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Full Length Research Paper

Antibiotic susceptibility profile of bacteria isolated from fomites in some day care centres in Ile-Ife, Nigeria

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This study was conducted by carrying out the isolation, identification and determination of the sensitivity profile to antibiotics in bacteria isolated from fomites in some day care centres in Ile-Ife, Osun State, Nigeria. A total of one hundred and twenty-four fomites were collected from selected seventeen day care centers within Ile-Ife. These were cultured on nutrient agar plates incubated at 37°C for 24 h using streak plate technique. Preliminary identification of bacterial isolates was performed using cultural, colonial and morphological characteristics of isolates on the agar plates such as relative size, colour, texture, consistency, pigment, elevation, edge and shape. Bacterial isolates were further characterized by physiological characteristics through biochemical reactions of the bacterial isolates to some reagents and media with reference to the Bergey's Manual of Determinative Bacteriology. Isolates were further identified with Microbact identification test kit. The antibiotype of the isolates was determined by the Kirby-Bauer's disk diffusion technique. Detection of extended spectrum beta-lactamase was done phenotypically by the double disc synergy test. Resistance to antibiotics varied greatly among the isolates. Resistance to cefuroxime, augmentin, cephalexin and ampicillin was notably high in *Bacillus* sp, *Staphylococcus* sp, *Corynebacterium* sp and *Staphylococcus aureus* from fomites. Meanwhile, all Enterobacteriaceae were susceptible to meropenem, ciprofloxacin, augmentin, trimethroprim, gentamycin, cotrimoxazole, chloramphenicol and ofloxacin. Multiple antibiotic resistance (MAR) was generally high among the Gram positive isolates with diversity of MAR patterns.

Key words: Fomites, bacteria, disk diffusion technique, antibiotic resistance.

INTRODUCTION

Day care can be defined as a facility, personal or relative(s) home, which provide care for infants and toddlers and preschoolers (Shahidul and Nasreen, 2015). Day care is also taking care of a child or multiple children at a time by nannies or babysitters, teachers, or other providers. Microorganisms are ubiquitous. They are found on the floor where the children play, toys, air, etc. Most of these microorganisms are *Proteus* sp, *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus* sp. *Bacillus* sp. and *Streptococcus*

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Table 1. Frequency and percentage distribution of the bacterial isolated from fomites in day care centers in Ile-Ife.

Bacterial Isolates (n=132)	No. of occurrence	Percentage
Staphylococcus aureus	7	5.30
Staphylococcus sp	18	13.64
Bacillus sp	54	40.90
Corynebacterium xerosis	29	21.97
Corynebacterium kutsceri	19	14.39
Enterobacter cloacae	1	0.76
Enterobacter agglomerans	1	0.76
Others	1	0.76
Providencia rettgeri	1	0.76
Proteus sp	1	0.76
Total	132	100

n = Total number of isolates.

faecalis (Olaitan and Adeleke, 2006). Fomites are non-living materials or surfaces which could harbor or spread fecal pathogens (Timothy et al., 2013). Inanimate objects (fomites) are known to transmit human pathogens via direct, surface-to-mouth, contacts, or indirectly, by contaminated fingers and oral transmission (Akinrotoye et al., 2018). The fomites includes baby toys, beddings, door handles, showers, toilet, hand lockers especially those found in day care, and restrooms (Bright et al., 2010). It is believed that inanimate objects are carriers of microorganisms emanating within the surrounding environment. These ubiquitous microorganisms could pose a bio-transfer potential that has the potential to be transferred to another substratum where growth is possible, for example on food, inanimate objects or on the human body (Joanna, 2012). The spread of infectious diseases through hand contact has been an area of major concern that should be looked into drastically for possible solutions. Surveys of the day care center environment have found contamination on the surfaces of toys, food areas, and diaper changing areas. The organisms thus picked from fomites can be transferred to another child as the fomites have shown to play a role in the transmission of organisms. This study therefore seek to isolate and determine the antibiotic susceptibility profile of bacteria of public health importance associated with fomites in selected day care centers located in Ile-Ife, Osun State, Nigeria.

MATERIALS AND METHODS

Samples were collected from 17 different Day care centers in Ile-Ife, Osun State. Sterile cotton swabs pre moistened with sterile normal saline was rotated around the baby toys, diaper changing tables, chairs, tables, mats, door handles and bed sheets. Preliminary identification of bacterial isolates was performed using cultural, colonial and morphological characteristics of isolates on the agar plates such as relative size, colour, texture, consistency, pigment, elevation, edge and shape (Olutiola et al., 2018). Bacterial isolates were further characterized by physiological characteristics through biochemical reactions of the bacterial isolates to some reagents and media with reference to the Bergey's Manual of Determinative Bacteriology. Isolates were identified to specie level using Microbact identification test kits (Oxoid).

ANTIBIOTIC SUSCEPTIBILITY TEST

Antibiotic susceptibility of the isolates was done using the Kirby-Bauer's disk diffusion method as described by Bauer et al. (1966) and interpreted according to the guidelines of Clinical Laboratory Standard (CLSI, 2013). An 18-24 h old broth culture of the inoculum was standardized (adjusted to 0.5 McFarland Standard - x 10^8 cfu/ml). The prepared standardized inoculum was seeded on the Mueller-Hinton susceptibility agar plates (Lab M, UK) with the aid of sterile swab stick and allowed to dry for 5-10 min. The Gram positive and Gram positive antibiotic disks (combined (Biomark Laboratories, India) containing varying and specific concentrations viz; gentamycin (10 µg), augmentin (30 µg), ceftazidime (30 µg), cephalexin (1.5 µg), cefuroxime (10 µg), erythromycin (5 µg), vancomycin (30 µg) cotrimoxazole (25 µg), ampicillin (10µg), tetracycline (30 µg), ciprofloxacin (5 µg), cefuroxime (10 µg) and ceftazidime (10 µg) and combined (Abtek) containing varying and specific concentrations which include gentamycin (10 µg), ceftazidime (30 µg), cefuroxime (30 µg), tetracycline (10 µg), meropenem (10 µg) cefixime (5 µg) ciprofloxacin (5 µg), trimethoprim (5 µg), nitrofurantoin (300 µg), ofloxacin (5 µg), augmentin (30 µg), Amoxicillin/clavulanic acid (30 µg), cefotaxime (30 µg) and ceftazidime (30 µg)) were used.

RESULTS

Frequency and percentage distribution of the bacterial isolated from fomites, in day care centers in Ile-Ife

Table 1 shows the overall distribution of bacteria isolated from fomites in day care centers. The distribution of the isolates recovered includes: *Staphylococcus aureus* 7

Table 2. Antibiotic susceptibility profile of the isolates cultured from fomites in day care centers in Ile-Ife.

