the terms of the Creative Commons Attribution License 4.0
Please refer to https://creativecommons.org/licenses/by/4.0/legalcode for details about Creative Commons Attribution License 4.0

Article Copyright

When an article is published by in the African Journal of Microbiology Research, the author(s) of the article retain the copyright of article. Author(s) may republish the article as part of a book or other materials. When reusing a published article, author(s) should; Cite the original source of the publication when reusing the article. i.e. cite that the article was originally published in the African Journal of Microbiology Research. Include the article DOI, Accept that the article remains published by the African Journal of Microbiology Research (except in occasion of a retraction of the article).

The article is licensed under the Creative Commons Attribution 4.0 International License.

A copyright statement is stated in the abstract page of each article. The following statement is an example of a copyright statement on an abstract page.

Self-Archiving Policy

The African Journal of Microbiology Research is a RoMEO green journal. This permits authors to archive any version of their article they find most suitable, including the published version on their institutional repository and any other suitable website.

Digital Archiving Policy

The African Journal of Microbiology Research is committed to the long-term preservation of its content. All articles published by the journal are preserved by Portico. In addition, the journal encourages authors to archive the published version of their articles on their institutional repositories and as well as other appropriate websites.
https://www.portico.org/publishers/ajournals/

Metadata Harvesting

The African Journal of Microbiology Research encourages metadata harvesting of all its content. The journal fully supports and implement the OAI version 2.0, which comes in a standard XML format. See Harvesting Parameter

Memberships and Standards

Academic Journals strongly supports the Open Access initiative. Abstracts and full texts of all articles published by Academic Journals are freely accessible to everyone immediately after publication.

All articles published by Academic Journals are licensed under the Creative Commons Attribution 4.0 International License (CC BY 4.0). This permits anyone to copy, redistribute, remix, transmit and adapt the work provided the original work and source is appropriately cited.

Crossref is an association of scholarly publishers that developed Digital Object Identification (DOI) system for the unique identification published materials. Academic Journals is a member of Crossref and uses the DOI system. All articles published by Academic Journals are issued DOI.

Similarity Check powered by iThenticate is an initiative started by CrossRef to help its members actively engage in efforts to prevent scholarly and professional plagiarism. Academic Journals is a member of Similarity Check.

CrossRef Cited-by Linking (formerly Forward Linking) is a service that allows you to discover how your publications are being cited and to incorporate that information into your online publication platform. Academic Journals is a member of CrossRef Cited-by.

Academic Journals is a member of the International Digital Publishing Forum (IDPF). The IDPF is the global trade and standards organization dedicated to the development and promotion of electronic publishing and content consumption.

Contact

Editorial Office: ajmr@academicjournals.org

Help Desk: helpdesk@academicjournals.org

Website: http://www.academicjournals.org/journal/AJMR

Submit manuscript online http://ms.academicjournals.org

Academic Journals
73023 Victoria Island, Lagos, Nigeria
ICEA Building, 17th Floor,
Kenyatta Avenue, Nairobi, Kenya.

Editors

Editors

Table of Content

Vol. 15(9), pp. 461-466, September 2021
DOI: 10.5897/AJMR2021.9561
Article Number: F9FA63767672
ISSN: 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Prevalence of aflatoxin M_1 in pasteurized and ultra-high temperature (UHT) milk marketed in Dar es Salaam, Tanzania

Hilda F. Mwakosya[1*] and Jovin K. Mugula[2]

[1]Tanzania Bureau of Standards, P. O. Box 9524, Dar es Salaam, Tanzania.
[2]Department of Food Technology, Nutrition and Consumer Sciences, College of Agriculture, Sokoine University of Agriculture, P. O. Box 3006, Morogoro, Tanzania.

Received 30 June, 2021; Accepted 10 August, 2021

The aim of this study was to determine the level of aflatoxin M_1 (AFM_1) in pasteurized and UHT milk marketed in Dar es Salaam, Tanzania. AFM_1 in pasteurized milk samples (75) and ultra-high temperature (UHT) milk (43) was determined by using immuno-affinity high performance liquid chromatography. AFM_1 was detected in 97% (115/118) of samples that consisted of 96% (72/75) of pasteurized milk samples and 100% (43/43) of UHT milk samples. About 82% of the contaminated pasteurized and UHT had AFM_1 levels above EU acceptable levels (0.05 µg/L). However, none of the contaminated pasteurized and UHT milk sample had levels of AFM_1 above the maximum recommended Codex limits (0.5 µg/L). The results indicate that the contamination of the samples with AFM_1 at such level could pose a serious public health problem. Thus, regular monitoring of AFM_1 levels in milk is important in order to protect consumers.

Key words: Aflatoxin M_1, ultra-high temperature (UHT), milk, pasteurized milk, food safety.

INTRODUCTION

Tanzania has the third largest livestock population in Africa comprising 25 million cattle out of which 98% are indigenous breeds (FAO, 2020). The dairy production in Tanzania is categorized into two systems: traditional system and dairy system (Munyaneza et al., 2019). Traditional system is the most dominant and it is based on both milk and meat products; dairy system is based mainly on milk production (URT, 2017). In the year 2018 about 934,628 tonnes of raw and heat-treated milk were produced in Tanzania. Milk production contributes to income, food security, nutrition and household livelihood (FAOSTAT, 2020). The sector contributes to 7.4% of total national GDP and the annual growth rate (2.2%) of the sector is considered low (FAO, 2020). Raw milk is a valuable nutritious food, highly perishable, with short shelf-life and it is an excellent medium for the growth of

*Corresponding author. E-mail: hmwakosya@gmail.com. Tel: 0765825585.

microorganisms, particularly harmful bacterial pathogens that can cause spoilage and diseases to consumers (FAO, 2021). Heat treatment of milk such as pasteurization and ultra-high temperature (UHT) allows the preservation of milk and helps to reduce food-borne illness (Melini et al., 2017).

According to FAO/WHO (1982), pasteurization is defined as a heat treatment process applied to a product such as milk with the objective of minimizing numbers of harmful micro-organisms to a level at which they do not constitute a significant health hazard with minimal chemical, physical and organoleptic changes in the product. It also extends the storage time for some products by reducing the number of spoilage micro-organisms in the product (FAO/WHO, 1982). Codex Alimentarius (2004), defined UHT treatment of milk and liquid milk products as the application of heat to a continuously flowing product using such high temperatures for such time that renders the product commercially sterile at the time of processing. When UHT treatment is combined with aseptic packaging, it results in a commercially sterile product at the heating range of 135 to 150°C for 1 s up to 4 s (Melini et al., 2017). AFM_1 is a heat stable compound that can survive heat treatment such as pasteurization, UHT technique and autoclaving but also AFM_1 may be reduced but not completely destroyed by heat treatments (Mahmoodi et al., 2019; Tahira et al., 2019).

Aflatoxins are amongst the most poisonous mycotoxins and are produced by *Aspergillus flavus, Aspergillus parasiticus* and *Aspergillus nomius* fungi found in soil and that can grow in plant, human food products and feeds (WHO, 2018). The most important aflatoxins in order of toxicity are B_I, B_2, G_1, and G_2 (Ismail et al., 2018; Tahira et al., 2019). Aflatoxins may also be found in the milk of animals that are fed contaminated feed, in the form of aflatoxin M_1, a hydroxylated metabolite of aflatoxin B_1, within 12 h of contaminated feed consumption (Langat et al., 2016). Once lactating cow consume contaminated feeds with aflatoxin B_1 it is absorbed into the gastrointestinal tract and biotransformation occurs in the liver by cytochrome P_{450} enzymes to form a 4-hydroxy metabolite known as aflatoxin M_1, a compound soluble in water and therefore it is easily excreted in milk during milking (Daou et al., 2020; Tahira et al., 2019).

Aflatoxin M_1 is a hepato-carcinogen, classified as a group 1 carcinogen by International Agency for Research on Cancer (IARC Monograph, 2018). Aflatoxin M_1 is heat-stable and can survive pasteurization, autoclaving and thermal inactivation (Zakaria et al., 2019). The contamination of milk and milk products by aflatoxin M_1 has been reported in various countries such as Morocco (Mannani et al., 2021), Iran (Mahmoodi et al., 2019), Lebanon (Daou et al., 2020), Turkey (Eker et al., 2019), Pakistan (Tahira et al., 2019), and Kenya (Langat et al., (2016).

The occurrence of aflatoxin M_1 in milk in Tanzania reported earlier indicated that 92% of raw cow milk retailed in Dar es Salaam city was contaminated with aflatoxin M_1 (Urio et al., 2006) and 83.8% of raw cow milk from households in Singida was contaminated with aflatoxin M_1 (Mohammed et al., 2016). However, there is no information on aflatoxin M_1 contamination of pasteurized and ultra-pasteurized (UHT) milk in Tanzania, as well as the awareness of contamination. Thus, the aim of this study was to carry out surveillance of the level of contamination of aflatoxin M_1 in pasteurized and ultra-pasteurized marketed milk in Dar-es-Salaam, commercial capital of Tanzania. The results of this study will provide information on level of milk contamination by aflatoxins and contribute to raise awareness and efforts of food control authorities in developing strategies to ensure public safety.

MATERIALS AND METHODS

Sample collection

A total of 118 milk samples both ultra-high temperature (UHT) and pasteurized were purchased randomly from different mini markets and supermarkets located in Kinondoni, Ilala, Temeke, Ubungo and Kigamboni districts of Dar es Salaam region a commercial city of Tanzania during December 2020 and January 2021. This region was selected on purpose since it is the largest urban consumer market with availability of milk brands from different regions in Dar es Salaam shops/outlets. Seventy-five samples of pasteurized milk and 43 UHT milk samples were collected. The larger number of pasteurized milks collected is due to the fact that most dairies produce pasteurized milk.

The collected samples originate from two climatic zones of Tanzania, hot humid coastal zone (Tanga, Morogoro, Dar es Salaam and Zanzibar) and temperate highland zone (Kilimanjaro and Iringa). All samples were randomly purchased, coded and transported in an ice box together with their original packaging prior to laboratory analysis at the Tanzania Bureau of Standards (TBS) food laboratory in Dar es Salaam.

Aflatoxin M_1 analysis

Reagents standards, chemicals, columns and other materials

HPLC grade acetonitrile, methanol and glacial acetic acid were obtained from Fisher Scientific UK. A standard aflatoxin M_1 (0.504 µg/L) solution was obtained from Biopure, Romer Labs Diagnostics GmbH, Tullin Austria. Distilled water was produced with a Milli-Q Integral 15 water purification system, France. Whatman Filter paper No. 4 (Whatman Inc., Clifton, NJ, USA) and AflaStar™ M1 R, Immunoaffinity Columns (IAC) for aflatoxin M_1 were from Romer Labs, Austria.

HPLC conditions

The HPLC system (Infinity II, Agilent technologies) with vial

sampler, Quant pump, MCT and FLD Spectra (model 1260) with excitation set at 365 nm and emission 450 nm was used. Instrument settings were: injection volume 50 µL, pump flow rate 0.8 mL/min, run time was 6 min, HPLC analytical column 4.6×150 mm (Waters® Spherisorb® 5 µm ODS1, Ireland) column oven set at 25°C, mobile phase comprised 2% acetic acid: acetonitrile: methanol (40:35:25) that was prior degassed for 20 min and run isocratically. Data acquisition and processing were done with OpenLab software (Version 3.4, Agilent technologies). Aflatoxin M_1 peak in the chromatogram was identified by comparing its retention time with that of the analyzed aflatoxin M_1 standard under the same conditions.

Standards preparation

Exactly 1985 µL of aflatoxin M_1 standard was added in 9015 µL of aflatoxin M_1 mobile phase to get a stock solution of 100 µg/L. This solution was used to prepare 5, 8 and 10 µg/L standards by dilutions with mobile phase. The 10 µg/L standard was further diluted with mobile phase to prepare 0.1, 0.5, 1 and 3 µg/L standard. These seven standards were used for validation and quality control of the method.

Extraction and quantification of aflatoxin M_1 by HPLC

The method for extraction, detection and quantification of aflatoxin M_1 in the milk samples was done in a dark room according to Behfar et al. (2012) with minor modifications. Fifty milliliters of the milk samples were measured in Teflon tubes, warmed up in the water bath (ThermoHaake IP30, Germany) set at 37°C. Samples were centrifuged at 4000 rpm for 15 min (Eppendorf 5810R, German) and the fat layer was removed completely and milk was filtered through filter paper (Whatman No. 4, UK). 10 mL of the filtered milk sample was passed through aflatoxin M_1 immuno-affinity column (AflaStar™ M1 R IAC column, Romer Labs, Austria) at steady flow rate of 2 to 3 mL/min. The column was washed with 10 mL distilled water (Milli-Q Integral 15 water purification system, France) two times. The column was dried by means of the syringe plunger and the toxins were eluted by 1 mL acetonitrile in two portions of 500 µL into test tubes. The extract was evaporated with nitrogen concentrator at 50°C to dryness gently with stream of nitrogen. The residues were reconstituted with 500 µL of mobile phase and vortexed ready for injection into HPLC system.

Method validation

Quality control

Linearity of the method was determined by running a seven-point calibration curve that was prepared from standard solutions having concentrations of 0.1, 0.5, 1, 3, 5, 8 and 10 µg/L each ten times. Peak area was plotted against concentration to give a regression equation which was used to determine aflatoxin M_1 concentrations. The calibration curve is described with the equation y = 0.4796x + 0.0044 (R^2 = 0.9992).

Recovery of aflatoxin M_1

The accuracy of the method was established based on the percentage recovery, and contaminated milk which was below the limit of detection was treated as blank sample and spiked with 1.0 µg/L aflatoxin M_1 standard solution, it was then run-in triplicate parallel with the samples. Recovery was calculated as:

$$\%Recovery = \frac{Observed\ concentration\ [\frac{ng}{mL}]}{Expected\ concentration\ [\frac{ng}{mL}]} * 100\%$$

Recovery in spiked sample was greater than 89% (89.8, 89.4 and 90.2%) with the average being 89.8% indicating the suitability and good performance of the HPLC.

Determination of the limit of detection and limit of quantitation of the HPLC method

The LOD and LOQ were established by analyzing successive lowest dilutions (0.1 µg/L) of the standard solution in the matrix. These LOD and LOQ values were related to the signal to noise ratio considering concentration that generated at 3 and 10 times, respectively of the lowest calibration point. The limits of detection (LOD) and quantification (LOQ) were 0.01 and 0.031 µg/L, respectively. Precision of the method was determined by running the lowest standard of 0.1 ng/mL ten times for three days and precision was determined by calculating their relative standard deviation. The measurement uncertainty, expressed as relative standard deviation (RSD) was 1.35%.

Statistical analysis

Data analysis was done with R Software (version 4.0.3, 2020), as shown in the equation:

$$Y_{ij} = \mu + \beta_i + \tau_j + \boldsymbol{\mathcal{E}_{ij}}$$

where Y_{ij} is the response (aflatoxin concentration) corresponding to the jth treatment (processing technique) in the ith zones, µ is the overall mean, τj is the jth treatment effect, and βi is the ith zones effect.

Skillings-Mack's test (Chatfield and Mander, 2009) by using 'Skillings.Mack' package in R was used for testing the significance variation interaction between process (UHT and pasteurized). Kruskal-Wallis test was used for testing effect of each treatment (sample type) while its pairwise comparisons was done by using Wilcoxon rank sum test with continuity correction. The $p < 0.05$ was considered significant. All data were summarized as mean and expressed in tables ±SE of the mean.

RESULTS AND DISCUSSION

Aflatoxin M_1 contamination in pasteurized and UHT milk

Among the 118 UHT and pasteurized milk samples analyzed in this study, 97.5% (115/118) samples were contaminated with AFM_1. This is similar to the study reported by Daou et al., (2020) in Lebanon that indicated 90.9% aflatoxin M_1 contamination in UHT and pasteurized milk. The results of the present study were higher than the study conducted by Nejad et al. (2019) in

Table 1. Aflatoxin M_1 contamination in pasteurized and UHT milk marketed in Dar es Salaam.

Milk type	Sample(N)	Contaminated, sample n(%)	Mean±SEM (µg/L)	Range (µg/L)
UHT	43	43 (100)	0.07±0.008[b]	<LOD-0.454
Pasteurized	75	72 (96)	0.144±0.015[a]	0.01-0.1

N is the total number of samples analyzed for each type of milk. n is the total number of contaminated samples for each type of milk. Mean with different superscripts are significant different at $p<0.05$.

Table 2. Incidence of aflatoxin M_1 contamination in pasteurized and UHT milk exceeding EU and Codex regulatory limits

Milk type	Sample (N)	Contaminated Sample (n)%	Exceed EU limits [n (%)]	Exceed codex limits [n (%)]	Range (µg/L)
Pasteurized	75	72 (96)	67 (93)	0 (0)	0.05-0.454
UHT	43	43 (100)	27 (63)	0 (0)	0.05-0.115
Total	118	115 (97)	94 (81.7)	-	0.05-0.454

Contaminated samples are all analyzed samples with value > limit of detection (LOD). N is the total number of analyzed samples for each type of milk. n is the total number of contaminated samples for each type of milk.