Name of organism	Antibiotics	No of isolates	Sensitive (%)	Intermediate (%)	Resistance (%)
Staphylococcus aureus	Cotrimoxazole (25 µg)	7	6(85.7)	0	1(14.3)
	Cefuroxime (10 µg)	7	3(42.86)	0	4(57.14)
	Gentamycin (10 µg)	7	7(100)	0	0
	Ciprofloxacin (5 µg)	7	7(100)	0	0
	Ampicillin (30 µg)	7	1(14.3)	0	6(85.7)
	Erythromycin (5 µg)	7	3(42.86)	2(28.57)	2(28.57)
	Tetracyline (30 µg)	7	6(85.7)	1(14.3)	0
	Augmentin (30 µg)	7	1(14.3)	0	6(85.7)
	Cephalexin (1.5 µg)	7	2(28.57)	0	5(71.43)
	Ceftaxidime (10 µg)	7	3(42.86)	0	4(57.14)
	Vancomycin (30 µg)	7	3(42.86)	0	4(57.14)
***Staphylococcus* sp.**	Cotrimoxazole (25 µg)	18	11(61.11)	0	7(38.89)
	Cefuroxime (10 µg)	18	2(11.11)	2(11.11)	14(77.78)
	Gentamycin (10 µg)	18	17(94.44)	1(5.56)	0
	Ciprofloxacin (5 µg)	18	18(100)	0	0
	Ampicillin (30 µg)	18	2(11.11)	0	16(88.89)
	Erythromycin (5 µg)	18	6(33.33)	4(22.22)	8(44.44)
	Tetracyline (30 µg)	18	9(50)	3(16.67)	6(33.33)
	Augmentin (30 µg)	18	3(16.67)	0	15(83.33)
	Cephalexin (1.5 µg)	18	5(27.78)	0	13(72.22)
	Ceftaxidime (10 µg)	18	4(22.22)	1(5.56)	13(72.22)
	Vancomycin (30 µg)	18	5(27.79)	0	13(72.22)
***Bacillus* sp.**	Cotrimoxazole (25 µg)	54	39(72.22)	1(1.85)	14(25.93)
	Cefuroxime (10 µg)	54	1(1.85)	0	53(98.15)
	Gentamycin (10 µg)	54	51(94.44)	0	3(5.56)
	Ciprofloxacin (5µg)	54	51(94.44)	1(1.85)	2(3.70)
	Ampicillin (30 µg)	54	1(1.85)	0	53(98.15)
	Erythromycin (5 µg)	54	25(46.29)	24(44.44)	5(9.30)
	Tetracyline (30 µg)	54	33(61.11)	5(9.26)	16(29.63)
	Augmentin (30 µg)	54	1(1.85)	0	53(98.15)
	Cephalexin (1.5 µg)	54	9(16.67)	0	45(83.33)
	Ceftaxidime(10 µg)	54	10(18.52)	0	44(81.48)
	Vancomycin(30 µg)	54	39(72.22)	0	15(27.78)
***Corynebacterium* sp.**	Cotrimoxazole (25 µg)	48	33(68.75)	0	15(31.25)
	Cefuroxime (10 µg)	48	4(8.33)	0	44(91.67)
	Gentamycin (10 µg)	48	38(79.17)	2(4.17))	8(16.66)
	Ciprofloxacin (5 µg)	48	41(85.41)	2(4.17)	5(10.42)
	Ampicillin (30 µg)	48	2(4.17)	0	46(95.83)
	Erythromycin (5 µg)	48	12(25)	8(16.67)	28(58.33)
	Tetracyline (30 µg)	48	27(56.25)	6(12.5)	15(31.25)
	Augmentin (30 µg)	48	6(12.50)	0	42(87.50)
	Cephalexin (1.5 µg)	48	41(85.42%)	0	7(14.58)
	Ceftaxidime (10 µg)	48	6(12.5)	0	42(87.5)
	Vancomycin (30 µg)	48	18(37.5)	0	30(62.5)
Enterobacteriaceae	Cotrimoxazole (30µg)	5	5(100)	0	0
	Chloramphenicol (30 µg)	5	5(100)	0	0
	Gentamycin (10 µg)	5	5(100)	0	0
	Cefotaxime (5 µg)	5	5(100)	0	0

Table 2. Cont'd.

	Ofloxacin (5 µg)	5	5(100)	0	0
	Augmentin (30 µg)	5	5(100)	0	0
	Nitrofurantion (300 µg)	5	4(80)	1(20)	0
	Ciprofloxacin (5 µg)	5	5(100)	0	0
	Tetracycline (30 µg)	5	2(40)	2(40)	1(20)
	Trimethoprim (5 µg)	5	3(60)	0	2(40)
	Meropenem (10 µ)g	5	5(100)	0	0

(5.30 %), *Staphylococcus* sp 18 (13.64%), *Bacillus* sp 54 (40.90%), *Corynebacterium xerosis* 29 (21.97%), *Corynebacterium kutsceri* 19 (14.39%), *Enterobacter clocae* 1 (0.76 %), *Enterobacter agglomerans* 1 (0.76%), other Enterobacteriacae 1 (0.76%), *Providencia rettgeri* 1 (0.76%) and *Proteus* sp 1 (0.76%).

Antibiotic susceptibility profile of the bacterial isolates cultured from fomites in day care centers in Ile-Ife

Table 2 shows the antibiotic susceptibility profiles of the bacterial isolates cultured from fomites. Resistance of bacterial isolates to antibiotics varies greatly. *Staphylococcus aureus* was resistant to ampicillin (85.7%), augmentin (85.7%), ceftazidime (57.14%), vancomycin (57.14%) and cephalexin (71.43%). However, the organism was sensitive to gentamycin (100%), ciprofloxacin (100%), cotrimoxazole (85.7%) and tetracycline (85.7%). Meanwhile, *Staphylococcus* sp was resistant to ampicillin (88.89%), augmentin (83.3%), ceftazidime (72.22%), cefuroxime (77.78%) and vancomycin (72.22%). However, *Staphylococcus* sp was sensitive to ciprofloxacin (100%), gentamycin (94.44%), cotrimoxazole (61.11%) and tetracycline (50%). *Bacillus* sp was resistant to cefuroxime (98.15%), ampicillin (98.15%) and augmentin (98.15%). However, the organism was sensitive to gentamycin (94.44%), ciprofloxacin (94.44%), tetracycline (61.11%) and cotrimoxazole (72.22%). The resistance profile of *Corynebacterium* sp, is as follows: cefuroxime (91.67%), ampicillin (95.83%), ceftaxidime (87.50%) and augmentin (87.50%). However, the organisms were susceptible to gentamycin (79.17%), ciprofloxacin (85.41%) and cotrimoxazole (68.75%). All Enterobacteriaceae were 100% susceptible to cotrimoxazole, gentamycin, cefotaxime, ofloxacin, augmentin, meropenem and ciprofloxacin.

Multiple antibiotic resistance profile of bacterial isolates from fomites in day care centers in Ile-Ife

The multiple antibiotic resistance patterns were calculated using the MAR index formular. The isolates with MAR index values higher than 0.2 were considered as multiple resistant. The classes of antibiotics used to investigate multiple resistance patterns include penicillins, macrolides, tetracyclines, sulfonamides, amino-glycosides, fluoroquinolones, beta lactams, glycopeptides and cephalosporins. The MAR (index) obtained from bacterial isolates from fomites in day care centers were observed to range from 0.18 to 1.0. The highest observed multiple resistant phenotype was found to exhibit resistance to 9 classes of antibiotics in *Bacillus* sp. All Gram positive strains exhibited different antibiotic resistance profiles with "AUG, AMP, CP, CRX AND CPZ" appearing most frequent. While for Gram negative, *Enterobacter agglomerans* was seen to be resistance to tetracycline and trimethoprim. This is shown in Table 3.

Frequency of ESBL - producing gram negative bacteria

The prevalence of ESBL by the double disk synergy test (DDST) showed that 5 (80%) from fomites were ESBL producing strains (Table 4).

DISCUSSION

Children, especially children in day care centers aged three years and under, have shown to have a high frequency of infectious disease than children cared for elsewhere. This could be because of direct transmission between children, workers, contaminated fomites, contact or respiratory droplet transmission (Ibfelt et al., 2015). An increased prevalence of antibiotic resistant organisms among children attending child care compared with children cared for at home may be expected considering the more frequent use of antibiotics, the gathering of large numbers of susceptible children, and the increased prevalence of infectious diseases in child-care settings (Adedire et al., 2016). The frequency of bacteria isolated from fomites was also very high. It was found to be 132 (27.22%). This corroborates with Risan (2017) who reported that opportunistic pathogens such as bacteria, viruses and fungi can survive on inanimate surfaces for long periods of time and items such as watches, pens, toys, floor, door handles and mobile phones are

Table 3. Multiple Antibiotic Resistance (MAR) Profile of Bacteria Isolates from Fomites in Day Care Centers in Ile-Ife.

Isolate	No. of antibiotics used (b)	No. of resistant isolates (a)	MAR Index a/b	Multiple resistance pattern	No. of MAR Pattern	Frequency	Total no of MAR isolates (%)
	11	2	0.18	AMP, AUG		1	6(4.92)
		3	0.27	AMP, AUG, CP	5	1	
S. aureus		6	0.55	AMP, AUG, CP, CPZ,CRX, VAN		2	
		7	0.64	AMP, AUG, CP, CPZ, CRX, ERY, VAN		1	
		8	0.73	COT, AMP, AUG, CP, CPZ, CRX, ERY, VAN		1	
		2	0.18	GEN, AMP	11	1	16(13.11)
		5	0.45	AMP, AUG, CP, CPZ, CRX		1	
		6	0.55	CRX, AMP, ERY, AUG, CP, VAN		2	
				COT, AMP, ERY, AUG, CP, VAN		1	
				COT, CRX, AMP, AUG, CP, VAN		1	
Staphylococcus sp.				AMP, AUG, CP, CRX, CPZ,VAN		1	
		7	0.64	AMP, AUG, CP, COT, ERY, TET, VAN		1	
				AMP, AUG, CP, CPZ, ERY, TET, VAN		3	
		8	0.73	AMP, AUG, CP.CPZ, CRX, COT, TET, VAN		3	
				AMP, AUG, CP,CPZ,CRX,ERY, TET, VAN		1	
		9	0.81	COT, AMP, AUG, CP,CPZ,CRX,ERY, TET, VAN		1	
		3	0.27	AMP, AUG, CP	23	1	53(43.44)
		4	0.36	AMP, AUG, CPZ, CRX		3	
				AMP, AUG, CRX, TET		2	
				AMP, AUG, CRX, COT		1	
				AMP, AUG, CP, CRX		1	
		5	0.45	AMP, AUG, CP, CRX, CPZ		16	
				AMP, AUG, CRX, TET, VAN		2	
				AMP, AUG, CP, CRX, TET,		1	
Bacillus sp.				AMP, AUG, CP, CRX, COT		1	
		6	0.55	AMP, AUG, CP, COT, CRX, VAN		8	
				AMP, AUG, CP,CRX, TET, VAN		3	
				AMP, AUG, CPZ, CRX, TET, VAN		1	
				AMP, AUG, CP, CPZ, CRX, VAN		1	
				AMP, AUG, CP, CRX, CPZ, ERY		2	
				AMP, AUG, CP, CRX, CPZ, TET		1	
		7	0.64	AMP, AUG, CP,CPZ,CRX, TET, VAN		1	
				AMP, AUG, CP, CPZ,COT, CRX, GEN		1	

Table 3. Cont'd.

			AMP, AUG, CP, CPZ, CRX, GEN, TET		1	
			AMP, AUG, CP, CPZ, CRX, GEN, VAN		1	
	8	0.73	AMP, AUG, CP,CPZ, CIP, CRX, ERY, VAN		2	
	9	0.82	AMP, AUG, CP, CPZ, COT, CRX, ERY, GEN, VAN		1	
			AMP, AUG, CP, CPZ, CRX, COT, ERY, TET VAN		1	
	10	0.90	AMP, AUG, CIP, CP, CPZ, CRX, COT, ERY, TET, VAN		1	
	2	0.18	AMP, CRX	26	1	46(37.70)
	3	0.27	AMP, CPZ, CRX		1	
			AMP, AUG, TET		1	
	4	0.36	AMP, AUG, CPZ, CRX		2	
			AMP, AUG, CP, CRX		1	
	5	0.45	AMP, AUG, CP, CPZ,CRX		2	
			AMP, AUG, CPZ, CRX, GEN		1	
			AMP, CP, CPZ,CRX, COT		1	
			AMP, AUG, CPZ, CRX, COT		1	
	6	0.55	AMP, AUG, CP, CRX, COT, VAN		1	
			AMP, CIP, CP, CPZ, COT, ERY		1	
			AMP, AUG, CP, CPZ, CRX, VAN		4	
			AMP, AUG, CP, CPZ, CRX, ERY		2	
Corynebacterium sp.			AMP, AUG, CP, CPZ, CRX, TET		1	
	7	0.64	AMP, AUG, CP, CPZ, CRX, ERY, VAN		8	
			AMP, AUG, CPZ, CRX, ERY, TET, VAN		1	
			AMP, AUG, CP, CPZ, COT, ERY, VAN		1	
	8	0.72	AMP, AUG, CP, CPZ, CRX, ERY, COT, VAN		3	
			AMP, AUG, CP, CPZ, CRX, ERY, TET, VAN		4	
			AMP, AUG, CP, CPZ, CRX, ERY, GEN, VAN		1	
	9	0.82	AMP, AUG, CRX, CP, CPZ, COT, ERY, GEN, VAN		1	
			AMP, AUG, CRX, CP, CPZ, COT, ERY, TET, VAN		1	
			AMP. AUG, CRX, CP, CPZ, COT, ERY, GEN, TET		1	
	10	0.90	AMP, AUG, CRX, CP, CPZ, COT, ERY, GEN, TET, VAN		1	
			AMP, AUG, CRX, CIP CP, CPZ, COT, ERY, TET, VAN		1	
	11	1.00	AMP, AUG, CRX, CIP CP, CPZ, COT, ERY, GEN, TET, VAN		3	
E. agglomerans	2	0.18	TET, TRI	1	1	1(0.82)

TET- Tetracycline, AMP- Ampicillin, AUG- Augmentin, CRX- Cefuroxime, CP- Cephalexin, CH- Chloramphenicol, COT- Cotrimoxazole, ERY- Erythromycin, GEN- Gentamycin, VAN- Vancomycin, CIP- Ciprofloxacin , TRI- Trimethoprim, MAR- Multiple antibiotic resistant, 'a'- number of antibiotics to which the isolates is resistant to, 'b' the number of antibiotic to which the isolates is exposed.

Table 4. Frequency of ESBL- producing bacteria isolated from fomites in day care centers in Ile-Ife.

Bacterial isolates	No. of isolates	ESBL positive (%)	ESBL negative (%)
Enterobacter clocae	1	1(1000	0
Enterobacter agglomerans	1	1(100)	0
Other *Enterobacteriaceae* sp.	1	1(100)	0
Providencia rettgeri	1	0	1(100)
Proteus sp.	1	1(100)	0
Total	5	4(80)	1(20)

permanent surfaces for transmission of these types of infections.

The prevalence of bacteria isolated from fomites in increasing order in this study is as follows. *Bacillus* sp 54 (40.90%), *C. xerosis* 29 (21.97%), *C. kutsceri* 19 (14.39%), *Staphylococcus* sp 18 (13.64%), *S. aureus* 7(5.30%), *E. clocae*, *E. agglomerans*, *P. rettgerri* and *Proteus* sp 1 (0.76%). *Bacillus* sp 54 (40.90%), was the highest bacteria recovered from the bacteria isolated from fomites in all the day care centers recruited for the study. This agrees with a study conducted by Ali et al. (2018) who reported *Bacillus* sp (66.66 %) to be the most commonly cultured bacteria from toys in child care centers, in Al-Rass city, Al-Qassim region. The reason for the high occurrence of *bacillus* sp in this study can be attributed to the fact that these microorganisms are spore forming organisms, rugged opportunistic bacilli and could be found easily in the environments. They are capable of forming endospores, which make them resistant to extreme conditions such as pressure, extreme heat or cold, drought, starvation, biocides, and UV irradiation (Gopal et al., 2015).

In this study, the frequency of gram-positive isolates was higher than gram-negative isolates. This is consistent with earlier studies conducted by Ayalew et al. (2019). The various bacteria such as *Bacillus* sp, *Staphylococus aureus*, *Staphylococus* sp, *Klebsiella* sp, *Proteus* sp, *Pseudomonas aeruginosa*, *Serratia* sp, *Escherichia coli* and *Enterobacter* sp isolated in this study are similar to the bacteria isolated by Adedire et al. (2016); except for *Corynebacterium* sp, which was found to be isolated in this study. No Vibrio sp was isolated in this study. The absence of this bacteria in this study corroborate with the findings of Adedire et al. (2016). The high occurrence of Gram positive bacteria over Gram negative bacteria agrees with the findings of Al-Harbi et al. (2017) who reported 80% of Gram positive bacteria and 20% Gram negative bacteria isolated from frequently used fomites in Kuwait. *Staphylococcus aureus* isolated from fomites was susceptible to gentamycin (100%), ciprofloxacin (100%), cotrimoxazole (85.7%) and tetracycline (85.7%) but resistance to ampicillin (85.7%), augmentin (85.7%); while *Bacillus* sp were 98.15% resistant to ampicillin, cefuroxime and augmentin. The results however agrees with the report of Afolabi et al. (2018) who reported 100% resistivity of *Staphylococcus aureus* to augmentin isolated from fomites in crèche. *Corynebacterium* sp were 87.50% and 95.83% to augmentin and ampicillin respectively. All Enterobacteriaceae isolated from fomites were susceptible to augmentin (100%), cefotaxime (100), nitrofurantoin (80%), ofloxacin (100%), gentamycin (100%), ciprofloxacin (100%), meropenem (100%), cotrimoxazole (100%) and chloramphenicol (100%). However; they were resistant to trimethoprim (40%) and tetracycline (20%). This result is different to the findings of Adedire et al. (2016).

The MAR index expressed by the Gram positive isolates was very high in all sampled locations and more than the 0.2 threshold value which is the set value for distinguishing low and high risk contamination (Krumperman, 1983).