Hamadan province of Iran who reported that 86.3% of pasteurized and UHT milk were contaminated with aflatoxin M_1. However, this study was contrary to results of the study conducted in Casablanca, El Jadida, Fez and Meknès cities in Morocco, which reported that 9 (13.4%) of pasteurized and UHT milk samples were contaminated with aflatoxin M_1 (Mannani et al., 2021). The discrepancy in AFM_1 levels might be due to differences in climatic conditions, hygiene, and precautions to prevent AFM_1 contamination of lactating cow feedstuff and dairy processing. The overall prevalence of aflatoxin M_1 contamination obtained in the present study was high which indicates the risk of chronic exposure to consumers. The high AFM1 concentrations might be due to poor storage of animal feeds and poor feeding practices observed, which resulted into aflatoxin B_1 contamination in feeds and eventually metabolized into aflatoxin M_1 in milk. A study carried out by Mohammed et al. (2016) in Singida region, Tanzania reported that aflatoxin M_1 was detected in raw milk from household cows fed with contaminated aflatoxin B_1 sunflower seedcakes.

Furthermore, this study (Table 1) showed that 96% (72/75) pasteurized milk samples analyzed, were found to be contaminated with AFM_1. A similar observation was made in a study conducted in Beijing and Shanghai in China where 96.2% pasteurized milk samples were contaminated with AFM_1 (Zheng et al., 2013). In the current study, all 100% (43/43) of UHT milk samples were contaminated with AFM_1. This was similar to the study conducted in Pakistan whereby all UHT milk samples 105 (100%) were contaminated by aflatoxin M_1 (Tahira et al., 2019). These results also confirmed the heat stable nature of aflatoxin M_1.

The highest mean for AFM_1 was in pasteurized milk with a significant difference between the means at $p<0.05$. The obtained mean value of AFM_1 contamination in pasteurized and UHT milk samples was 0.144±0.015 and 0.07±0.008 µg/L, respectively, while concentration range of pasteurized and UHT milk was <LOD - 0.454 and 0.01-0.1 µg/L, respectively, shown in Table 1. This was similar to the studies reported by Lindahl et al. (2018) in Nairobi, Kenya and Xiong et al. (2018) in Henan, Hubei and Hunan provinces in China whose results indicated low mean concentration of AFM_1 in UHT milk and high mean concentration of AFM_1 in pasteurized milk. These observations might be due to the fact that, UHT milk is subjected to high temperature (above 135°C) treatments to kill harmful microbes and to increase the shelf life of milk. The UHT heat treatment may reduce AFM_1 concentration. This is supported by a study conducted by Omeiza et al. (2018) in Nigeria reported that high temperature treatments reduce AFM_1 up to 58.8% but could not be removed completely.

Ninety three percent (93%) of AFM_1 contaminated pasteurized milk sample in this study were found to exceed the EU regulatory limits (0.05 µg/L) and 63% of UHT contaminated milk sample were found to exceed the EU regulatory limits (0.05 µg/L) (Table 2). However, none of the contaminated samples of pasteurized and UHT milk were above the maximum Codex limit (0.5 µg/L) for AFM_1.

The results obtained in this study indicated that mean values for aflatoxin M_1 contamination for pasteurized and UHT milk samples from hot humid coastal zone (Dar es Salaam, Tanga, Zanzibar and Morogoro) and temperate

Table 3. Mean concentration of contaminated UHT and pasteurized milk samples marketed in Dar es salaam from various climatic zones.

Climatic zone	Milk type	Sample (N)	Mean±SEM (µg/L)
Hot humid coastal	Pasteurized	57	0.15±0.019[a]
Hot humid coastal	UHT	30	0.08±0.011[b]
Temperate highland	Pasteurized	18	0.11±0.009[ab]
Temperate highland	UHT	13	0.05±0.005[b]

Means across the column with different statistical letters indicates statistical different at 5% significant level according to Wilcoxon rank sum test with continuity correction. N is the total number of samples analyzed for each zone.

highland zone (Kilimanjaro and Iringa) ranged from 0.05±0.005 to 0.15±0.019 µg/L. The highest aflatoxin M1 mean value was in the hot humid coastal zone, while temperate highland zone had the lowest contaminated sample. In all samples from climatic zones, pasteurized milk samples had statistically higher mean values ($p<0.05$) of aflatoxin M1 than UHT milk samples (Table 3). Higher AFM_1 concentration from hot humid coastal zones might be due to the fact that hot humid zones are characterized by high temperature and humidity which are favorable environmental conditions for fungal growth in animal feeds and production of aflatoxin B_1 which in turn are responsible for high levels of AFM_1 in milk. This is supported by the study done by Khaneghahi et al. (2019) from Iran who reported that milk samples obtained from hot humid climate areas were significantly higher in AFM_1 content. Hot humid climates are more favorable for the growth of aflatoxigenic fungi (*A. flavus* and *A. parasiticus*) and aflatoxin production than temperate climate (Benkerroum, 2020).

Conclusion

In the current study, high AFM_1 levels were found in both UHT and pasteurized milk samples collected from supermarkets and dairy shops in Dar es Salaam city. Aflatoxicosis is still one of the main public health concerns in Tanzania that lead to health hazard in all population particularly children. There is need to reduce AFM_1 transmission in milk by controlling aflatoxin B_1 contamination in animal feed and feed ingredients by adopting Good Agriculture Practices (GAP) at farm level as well as improved storage conditions. It is important that farmers and other stakeholders of the dairy industry be educated on the potential harmful effects of AFM_1 on human health.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors appreciate the financial support from Tanzania Bureau of Standards and are grateful to Mr. Roman Fortunatus of Tanzania Bureau of Standards (TBS) for technical assistance during samples extraction and analysis of AFM_1.

REFERENCES

Behfar A, Khorasgani ZN, Alemzadeh Z, Goudarzi M, Ebrahimi R, Tarhani N (2012). Determination of Aflatoxin M_1 levels in produced pasteurized milk in Ahvaz City by using HPLC. Jundishapur Journal of Natural Pharmaceutical Products 7(2):80-84.

Benkerroum N (2020). Aflatoxins. Producing-molds, structure, health issues and incidence in Southeast Asian and Sub-Saharan African countries. International Journal of Environmental Research and Public Health 17(4):1-40.

Chatfield M, Mander A (2009). The Skillings–Mack test (Friedman test when there are missing data). The Stata Journal 9(2):299-305.

Codex Alimentarius (2004). Code on Hygienic Practice for Milk and Milk Products. Standard CAC-RCP57-2004. [http://www.fao.org/fao-who-codexalimentarius/sh-proxy/pt/] site visited on 07/06/2021.

Daou R, Afif C, Joubrane K, Khabbaz LR, Maroun R, Ismail A, El Khoury A (2020). Occurrence of aflatoxin M_1 in raw, pasteurized, UHT cows' milk, and dairy products in Lebanon. Food Control 111:1-543.

FAOSTAT (2020). Raw and heat-treated milk production in Tanzania 2018. [http://www.fao.org/faostat/en/#data/QP] site visited on 07/06/2021.

Food and Agriculture Organization (FAO) (1982). Joint FAO/WHO Expert Committee on the code of principles concerning milk and milk products [meeting held in Rome Italy from 26 to 30 April 1982]. twentieth report. World Health Organization.

Food and Agriculture Organization (FAO) (2020). Tanzania at a glance. Food and Agriculture Organization of the United Nations,[http://www.fao.org/tanzania/fao-in-tanzania/tanzania-at-a-glance/en/] site visited on 22/9/2020.

Food and Agriculture Organization (FAO) (2021). Gateway to dairy production and products - Milk processing. [http://www.fao.org/dairy - production-products/processing/en/] site visited on 21/5/2021.

International Agency for Research on Cancer (IARC) (2018). Monograph 100F Aflatoxins.[https://monographs.iarc.fr/wpcontent/uploads/2018/06/mono100F-23.pdf] site visited on 15/7/2020.

Ismail A, Gonçalves BL, de Neeff DV, Ponzilacqua B, Coppa CF, Hintzsche H, Oliveira CA (2018). Aflatoxin in foodstuffs. Occurrence and recent advances in decontamination. Food Research

International 113:74-85.
Khaneghahi AH, Bahonar A, Noori N, Yazdanpanah H, Shojaee Aliabadi MH (2019). Aflatoxin M_1 in raw, pasteurized and UHT milk marketed in Iran. Food additives and contaminants Part B 12(4):236-244.
Langat G, Tetsuhiro M, Gonoi T, Matiru V, Bii C (2016). Aflatoxin M_1 contamination of milk and its products in Bomet County, Kenya. Advances in Microbiology 6(07):528-536.
Lindahl JF, Kagera IN, Grace D (2018). Aflatoxin M_1 levels in different marketed milk products in Nairobi, Kenya. Mycotoxin Research 34(4):289-295.
Mahmoodi M, Mazaheri M, Talebi Mehrdar M (2019). Determination of Aflatoxin M_1 in pasteurized liquid and powdered milk products imported to Iran. Iranian Journal of Toxicology 13(2):19-23.
Mannani N, Tabarani A, El Adlouni C, Zinedine A (2021). Aflatoxin M_1 in pasteurized and UHT milk marked in Morocco. Food Control 124:1-12.
Melini F, Melini V, Luziatelli F, Ruzzi M (2017). Raw and heat-treated milk. From public health risks to nutritional quality. Beverages 3(4):1-33.
Mohammed S, Munissi JJ, Nyandoro SS (2016). Aflatoxin M_1 in raw milk and aflatoxin B_1 in feed from household cows in Singida, Tanzania. Food Additives and Contaminants Part B 9(2):85-90.
Munyaneza C, Kurwijila LR, Mdoe NS, Baltenweck I, Twine EE (2019). Identification of appropriate indicators for assessing sustainability of small-holder milk production systems in Tanzania. Sustainable Production and Consumption 19:141-160.
Nejad ASM, Heshmati A, Ghiasvand T (2019). The occurrence and risk assessment of exposure to aflatoxin M_1 in ultra-high temperature and pasteurized milk in Hamadan province of Iran. Osong Public Health and Research Perspectives 10(4):228-233.
Omeiza GK, Mwanza M, Enem SI, Godwin E, Adeiza MA, Okoli C (2018). Reducing efficiencies of the commonly used heat treatment methods and fermentation processes on aflatoxin M_1 in naturally contaminated fresh cow milk. Open Journal of Veterinary Medicine 8(8):134-145.
Tahira I, Sultana N, Munir A, Hasan SM, Hanif NQ (2019). Occurrence of Aflatoxin M_1 in raw and processed milk consumed in Pakistan. Pakistan Journal of Pharmaceutical Sciences 32(3):1097-1101.
United Republic of Tanzania (URT) (2017). Tanzania livestock master plan (2017/2018 – 2021/2022). Ministry of Livestock and Fisheries.
Urio E, Juma A, Mwanyika S, Mlingi N, Ndunguru G, Ndossi G (2006). The occurrence of aflatoxin M_1 in fresh milk retailed in Dar es Salaam, Tanzania In. Mycotoxins and phytotoxins. (Edited by Njapau H, Trujillo S, van Egmond HP and Park DL), Advances in determination, toxicology and exposure management. Wageningen Academic Publishers, Netherlands pp. 202-207.
World Health Organization (WHO (2018). Mycotoxins. World Health Organization.[https://www.who.int/news-room/fact-sheets/detail/mycotoxins] site visited on 6/6/2020.
Xiong J, Xiong L, Zhou H, Liu Y, Wu L (2018). Occurrence of aflatoxin B_1 in dairy cow feedstuff and aflatoxin M_1 in UHT and pasteurized milk in central China. Food Control 92:386-390.
Zakaria AM, Amin YA, Khalil OSF, Abdelhiee EY, Elkamshishi MM (2019). Rapid detection of aflatoxin M_1 residues in market milk in Aswan Province, Egypt and effect of probiotics on its residues concentration. Journal of Advanced Veterinary and Animal Research 6(2):197-201197.
Zheng N, Sun P, Wang JQ, Zhen YP, Han RW, Xu XM (2013). Occurrence of aflatoxin M_1 in UHT milk and pasteurized milk in China market. Food Control 29(1):198-201.

Vol. 15(9), pp. 467-474, September 2021
DOI: 10.5897/AJMR2021.9566
Article Number: 04985AF67710
ISSN: 1996-0808

http://www.academicjournals.org/AJMR

ACADEMIC JOURNALS
expand your knowledge

African Journal of Microbiology Research

Full Length Research Paper

Hazard assessment and resistance profile of *Escherichia coli* strains isolated from bovine carcasses at the main slaughterhouses of Dakar, Senegal

Luc Loubamba[1]*, Bellancille Musabyemariya[1], Rachel Moyen[2], Alpha Amadou Diallo[3], Khalifa S. Babacar Sylla[4], Pouwedéou André Bedekelabou[1] and Rianatou Bada Alambédji[1]

[1]Ecole Inter Etats des Sciences et Médecine Vétérinaires, Dakar, Sénégal.
[2]Faculté des Sciences et Techniques, Université Marien NGOUABI, B.P. 69 Brazzaville, Republic of Congo.
[3]Laboratoire National de l'Elevage et de Recherches Vétérinaires (LNERV), Dakar, Senegal.
[4]Université du Sine-Saloum El-Hâdj Ibrahima NIASS, Kaolack, Senegal.

Received 16 July, 2021; Accepted 23 August, 2021

Foodborne bacterial hazards and antibiotics resistance are a public health threat. This study aimed at assessing the slaughter conditions, the bacteriological quality of bovine carcasses and at determining the antimicrobial resistance of *Escherichia coli* strains isolated from bovine carcasses. A questionnaire was administered to 41 stakeholders on the slaughter line. Swabbed carcasses were analyzed according to the French standard NF ISO 17604. *E. coli* were plated on Tryptone Bile X Glucuronide and their sensitivity to 15 antibiotics was tested by the disk diffusion method. The results indicate deficiencies in slaughter practices, control of critical operations such as evisceration and also at the level of the training of stakeholders. The prevalence of contaminated carcasses was 99% with an average of 3.03 log10 CFU/cm^2. Susceptibility testing showed *E. coli* to be resistant to tetracycline (32%), colistin (26%), cefepim (12%), ceftazidime (9%) and ciprofloxacin (5%). However, all *E. coli* strains were susceptible to cefotaxime, imipenem and norfloxacin. It is concluded that bovine carcasses from Dakar slaughterhouses represent a potential risk to public health due to the occurence of *E. coli* that are possible indicators of enteropathogenic agents. It also suggests the presence of *E. coli* resistant to critical betalactamins such as third and fourth generation cephalosporins.

Key words: Antibiotic resistance, indicator bacteria, cattle, slaughter hygiene, risk factors.

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) is a serious public health concern and serotype O157:H7 is one of the dangerous strains of *E. coli* that can be fatal, causing severe illness, permanent kidney and brain damage and even death in humans (Adingra et al., 2011). Cattle are known to be the main reservoir of STEC strains and the cattle bovine carrying the strain do not often show signs of clinical disease (Naylor et al., 2005).

*Corresponding author. E-mail: loubluc@yahoo.fr. Tel: (221) 33 865 10 08. Fax:(221) 33 825 42 83.

Epidemiological and analytical studies conducted on these pathogenic *E. coli,* in particular serotype O157:H7, have shown that human infection occurs mainly through consumption of contaminated food or water, direct contact with contaminated animal products, human carriers, or contaminated objects (Bruyand et al., 2018). Studies such as Bibbal et al. (2015) noted that the prevalence of potentially pathogenic *E. coli* varies between farms, and is estimated to be 4.5% in young dairy cattle, 2.4% in young beef cattle, 1.8% in dairy cows and 1% in beef cows.

While it is true that macroscopic inspection in slaughterhouses remains the standard means of public health protection, the fact remains that this method has its limitation in terms of detecting carcasses carrying bacterial agents responsible for foodborne diseases. In the meat value chain, previous studies have shown that carcasses from fecal contaminated cattles had a higher contamination load than those from clean cattle (Barco et al., 2015). Indeed, when slaughter hygiene is poor, dressing and evisceration operations are regarded to be stages offering multiple possibilities of contamination (Savoye, 2011). In slaughterhouses, the presence of *E. coli* is a good indicator to assess enteric contamination along the slaughter process (Ghafir et al., 2008).

Alongside the food poisoning risk posed by *E. coli* in the beef value chain, these last years have seen the emergence of extended spectrum β-lactamase (ESBLs) producing *E. coli* isolates, regarded as one of the most serious threats for animal and human health worldwide (Sihem et al., 2015). In addition, resistance to the most widely used antibacterials for the treatment of *E. coli* urinary tract infections, fluoroquinolones (FQLs) and some β lactams (3rd and 4th generation cephalosporins), have become a public health concern (Um, 2016). Given the importance of beef consumption in the Dakar region, the monitoring of the hygiene of slaughtering processes through the indicator of fecal contamination (*E. coli*) is necessary for food safety. Furthermore, monitoring of antibacterial resistance at the human-animal interface in relation with the "one health" approach provides useful knowledge at the national level to reduce the emergence and transmission of antibiotic resistant bacteria. The study aimed at assessing the slaughter conditions to evaluate the bacteriological quality of bovine carcasses and to determine the antimicrobial resistance of *E. coli* strains isolated from these carcasses in Dakar slaughterhouses.