Conclusion

Considering the various findings, the result of this study confirms that fomites in day care centers could serve as media for transmission of the disease. The micro-organisms pose health risk for immunocompromised children. These environments must improve the suitable hygiene procedures for protecting the children by ensuring that the workers observe stringent guidelines on proper washing and regular disinfecting of toys and beddings. The study concluded that the incidence of multiple antibiotic resistant bacteria isolated from fomites was high. Hence, this calls for great concern considering its implications in the day care centers studied.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest

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Prevalence of anemia and associated factors in HIV-1 infected children before and after initiation of antiretroviral therapy in Burkina Faso: A retrospective study

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Anemia is a public health problem and has significant adverse health consequences in HIV-infected persons. In Burkina Faso, there are little data on anemia in HIV-1 infected children. This study aimed to describe the prevalence of anemia and its associated factors in HIV-infected children before and after their highly active antiretroviral therapy (HAART). This was a retrospective study that involved a cohort of 151 HIV-1 infected children on HAART at the pediatric service of Saint Camille Hospital, from January 2018 to October 2018. Data were collected before and after their HAART initiation and analyzed using SPSS version 21.0. The prevalence of anemia was 81.46% before treatment and 48.34% 12 months after initiation of treatment. There was a significant association between gender, BMI, TB, and CD4 counts before HAART initiation While, after HAART initiation, only gender, age, and CD4 T cell count were significantly associated with anemia. Children with a CD4 count <200 Cell/µl had a risk of developing anemia before HAART initiation but no longer had a risk of developing anemia 1 year after HAART initiation [OR=0.35 (0.14-0.89); p=0.028 vs. OR=1.54 (0.67-3.51); p=0.300].This study showed that antiretroviral treatment contributes strongly to improving the hemoglobin level in persons living with HIV.

Key words: HIV, anemia, prevalence, highly active antiretroviral therapy (HAART), Children, Burkina Faso.

INTRODUCTION

The number of children (0-14 years) infected with HIV was estimated to be 1.7 million [1.2 million- 2.2 million] in 2020 (UNAIDS, 2021). HIV infection causes several complications, including anemia (Cao et al., 2022). Anemia defined as reduced hemoglobin levels of red blood cells may carry less oxygen to skeletal muscle and impair physical performance (Tsai et al., 2019). Anemia is a risk factor for death in HIV-1 infected individuals (Harding et

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al., 2020). In Burkina Faso, the prevalence of anemia in children aged 6-59 months was 87.0% in 2020 (Muriuki et al., 2020). In children, depending on the hemoglobin level, anemia can be considered severe (Hb level<7 g/dL), moderate (Hb level [7-9.9] g/dL), and mild (Hb level [10-11] g/dL). According to WHO, Africa represented the continent with the highest rate of anemia in children aged 6-59 months (60.2%), followed by Southeast Asia (49.0%) (WHO, 2021a).

In Africa, anemia remains the most frequently observed hematologic abnormality and an independent predictor of disease progression in people living with HIV, particularly in children and most people infected with HIV-1 are anemic (Abioye et al., 2020; Duffy et al., 2020). However, the risk of anemia is reduced after the initiation of highly active antiretroviral therapy (HAART) (Yesuf et al., 2019). Indeed, studies have shown that antiretroviral therapy helps to improve anemia and reduce mortality in children (Haider et al., 2019; Duffy et al., 2020). Moreover, antiretroviral treatment (ART) is known to profoundly suppress viral replication. It increases CD4 cell count, delays disease progression and death; patients on highly active antiretroviral therapy commonly suffer from side effects of the drugs (Geletaw et al., 2017; Mouhari-Toure et al., 2018). Each antiretroviral (ARV) drug is associated with specific adverse effects and among the ARV drugs, zidovudine (AZT) remains the most widely used drug, resulting in myelosuppression (Leroi et al., 2017) and has been associated with anemia (Tamir et al., 2018).

Studies have identified factors associated with anemia in HIV-infected children such as age, low CD4 percentage, WHO clinical stage, and Iron deficiency (Bisong et al., 2017; Lai et al., 2018; Huibers et al., 2020). A study in Uganda showed that in patients who started or continued ARV therapy, CD4 counts increased significantly over 18 months of follow-up, and the improvement did not differ by baseline ferritin level or anemia status (Ezeamama et al., 2019).

In Burkina Faso, there are little data on the effect of ARV treatment on anemia in HIV-1 infected children as well as the associated factors. However, having information on this would help in the better therapeutic and clinical management of children. So, the objective of this study was to describe the prevalence and associated factors of anemia in HIV-infected children before and after their HAART.

MATERIALS AND METHODS

Study population and data collection

This was a retrospective study whose data were collected from the records of HIV-1 children on HAART at the pediatric ward of Saint Camille Hospital in Ouagadougou, Burkina Faso during the period from January 2018 to October 2018. This study involved 151 HIV-1 infected children. Data were collected before and 12 months after initiation of HAART and included: socio-demographic, clinical, hemoglobin (Hb), and immunologic characteristics. Hemoglobin and CD4 T cell counts were determined using the CELL-DYN Ruby (Abbott, Illinois, USA) and the BD FACSCOUNT (Becton Dickenson, California, USA) respectively. To ensure good quality results, the data were collected through a questionnaire form and incomplete data were eliminated during processing.

Definition of variables and data analysis

Based on hemoglobin level, anemia was classified as severe (Hb level<7 g/dL), moderate (Hb level [7-9.9] g/dL), and mild (Hb level [10-11] g/dL). Body mass index (BMI) was classified into normal body weight (18.5 - 24.9), underweight (<18.5), and overweight (≥25) according to WHO, and the calculation was based on the formula BMI= (weight in Kg/height in m^2) (WHO, 2021b). Bivariate analysis and multivariate logistic regression were performed using IBM SPSS version 21.0 software and any value was considered statistically significant for $p<0.05$. Odds ratios with confidence intervals (95% CI) were used to determine the association between anemia and potential risk factors.

RESULTS

General characteristics of the study population

The socio-demographic and clinical characteristics of the study population at the time of initiation of ARV treatment are shown in Table 1. Female children were more represented (54.3%) than males (45.7%) with a sex ratio of 0.84 and the mean age was 10.04±3.7 years. The most represented age group was 5-11 years (58.28%). The majority of HIV-1 infected children (70.86%) were underweight at the start of ARV treatment. Approximately, 64.9% of the children were in WHO clinical stages I and II and 25.83% of them had a CD4 count below 200 cells/µl (Table 1).

Prevalence of anemia before and after HAART initiation

Figure 1 shows the severity of anemia in HIV-1 infected children before and after initiation of HAART. The prevalence of anemia was 81.46% (123/151) before treatment and 48.34% (73/151) 12 months after initiation of treatment. About 5.96% of the children had severe anemia, 50.33% had moderate anemia and 25.16% had mild anemia before initiation of HAART (Figure 1). Among the children with anemia before starting ARV treatment, 97.10% (67/69) were male and 68.29% (56/82) were female. This study shows that all children under 5 years of age (100%) and 86.92% of those who were underweight developed anemia. Also, anemia was found in all HIV-1 infected children with CD4 T cell count between 200 and 350 cells/µl (Table 2). In contrast, 12 months after initiation of HAART, the prevalence of anemia decreased overall. It was 63.49% (40/63) in male children and 37.5% (33/88) in female children. The age group of 5-11 years was the most anemic (60.23%).

Table 1. Sociodemographic and clinical characteristics of HIV-1 infected children.

Characteristics		Number	Percentage
Sex	Male	69	45.7
	Female	82	54.3
Age (years)	< 5	16	10.6
	5-11	88	58.28
	12-14	47	31.12
WHO clinical stage	I and II	98	64.9
	III and IV	53	35.1
BMI (Kg/m^2)	Normal weight	37	24.5
	Underweight	107	70.86
	Overweight	7	4.64
Tuberculosis	Yes	7	4.64
	No	144	95.36
CD4 (cell/µL)	< 200	39	25.83
	200-350	33	21.85
	≥ 350	79	51.32

BMI; Body Mass Index.
Source : Serge Theophile Soubeiga.