The results will stand as value to the staff of the Dakar slaughterhouse, local research structures (EISMV, ISRA) Ministry of Livestock and the whole scientific community throughout the world.

MATERIALS AND METHODS

Survey and data collection

A survey on slaughter hygiene was carried out at the main modern slaughterhouse of Dakar, Senegal, which is supplied with a preparation line consisting of suspension rails, fixed or movable plateforms and elevated or lowered plateforms. It has a slaughter capacity of about 300 cattle heads per day. For this survey, observations were made on the conditions and method of slaughter, in particular the execution of the various technological operations from the holding pens of the live animals until to the obtaining of the carcasse. Then, a questionnaire was administered in the form of individual interviews. The questionnaire collected information on slaughtering practices such as the cleaning hands during the slaughter process and the knowledge and perception towards contamination factors. The target group for this survey was the staff working in the slaughter line which has an impact on the safety of prepared animals. Thus, 41 workers were selected by a simple random method and with their prior consent.

Escherichia coli sample collection

To ensure the consistency of the observed parameters over time, *E. coli* sampling was taken over a 3-month period (July to September, 2020). Samples were taken once a week, changing the collection day to be able to cover all days of the week. On each collection day, ten (10) bovine half carcasses were randomly sampled prior to chilling. The carcasses numbered 26th, 51st, 76th, 101st, 126th, 151st and so on were selected. As a sampling method, the study resorted to the non-destructive method using swabs in accordance with the provisions of French Standard NF ISO 17604: "Microbiology of foodstuffs: taking samples from carcasses for microbiological analysis". Four samples were collected per carcass, constituting a single sample as indicated by Regulation (EC) No 2073/2005. The sampling sites on the carcasses are those indicated by French Standard NF ISO 17604. Thus, four (4) anatomical sites (shoulder, flank, thigh and rump) were swabbed according to the locations indicated in Figure 1. After collecting, the samples were transported in a cooler boxes (between 0 and 5°C) and immediately analysed upon arrival at the food microbiology laboratory of the EISMV (Ecole Inter-Etats des Sciences et Médecine Vétérinaires) of Dakar/Senegal.

Analysis

The enumeration of beta-glucuronidase-positive *E. coli* was done according to ISO 16649-2: "Horizontal method for the enumeration of beta-glucuronidase-positive *E. coli*". Successive decimal dilutions (10^{-1}, 10^{-2}, and 10^{-3}) were plated on TBX agar. After 24 hours of incubation, all characteristic colonies were counted and the results were expressed in colony forming units per cm^2 (cfu/cm²) and then in $\log_{10}$ cfu/cm². Two to three characteristic *E. coli* colonies were collected per Petri dish and placed in an Eppendhorf tube containing 500µL of Luria Bertani (LB) broth (Invitrogen, Paisley, Scotland). The tubes were incubated to allow the *E. coli* to grow overnight at 37°C and transported in a cooler (between 0 and 5°C) to LNERV (Laboratoire National d'Elevage et Recherches Vétérinaires) for storage at -80°C in 20% glycerol until the time of antibiotic susceptibility testing.

Antibiogram

After thawing the strains stored at -80°C, they were incubated overnight at 37°C on regular agar to obtain fresh colonies. The Mueller-Hinton agar medium (MH) diffusion method was used according to the standards and recommendations of the antibiogram committee of the French Society of Veterinary Specialty Microbiology (CA-SFM-vet, 2019). Fifteen antibiotics (Bio-Rad) from six different families were individually added to the MH agar plates and selected on the basis of a survey of antimicrobials, of which

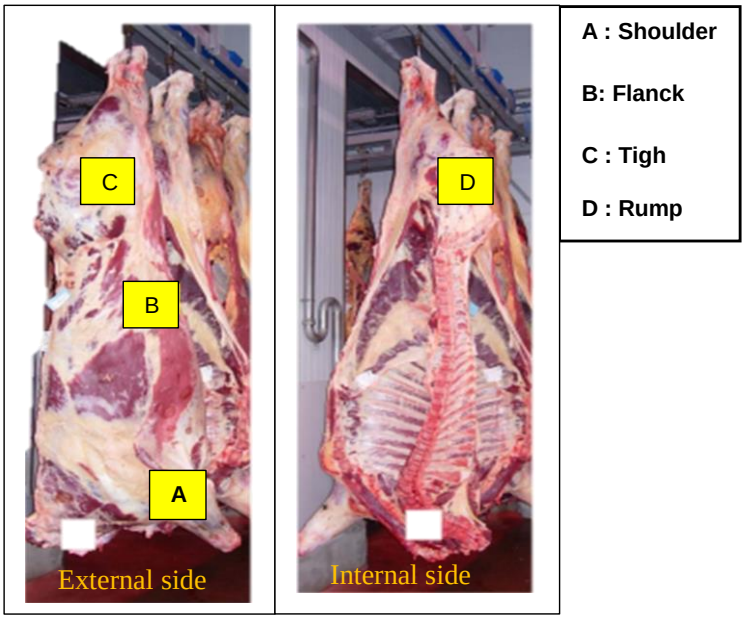


Figure 1. Carcass collection sites for *E. coli*.

some are most widely used in veterinary and human medicine in Senegal. These include: (1) Betalactamins: Ampicillin (AMP, 10 µg), Cefotaxime (CTX, 30 µg), Cefoxitin (FOX, 30 µg), Cefepime (CEF, 30 µg), Amoxicillin+clavulanic acid (AMC, 20/10 µg), Ceftazidime (CAZ, 30 µg), Cephalotin (CEF, 30 µg); (2) Carbapenems: Imipenem (IPM, 10 µg); (3) Aminosides: Gentamicin (GEN, 10 µg), Kanamycin (KAN, 30 µg); (4) Polypeptides: Colistin (COL, 50 µg); (5) Tetrayclines: Tetracycline (TET, 30 µg); (6) Quinolones and fluoroquinolones: Nalidixic acid (NAL, 30 µg) and Norfloxacin (NOR, 10 µg), Ciprofloxacin (CIP, 5 µg). The reading and measurement of the zone of inhibition diameters were done after 24 h of incubation at 37°C. Interpretation of the results was done according to the recommendations of CA-SFM-vet (2019) which classified the isolates as susceptible, intermediate and resistant. All intermediate isolates were regarded as resistant.

In addition, the study used the double disc synergy technique to detect the strains producing Extended Spectrum β-lactamases (ESBL). Agar diffusion is not the preferred method for colistin, but was used for the evaluation of colistin resistance in strains (Belloc et al., 2008).

Data analysis

The data were entered into Microsoft office Excel (2010 for windows) spreadsheet software for graphing and transforming the data into percentages. The data was then transferred to SPSS® version 24 for statistical analysis. In order to compare the rates of contaminated carcasses according to collection days, student (*t*) test and ANOVA were used. *P* value <0.05 was considered statistically significant.

Ethical consideration

For this study, authorization was obtained from slaughterhouses managers, while consent was sought before interviewing staff on the slaughter line.

RESULTS

Observations on slaughter conditions

Observations made on the cattle slaughter line revealed hygiene shortcomings in the holding pens, in particular due to the lack of water for cleaning of the floor after the passage of a herd. Cattle with diarrhea and hindquarter soiling were not sorted and separated. Animal mistreatment was observed, causing stress which eventually caused defecation. After bleeding by halal method, the evicted animals from the killing box were stacked on top of each other. The animals were also in contact during and after dressing of carcasses.

There are no functional hygiene stations to sterilize the unsuitable equipment, such as the sharp-tipped knives used at the evisceration station, which cause perforations of the intestines, pre-stomach and stomach. Ligation of the oesophagus and rectal colon is not systematic during evisceration. Partial automation of slaughter line leads to manual handling of carcasses to the refrigerating rooms. The difficulties in obtaining a supply of quality water at almost all workstations and in the sanitary facilities add to the negative conditions observed. Front of these shortcomings, nothing is done to apply preventive measures or adopt an attitude that would prevent a risk from occurring in the food chain.

Interview information

As for the staff, a total of 41 workers were interviewed on the knowledge, practices and perception of the cattle preparation hygiene. Nearly all of them (95.83%) noted

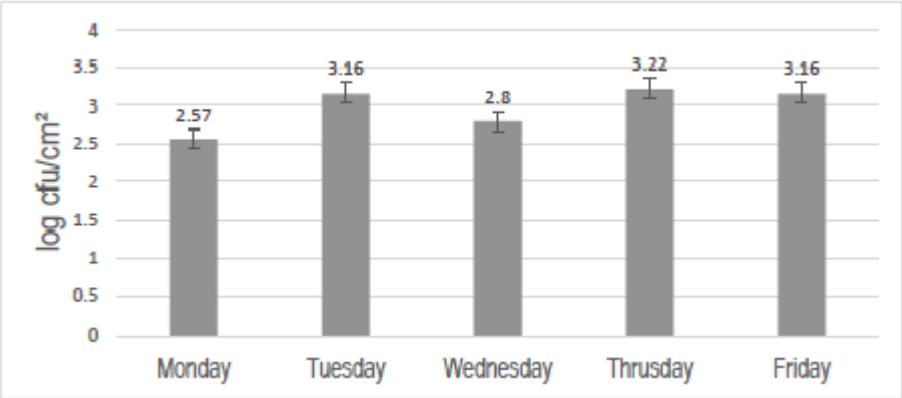


Figure 2. Average loads of bacterial flora (*E. coli*) isolated from 120 bovine carcasses according to the day of slaughter.

the importance of awareness-raising and training sessions on Good Hygiene Practices (GHP), in order to maintain the hygiene of the preparation at an acceptable level. However, most of them indicated that they did not receive any capacity building training to reinforce their knowledge, particularly regarding hygiene standards and food-related hazards. With regard to compliance with GHP, all respondents stated that the lack of hygiene in the beef meat preparation chain can be a source of contamination that can have negative consequences on the quality of the carcasses. As for the hygiene conditions of the premises, 51.2% found them acceptable. With regard to the risk of contamination, 54.2% indicated that contact with the carcasses on the slaughter line was conducive to contamination. The same is true for the absence of water at the work stations, which is considered to lead to the contamination of carcasses. The presence of water and soap in the sanitary facilities was regarded by more than 62.5% to be very important in reducing the risk of cross-contamination of carcasses. The respondents also stated that the hygiene of the premises (62.5%) and the sanitary facilities (66.7%), the cleanliness of the hands of the staff (2.5%) and of the equipment (66.7%), are factors that help to reduce the contamination of the carcasses. During the work, if the hands of the handlers were soiled, 79.2% said that they washed their hands before continuing the work. In relation to the slaughtering/dressing operations, 66.7% of the respondents indicated that the presence of leather on the carcass is a source of contamination. The same was true for 79.2% and 41.7% of the respondents who respectively confirmed that the presence of stomach contents and feces on the carcasses are sources of contamination. The automatic conveying of carcasses was considered by 20% of the respondents to be a less risky practice compared to manual conveying, that almost 40% of the staff considered this practice to have no impact on the contamination of carcasses. The others had no opinion on the matter. Animal mistreatment was considered by almost 57.14% of them having no impact on carcass quality.

Occurrence of *E. coli* in samples

Bacteriological analyses were performed on a total of 120 bovine carcasses. The prevalence of *E. coli* β-glucuronidase-positive carriage on the sampled carcasses was 99% for an overall average contamination of 3.03 log10 CFU/cm^2 +/- 0.80. The analysis of the average bacterial loads isolated per day indicated that overall carcass contamination rates were low at the beginning of the week and climbed throughout the week. (Figure 2).

The analysis revealed a statistically significant difference ($P<0.05$) between the contamination averages of Monday and the rest of the days (Tuesday, Wednesday, Thursday and Friday). However, there was no statistically significant difference ($P>0.05$) between the Thursday and Friday averages.

Antibiotic resistance profile

All 57 isolates of *E. coli* were tested for their antibiotic resistance profiles against 15 different antimicrobial agents (Figure 3). All *E. coli* isolates were non-ESBL-producers. Among non-ESBLs-producing strains, 9% (5/57) were resistant to third generation cephalosporin, in particular to ceftazidime but none were resistant to cefotaxime. The data revealed also a higher prevalence of *E. coli* strains resistant to tetracycline (32%), followed by colistin (26%), cefepime (12%), cefoxitin (12%), ampicillin, gentamicin and amoxicillin + clavulanic acid with a proportion of 11%, and kanamycin (9%). Low rates of resistance were observed with nalidixic acid (2%), cephalotin (4%) and ciprofloxacin (5%). In contrast all of them were susceptible to cefotaxime, imipenem and norfloxacin (Figure 3).

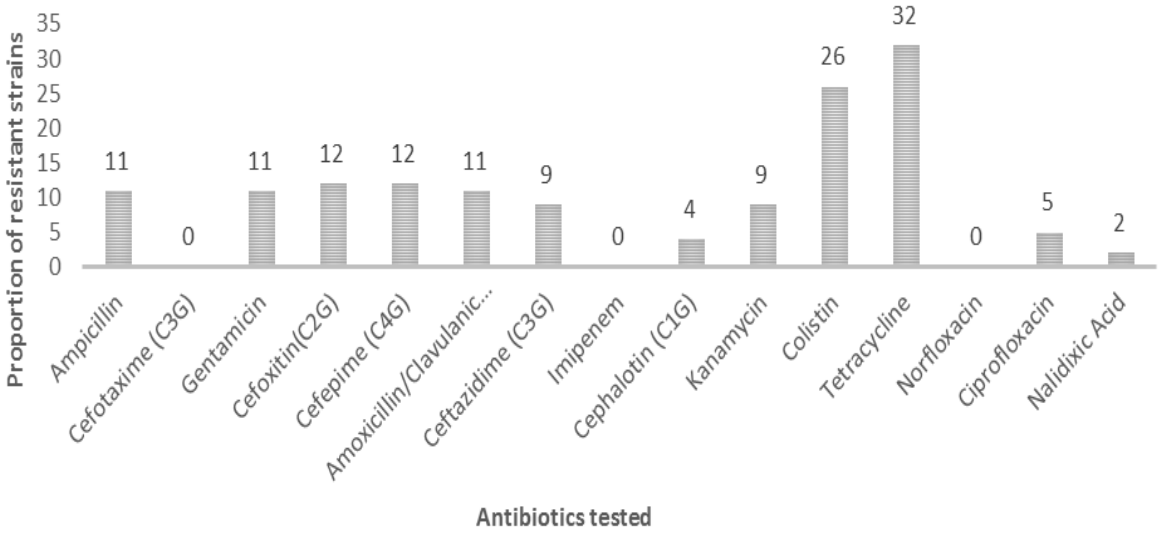


Figure 3. Variability in antibiotic resistance of *E. coli* strains isolated from bovine carcasses.

DISCUSSION

Observations on slaughter conditions

The slaughtering conditions of cattle observed at the Dakar slaughterhouses are not adequate to prevent contamination of carcasses, particularly due to poor slaughtering practices. According to the FAO/OIE (2009) guidelines on food safety, there are hygiene standards to be complied with. Indeed, these standards cover all essential aspects of production such as cleanliness and welfare of the animals, adequate facilities for hand washing, cleaning and disinfection of instruments, water supply, control of sensitive operations (dressing/ evisceration) and adequate sanitary facilities. However, in our context the conditions do not meet the pre-established standards to ensure the production of safe meat for human consumption. Similar results were also reported by Dieye (2011) and Yougbare (2014) in slaughterhouses in Dakar. Therefore, these observations suggest that the carcasses of slaughtered animals represent a risk to public health. This indicator should lead to the implementation of actions to improve the general hygiene of slaughtering conditions, in order to prevent and reduce the factors that contaminate cattle carcasses in Dakar slaughterhouses.

Interview information

The evaluation of the knowledge, attitudes, practices and perceptions of the slaughterhouse workers revealed a significant need for capacity building on GHP. Indeed, some obvious contamination situations are considered by some workers as having no impact on the bacteriological quality of carcasses. Furthermore, nothing is done by the workers to apply preventive measures or adopt an attitude that would prevent a risk from occurring in the food chain, as indicated in the results of observations section. These findings were also noted by Cadmus et al. (2008) who indicated that slaughterhouse workers in Nigeria were engaging in unhygienic practices that put meat consumers directly at risk. The low level of hygienic training may be the cause, as well as the lack of training in the hygiene of slaughter processes for butchers. In fact, there is no training plan established and executed according to a predefined periodicity to reinforce the level of knowledge of the staff, particularly with regard to hygiene standards and food-related hazards. These results are in line with those of Okoli et al. (2006), who found that most butchers lack professional training in Nigeria. In principle, staff undertaking meat hygiene activities should be trained or instructed so that they have the required knowledge and skills (FAO, 2006). Failure to do so can lead to serious consequences for public health. Hence, there is a need to implement a training plan for staff to change their slaughtering practices.

Occurrence of *E. coli* in samples

The many shortcomings observed during cattle slaughtering operations have consequences on the contamination of cattle carcasses. The present study showed that the carriage rate of cattle carcasses with *E. coli* was of 99%. This is significantly higher than the 10% reported by Phillips et al. (2001) in Australia and the 57.92% by Ahouandjinou et al. (2016) in Benin. The average contamination observed (3.03 $\log_{10}$ CFU/cm^2) in this study is also much higher than the one found by Dieye (2011) (0.13 $\log_{10}$ CFU/cm^2). This level of

contamination reflects poor hygiene practices during slaughtering operations. According to Ray (2001) and Savoye (2011), the main source of *E. coli* contamination of meat is the intestinal tract of animals. Their presence corresponds to a defect in the slaughter technique. Consequently, the *E. coli* isolated in this study probably originate from the absence of ligature of the oesophagus and rectal colon during evisceration, which favours the soiling of carcasses by feces, or by the leakage of gastric contents after accidental perforation of the gastric sacs by the operator. But *E. coli* strains can also come from meat handlers. The variability observed in the average bacterial loads isolated according to the day of slaughter has made it possible to identify two main trends: Firstly, a level of contamination that is lower at the beginning of the week and higher at the end of the week. This can be explained by the state of fitness of the workers, who resume work after the Sunday break (slaughterhouses operate 6 days a week), as well as the low throughput slaughtering at the beginning of the week. Secondly, we can mention the purchases which are more often made on weekend for most households. Despite this development, the end of the week is marked by the fatigue among workers who will have been working since the beginning of the week, but also by the increase in slaughtering at the end of the week in order to ensure the availability of meat on the weekend. The contamination of bovine carcasses on the weekend represents a significant human health threat, especially if the meat is consumed undercooked. This could lead to foodborne outbreaks and sporadic cases of benign diarrhea, but some can progress towards severe disease such as hemolitic uremic syndrome (HUS). In order to protect public health, the consumers should sufficiently cook the meat from the slaughterhouses of Dakar, and two different teams of well-trained workers can alternate for slaughter operations to avoid having only one team to do the work the whole week. This can improve the quality of carcasses throughout the week, especially on Fridays.

Antibiotic resistance profile

The results of the antibiotic susceptibility testing of *E. coli* strains showed a variability among the fifteen antibiotics tested. The *E. coli* strains were resistant to tetracyclines (32%), colistin 26%, cefoxitin and cefepime12%, ampicillin, gentamicin and amoxicillin+clavulanic acid 11% and kanamycin 9%. These percentages are significantly lower than those reported by Kohansal and Ghanbari (2018) who obtained on a total of 52 isolates of bovine origin a resistance to ampicillin of 73% and tetracycline of 65%. The present results are not similar with those of Kohansal and Ghanbari (2018) because, in their study they have tested clinical strains from sick cows, which had probabily been in contact with antibiotics. The resistance to tetracyclines can be explained by their wide range of uses in treating animal diseases due to their broad spectrum of activity. The results are also lower than those of Ahouandjinou et al. (2016) who obtained ampicillin 87.77%, cefoxitin 20.80% and amoxicillin+clavulanic acid 66.19%. This difference in the results can be explained by the fact that fewer *E. coli* strains (57) were tested than in the study by Ahouandjinou et al. (2016) who used 150 strains of *E. coli*. However, our results are higher than those of Sarr (2012) who noted very low levels of resistance to kanamycin (2.5%) and tetracycline (2.5%). This may be related to the higher number of *E. coli* strains tested compared to the study of Sarr (2012). They also do not agree with the results of Martel et al. (1983) in France who obtained very high levels of resistance exceeding 50% for all *E. coli* strains of bovine origin tested against ampicillin, kanamycin and tetracycline. In the previous study, clinical *E. coli* strains from cows and their newborn calves treated with prophylaxis were also tested. That is why, levels of resistance in this study are higher than our context. Low resistance to ceftazidime (9%) and sensitivity of all isolates to cefotaxime (0%) were noted both of which are third generation cephalosporins. Studies, such as the one by Sarr (2012), have shown that *E. coli* isolates sensitive to ceftazidime were also sensitive to cefotaxime. However, Abayneh et al. (2019) reported in their study that among the seven non-ESBL producing strains, six were resistant to ceftazidime and only one was resistant to cefotaxime. Our results seem to be closer to this second study. Therefore, the low resistance of *E. coli* strains to third generation cephalosporins could be explained by the high cost of the latter, which would limit the related prescriptions to veterinary settings or extensive farming systems. Furthermore, we did not detect ESBL producing strains in this study, which could be due to the small number of tested strains. Resistance to colistin was determined by the disk diffusion method. According to Belloc et al. (2008) this method can be used for the evaluation of colistin resistance. However, Wasyl et al. (2013) found a prevalence of 0.9% of colistin resistant strains by the reference method. This rate is much lower than the 26% observed in the present study. This may be due to the fact that we did not use the same method. All strains tested were sensitive to imipenem and norfloxacin. They were most active on *E. coli* strains. According to Mendes et al. (2009), the frequency of carbapenem resistance is low, affecting less than 2% of the strains isolated. Our results are similar to this observation. This can be explained by the fact that these are last-line molecules and therefore practically not used in human and veterinary medicine. The low rates of resistance observed with quinolones/fluoroquinolones show that despite their use in cattle farming, these molecules remain effective on *E. coli* strains. The profile of resistance to antibiotic observed in this study can help to determine the origin of the animals farming system.

Because of the low resistance rates, these animals

probabily come from extensive farming systems, where antibiotic therapy is not used as in intensive farming systems. According to Van Boeckel et al. (2015), the intensive system is marked by the widespread and unprecedented use of antimicrobials at subtherapeutic doses to stimulate growth or prevent disease. This leads to the emergence of resistant bacteria in this system. In terms of human health, data indicate on the one hand a less alarming resistance phenomenon and on the other hand some significant classes of antibiotics which have been most active, as third generation cephalosporins, carbapenem and quinolones. These latter can be used in the case of *E. coli* diseases. However, their rational use should be ensured by veterinary and human health professionnals in order to combat the spreading of antibiotic resistance.

Conclusion

The observed conditions during the preparation of the cattle and their consequences on the fecal contamination of the carcasses particulary *E. coli*, seem to be a public health concern. This study highlights the place of *E. coli* as fecal indicator in the processing of beef cattle at the main abattoir in Dakar during the warmer months. Thus, to reduce their impact on public health, slaughtering conditions should be improved by upgrading the general hygiene of cattle slaughter preparation. It is also important to identify the training needs of the staff and to define a training plan enabling each staff member to be trained in food hygiene. Furthermore, the analysis of antimicrobial profile concluded to a phenomenon of antibiotic resistance of *E. coli* isolated from beef carcasses. This finding implies that the meat prepared in Dakar slaughterhouses is likely to play the role of a vector in the dissemination of resistant bacteria to humans. It is necessary to break the chain of fecal contamination of carcasses. Then, antimicrobial surveillance plans should also be implemented at the primary production and slaughterhouse levels to better understand the risks of human exposure to resistant *E. coli* via meat of beef cattle. Finally, in research, it would be necessary to characterize the virulence genes of the isolated *E. coli* strains and to search for serogroups that are potentially dangerous for humans, such as: O103, O145, 308 O26 and O111.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Abayneh M, Tesfaw G, Woldemichael K, Moti Y, Abdissa A (2019). Assessment of extended-spectrum βlactamase (ESBLs) – producing *Escherichia coli* from minced meat of cattle and swab samples and hygienic status of meat retailer shops in Jimma town, Southwest Ethiopia. BMC Infectious Diseases 19:897.

Adingra AA, Kouadio AN, Kouassi AM (2011). Les *Escherichia coli* entérohémorragiques (EHEC) O157:H7:Un problème de santé publique. F. Tech. & Doc. Vulg. 22-27. Disponible en ligne sur http://hdl.handle.net/1834/5806

Ahouandjinou H, Gbaguidi B, Sina H, Adeoti K, Mousse W, Nepouadjeu-Wouansi S, Toukourou F, Soumanou M, Baba-Moussa L (2016). Antibiorésistance et facteurs de virulence des souches d'*Escherichia coli* isolées des carcasses bovines du bénin. European Scientific Journal 12(33):493.

Barco L, Belluco S, Roccato A, Ricci A A (2015). A systematic review of studies on *Escherichia coli* and Enterobacteriaceae on beef carcasses at the slaughterhouse. International Journal of Food Microbiology 207:30-39.

Belloc C, Lam ND, Laval A (2008). Low occurrence of colistin-resistant *Escherichia coli* in faecal content of pigs in French commercial herds. Revue de Medecine Veterinaire 159(12):634-637.

Bibbal D, Loukiadis E, Kerouredan M, Ferre F, Dilasser F, Peytavin de Garam C, Cartier P, Oswald E, Gay E, Auvray F, Brugère H (2015). Prevalence of carriage of Shiga toxin-producing *Escherichia coli* serotypes O157:H7, O26:H11, O103:H2, O111:H8, and O145:H28 among slaughtered adult cattle in France. Applied and Environmental Microbiology 81(4):1397-1405.

Bruyand M, Mariani-Kurkdjian P, Gouali M, de Valk H, King LA, Le Hello S, Bonacorsi S, Loirat C (2018). Hemolytic uremic syndrome due to Shiga toxin-producing *Escherichia coli* infection. Medicine and Infectious Diseases 48(3):167-174.

Cadmus SIB, Adesokan HK, Awosanya AEJ (2008). Public health issues and observations made during meat inspection at Bodija Municipal abattoir, Ibadan, Oyo State, Nigeria. Nigerian Veterinary Journal 29(2):4347.

Dieye A (2011). Etude de l'hygiène de la préparation des bovins aux abattoirs de Dakar. Thèse: Méd. Vét. E.I.S.M.V: Dakar 13:92.

Food and Agriculture Organization (FAO) (2006). Bonnes pratiques pour l'industrie de la viande.- Rome: FAO.- Production et santé animale.

FAO/OIE (2009). Guide des bonnes pratiques d'élevage visant à assurer la sécurité sanitaire des denrées d'origine animale.- Rome: FAO.

Ghafir Y, China B, Dierick K., De Zutter L, Daube G (2008). Hygiene indicator microorganisms for selected pathogens on beef, pork, and poultry meats in Belgium. Journal of Food Protection 71(7):35-45.

Kohansal M, Ghanbari AA (2018). Molecular analysis of Shiga toxin-producing *Escherichia coli* O157:H7 and non-O157 strains isolated from calves. Onderstepoort Journal of Veterinary Research 85(1):1-7.

Martel JL., Moulin G, Guillot JF, Libmann M (1983). Mise en évidence de *Escherichia coli* K99+ dans les fèces des vaches et de leurs veaux. Annales de Recherches Vétérinaires 14(2):121-127.

Mendes Re, Bell JM, Turnidge JD, Castanheira M, Jones RN (2009). Emergence and widespread dissemination of OXA-23, -24/40 and -58 carbapenemases among Acine-tobacter spp. in Asia-Pacific nations: report from the SENTRY Surveillance Program. Journal of Antimicrobial Chemotherapy 63(1):55-59.

Naylor SW, Gally DL, Low JC (2005). Enterohaemorrhagic *E. coli* in veterinary medicine. International journal of Medical Microbiology 295(6-7) :419-441.

Okoli CG, Okoli IC, Okorondu UV, Opara MN (2006). Environmental and public health issues of animal food products delivery system in Imo State, Nigeria. Online Journal of Health and Allied Sciences 5(2). http://www.ojhas.org/issue18/2006-2-2.htm.

Organisation Internationale de Normalisation ISO 17604: microbiologie des aliments : prélèvement d'échantillons sur des carcasses en vue de leur analyse microbiologique. Organisation internationale de Normalisation : Genève, 2003d. P 15.

Phillips D, Sumner J, Alexander JF, Dutton KM (2001). Microbiological quality of Australian beef. Journal of Food Protection 64(5):692-696.

Ray B (2001). Indicators of bacterial pathogens. In: Ray B. (Ed.), Fundamental food microbiology. CRC Press: Boca Raton pp. 409-417.

Sarr MM (2012). Prévalence des souches d'*Escherichia coli* porteuses de gènes de virulence associés aux *Escherichia coli*

entérohémorragiques (EHEC) et/ou résistantes aux antibiotiques dans les effluents de la station d'épuration de Cambérène et des abattoirs de Dakar. Thèse: Méd. Vét., Dakar 39:60.

Savoye F (2011). Optimisation du protocole de recherche des *Escherichia coli* producteurs de Shigatoxines (STEC) dans les aliments. Thèse doctorale de l'université de Bourgogne 197(54).

SFM (Société Française de Microbiologie) (2019). Recommandations 2019 du comité français de l'antibiogramme vétérinaire de la Société Française de Microbiologie, disponible sur le site: http://www.sfm-microbiologie.org/UserFiles/

Sihem M, Salah H, Riadh M, Salah AM (2015). Overview of ESBL-producing *Escherichia coli* of Animal Origin in Tunisia: In the Way of the Global Spread of CTX-M β-Lactamases. Archives of clinical microbiology 6(2:4):1-7.

Um MM (2016). *Escherichia coli* entérohémorragiques et/ou résistantes aux antibiotiques : contamination des effluents d'origine bovine. Thèse doctorale de l'université Toulouse 3 Paul Sabatier (UT3 Paul Sabatier). P 218.

Van Boeckel TP, Brower C, Gilbert M, Grenfell BT, Levin SA., Robinson TP, Teillant A, Laxminarayan R (2015). Global trends in antimicrobial use in food animals, Proceedings of the National Academy of Sciences 112(18):5649-5654.

Wasyl D, HoszowskI A, Zajac M, Szulowski K (2013). Antimicrobial resistance in commensal *Escherichia coli* isolated from animals at slaughter. Frontiers in Microbiology 4(221):12.

Yougbare B (2014). Appréciation des risques de contamination microbienne de la viande de petits ruminants dans les abattoirs et dibiteries de Dakar, Sénégal. Mémoire master en santé publique vétérinaire, spécialité: EGRS. Eismv: Dakar (14):45.

Vol. 15(9), pp. 475-481, September 2021
DOI: 10.5897/AJMR2021.9552
Article Number: 6030DF067720
ISSN: 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Candida spp. associated with hot beverages of coffee and tea sold on street in Côte d'Ivoire

Atobla Koua[1*], Blé Yatanan Casimir[2], Ouattara Mohamed Baguy[3], Touré Naka[4], Adjehi Dadié[5] and Niamké Sébastien[1]

[1]Laboratory of Biotechnologies, Department of Biosciences, Felix Houphouet-Boigny University, 22 BP 582 Abidjan 22, Côte d´Ivoire.
[2]Department of Agronomic Forest and Environmental Engineering, University of Man, BP 20 Man, Côte d'Ivoire.
[3]Department of Bacteriology and Virology, National Reference Center for Antibiotics, Pasteur Institute, Abidjan, 01 BP 490 Abidjan 01, Côte d'Ivoire.
[4]Laboratory of Biochemical and Microbiology of Biology Sciences of Korhogo, Peleforo Gon Coulibaly University, BP 1328 Korhogo, Côte d'Ivoire.
[5]Department of Food Sciences and Technologies (STA), Laboratory of Microbiology and Biotechnology, Nangui Abrogoua University, 02 BP 801 Abidjan 02, Côte d'Ivoire.

Received 13 June, 2021; Accepted 27 July, 2021

Some *Candida* species are considered as human opportunistic pathogens and can play an important role in spontaneous fermentations, but also as beverage-spoiling microorganisms. The aim of this pioneering study was to investigate *Candida* spp. in hot beverages, which is consumed mostly on the streets in Côte d'Ivoire. Yeast strains were isolated from 400 hot beverages of tea (200) and coffee (200) samples. Yeast cultures were identified at genus and species level by MALDI-TOF mass spectrometry at the biobank laboratory of the Pasteur Institute of Côte d'Ivoire. A total of 37 *Candida* isolates were clearly identified by MALDI-TOF (MS) and revealed 11 species of *Candida*: *C. krusei* (21.6%), *C. tropicalis* (18.9%), *C. parapsilosis* (16.2%), *C. guilliermondii* (16.2%), *C. pelliculosa* (8.1%), *C. dubliniensis* (5.4%), *C. rugosa* (2.7%), *C. kefyr* (2.7%), *C. silvicola* (2.7%), *C. lusitaniae* (2.7%) and *C. orthopsilosis* (2.7%). The results showed that *C. krusei* and *C. tropicalis* were the dominant yeasts in hot beverages from street vendors. *Candida* species were more isolated in tea (10%) than in coffee (8.5%). *C. tropicalis*, *C. pelliculosa* and *C. krusei* were more isolated in Cocody. *C. guilliermondii* and *C. parapsilosis* were more isolated in Port-Bouët. *C. dubliniensis* was only isolated in Yopougon town. The presence of *Candida* spp. in street hot beverages could cause a sanitary risk to consumers or be used as a novel source for biotechnological uses to be explored in future work.

Key words: Street hot beverages, *Candida* spp., coffee, tea, food safety.

INTRODUCTION

Yeasts are unicellular eukaryotes microorganisms that belong to the Kingdom of Fungi and play various roles in affecting the quality and safety of food products (Khattab et al., 2016). They are ubiquitous, and commonly spoilage fruits, vegetables and other plant materials, in addition to, an association with soil and insects (Bekatorou et al.,

*Corresponding author. E-mail: atobla@yahoo.fr.