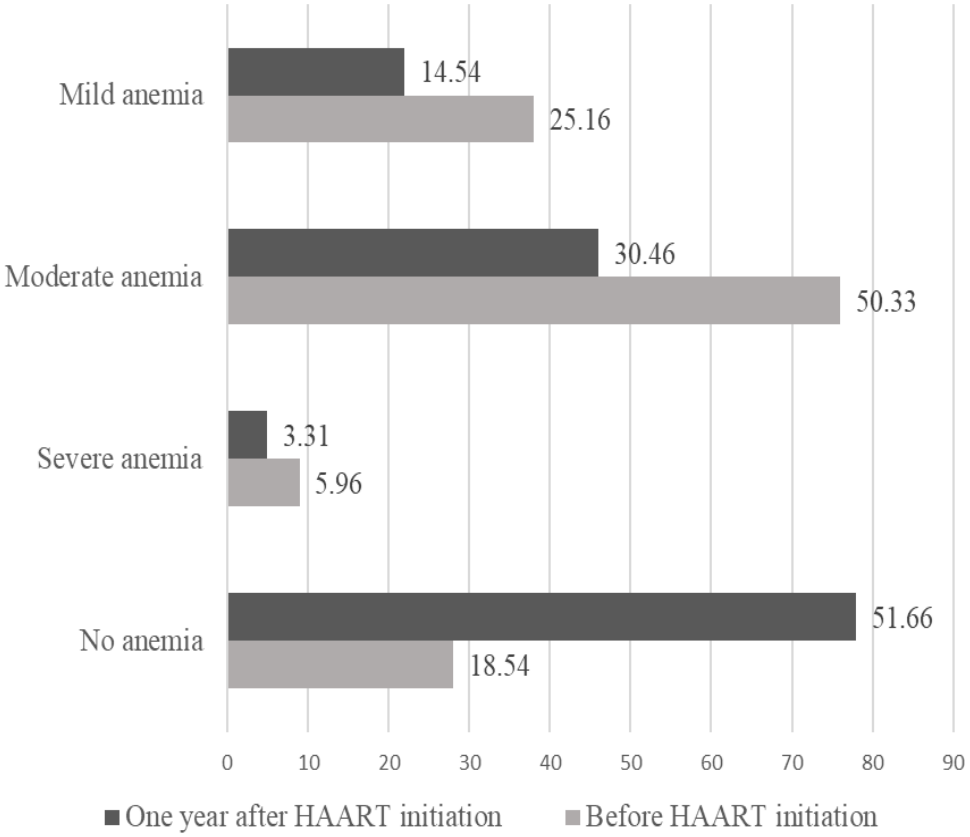

Figure 1. Severity of anemia in HIV-1 infected children before and after HAART initiation.

Table 2. Sociodemographic and clinical characteristics by anemia before ART initiation in HIV-1 infected children.

Characteristics		Anemic (n=123)	Non- anemic (n=28)	p-value
Sex	Male	67 (97.10)	2 (2.90)	<0.001
	Female	56 (68.29)	26 (31.71)	
Age (years)	< 5	16 (100)	0 (0,00)	0.110
	5-11	71 (80.7)	17 (19.3)	
	12-14	36 (76.6)	11 (23.4)	
WHO clinical stage	I and II	81 (82.65)	17 (17.35)	0.607
	III and IV	42 (79.25)	11 (20.75)	
BMI (Kg/m^2)	Normal weight	17 (79.91)	6 (26.09)	0.054
	Underweight	102 (85.00)	18 (15.00)	
	Overweight	4 (57.14)	3 (42.86)	
Tuberculosis	Yes	3 (42.86)	4 (57.14)	0.028
	No	120 (81.08)	24 (18.92)	
CD4 (cell/µl)	< 200	31 (79.5)	8 (20.5)	0.007
	200-350	33 (100)	0 (0.00)	
	≥ 350	59 (74.7)	20 (25.3)	

Source : Serge Theophile Soubeiga.

Similarly, 46.97% (31/68) of HIV-1 infected children in whom treatment was not AZT-based developed anemia (Table 3).

Factors associated with anemia before and after HAART

Analysis of the data showed a significant association between gender, BMI, TB, and CD4 counts before HAART initiation, but there was no association between age, WHO clinical stage, and anemia (Table 2). After HAART initiation, gender, age, and CD4 T cell count were significantly associated with anemia. However, there was no association between WHO clinical stage, BMI, ARV treatment, and anemia (Table 3). Female children had a 16-fold [OR=16.07 (3.65-70.71; p<0.001] risk of developing anemia before HAART initiation but this risk significantly decreased 1 year after HAART [OR=0.41 (0.19-0.85), p=0.017]. Children with a CD4 count <200 Cell/µl had a risk of developing anemia before HAART initiation but no longer had a risk of developing anemia 1 year after HAART [OR=0.35 (0.14-0.89); p=0.028 vs. OR=1.54 (0.67-3.51); p=0.300] (Table 4).

DISCUSSION

This study showed a high prevalence (81.46%) of anemia in HIV-1 infected children before initiation of HAART, which is lower than the prevalence of 87.0% found in children in 2020 from Burkina Faso in a study conducted in African children but higher than the prevalence of anemia found other countries (70.0% in Kenya; 49.7% in Uganda; 60.1% in Gambia) (Muriuki et al., 2020). This high prevalence of anemia confirms that the situation remains concerning in Central and Western Africa. Indeed, children in developing countries have a high prevalence of anemia due to poverty, a high burden of infectious diseases whose inflammation is a primary contributor to anemia (Mantadakis et al., 2020). In this study, the prevalence of anemia decreased 12 months after treatment initiation. It decreased from 81.46 to 43.34%, that is, a reduction of 33.90% in one year. This prevalence is nevertheless higher than that found in several countries: 11.4% in Southern Ethiopia (Fenta et al., 2020); 54.2% in Nigeria (Ahumareze et al., 2016) but remains lower than 49.6% found in Cameroon (Bate et al., 2016) and 88% in Mozambique (Duffy et al., 2020). These differences could be due to the methodologies used, the heterogeneity of the populations, and the nutritional status of the children. Indeed, a study conducted in Sub-Saharan Africa among children aged 6-59 months revealed a difference in the prevalence of anemia between countries. West Africa had the highest prevalence (70-88%), followed by Central Africa (63-67%) and East Africa (38-69%) (Tesema et al., 2021). In this study, the decrease in anemia after 1 year of HAART truly demonstrates the involvement of ARV therapy in improving hemoglobin levels. Indeed, studies have shown that ARV therapy contributes to improved anemia

Table 3. Sociodemographic and clinical characteristics according to anemia 12 months after initiation of HAART in HIV-1 infected children.

Characteristics		Anemic (n=73)	Non- anemic (n=78)	p-value
Sex	Male	40 (63.49)	23 (36.51)	0.001
	Female	33 (37.5)	55 (62.5)	
Age (years)	< 5	5 (45.45)	11 (54.55)	0.002
	5-11	53 (60.23)	35 (39.77)	
	12-14	15 (31.91)	32 (68.09)	
WHO clinical stage	I and II	43 (45.74)	51 (54.26)	0.411
	III and IV	30 (52.63)	27 (47.37)	
BMI (Kg/m^2)	Normal weight	56 (44.07)	62 (55.93)	0.627
	Underweight	13 (56.52)	10 (43.48)	
	Overweight	4 (40.0)	6 (60.0)	
Treatment	AZT based	42 (49.41)	43 (50.59)	0.501
	Non AZT based	31 (46.97)	35 (53.03)	
CD4 (Cell/µl)	< 200	25 (65.79)	13 (34.21)	0.001
	200-350	12 (27.27)	32 (72.73)	
	≥ 350	36 (52.17)	33 (47.83)	

Source : Serge Theophile Soubeiga.

Table 4. Logistic regression of risk factors for anemia in HIV-1 infected children before and 1 year after initiation of HAART.

Characteristics		Before HAART initiation		1 year after HAART initiation	
		Odds ratio (IC 95%)	p-value	Odds ratio (IC 95%)	p-value
Sex	Male	1.00		1.00	
	Female	16.07 (3.65-70.71)	**<0.001**	0.41 (0.19-0.85)	**0.017**
WHO clinical stage	I and II	1.00		1.00	
	III and IV	0.80 (0.34-1.86)	0.667	1.48 (0.61-3.56)	0.382
CD4 (cell/µl)	≥350	1.00		1.00	
	200-350	0.50 (0.10-2.50)	0.403	5.76 (1.83-18.11)	**0.003**
	<200	0.35 (0.14-0.89)	**0.028**	1.54 (0.67-3.51)	0.300

Source : Serge Theophile Soubeiga.

and reduced mortality in children (Haider et al., 2019). This is confirmed by the decrease in the rate of severe anemia (5.96% before HAART vs 3.31% after HAART). Our results are in agreement with other studies (Geletaw et al., 2017; Beletew et al., 2020). Authors have shown the reduction of anemia 6 months, 12 months, 18 months, 24 months, and 30 months after AZT-based ART treatment (Getaneh et al., 2021). While our study found a higher rate of anemia in children on HAART with AZT (64.2%) than in those on non-AZT HAART (51.11%). The factor that may have contributed to the observed high prevalence of anemia is the possible effect of AZT. Side effects such as myelotoxicity, mitochondrial toxicity, myopathy, and incidence of anemia associated with AZT use in people living with HIV have been reported in several studies (Tamir et al., 2018). AZT is known to be associated with life-threatening hematological toxicity like anemia due to early long-term higher-dose therapy. AZT also causes bone marrow suppression, which causes anemia (Getaneh et al., 2021).