2006). Yeasts are the major producer of biotechnology products worldwide, exceeding production in capacity and economic incomes than other groups of industrial microorganisms. Yeasts have wide ranging fundamental and industrial importance in scientific, food, medical and agricultural sciences (Johnson, 2013). Traditionally, yeasts have been used for several fermentations such as alcoholic beverages, biomass production and other fermented food. Fermented foods and beverages play an important role in the diet of African people, and the most often, these foods and beverages are produced at household level or at small industrial scale and are consequently often of varying quality and stability (Mogmenga et al., 2017; Tankoano et al., 2017).

Fruit juices and soft drinks constitute suitable environment for growth of most microorganisms. Actually, beverages are excellent substrates for supporting the growth of yeasts, where the highest amount of nitrogenous compounds and vitamins promote occurrence of yeasts (Khattab et al., 2016).

The genus *Candida* includes around 154 species. Among these, six are most frequently isolated in human infections. While *Candida albicans* is the most abundant and significant species, *Candida tropicalis, Candida glabrata, Candida parapsilosis, Candida krusei,* and *Candida lusitaniae* are also isolated as causative agents of *Candida* infections (Aggarwal et al., 2018). Non-*Saccharomyces* yeasts could be used as biocontrol agents against moulds and in the treatment of wastewaters contaminated by heavy metals (Ubeda et al., 2014). Hence, a comprehensive understanding, linking intrinsic and extrinsic factors to microbial diversity and successions is of outmost importance for upgrading indigenous sub-Saharan African fermented food and beverages (Johansen et al. (2019). The suspicion or suggestion that beverage-spoiling *Candida* species can be (opportunistic) pathogens is not well-documented in microbiological tests performed on beverages and as a consequence is not given much attention in the relevant literature (Hutzler et al., 2012).

The aim of this work was to investigate *Candida* spp. of street hot beverages of tea, coffee and to perform phenotypic characterization of the isolated yeasts in order to contribute to the body of knowledge of yeasts in beverage food, and to add new information for a previously unexplored geographical area.

MATERIALS AND METHODS

Sampling procedure

A total of 400 hot beverages of coffee (200) and tea (200) samples were collected from street vendors in Abidjan according to the method of Atobla et al. (2020). This study was conducted from July to December 2020.

Isolation, purification and storage of yeasts

According to the prescriptions of the standards used, one milliliter (1 mL) of beverage sample is aseptically transferred to a Petri dish. Enumeration of yeasts on Sabouraud Chloramphenicol agar (SCA, Biokar Diagnostics, France) was carried out according to the NF/ISO 16212: 2011 standard. All the Petri dishes were then incubated in an incubator at 30°C for 48 h for the enumeration of yeasts. Colonies were firstly selected based on colony morphology, aiming at selecting colonies of varying morphology, and colonies were randomly selected. The appearance of white to yellowish colonies would indicate the presence of yeasts. Colonies identified as yeasts by their macroscopic aspects and their microscopic observations in the fresh state were purified by striae on Sabouraud agar (SA, Biokar Diagnostics, France). One hundred and twenty-one (121) purified isolates were obtained and stored at -20°C in MYPG broth supplemented with glycerol 20% (v/v). Colonies were identified as yeasts by their macroscopic aspects on agar medium and their microscopic observations in the fresh state on an optical microscope at magnification 40X then at 100X.

Identification of yeasts by MALDI-TOF mass spectrometry

Yeast cultures were identified at genus and species level by MALDI-TOF MS at the biobank laboratory of the Pasteur Institute of Côte d'Ivoire. The identification of yeasts was done at the genus and species level based on mass of ribosomal proteins by the MALDI-TOF (Vitek MS BioMerieux) MS, which is a spectrometer using a matrix-assisted laser ionization source and a time-of-flight analyzer. MALDI-TOF identification was done in three steps: sample preparation followed by sample analysis and data processing (Lo et al., 2017). For samples preparation, yeasts isolated from street hot beverages were cultured in Sabouraud agar for 48 h at 30°C. Afterwards, a colony of the calibrating strain of *Escherichia coli* ATCC 8739 (positive control), was put on the MALDI-TOF plate with 1 µL of CHCA matrix (α-cyano-4-hydroxycinnamic acid, BioMerieux SA, ref 411071). Then, using a sterile oese calibrated at 1 µL, a colony of yeast to be tested was collected and put on the target wells. The sample was tested in duplicate. On both deposits, 0.5 µL of 25% formic acid (vitek MS-FA, BioMerieux SA, ref 411072) was added. After air drying, 1 µL of CHCA matrix was put on each spot and dried again. Subsequently, the deposition plate was introduced into the Vitek MS for sample analysis after transferring the data from the Prep Station, which is a module consisting of a computer and an optical scanner used to introduce the sample data and their location on the slide to the Vitek MS. The interpretation of the results involved two important parameters: the percentage confidence degree and the confidence level displayed by different colours.

Data analysis

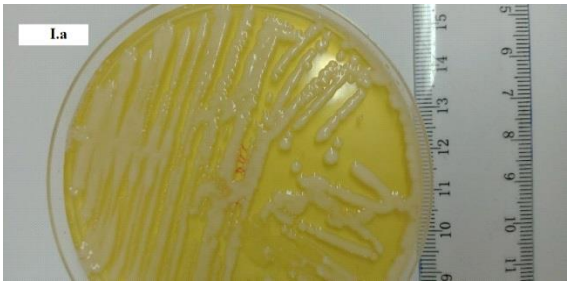
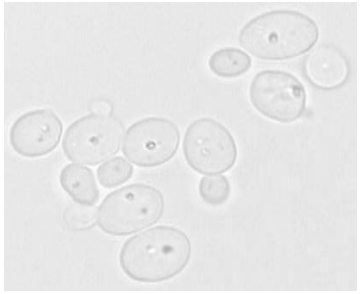
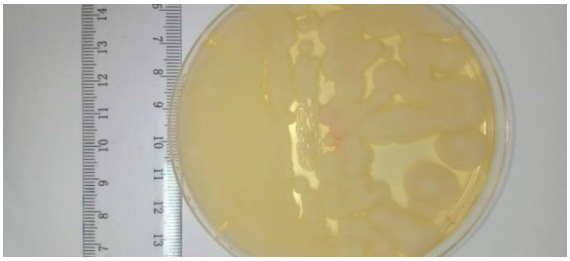
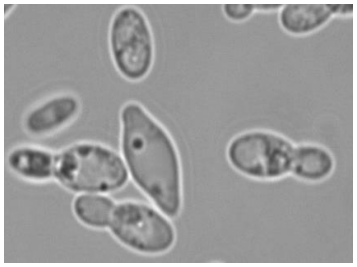
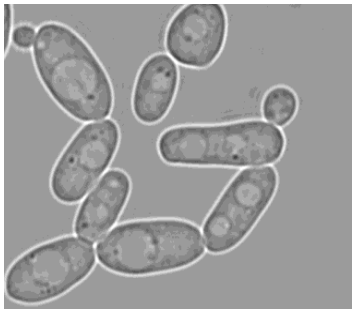
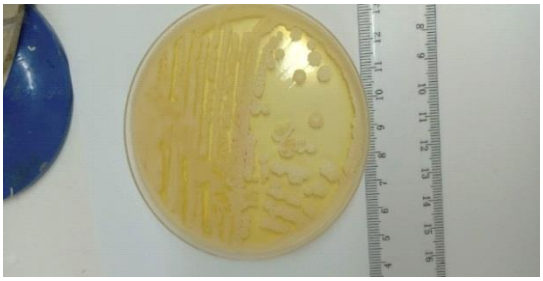
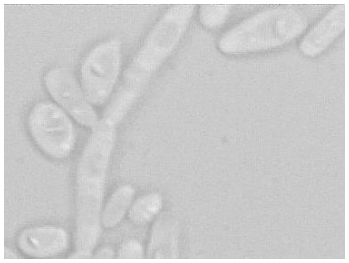
The data were exported to Microsoft Excel to calculate the various scores. Descriptive statistics were used to summarise the variables of interest and determine relationships between them. The Chi-square test was used to test the relationships between the variables. The difference between the variables was considered significant at $p < 0.05$.

RESULTS

Morphologies of strains and cell of yeasts

Yeast strains were isolated from hot beverages of tea and coffee. The aspect of colonies on Sabouraud agar showed creamy or dry, smooth or rough colonies. Microscopic morphology of the fresh isolates revealed

Table 1. Morphologies of strains and yeast cell of isolates.

Group (%)	**Aspect of colonies on Sabouraud agar**	**Microscopic morphology of yeast cell**
I (25.5%)	 Creamy white colonies, bright and smooth	 spherical and budding cell
II (38.1%)	 Creamy white colonies smooth and dull surface of regular contour	 Ovoid and budding cell
III (10.7%)	 Creamy white colonies, rough surface, regular contour	 Ovoid elongated and budding cell
IV (2.5%)	 Dry white colonies, with a rough surface	 Ovoid elongated, pseudohypha and budding cell

Group I, II, III and IV: morphotype groups of isolated yeast strains. Magnification: M x100.

spherical, ovoid, elongated cells, single or connected in pairs or chains, and propagates by budding. Based on the macroscopic and microscopic characters, four morphotypes yeast were isolated. Creamy white colonies, smooth, dull surface and regular contour (group II) with a rate of 38.1%, which were the most prevalent, followed by the creamy white colonies, bright and smooth (group I) with the rate of 25.5% (Table 1).

Identification of *Candida* species

Candida species were identified by MALDI-TOF MS (Table 2). A total of 37 *Candida* isolates were clearly

Table 2. *Candida* species identified by MALDI-TOF MS.

Candida species	Laboratory code	Score	Rate of isolation : n (%)
C. tropicalis	I7C2, IV3T1, IV7T2, IV11T3, IV12T2, IV14T2, IV14T4	99.9	7 (18.9)
C. parapsilosis	II1T4, II5T3, V5T4, V5T5, V8C2, V8T4	99.9	6 (16.2)
C. pelliculosa	IV2T1, IV2T1, IV8T4	99.9	3 (8.1)
C. krusei	I7C1, I7C5, III3T1, IV1C2, IV10C1, IV10C2, IV13T2, V1C2	99.9	8 (21.6)
C. guilliermondii	V8C4, V9C2, V11C1, V12C2, V15C1, V15C2	99.9	6 (16.2)
C. dubliniensis	III13T2, III13T3	99.9	2 (5.4)
C. rugosa	IV5C2	88.9	1 (2.7)
C. kefyr	V13T2	99.9	1 (2.7)
C. silvicola	V9C5	99.5	1 (2.7)
C. lusitaniae	IV11C1	85.8	1 (2.7)
C. orthopsilosis	V5T1	88.2	1 (2.7)
Total			**37 (100)**

Table 3. Presence of *Candida* species in hot beverages on street.

Hot beverages	Number	Presence of *Candida* species in hot beverages: n (%)	Prevalence of *Candida* species:n (%)
Coffee	200	17 (8.5)	17 (45.9)
Tea	200	20 (10.0)	20 (54.1)
Total	400	37 (9.2)	37 (100)

identified and showed 11 species: *C. tropicalis, C. parapsilosis, C. pelliculosa, C. krusei, C. guilliermondii, C. dubliniensis, C. rugosa, C. kefyr, C. silvicola, C. lusitaniae* and *C. orthopsilosis.* The major species included *C. krusei* (21.6%), *C. tropicalis* (18.9%), *C. parapsilosis* and *C. guilliermondii* (16.2 %) followed by a low proportion of *C. pelliculosa* (8.1%), *C. dubliniensis* (5.4%), *C. rugosa* (2.7 %), *C. kefyr* (2.7 %), *C. silvicola, C. lusitaniae* and *C. orthopsilosis* (2.7%).

Distribution of *Candida* species in hot beverages

From total of 400 hot beverage samples analyzed, *Candida* species were more isolated in tea than in coffee. The rate of the presence of *Candida* species in tea was 10%, whereas in coffee it was 8.5%. The prevalence of *Candida* species in tea was 54.1% (Table 3). The Chi-square test (Chi 2 = 0.268) showed that the presence of *Candida* species in hot beverage of coffee and tea was not significantly linked to the sample analyzed (p-value = 0.605). *C. krusei* is mostly isolated in coffee than in tea, while *C. tropicalis* is more isolated in tea than in coffee. *C. guilliermondii, C. rugosa, C. silvicola* and *C. lusitaniae* were only isolated in coffee while *C. pelliculosa*, *C. dubliniensis*, *C. kefyr* and *C. orthopsilosis* were isolated only in tea (Figure 1).

Distribution of *Candida* spp. according to the location

C. tropicalis (n = 6), *C. pelliculosa* (n = 3) and *C. krusei* (n = 4) were more isolated in Cocody. *C. guilliermondii* (n = 6) and *C. parapsilosis* (n = 4) were more isolated in Port-Bouët. *C. dubliniensis* was only isolated in Yopougon town (Figure 2).

DISCUSSION

The aspect of colonies on Sabouraud agar showed creamy or dry colonies, smooth or rough. Microscopic observation of the fresh isolates revealed spherical, ovoid, elongated cells. In the study of Sipiczki (2011), *Candida citri* vegetative cells are ovoid or nearly spherical, single or connected in pairs or chains, and propagated by multipolar budding. It is important to bear in mind that while pseudohyphae may appear physically more similar to hyphae, they actually share far more properties with yeasts and might be better described as elongated, attached yeast cells (Sudbery et al., 2004; Delma et al., 2011). In addition, these results suggest that in the case of *Candida* species, morphology may have evolved in a stepwise fashion from yeast to pseudohyphae to hyphae (Bastidas and Heitman, 2009). Consistent with this hypothesis, while many *Candida* species are capable of forming yeast and pseudohyphae, only three species, which are phylogenetically closely related (*C. tropicalis*, *C. dubliniensis*, and *C. albicans*), are known to form hyphae as well (Moran et al., 2002; Delma et al., 2011). It is therefore likely that the earliest *Candida* species also possessed a very weak ability to form pseudohyphae and over time evolved to undergo this morphological transition more frequently in response

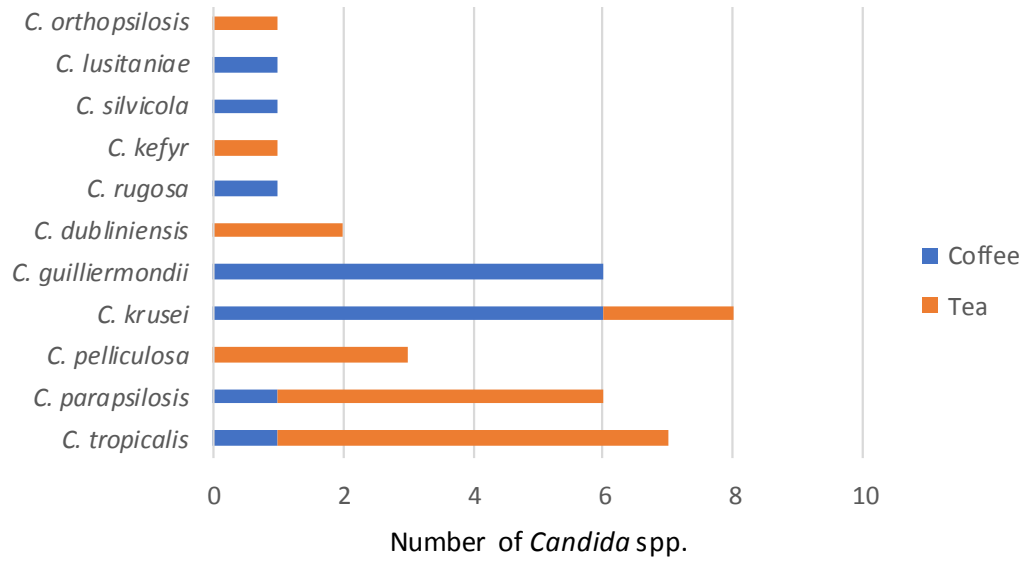


Figure 1. Distribution of *Candida* spp. in beverages of tea and coffee.

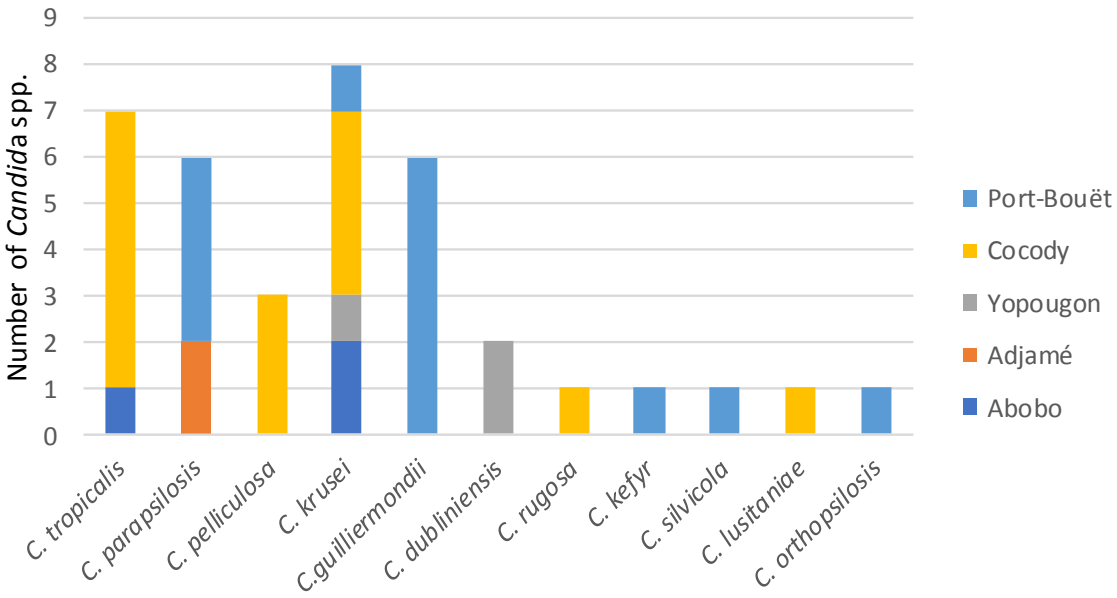


Figure 2. Distribution of *Candida* spp. according to the location.

to a broader array of host environmental conditions and niches (Delma et al., 2011). Over evolution, *Candida* species are believed to have acquired the ability to form pseudohyphae more frequently and in response to a broader range of conditions in the host environment (Delma et al., 2011). In contrast, hypha-forming *C. albicans*, *C. dubliniensis*, and *C. tropicalis* appear to have an increased ability to adhere to host cells and secrete proteases relative to other *Candida* species, which lack the ability to form hyphae (Moran et al., 2002). The stressful microbial environments in fermented food and beverages result in a high selection pressure, which can lead to development of new strains better adapted to the fermentation process. As a consequence, species occurring in spontaneously fermented food and beverages might, over time, differentiate into populations of strains of the same species (Suzzi, 2011).

Candida species were identified by MALDI-TOF in our study. *Candida* isolates were clearly identified and showed 11 species: *C. tropicalis, C. parapsilosis, C. pelliculosa, C. krusei, C. guilliermondii; C. dubliniensis; C. rugosa, C. kefyr, C. silvicola, C. lusitaniae* and *C.*

orthopsilosis. According to Bader et al. (2011), MALDI-TOF MS analysis was able to differentiate closely related species when conventional biochemical methods were not such as species of the parapsilosis complex (*C. parapsilosis, C. orthopsilosis* and C. *metapsilosis*). Furthermore, MALDI-TOF MS can detect 95.7 – 100% of common *Candida* species like *C. albicans, C. glabrata, C. dubliniensis,* and *C. tropicalis* (Bader et al., 2011; Bille et al., 2012; Iriart et al., 2012). Accuracy is lower for uncommon species like *C. inconspicua*, *C. rugosa*, and *C. norvegensis* (73.6–88.9%). However, when databases are sufficiently extensive and regularly updated, MALDI-TOF MS could detect these species, whereas the classical identification method could not (Santos et al., 2011; Posteraro et al., 2013). For Johansen et al. (2019), these techniques have so far not been used for identification of yeasts from indigenous sub-Saharan African fermented food and beverages.

Candida species were more isolated in tea (10.0%) than in coffee (8.5%). Atobla et al. (2021) also isolated more yeasts in hot beverages of tea than in coffee. It is well known that final product quality in industries such as wine-making, sausage production, cheese ripening, bakery, and the “fermentations” of cacao and coffee beans is affected directly by the development of spoilage microorganisms (Romano et al., 2006; Viljoen, 2006). *C. krusei* (21.6%) is the most isolated yeast in hot beverages of coffee and tea. Likewise, in the study of Jesperson et al. (2005), *C. krusei* is also the dominant yeast present in chocolate production and cocoa bean fermentation in West African cocoa beans. Heap fermentation in particular is most effective with *C. krusei* (Jesperson et al., 2005).

C. tropicalis with a rate of 18.9% was more isolated in tea and *C. guilliermondii* was only isolated in coffee in our study. Likewise, Johansen et al. (2019) reported that *C. tropicalis* is frequently identified in indigenous sub-Saharan African fermented food and beverages, including akyeke, amasi, attiéké, bandji, fufu, fura, gari, gowé, kaffir, lafun, mawè, mukumbi, nunu, palm wine, pito, sethemi, tchapalo and teffinjera (Coulin et al., 2006; Oyewole, 2001; Greppi et al., 2013). For the production of ‘‘fufu’’, Oyewole (2001) reported that six different yeast species, *Pichia saitoi, Pichia anomala, C. krusei, C. tropicalis, Zygosaccharomyces bailii* and *S. cerevisiae* have been identified. For Johansen et al. (2019), yeasts are most probably occurring as a result of contamination during processing due to improper human handling. As indigenous sub-Saharan African food and beverages predominantly are produced by spontaneous fermentation, the consumers may be exposed to large populations of different yeast species of often unknown origin (Ogunremi et al., 2017).

C. tropicalis, C. pelliculosa and *C. krusei* are more isolated in Cocody town. *C. pelliculosa, C. guilliermondii, C. kefyr, C. silvicola* and *C. orthopsilosis* were more isolated in Port-Bouët town. For Turner and Butler (2014), the incidence varies substantially with geographical location. *C. glabrata* is the highest in Asia-Pacific and the European Union (EU); whereas the incidence of *C. tropicalis* infection in Africa and the Middle East is approaching three times that of the European Union; on the other hand, *C. parapsilosisis* is highest in North America and Latin America (Pfaller and Diekema, 2004; Klevay et al. 2008; Alexander et al., 2013). From a local kombucha in Saudi Arabia, Ramadani and Abulreesh (2010) isolated *Candida* sp. in kombucha beverages and identified four yeasts: *C. guilliermondi, C. colleculosa, C. kefyr*, and *C. krusei*.

According to Johansen et al. (2019), the market size of indigenous sub-Saharan African fermented food and beverages are growing among others due to their ability to be used as convenient food by consumers. Additionally, fermentation is an affordable and sustainable way of processing that can be easily used to improve the quality and safety of food and beverages. Unfortunately, uncontrolled processing, handling and selling on streets may induce contamination and growth of harmful microorganisms.

Conclusion

To our knowledge, this is the first study to assess *Candida* species in hot beverages in Côte d’Ivoire. The present study has proven that a significant number of *Candida* species are involved in hot beverages on street. Consequently, further research is needed to optimize fermentation conditions to eliminate opportunistic pathogenic yeasts during processing and equally important, improved hygienic conditions need to be ensured in order to prevent cross-contamination from human handling during food processing.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

ACKNOWLEDGEMENTS

The authors appreciate the hot beverage street vendors. This work was supported by ASCAD (Académie des Sciences, des Arts, des Cultures d'Afrique et des Diasporas Africaines) funds.

REFERENCES

Aggarwal N, Bhateja S, Arora G, Yasmin T (2018). Candidiasis- The Most Common Fungal Infection of Oral Cavity. Biomedical Journal of Scientific and Technical Research 8(3):6487-6491.

Alexander BD, Johnson MD, Pfeiffer CD, Jimenez-Ortigosa C, Catania J, Booker R, Castanheira M, Messer SA, Perlin DS, Pfaller MA (2013). Increasing echinocandin resistance in *Candida glabrata*: Clinical failure correlates with presence of FKS mutations and elevated minimum inhibitory concentrations. Clinical Infectious

Diseases 56(12):1724-1732.
Atobla K, Kouadio-Ngbesso N, Oumarou TF, Dadié A, Niamké S (2020). Safety Assessment of Street Hot Beverages Made of Coffee, Tea, Milk or Cocoa Consumed in Abidjan City. American Journal of Food and Nutrition 8(2):23-31.
Atobla K, Blé YC, Kouamé ND, Benié CKD, Adjehi D, Niamké S (2021). Gender Consumption, Microbial Profile and Potential Risk of Hot Beverages of Coffee, Tea, Milk or Cocoa Sold by Street Vendors in Abidjan, Côte d'Ivoire. Food Science and Quality Management 105:32-41.
Bader O, Weig M, Taverne-Ghadwal L, Lugert R, Groß U, Kuhns M (2011). Improved clinical laboratory identification of human pathogenic yeasts by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clinical Microbiology and Infection 17(9):1359-1365.
Bastidas RJ, Heitman J (2009). Trimorphic stepping stones pave the way to fungal virulence. Proceedings of the National Academy of Sciences of the United States of America 106(2):351-352. DOI: 10.1073/pnas.0811994106.
Bekatorou A, Psarianos C, Koutinas AA (2006). Production of food grade yeasts. Food Technology and Biotechnology 44(3):407-415.
Bille E, Dauphin B, Leto J, Bougnoux, M-E, Beretti J-L, Lotz A. Suarez S, Meyer J, Join-Lambert O, Descamps P, Grall N, Mory F, Dubreuil L, Berche P, Nassif X, Ferroni A (2012). MALDI-TOF MS andromas strategy for the routine identification of bacteria, mycobacteria, yeasts, aspergillus spp. and positive blood cultures. Clinical Microbiology and Infection 18(11):1117-1125.
Coulin P, Farah Z, Assanvo J, Spillmann H, Puhan Z (2006). Characterisation of the microflora of attiéké, a fermented cassava product, during traditional small-scale preparation. International Journal of Food Microbiology 106(2):131-136.
Delma ST, Patricia LC, David K (2011). Coevolution of Morphology and Virulence in *Candida* Species. Eukaryotic Cell 10(9):1173-1182.
Greppi A, Rantisou K, Padonou W, Hounhouigan J, Jespersen L, Jakobsen M, Cocolin L (2013). Yeast dynamics during spontaneous fermentation of mawè and tchoukoutou, two traditional products from Benin. International Journal of Food Microbiology 165:200-207.
Hutzler M, Riedl R, Koob J, Jacob F (2012). Fermentation and Spoilage Yeasts and their Relevance for the Beverage Industry – A Review. Brewing Science 65(3):33-52.
Iriart X, Lavergne R.-A, Fillaux J, Valentin A, Magnaval J-F, Berry A, Cassaing S (2012). Routine identification of medical fungi by the new vitek MS matrixassisted laser desorption ionization–time of flight system with a new time effective strategy. Journal of Clinical Microbiology 50(6):2107-2110.
Jesperson L, Nielson DS, Hønholt S, Jakobsen M (2005). Occurrence and diversity of yeasts involved in fermentation of West African cocoa beans. FEMS Yeast Research 5(4-5):441-453.
Johansen PG, Owusu-Kwarteng J, Parkouda C, Padonou SW, Jespersen L (2019). Occurrence and Importance of Yeasts in Indigenous Fermented Food and Beverages Produced in Sub-Saharan Africa. Frontiers in Microbiology 10(1789):1-22.
Johnson EA (2013). Biotechnology of non-*Saccharomyces* yeasts — the basidiomycetes. Applied Microbiology and Biotechnology 97(17):7563-7577.
Khattab RMS, Abdel-Hadi MA, Abo-Dahab FN, Atta MO (2016). Isolation, Characterization, and Identification of Yeasts Associated with Foods from Assiut City, Egypt. British Microbiology Research Journal 13(1):1-10.
Klevay MJ, Ernst EJ, Hollanbaugh JL, Miller JG, Pfaller MA, Diekema DJ (2008). Therapy and outcome of *Candida glabrata* versus *Candida albicans* bloodstream infection. Diagnostic Microbiology and Infectious Disease 60(3):273-277.
Lo CI, Fall B, Sambe-Ba B, Flaudrops C, Faye N, Mediannikov O, Sokhna C, Wade B, Raoult D, Fenollar F (2017). Value of matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry in clinical microbiology and infectious diseases in Africa and tropical areas. African Journal of Microbiology Research 11(35):1360-1370.
Mogmenga I, Somda KM, Keita I, Traoré AS (2017). Evaluation of hygienic quality of ferment of local beer — dolo ǁ used as condiment in Burkina Faso. African Journal of Biotechnology 16(26):1449-1456.
Moran GP, Sullivan DJ, Coleman DC (2002). Emergence of non-*Candida albicans Candida* species as pathogens, pp. 37–54. *In* Calderone RA (ed.), *Candida* and candidiasis. ASM Press, Washinton, DC.
Ogunremi OR, Banwo K, Sanni AI (2017). Starter-culture to improve the quality of cereal-based fermented foods: trends in selection and application. Current Opinion in Food Science 13:38-43.
Oyewole OB (2001). Characteristics and significance of yeasts involvement in cassava fermentation for fufu production. International Journal of Food Microbiology 65(3):213-218.
Pfaller MA, Diekema DJ (2004). Twelve years of fluconazole in clinical practice: Global trends in species distribution and fluconazole susceptibility of bloodstream isolates of *Candida*. Clinical Microbiology and Infection 10(1):11-23.
Posteraro B, De Carolis E, Vella A, Sanguinetti M (2013). MALDI-TOF mass spectrometry in the clinical mycology laboratory: identification of fungi and beyond. Expert Review of Proteomics 10(2):151-164.
Ramadani SH, Abulreesh HH (2010). Isolation and Identification of Yeast Flora in Local Kombucha Sample: AL NABTAH. Umm Al-Qura University. Journal of Applied Sciences 2(1):42-51.
Romano P, Capece A, Jespersen L (2006). Taxonomic and ecological diversity of food and beverage yeasts, in The Yeast Handbook, eds. Querol A, Fleet G (Berlin: Springer) pp. 13-53.
Santos C, Lima N, Sampaio P, Pais C (2011). Matrix-assisted laser desorption/ionization time-of-flight intact cell mass spectrometry to detect emerging pathogenic *Candida* species. Diagnostic Microbiology and Infectious Disease 71(3):304-308.
Sipiczki M (2011). Dimorphic cycle in *Candida citri* sp. nov., a novel yeast species isolated from rotting fruit in Borneo. FEMS Yeast Research 11(2):202-208.
Sudbery P, Berman J, Gow NAR (2004). The distinct morphogenic states of *Candida albicans*. Trends in Microbiology 12(7):317-324.
Suzzi G (2011). From wild strain to domesticated strain: the philosophy of microbial diversity in foods. Frontiers in Microbiology 2:169.
Tankoano A, Sawadogo-lingani H, Savadogo A, Kabore D, Traore Y (2017). Study of the process and microbiological quality of Gappal, a fermented food from Burkina Faso based on milk and millet dough. International Journal of Multidisciplinary and Current Research 5:104-110.
Turner SA, Butler G (2014). The *Candida* Pathogenic Species Complex. Cold Spring Harbor Perspective in Medicine 4(9):a019778:1-17.
Ubeda JF, Maldonado M, Briones AI, Gonzalez FJ (2014). Bio-prospecting of Distillery Yeasts as Bio-control and Bioremediation Agents. Current Microbiology 68(5):594-602.
Viljoen B (2006). Yeast ecological interactions. Yeast'yeast, yeast'bacteria, yeast'fungi interactions and yeasts as biocontrol agents, in yeasts in food and beverages, eds. A. Querol and G. Fleet (Berlin: Springer) pp. 83-110.

Vol. 15(9), pp. 482-489, September 2021
DOI: 10.5897/AJMR2021.9510
Article Number: BCDB70267872
ISSN: 1996-0808

http://www.academicjournals.org/AJMR

ACADEMIC JOURNALS
expand your knowledge

African Journal of Microbiology Research

Full Length Research Paper

Human immunodeficiency virus type 1 (HIV-1) subtype diversity in Busia, Western Kenya

James Munyao Kingoo[1,2]*, Anne WT Muigai[2], Viviene Matiru[4] and Samoel A. Khamadi[3]

[1]Department of Biochemistry and Biotechnology, School of Life and Biological Sciences, The Technical University of Kenya, Nairobi, Kenya.
[2]Department of Botany, School Biological Sciences, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya.
[3]Centre for Virus Research, The Kenya Medical Research Institute, Nairobi, Kenya.
[4]Department of Medical Microbiology, School of Biological Sciences, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya.

Received 2 February, 2021; Accepted 10 June, 2021

HIV infection is currently the single biggest epidemic globally. HIV the etiologic agent for AIDS is divided into two types: HIV-1 and HIV-2. HIV-2 is rare and is mainly found in some parts of West Africa. HIV-1 accounts for most cases of AIDS reported globally. HIV-1 strains can be classified into four groups: The "major" group M, group O, group N and the most recent group P. All of which may represent separate introductions of SIVs into humans. This cross sectional study determined the HIV-1 subtype diversity in Busia, Western Kenya. Briefly, participants were consented into the study based on pre-determined inclusion criteria. Viral RNA quantification was performed to select participants with virologic failure for drug resistance testing. HIV drug resistance testing (DRT) was performed and sequences obtained were used to determine circulating HIV-1 subtypes using the REGA HIV-1 Subtyping Tool Version 3.0. Phylogenetic analysis was performed using MEGA software V7.0 to confirm the circulating HIV subtypes. Out of 915 participants screened, 146 participants had virologic failure although 140 were successfully sequenced. Subtype A1 was the most prevalent subtype present in 52.9% of the participants followed by subtype D (20.7%), CRF A1_D (7.1%) subtype C and subtype B (4.3%) and subtype A2 (3.6%). Sequences within the same subtype and CRF clustered close together on the phylogenetic tree. An increase in CRFs in the population compared to previous studies. Circulating HIV subtypes should be continually monitored in Busia to determine trends in transmission and map the circulating recombinant forms for epidemiological purposes.