In this study, the under-5 years' age group was most affected by anemia before HAART initiation. This confirmed that children under 5 years of age in developing countries are more vulnerable to severe anemia (Kejo et al., 2018) certainly due to malnutrition, malaria, and opportunistic infections (Bate et al., 2016; Wagnew et al., 2019). The 5-11-year-old was most affected 1 year after HAART treatment in contrast to the under 5-year-old. This was also found in a study conducted in Cameroon (Bate et al., 2016). There was an association between gender, BMI, tuberculosis, and CD4 T-cell count with anemia before initiation of HAART in children. Indeed, opportunistic infections and immune system failure are factors that can promote anemia. One study reported that tuberculosis and malaria were associated with anemia in HIV-1 infected children (Huibers et al., 2020). In terms of BMI, anemia was found most in underweight HIV-1 infected children. Indeed, the hemoglobin level is an important biological parameter that, when it is reduced, can affect growth, especially in children (Ibrahim et al., 2017).

Children with CD4 counts < 200 Cell/µL and 200-350 Cell/µl had a 0.35- and 5.76-fold risk of developing anemia before initiation and 1 year after HAART, respectively, as a study in Ethiopia showed that immunocompromised children were at higher risk of developing anemia than those with normal immunity (Techane et al., 2020). Tuberculosis, zidovudine-based drugs, severe immunosuppression, and undernutrition have remained predictors of anemia among children on antiretroviral therapy (Atalell et al., 2018; Techane et al., 2020).

In general, HAART reduces the incidence of anemia by suppressing viral replication and increasing CD4 cell counts (Beletew et al., 2020; Getaneh et al., 2021). In addition to HAART, all HIV-infected children received Cotrimoxazole prophylaxis to prevent other opportunistic infections. This treatment could contribute to the reduction of anemia in children. Indeed, cotrimoxazole may have a direct impact on erythropoiesis by reducing the number of red blood cells (Bouyou Akotet et al., 2018). This study showed important results but has presented some limits. Outside of tuberculosis, there was no existing data on other diseases that could influence the hemoglobin level of children before their treatment and we did not identify the exact cause of anemia. This study confirmed with many authors that antiretroviral treatment contributes strongly to improving the hemoglobin level in persons living with HIV. However, there is the need to enhance the management of HIV-infected children to restore their immune system, monitor their nutritional status, and prevent opportunistic infections.

Ethics approval

This study received approval from the Burkina Faso Health Research Ethics Committee (deliberation N° 2014-7-084). Anonymity and confidentiality were respected and the results were used to improve the clinical management of the children included in the study.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Morphological and molecular characterisation of termite species in Taita Taveta County, Kenya

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The diversity of termites remains obscure despite having seized the equatorial ecological community with numerous effects both environmental and solvent. In the current study, morphology and DNA based techniques were used to appraise the heterogeneity of termites in Taita Taveta County. Soldier termites were arbitrarily fragmented from the five main plant lives in the study region. A dissecting microscope was used to discern the external features of the termites and was noted. The features were compared to the identification keys and then transformed into quasi-characters to generate a dendrogram. DNA was isolated from the 31 specimens from which mitochondrial cytochrome oxidase II (COX II) gene was sequenced and probed. We tested whether or not molecular characterisation underpins morphological characterization and then obtained similar sequences from the Genebank Repository of National Center for Biotechnology Information (NCBI) and used them together with our sequences for the phylogenetic tree construction. The results were supported by genetic specifications such as nucleotide composition and dichotomized genetic range. Termite of three different genera; *Macrotermes, Amitermes* and *Odontotermes* were identified based on their mound structure and soldier morphology. Phylogenetic analysis also placed the termites into three clusters, which were affiliated with the genera above. Genera *Amitermes* and *Odontotermes* were confined to distinct vegetation whereas the genus *Macrotermes* were all-pervasive. The study confirmed that both approaches identified the termite genera but a combination of both is necessary for confirmation and higher taxonomic classification.

Key words: COII, diversity, Macrotermitinae, termites, phylogeny.

INTRODUCTION

Termites (Isoptera) are known both as ecological drivers and significant menace to the environment (Vidyashree et

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al., 2018). They contribute to various ecosystem processes such as decomposition of organic matter into nutrients, and also modify soil properties decomposition of organic matter and nutrient cycling (Arif et al., 2019). Moreover, their termitaria provide special microhabitat for a number of organisms (Enagbonma and Babalola, 2019). However, they are also pests of agricultural crops and household items made of cellulosic material, a trait that tends to conceal their role in offering ecosystem services (Jouquet et al., 2018). Both positive and negative effects of termites arise from their feeding activities abetted by symbiotic microorganisms such as fungi and bacteria which facilitate cellulose digestion (Adaobi and Abiye, 2019).

Identification of termites still poses challenges due to inadequate taxonomic knowledge as well as their eusocial behavior coupled with distinct caste system (Murthy, 2020). The latter is because termites present limited or no species-specific morphological traits which allows identification only up to the genus level (Korb et al., 2019). In addition, there is a shortage of experts in most of the equatorial and subtropical countries (Mugerwa et al., 2014). As a result, there is scanty information on prevalence, identity, diversity and distribution of termites in Kenya and the African continent as a whole. Research leading to character level designation is thus necessary for accurate biodiversity assessment (Murthy et al., 2017).

Currently there are about 3106 sentient and remnant identified termite species (Effowe et al., 2021) allied to 330 genera, 12 families, 21 subfamilies and eleven families (Krishna et al., 2013). The order Isoptera is formally divided into inferior (underdeveloped) and superior (more advanced) termites (Zhu et al., 2012). The latter belong to the family Termitidae which have varying and sometimes conflicting characteristics. Members in the family Termitidae are capable of building termitaria albeit some just make galleries and tunnels underground. As a matter of fact, the family Termitidae contains the greatest number of studied genera and described species that is more than 2/3 in each case (Kanwal et al., 2011). The African continent leads in the number of species and this is due to its favourable climatic conditions (van Huis, 2017). There are over 650 species in the family Termitidae. The subfamilies under this family have grown to eight over the years (Engel, 2011). Majority of the described species belong to the eight families. East Africa alone has over 177 termite species and more could yet be identified (Ahmed et al., 2011). The lower termites which are majorly dependent on protozoa and bacteria (Huda et al., 2019) are*; Rhinotermitidae, Kalotermitidae, Hodotermitidae, Termopsidae, Archotermopsidae, Stolotermitidae, Stylotermitidae, Serritermitidae and Mastotermitidae.* The number of species in these families reduces respectively with the last two families having only one species each (Namanda, 2015; Nkunika, 1998).

External morphology of termites has for a long time been given importance in systematics and classification studies (Mandal et al., 2014). Taxonomic characters found in soldier caste present conspicuously reliable features for diversity investigations (Himmi et al., 2020). However, without keen conduction, morphological identification could present some ambiguity when solely applied. It should therefore be complemented with other methods (Patel and Jadhav, 2019). Many studies have opted to couple morphological identification with molecular characterization to avoid opaque identification (Schyra et al., 2019). The use of mitochondrial DNA (mtDNA) sequences has been successfully complemented with morphological characterisation for species identification. Mitochondrial COII gene in particular has been established to be reliable in species identification of members of different invertebrate groups as well as in establishing phylogenetic relationships among these groups (Garrick et al., 2015). To analyze mtDNA, little DNA samples is required and gives accurate results (Kapli et al., 2017); thus it remains a marker of choice for species identification. In addition, the COII gene has adequate representation of posted sequences in the GenBank database (Ghesini et al., 2020). This study aimed to determine termite colonies of the equatorial savannah of Taita Taveta County, Kenya based on the two approaches.

MATERIALS AND METHODS

Study site and sample collection

Collection of termites was done in four main districts of Taita Taveta County. The county is situated northwesterly of Mombasa city in the coast and 360 km southeast of Nairobi. Its expanse stretches an area of about 17,000 km^2 and is within a tropical savannah with various plant life systems. We highlighted Herbaceous Grassland (HG) [Mwatate district Latitude: -3° 29′ 59.99″ S, Longitude: 38° 22′ 59.99″ E]; Rain Forest (FS) [Wundanyi district Latitude: -3° 24′ 6.95″ Longitude 38° 21′ 50.47″]; Shrub Savanna (SV) [Voi district Latitude: -3° 23′ 45.78″ S, Longitude: 38° 33′ 21.92″ E]; Rain Fed Trees (RFT) [Taveta district Latitude: -3° 39′ 63.20″ S, Longitude: 37° 67′ 36.20 E″]; and Disturbed Areas (DL) all over of the county.