Key words: HIV-1, Busia county, subtype diversity, reverse transcriptase.

INTRODUCTION

The human immunodeficiency virus (HIV), can be divided into two types: HIV type 1 (HIV-1) and HIV type 2 [HIV-2] (Butler et al., 2006; Faria et al., 2014; Seitz, 2016; Sharp and Hahn, 2011; Weidle et al., 2000). The origin of HIV-1 among non-human primates has been traced to the simian immunodeficiency viruses (SIVs), which infected

*Corresponding author. E-mail: Israeldominion2015@gmail.com. Tel: +254784682788.

several geographically isolated chimpanzee communities in southern Cameroon and it has been postulated that both HIV types are as a result of multiple cross-species transmissions with SIVs (Faria et al., 2014; Sharp and Hahn, 2011; Taylor et al., 2008). HIV-1 accounts for most cases of HIV infections globally (Faria et al., 2014; Taylor et al., 2008). The strains of HIV-1 can be classified into four groups based on the genotypic variation in the *env* region: the "major" group M, the "outlier" group O, and the "new" group N and the most recent group P (Abongwa et al., 2019; Buonaguro et al., 2007; Faria et al., 2014; Ragupathy et al., 2011; Santoro and Perno, 2013; Tebit et al., 2016).

These four groups may represent the four separate introductions of SIVs into humans (Faria et al., 2019; Taylor et al., 2008). Within group M, there are known to be at least nine genetically distinct subtypes (or clades) of HIV-1 namely subtypes A, B, C, D, F-H, J, K (D'Arc et al., 2015; Faria et al., 2014; Taylor et al., 2008). The global HIV pandemic has been dominated by group M viruses since its discovery with the other groups N, O and P being poorly disseminated (Bbosa et al., 2019). Occasionally, two or more viruses of different subtypes can meet in the cell of an infected person and combine their genetic material to create a new hybrid virus (a process similar to sexual reproduction, and sometimes called "viral sex"), resulting in inter-subtype recombinants (Recordon-Pinson et al., 2018; Reis et al., 2019; Tongo et al., 2016). When the recombinants are transmitted and spread within a population, they are recognized as circulating strains in the HIV epidemic and are classified as circulating recombinant forms (CRFs) while unique recombinant forms (URFs), on the other hand, have been sampled only once from a single multiply-infected individual (Reis et al., 2019; Tongo et al., 2016). It is worth noting that in East Africa, where HIV-1 subtypes A, D and C are predominant, several AD and AC recombinants have been described (Giovanetti et al., 2020).

The HIV-1 subtype present in an individual has been shown to play a major role in the development of resistance towards antiretroviral therapy [ART] (Conroy et al., 2010; Kiwanuka et al., 2009; Santoro and Perno, 2013), viral transmission rates (Kiwanuka et al., 2009; Santoro and Perno, 2013) as well as progression to disease with subtype D showing faster progression to disease and higher mortality rates than subtype A1 (Baeten et al., 2007; Conroy et al., 2010; Santoro and Perno, 2013; Ssemwanga et al., 2013). The fast-increasing emergence of HIV recombinant forms has been shown to greatly impact accurate diagnosis, phylogenetic reconstruction, antiretroviral treatment and vaccine development. Several studies have demonstrated significant differences in the acquisition of HIV drug resistance, the majority noting that subtype D and C are more vulnerable to the development of drug resistance than subtype A (Santoro and Perno, 2013; Wallis et al., 2017). This study sought to determine the circulating HIV subtypes in Busia County, Kenya.

METHODOLOGY

Study site

The study was conducted at the Busia County Referral Hospital, Busia County, Kenya. All participants were on first-generation nucleoside reverse transcriptase inhibitor (NRTI) and non-nucleoside reverse transcriptase inhibitor (NNRTI) anti-HIV regimens at the hospital's HIV comprehensive care center (CCC) which provides services to over 20,000 HIV infected patients. All the patients were on ART under the Kenyan guidelines for 12 months or more at the time of the study.

Study participants

The study participants were HIV-1 positive adult patients (aged between 21 and 67 years). All participants were receiving a standard triple first-line ART regimen from Busia County Referral Hospital's CCC for 12 months or more. Participants who had attended the clinic at least once within the previous 6 months were included in this cross-sectional single visit study. All participants gave voluntary informed consent to participate in the study. Study participants who demonstrated virologic failure as per the Kenyan guidelines with viral loads of >1,000 copies/mL had drug resistance testing performed.

Ethical considerations

This study was approved by the Kenya Medical Research Institute/National Scientific and Ethical Review Committees. Written informed consent was obtained from each participant before conducting any study procedure. The study was conducted according to good clinical laboratory practices.

Laboratory testing

HIV-1 RNA viral load testing

Plasma samples for HIV-1 viral RNA and drug resistance testing stored at -70°C were retrieved and thawed at room temperature. A total of 925 patients qualified for viral load testing based on the above inclusion criteria. Viral load testing was performed using Abbott M2000SP/RT (Abbott Molecular, Inc., Des Plaines, IL, USA) viral load testing assay, whose lower detection limit was 40 copies/mL.

HIV-1 drug resistance testing

Reverse transcription (RT) polymerase chain reaction (PCR), nested PCR and genotyping was performed for all participants having a viral load of >1,000 copies/mL (n=146). Briefly, blood was collected from these participants, and viral ribonucleic acid (RNA) extracted from blood plasma using Qiagen RNA MiniAmp kit (Qiagen, Valencia, CA, USA). Reverse transcription of the extracted RNA was performed to obtain complimentary deoxyribonucleic acid (cDNA) followed by nested polymerase chain reactions to produce millions of copies of the cDNA for sequencing. Successful amplification was confirmed by gel electrophoresis.

HIV-1 genotyping assay, which sequences the HIV-1 pol gene(base pairs covering PR region: codons 4 – 99 and RT region: codons 38 – 247), was performed on the amplicons using an

Table 1. Participant demographic characteristics.

REGA subtype	Mean viral load (copies/ml)	Mean age (Years)	Mean CD4	Total number of participants	Number with DRAMs
A1	97,285	38.7	159	74	43
D	97,625	39	161	29	22
CRF A1_D	93,356	38.5	164	10	4
C	11,959	40.3	342	6	0
B	184,544	38	125	6	6
A2	30,057	33.25	182	4	2
CRF A1_C	27,549	38.5	171	4	4
G	324,401	43.5	119	2	2
CRF A1_J	65,636	29	198	2	2
CRF B_C	54,456	43	282	1	1
CRF A1_F1	67,186	29	245	1	1
CRF A2_H	186,098	32	118	1	1

automated ABI 310 sequencer (Applied Biosystems, Foster City, CA, USA). Sequence quality control was performed using the Los Alamos HIV-1 sequence quality assurance tool on https://www.hiv.lanl.gov/content/sequence/QC/index.html?sample_input=1. HIV-1 subtyping was performed using the automated REGA HIV-1 Subtyping Tool Version 3.0 available on http://dbpartners.stanford.edu:8080/RegaSubtyping/stanford-hiv/typingtool/. The FASTA formatted sequences were copied and pasted into the REGA HIV-1 Subtyping Tool Version 3.0 and submitted for subtype analysis. The subtypes obtained and their proportions were downloaded in excel format. Similarly, the sequences were pasted onto the Stanford University HIVdb program version 8.8 on https://hivdb.stanford.edu/hivdb/by-sequences/ to obtain subtypes for comparative study. The HIV-1 subtypes obtained from the Stanford University HIVdb were transcribed onto the excel spreadsheet pending analysis. HIV-1 resistance-associated mutations and phenotypic drug resistance profiles were obtained from the Stanford University HIV database available on https://hivdb.stanford.edu/hivdb/by-sequences/. Phylogenetic analysis was performed using the Molecular Evolutionary Genetics Analysis (MEGA) software version 7.0 at 1,000 bootstrap replications and the Jukes-Cantor model. Once the computation was complete, the Tree Explorer displayed two tree tabs: the original Maximum Likelihood tree and the Bootstrap consensus tree. The results were exported and saved as a .mas file for further review and analysis.

Statistical analysis

Descriptive statistics (median, interquartile range [IQRs], mean, and percentages) were used to summarize the demographic and clinical characteristics of the study participants in this study. The proportions of the different subtypes were analyzed by Pearson's Chi-squared test. Comparisons between different ages and gender were carried out using the Wilcoxon signed-rank test. Pearson's correlation coefficients were calculated. A p-value of less than 0.05 was considered statistically significant.

RESULTS

A total of 140 participants were successfully sequenced hence their subtypes determined (GenBank accession numbers MW618176-MW618315). The age range was 21 to 67 years, with a mean age of 38.96 and a median age of 38 (CI=95%, IQR=14). Seventy-eight (55.7%) of the participants were females with 44.3% being males. The participants were either on EFV or NVP based regimens with 112 (80%) and 28 (20%) being on EFV and NVP based regimens, respectively. The majority of the participants (72%) were on the TDF+3TC+EFV combination regimen.

A look at the circulating HIV-1 subtypes from the REGA HIV subtyping tool revealed that subtypes A1, A2, B, C, D, G and circulating recombinant forms (CRFs) A1_D, A1_C, C_B, A2_H, and A1_F1 were present in the study population. Subtype A1 was the most prevalent subtype in the population, present in 74 (52.9%) of the participants. Subtype D was present in 29 (20.7%), CRF A1_D was present in 10 (7.1%) of the population, subtype C and subtype B were both present in 6 (4.3%), of the population while subtype A2 was present in 5 (3.6%) of the population. CRF A1_C was present in 4 (2.9%) of the study population. Subtype G was present in 2 (1.4%), whereas CRF B_C, CRF A1_F1, and CRF A1_J were all present in 1 (0.7%) of the population (Table 1). CRFs accounted for 13.6% of the circulating HIV-1 subtypes with a majority of these CRFs (~79%) containing subtype A1. CRF A1_D was the most prevalent CRF within the study population. There were significant differences in the availability of the different subtypes and CRFs within the study population ($p=<0.0001$).

Although discordance was reported between subtypes obtained from COMET, REGA, Los Alamos National Library (lanl), and the Stanford University HIVdb subtyping software, the differences were not statistically significant ($p=0.9241$). There were subtype discordances between the REGA subtyping tool, Los Alamos National Library (lanl) and the Stanford University HIVdb subtyping software in reporting all other subtypes except subtypes G, C and D.

Table 2. Distribution of the HIV-1 subtypes circulating in the population generated from REGA, Los Alamos National Library (lanl) and the Stanford University HIVdb subtyping software.

Subtype assignment	REGA numbers (%)	LANL numbers (%)	Stanford numbers (%)
HIV-1 Subtype A1	74 (52.9)	86 (61.4)	86 (61.4)
HIV-1 Subtype D	29 (20.7)	29 (20.7)	25 (17.9)
CRF A1_D	10 (7.1)	0/1/00	0
HIV-1 Subtype C	6 (4.3)	7 (5)	7 (5)
HIV-1 Subtype B	6 (4.3)	12 (8.6)	14 (10)
HIV-1 Subtype A2	4 (2.9)	0/1/00	4 (2.9)
CRF C_A1	4 (2.9)	0/1/00	0
HIV-1 Subtype G	2 (1.4)	2 (1.4)	2 (1.4)
CRF A1_J	2 (1.4)	0/1/00	0
CRF C_B	1 (0.7)	0/1/00	0
CRF A1_F1	1 (0.7)	0/1/00	0
CRF A2_H	1 (0.7)	0/1/00	0
HIV-1 Subtype A6	0	1 (0.7)	0
CRF A1BC	0	1 (0.7)	0
CRF A1_B	0	1 (0.7)	0
CRF 01_AE	0	1 (0.7)	0
CRF10_CD	0	0/1/00	2 (1.4)
Total	140 (100)	140 (100)	140 (100)

REGA subtyping tool reported the highest number of CRFs (25), COMET reported 19 CRFs while lanl and Stanford University HIVdb reported 3 and 2 CRFs, respectively. There were however no significant differences in the number of CRFs generated by the different subtyping tools (p=0.3143). Interestingly, both lanl and the Stanford University HIVdb subtyping software did not report any CRF A1_D, the most prevalent CRF in the study population (Table 2). Only the lanl subtyping reported CRF A1_B_C, CRF A1_B, and CRF 01_AE.

It was further noted that of the 53 patients who did not possess any DRAMs hence no resistance to any of the available medications, 32 (60.4%) were subtype A1, 7 (13.2%) were subtype D, 6 were CRF A1_D, 6 (11.3%) were subtype C, while 3 (5.7%) were subtype A2. Of the 87 participants with at least one DRAM, 42 (48.3%) were subtype A1, 21 (39.6%) were subtype D, 20 (23%) were CRFs, 5 (5.7%) were subtype A2, 5 (5.7%) were subtype C while 4 (4.6%) were subtype A2. It is important to note that whereas none of the subtype C sequences had any DRAMs, all subtype B sequences had DRAMs conferring resistance (Figure 1). Interestingly, the 6 participants with E138 series mutations (mutations previously associated with ETR and RPV resistance only in subtype B) were all subtype A1. There were 13 sequences with K65R mutations (mutations selected by TDF, ABC, d4T, ddI, and rarely 3TC), of which 10 were subtype A1 while 3 were subtype D. All the 37 sequences had at least one thymidine analog mutations (TAM), 23 (62.2%) were subtype A1 or subtype A1 containing CRFs, 9 (24.3%) were subtype D or subtype `D containing CRFs while 4 (10.8%) were subtype B. For this study, there was no significant difference between the subtypes in relation to the acquisition of DRAMs (p = 0.2766). Seven participants had PI DRAMs, 5 of which were subtype A1 while the other 2 were subtype B. Of the 82 participants with NRTI DRAMs, 53 (64.6%) were subtype A1 or A1 containing CRFs, 23 were subtype D or D containing subtypes, 6 were subtype B while 2 were A2 and G, respectively. NNRTI DRAMs, present in 82 participants, 53 (64.6%) were subtype A1 or A1 containing CRFs, 25 (30.4%) were subtype D, 4 were subtype B, 2 were subtype A2 while 2 were subtype G.

While comparing the prevalence of different subtypes, female participants had significantly higher probabilities of having subtypes A1, D and A1_D, G and A2 (p=0.0211) while male participants had a higher prevalence of subtypes B and C (Table 3). Interestingly, 18 (94.7%) of the 19 participants with CRFs were females; hence, only 1 (5.3%) had CRFs.

DISCUSSION

From this study, we confirmed the complexity of the HIV-1 subtypes circulating in the population with subtype A1 being the most predominant (52.9%). Many other studies have also supported the predominance of HIV subtype A1 in most of the Kenyan HIV-positive populations. Gounder et al. (2017), in a study evaluating the complex subtype diversity of HIV-1 among drug users in major Kenyan cities also reported subtype A1 to be the predominant subtype (44.4%); a slightly lower prevalence

Table 3. Distribution of subtypes among male and female participants within the study population.

REGA subtype	Female	Male	Grand Total
A1	51	23	74
D	17	12	29
A1_D	9	1	10
B	4	2	6
C	2	4	6
A1_C	4	0	4
A2	3	1	4
A1_J	2	0	2
G	2	0	2
A1_F1	1	0	1
A2_H	1	0	1
B_C	1	0	1
Grand Total	97	43	140

There were statistically significant differences in the distribution of different subtypes between males and females (p=0.0012) with females having a high prevalence of each of the subtypes except B and C.

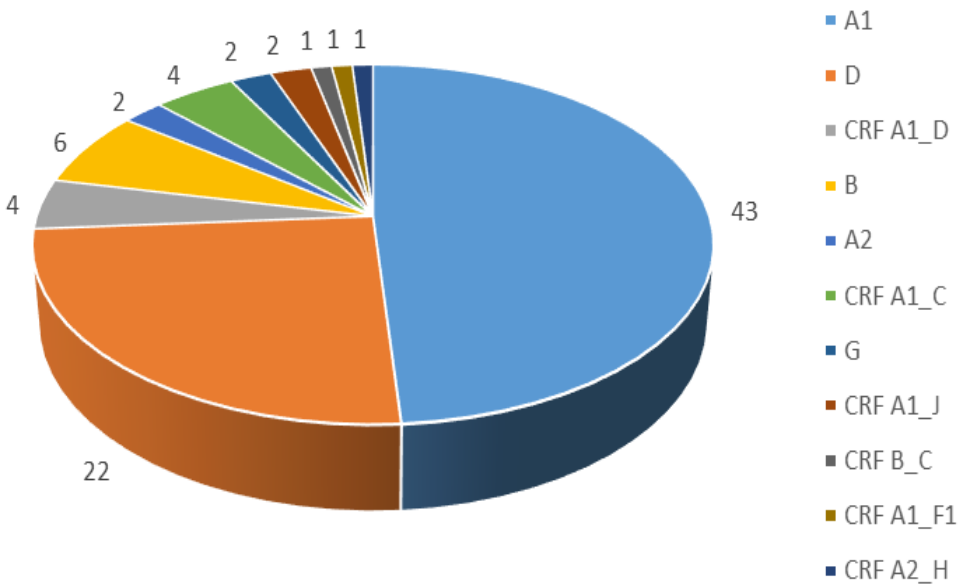


Figure 1. Distribution of different DRAMs among subtypes within the study population.

than that obtained from our current study (52.9%). According to the results of this study, subtype D was the second most predominant subtype in the population (20.7%), thereby differing with the findings by Gounder et al. (2017), who reported subtype C as the second most predominant subtype among drug users in Kenya, but agreeing with results from studies by Khoja et al. (2008), Lihana et al. (2009) and Kantor et al. (2014).