Aggregately, 54 active termite assemblages from the highlighted plant life systems were haphazardly chosen for sampling. Within each vegetation type we sampled mounds, which were at most 100 m apart. To obtain the exact points of reference of the collection points, Gamin software was installed in the phone and used to take the points (Table 1). Live termitaria were able to be distinguished by the absence of plant establishment as they cause developing soils around to harden and become impregnable (Yamashina, 2013). In order to get to the termite territory, the mounds had to be dug to a vertical extent of 50 cm downwards or depending on how far the nest was. 20 soldier termites were picked using pincers and preserved in sterile falcon tubes containing absolute ethanol. The collected samples were put in a cool box after which they were taken to the institution's research laboratory. In the laboratory, they were kept in the refrigerator for subsequent morphological and molecular characterization.

Table 1. GPS co-ordinates of termite sampling locations in Taita Taveta County in July 2013 and February 2014.

Sample site	Vegetation type	Latitude	Longitude
Mwatate TTUC	Disturbed land	3° 25' 17.24″ S	38° 30' 13.15″ E
Mwatate TTUC	Disturbed land	3° 25' 10.21″ S	38° 30' 07.98″ E
Mwatate TTUC	Herbaceous grassland	3° 25' 10.21″ S	38° 30' 07.98″ E
Mwatate TTUC	Disturbed land	3° 25' 10.22″ S	38° 30' 07.75″ E
Mwatate TTUC	Disturbed land	3° 25' 09.10″ S	38° 30' 07.11″ E
Mwatate KHS	Disturbed land	3° 29' 29.92″ S	38° 30' 08.44″ E
Mwatate KHS	Disturbed land	3° 22' 28.81" S 38°27'38.39'' E	38° 22' 40.40" E
Tausa	Rain fed trees	3° 20' 07.08" S	38° 29′ 41.02″ E
Konenyi	Rain fed trees	3° 20' 07.08" S	38° 29' 42.02 " E
Tausa	Rain fed trees	3° 20′ 06.59″ S	38° 29′ 41.94″ E
Konenyi	Rain fed trees	3° 22′ 28.81″ S	38° 27′ 38.39″ E
Mwambirwa	Forest	3° 21′ 11.36″ S	38° 25′ 54.36″ E
Taveta town	Disturbed land	3° 23′ 47.54″ S	37° 40′ 42.60″ E
Malukiloriti	Shrub savanna	3° 21′ 59.60″ S	37° 42′ 24.42″ E
Malukiloriti primary school	Disturbed land	3° 21′ 47.51″ S	37° 42′ 22.99″ E
Timbila	Rain fed trees	3° 23′ 52.49″ S	37° 43′ 05.09″ E
Luduwhai	Rain fed trees	3° 25′ 13.28″ S	38° 10′ 09.94″ E
Salaita	Shrub savanna	3° 25′ 13.28″ S	37° 45′ 51.02″ E
Bura forest	Forest	3° 30′ 30.24″ S	38° 22′ 41.01″ E
Mwaktau (Tsavo west)	Shrub Savanna	3° 25′ 13.28″ S	38° 10′ 19.94″ E
Tsavo east	Shrub savanna	3° 20′ 07.08″ S	38° 29′ 42.02″ E
Tsavo east	Shrub savanna	3° 22′ 28.81″ S	38° 27′ 39.38″ E
Buguta	Disturbed land	3° 40′ 59.35″ S	38° 29′ 41.94″ E
Tsavo East	Herbaceous grassland	3° 22′ 05.48″ S	38° 35′ 01.11″ E

Assessment of mound characteristics, morphological characterisation and analysis

Termite mound structure and features including height; size, presence and absence of ventilations, as well as shapes of the mounds were observed and noted. These features were collated with the identification features posted by Roonwal (1977). Morphological characterisation of soldier termites was conducted under a dissecting microscope. We measured the breath and linear distance of the soldier heads, entire linear distance, breath and linear distance of prothorax as well as the shape, and similarly for the mesothorax and metathorax. Apart from these characteristics, we also used; sensory features on the head by counting them and the parts thereof, head features including size and shape and maxillary characteristics. A combination of these characteristic features was employed in the generic level classification and identification of the termite samples. From every active mound sampled, only four termites were used to take measurements and features, and an average of the four was worked out. A high accurate vernier caliper (Mitutoyo 530-312) was used for taking measurement. We used the criterion published by Sornuwat et al. (2004) and Wijerathna and Dias (2012) respectively to ultimately identify the termites to the genus level. Further, Euclidean distance measure was employed and a hierarchical cluster analysis with average linkage method was performed. The impact brought about by scale was avoided by standardization of crude phonological information observed to zero mean and unit variance. The resulting covariance was employed in working out the interrelation amidst termite communities with cluster examination through hierarchical cluster analysis (HCA) with UPGMA (unweighted pair-group method using arithmetic averages). Paired range for all the sample (covariant equal advantage) were calculated. This was in a bid to establish those specimens with the smallest genetic range, which was done repeatedly for successive couples of specimens having trivial pair-wise range. These ranges are thus grouped repeatedly in a continuous manner. Proceeds from this continuous clustering were concocted to generate a rooted dendrogram with branching values using Euclidean diversity estimate in DARwin v.6.1 software. The dendrogram was generated using GenStat 16th Edition statistical software. Quality pictures of the exemplary termite galleries and termite samples were taken with an electric camera (OPPO A 93). A comparison of our findings with the records available in the National Museum of Kenya was done after which our representative specimens bearing the name and identity number of the researchers were conserved at the Entomology Department of the Kenyan archives.

DNA based delineation

Extraction of DNA

Slightly moderated Phenol:Chloroform method (Sambrook et al., 1989) of DNA extrication was used to extract complete DNA from

the heads of soldier termites. The heads were sterilized, combined and crushed in 200 µl of TE (Tris EDTA pH 8) with mortar and pestle. For easy and faster disintegration of the tissues, we used 500 µl of lysis buffer (400 µl of TE and 100 µl of 5% SDS) and 10 µl of Proteinase K. The blend was inoculated at 65°C for 1 h. A mixture of 120 µl of phenol: chloroform: isoamyl alcohol (25: 24: 1) was put in the tubes followed by swirling for 30 s, and then centrifugation for 10 min at 10,000 rpm. The uppermost watery film was cautiously taken and put in a sanitized Eppendorf tube ensuring that the protein layer remained intact at the interphase. For precipitation of DNA, 500 µl of isopropanol was appended to the separated watery film and stored at -4°C for a whole night after which centrifugation at 12,000 rpm for 10 min was done. This led to the formation of a pellet which was cleaned severally by first getting rid of the supernatant followed by rinsing with 70% ethanol and sterile water. The resulting pellet was dried in air for half an hour or thereabout and eluted in 40 µl of TE buffer to stabilize it against disintegration. To confirm whether the gDNA was up to standard, we ran the extract in 0.8% agarose gel. The DNA was stored at -20°C for subsequent use (Sambrook et al*.,* 1989).

Amplification and sequencing of COII gene

Using these set of primers; A-tLeu (5'-CAG ATA AGT GCA TTG GAT TT-3') and B-tLys (5'-GTT TAA GAG ACC AGT ACT TG-3'), forward and reverse directions respectively (Miura et al., 1998), the extracted genomic DNA was used as a prototype to multiply the targeted COII gene portion. The mix therefore comprised of 2.5 µl of 10X PCR buffer, 2.0 µl $MgCl_2$ (2.5 mM), 2.0 µl dNTPs (200 µM), 0.25 µl of *Taq* Polymerase (5 U/µl), 1 µl of each forward and reverse (5 Pico moles) and 0.5 µl of DNA. This was topped up with 15.75 µl of PCR water making a final volume of 25 µl. A reaction check with all the components except the prototype was also set for internal validity. Temperature cycles consisted of; primary denaturation at 95°C for five minutes, denaturation at 95°C for 30 s, annealing at 52°C for 45 s, and extension at 72°C for 1 min using automatic PCR unit. This was repeated 30 times, followed by 5 min of extension at 72°C. The resulting PCR by-products were authenticated by gel electrophoresis using 1.5% agarose in 1x TAE buffer stained with ethidium bromide and visualized under ultraviolet light. The by-products were refined using the QIAquick PCR purification Kit protocol (Qiagen, Germany). The refined by-products were preserved at - 20°C. This was then sent for sequencing through international courier services to Macrogen Netherlands, which is a commercial service provider (http://www.macrogen.com). The amplified DNA fragments were sequenced employing Sanger sequencing, the most common dideoxy method using COII universal primers, whereby there was use of redesigned dideoxy bases (ddNTP's). The sequence data of the termites were recovered in the form of chromatograms which were extracted and saved for editing and further analyses.

Phylogenetic analyses

Out of the effectual 31 sequences amplified, only four were utilized in the eventual probe to maximize on the quality of the results. These sequences were manually edited in CLC main workbench version 7.2.6. It was done by first trimming the sequences to eliminate reads of poor quality, followed by assembling the forward and reverse sequences to align them where they overlap to get a contiguous sequence. Conflicts arising as a result of mismatch in overlapping regions of the forward and reverse sequences were assembled. Using Mallard software (Ashelford et al*.,* 2006), we looked out for any chimerical structures and artefacts present. The edited sequences were submitted to the Genebank to obtain the following accession numbers; KT845956, KT845957, KT845958, KT845959, KT845960, KT845961, KT845962 and KT845963. Further, we did an exploration of identical sequence arrangement using BLASTN. Closest relatives of the sequences with 99-100% identity were retrieved from the GenBank for subsequent phylogenetic analysis. The sequences were aligned with the help of CLUSTAL Omega program (http://www.clustal.org). The evolutionary history was hypothesized employing UPGMA formula (Sneath and Sokal, 1973) after which it was possible to highlight optimal tree with total branch length that was equal to 1.77017127. The rate percent of duplicate trees in which the related species were grouped simultaneously in the bootstrap test (1000 replicates), were presented above the nodes (Tamura and Kumar, 2002). The tree was drawn to scale, using similar units like the units of the branch length in evolutionary distances for phylogenetic tree inference. Using Maximum Composite Likelihood formula (Tamura et al., 2004), we determined the genetic range in the units of the number of base interchanges in each site. The differentiation in constitution partiality among sequences was given consideration in evolutionary differentiation (Tamura and Kumar, 2002). We incorporated 15 homolog sequences. Codon sites considered were 1st+2nd+3rd. For every set of sequences, all obscure sites were eliminated leaving 682 sites in the eventual data-set. MEGA 7 (Kumar et al., 2016) was used to do the evolutionary analysis. To construct the tree, we exported the aligned sequences to MEGA and converted them into Mega format. We then set the analysis parameters like bootstrapping values (in this case 1000 times) as well as appropriate substitutional model and left it to compute and finally saved the generated tree. All prototypical sequences were retained in a collective database (GenBank) for easy access by interested stakeholders.

RESULTS

Morphological identification of termites

In this study it was observed that termite mounds were present in all the types of plant life surveyed. Collectively, 54 active termitaria were documented. Of the total number, 17 were located in the herbaceous grassland vegetation, seven in the rain-fed trees, another seven mounds in the shrub savannah vegetation and six mounds forested in the vegetation. The disturbed land recorded 17 active termite mounds. It was noted that in the disturbed areas such as the area occupied by the institutions, there were more active mounds compared to farmlands, which had mounds that were either destroyed or inactive. Despite encountering few mounds in the rain-fed trees and shrub savanna vegetation, occurrence of inactive mounds was uncommon. In the herbaceous grassland vegetation type (the most dominant vegetation in the County), the active mounds were dominant.

Mound architectural characteristics

A comprehensive analysis of the mound characteristics revealed the presence of three types of mounds. These were regular and irregular conoid mounds with several

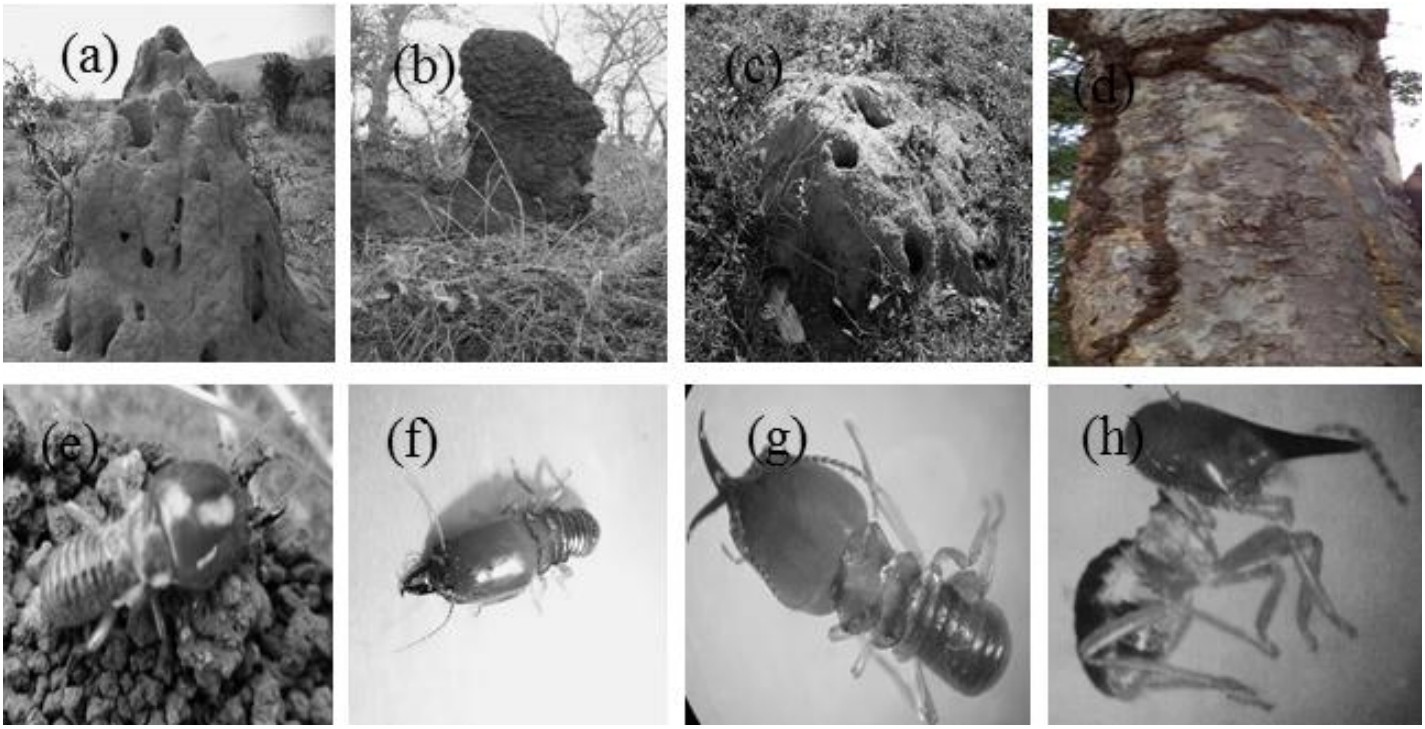

Figure 1. Representatives of termite mounds and termite species identified in Taita Taveta county Kenya. (a) and (b) are open and closed cone and dome shaped *Macrotermes* mounds, (c) slightly raised above the ground *Odontotermes* mound, (d) subterranean gallery of *Amitermes* species (e) and (f) rounded and oval shaped head capsule of *Macrotermes* species (g) Flattened semi rectangular *Odontotermes* head capsule and (h) single mandibular snouted head capsule of *Amitermes* species.

vents, elongated closed dome-shaped mounds and above ground mounds. Of these mounds, 32 were huge irregularly conoid with several vents. The elongated closed dome shaped mounds were 17, the slightly above ground mounds were three while the arboreal nests were two (Figure 1).

Termite identification

Termite samples identified from this study clustered into three groups based on the mound structures they construct and the observed termite characters noted, including the head capsule, antennae segments, mandibular features, pronotum, labrum, mesonotum and metanotum (Table 2).

Cluster analysis of morphological characters

There were five clusters distinguished as a result of examining the phenotypic characteristics of the termites when a dendrogram was generated (Cluster I, II, III, IV and V). Most samples (41 members) clustered together in Cluster I (Figure 2). The populations in this cluster were a mixture from the different vegetation types; DL (37%), RFT (15%), HG (24%), shrub savannah (20%) and forest (4%). Sample 12DL formed a cluster of its own in Cluster II. Cluster III comprised six members, namely 18RFT, 19FS, 26FS, 21HG, 32SV, and 38RFT. Furthermore, five samples (24SV, 25FS, 29FS, 51RFT, 28FS, and 27FS) formed Cluster IV whereas 27FS formed a singleton in Cluster V. Clustering of the termites based on morphological characters indicated that the soldier characters were not in any way dependent on vegetation type as some characters were shared by members of different vegetation types. Members sampled from the same plant life system also clustered separately, which is an indication of diversity among them. It also pointed to heterogeneity between members of the same vegetation and those of other vegetation types.

Molecular identification of termites

Nucleotide analysis, genetic distances and sequence divergence

The approximately 750 bp fragment of COII gene of the termite specimen amplified, suggests that there was no transfer of mitochondrial DNA to the nucleus which were

Table 2. Possible genus identity of termites collected from Taita Taveta County studied in 2013 and 2014.

Sample code	