A study by Oyaro et al. (2018), on adults on ART in Western Kenya reported comparable HIV-1 subtype A1 prevalence to this study (51.4% vs 52.8% for our study) but reported lower subtype D prevalence (9.4% vs 20.7% for this study). On the other hand, Adungo et al. (2014), reported an HIV-1 subtype A1 prevalence similar to our reported figures (53.3% vs 52.9% for our study) but reported a slightly higher prevalence of subtype D (28% vs 22.7% for this study) and a comparatively lower prevalence of HIV-1 subtype A1_D (1.3% vs 7.1% for our study). This study further confirms the predominance of HIV-1 subtype A1 in Western Kenya, with an increasing

HIV-1 subtype D and CRF A1_D within the population. It was postulated that the lower subtype D proportions in our study were compensated by comparatively higher A1_D CRFs compared to the study by Adungo et al. (2014). Secondly, our higher sample size (140 compared to 75 in Adungo's study), could have been more representative of the population.

Lel et al. (2014), while looking at transmitted HIV-1 subtypes in children from Busia, reported a higher prevalence of HV-1 subtype A1 (69.8%) compared to 52.9% prevalence in this study. They also reported a higher prevalence of HIV-1 subtype D (22.6%) compared to the 20.7% prevalence reported in this study. They did not report any CRF A1_D. Important to note is the fact that the study looked at transmitted HIV-1 subtypes in infants whereas the current study looked at HIV-1 subtypes in adults hence the glaring differences in the circulating subtypes. The differences in subtype prevalence between the two studies could have resulted from participant age differences.

The HIV-1 subtype present in an individual has been shown to play a major role in the development of resistance towards ART (Chaplin et al., 2011; Santoro and Perno, 2013; Wainberg et al., 2011), viral transmission rates (Conroy et al., 2010; Kiwanuka et al., 2009; Santoro and Perno, 2013; Shaw and Hunter, 2012) as well as progression to disease with subtype D showing faster progression to disease and higher mortality rates than subtype A1 (Baeten et al., 2007; Conroy et al., 2010; Santoro and Perno, 2013; Ssemwanga et al., 2013). Several studies have demonstrated that subtypes D and C are more vulnerable to the development of drug resistance than subtype A (Clutter et al., 2016; Lessells et al., 2012; Santoro and Perno, 2013). These findings were partly supported by the current study where 75.9% of subtype D sequences had DRAMs compared to 53.4% subtype A1 sequences. None of the subtype C sequences in this study possessed any DRAMs, contradicting findings by Santoro and Perno (2013) who recorded higher resistance in subtype C than any other subtype.

Studies highlighting the differences in HIV-1 subtype diversity between males and females are scanty. One study contacted in Kenya showed marked differences in the diversity of transmitted virus between male and female participants where women from Kenya were found to be infected with multiple HIV-1 variants whereas their male counterparts were not (Long et al., 2000). The differences in the diversity of the transmitted virus could explain the emerging differences in subtype diversity between male and female participants in this study.

World Health Organization (2012), reported higher rates of mother-to-child transmission of HIV-1 in mothers having subtypes C and D than in those with subtype A and CRFs. HIV-1 subtype D has also been reported to progress faster to disease and have lower transmissibility than subtype A (Conroy et al., 2010; Ssemwanga et al., 2013). Subtype A has been shown to have higher viral transmission rates than subtype D (Kiwanuka et al., 2009). These findings imply that the high prevalence of subtype A1, D, and CRF A1_D, as confirmed in this current study, may pose a challenge to HIV-1 treatment and prevention strategies including PMTCT.

The present study demonstrated a higher prevalence of subtype D (20.7%) and CRF A1_D (7.1%) compared to most other studies in Kenya (Gounder et al., 2017; Kantor et al., 2014; Khoja et al., 2008; Koigi et al., 2014). HIV-1 subtype D has been reported to be more prevalent in Uganda (Collinson-Streng et al., 2009; Conroy et al., 2010; Kapaata et al., 2013). A higher prevalence of subtype D and its associated CRFs in the border town of Busia could therefore be indicative of cross-border infections from Uganda.

Circulating HIV subtypes in Busia county are highly diverse with reported increased subtype D and its recombinants. There is also an increased prevalence of HIV-1 CRFs within the population, the majority of which are found in females which could be indicative of higher rates of multiple infections in women than men within the study population. Continuous monitoring of the circulating HIV-1 subtypes and CRFs, therefore, is key to formulating strategies aimed at reducing the prevalence of HIV-1 within the population, reducing transmission rates, monitoring and controlling HIV-1 drug resistance testing as well as taming morbidity and mortality associated with HIV infection for the realization of WHO's 90:90:90 HIV treatment targets by the year 2030 and beyond.

Limitations of the study

The small sample size and the cross-sectional nature of the study to track virologic failure in the treated HIV patients and inclusion of infected individuals at different times and stages of HIV infection may not be representative of the epidemic drive in the region. As such the data should therefore be interpreted with caution. Additionally, recombination patterns in a heterogeneous epidemic are complex and will require another generation of genome data level to fully understand the changing dynamics of viral genotypes in this region of Kenya.

CONFLICTS OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors sincerely thank the participants who whole heartedly devoted themselves to make the study a success.

We acknowledge the support given by the Kenya Medical Research Institute, Busia towards the completion of this project and Jomo Kenyatta University of Agriculture and Technology and the Technical University of Kenya.

REFERENCES

Abongwa LE, Nyamache AK, Torimiro JN, Okemo P, Charles F (2019). Human immunodeficiency virus type 1 ((HIV-1) subtypes in the northwest region, Cameroon. Virology Journal 16(1):1-7.

Adungo FO, Gicheru MM, Adungo NI, Matilu MM, Lihana RW, Khamadi SA (2014). Diversity of Human Immunodeficiency Virus Type-1 Subtypes in Western Kenya. World Journal of AIDS 4(4):365-372.

Baeten JM, Chohan B, Lavreys L, Chohan V, McClelland RS, Certain L, Mandaliya K, Jaoko W, Overbaugh J (2007). HIV-1 Subtype D Infection Is Associated with Faster Disease Progression than Subtype A in Spite of Similar Plasma HIV-1 Loads. The Journal of Infectious Diseases 195(8):1177-1180.

Bbosa N, Kaleebu P, Ssemwanga D (2019). HIV subtype diversity worldwide. Current Opinion in HIV and AIDS 14(3):153-160.

Buonaguro L, Tornesello ML, Buonaguro FM (2007). Human Immunodeficiency Virus Type 1 Subtype Distribution in the Worldwide Epidemic: Pathogenetic and Therapeutic Implications. Journal of Virology 81(19).

Butler I, Pandrea I, Marx P, Apetrei C (2006). HIV Genetic Diversity: Biological and Public Health Consequences. Current HIV Research 5(1):23-45.

Chaplin B, Eisen G, Idoko J, Onwujekwe D, Idigbe E, Adewole I, Gashau W, Meloni S, Sarr AD, Sankalé JL, Ekong E, Murphy RL, Kanki P (2011). Impact of HIV type 1 subtype on drug resistance mutations in Nigerian patients failing first-line therapy. AIDS Research and Human Retroviruses 27(1).

Clutter DS, Jordan MR, Bertagnolio S, Shafer RW (2016). HIV-1 drug resistance and resistance testing. In Infection, Genetics and Evolution 46:292-307.

Collinson-Streng AN, Redd AD, Sewankambo NK, Serwadda D, Rezapour M, Lamers SL, Gray RH, Wawer MJ, Quinn TC, Laeyendecker O (2009). Geographic HIV type 1 subtype distribution in Rakai district, Uganda. AIDS Research and Human Retroviruses 25(10):1045-1048.

Conroy SA, Laeyendecker O, Redd AD, Collinson-Streng A, Kong X, Makumbi F, Lutalo T, Sewankambo N, Kiwanuka N, Gray RH, Wawer MJ, Serwadda D, Quinn TC, SA, C, O, L, AD, R, A, CS, X, K, F, M, D, S (2010). Changes in the distribution of HIV type 1 subtypes D and A in Rakai District, Uganda between 1994 and 2002. AIDS Research and Human Retroviruses 26(10):1087–1091.

D'Arc M, Ayouba A, Esteban A, Learn GH, Boué V, Liegeois F, Etienne L, Tagg N, Leendertz FH, Boesch C, Madinda NF, Robbins MM, Gray M, Cournil A, Ooms M, Letko M, Simon VA, Sharp PM, Hahn BH, Peeters M (2015). Origin of the HIV-1 group O epidemic in western lowland gorillas. Proceedings of the National Academy of Sciences of the United States of America 112(11):E1343-E1352.

Faria NR, Rambaut A, Suchard MA, Baele G, Bedford T, Ward MJ, Tatem AJ, Sousa JD, Arinaminpathy N, Pépin J, Posada D, Peeters M, Pybus OG, Lemey P (2014). The early spread and epidemic ignition of HIV-1 in human populations. Science 346(6205):56-61.

Faria NR, Vidal N, Lourenco J, Raghwani J, Sigaloff KCE, Tatem AJ, Van De Vijver DAM, Pineda-Peña AC, Rose R, Wallis CL, Ahuka-Mundeke S, Muyembe-Tamfum JJ, Muwonga J, Suchard MA, De Wit TFR, Hamers RL, Ndembi N, Baele G, Peeters M, Dellicour S (2019). Distinct rates and patterns of spread of the major HIV-1 subtypes in Central and East Africa. PLoS Pathogens 15(12):e1007976.

Giovanetti M, Ciccozzi M, Parolin C, Borsetti A (2020). Molecular epidemiology of hiv-1 in african countries: A comprehensive overview. Pathogens 9(12):1-10.

Gounder K, Oyaro M, Padayachi N, Zulu TM, De Oliveira T, Wylie J, Ndung'u T, de Oliveir T, Wylie J, Ndung'u T (2017). Complex Subtype Diversity of HIV-1 Among Drug Users in Major Kenyan Cities | AIDS Research and Human Retroviruses. AIDS Research and Human Retroviruses 33(5):500-510.

Kantor R, DeLong A, Balamane M, Schreier L, Lloyd RM, Jr Injera W, Kamle L, Mambo F, Muyonga S, Katzenstein D, Hogan J, Buziba N, Diero L (2014). HIV diversity and drug resistance from plasma and non-plasma analytes in a large treatment programme in western Kenya. Journal of the International AIDS Society 17(1):19262.

Kapaata A, Lyagoba F, Ssemwanga D, Magambo B, Nanyonjo M, Levin J, Mayanja BN, Mugasa C, Parry CM, Kaleebu P (2013). HIV-1 subtype distribution trends and evidence of transmission clusters among incident cases in a rural clinical cohort in Southwest Uganda, 2004-2010. AIDS Research and Human Retroviruses 29(3):520-527.

Khoja S, Ojwang P, Khan S, Okinda N, Harania R, Ali S (2008). Genetic analysis of HIV-1 subtypes in Nairobi, Kenya. PLoS ONE 3(9):e3191.

Kiwanuka N, Laeyendecker O, Quinn TC, Wawer MJ, Shepherd J, Robb M, Kigozi G, Kagaayi J, Serwadda D, Makumbi FE, Reynolds SJ, Gray RH (2009). HIV-1 subtypes and differences in heterosexual HIV transmission among HIV-discordant couples in Rakai, Uganda. AIDS 23(18):2479-2484.

Koigi P, Ngayo MO, Khamadi S, Ngugi C, Nyamache AK (2014). HIV type 1 drug resistance patterns among patients failing first and second line antiretroviral therapy in Nairobi, Kenya. BMC Research Notes 7:890.

Lel R, Ngaira J, Lihana R, Khamadi S (2014). HIV-1 drug resistance mutations among infants born to HIV-positive mothers in Busia, Kenya. AIDS Research and Human Retroviruses 30(12):1236-1238.

Lessells RJ, Katzenstein DK, De Oliveira T (2012). Are subtype differences important in HIV drug resistance? Current Opinion in Virology 2(5):636-643.

Lihana RW, Khamadi SA, Lwembe RM, Kinyua JG, Muriuki JK, Lagat NJ, Okoth FA, Makokha EP, Songok EM (2009). HIV-1 subtype and viral tropism determination for evaluating antiretroviral therapy options: An analysis of archived Kenyan blood samples. BMC Infectious Diseases 9(1):1-8.

Oyaro M, Mbithi J, Oyugi F, Laten A, Anzala O, Engelbrecht S (2011). Molecular characterization of HIV type 1 among HIV-infected respondents in a cohort being prepared for HIV phase III vaccine clinical trials, Western Kenya. AIDS Research and Human Retroviruses 27(3):257-264.

Oyaro M, Wylie J, Chen CY, Ondondo RO, Kramvis A (2018). Human immunodeficiency virus infection predictors and genetic diversity of hepatitis B virus and hepatitis C virus co-infections among drug users in three major Kenyan cities. Southern African Journal of HIV Medicine 19(1):a737.

Ragupathy V, Zhao J, Wood O, Tang S, Lee S, Nyambi P, Hewlett I (2011). Identification of new, emerging HIV-1 unique recombinant forms and drug resistant viruses circulating in Cameroon. Virology Journal 8(1):185.

Recordon-Pinson P, Alves BM, Tumiotto C, Bellecave P, Bonnet F, Neau D, Soares EA, Soares MA, Fleury H (2018). A New HIV-1 Circulating Recombinant Form (CRF98-cpx) Between CRF06-cpx and Subtype B Identified in Southwestern France. AIDS Research and Human Retroviruses 34(11):1005-1009.

Reis MNG, Guimarães ML, Bello G, Stefani MMA (2019). Identification of new HIV-1 circulating recombinant forms CRF81-cpx and CRF99-BF1 in central Western Brazil and of unique BF1 recombinant forms. Frontiers in Microbiology 10:97.

Santoro MM, Perno CF (2013). HIV-1 Genetic Variability and Clinical Implications. ISRN Microbiology Article ID 481314, 20 p.

Seitz R (2016). Human Immunodeficiency Virus (HIV). Transfusion Medicine and Hemotherapy 43:203-222.

Sharp PM, Hahn BH (2011). Origins of HIV and the AIDS pandemic. Cold Spring Harbor Laboratory Press https://doi.org/10.1101/cshperspect.a006841

Shaw GM, Hunter E (2012). HIV transmission. Cold Spring Harbor Laboratory Press https://doi.org/10.1101/cshperspect.a006965

Ssemwanga D, Nsubuga RN, Mayanja BN, Lyagoba F, Magambo B, Yirrell D, van der Paal L, Grosskurth H, Kaleebu P (2013). Effect of HIV-1 Subtypes on Disease Progression in Rural Uganda: A Prospective Clinical Cohort Study. PLoS ONE 8(8):e71768.

Taylor BS, Sobieszczyk ME, McCutchan FE, Hammer SM (2008). The challenge of HIV-1 subtype diversity. In New England Journal of Medicine 358:1590-1602.

Tebit DM, Patel H, Ratcliff A, Alessandri E, Liu J, Carpenter C, Plantier

JC, Arts EJ (2016). HIV-1 Group O Genotypes and Phenotypes: Relationship to Fitness and Susceptibility to Antiretroviral Drugs. AIDS Research and Human Retroviruses 32(7):676-688.

Tongo M, Dorfman JR, Martin DP (2016). High Degree of HIV-1 Group M (HIV-1M) Genetic Diversity within Circulating Recombinant Forms: Insight into the Early Events of HIV-1M Evolution. Journal of Virology 90(5):2221-2229.

Wainberg MA, Zaharatos GJ, Brenner BG (2011). Development of Antiretroviral Drug Resistance. New England Journal of Medicine 365:637-646.

Wallis CL, Godfrey C, Fitzgibbon JE, Mellors JW (2017). Key Factors Influencing the Emergence of Human Immunodeficiency Virus Drug Resistance in Low-And Middle-Income Countries. Journal of Infectious Diseases 216(suppl_9):S851–S856.

Weidle PJ, Ganea CE, Irwin KL, Pieniazek D, McGowan JP, Olivo N, Ramos A, Schable C, Lal RB, Holmberg SD, Ernst JA (2000). Presence of Human Immunodeficiency Virus (HIV) Type 1, Group M, Non-B Subtypes, Bronx, New York: A Sentinel Site for Monitoring HIV Genetic Diversity in the United States. The Journal of Infectious Diseases 181(2):470-475.

Related Journals: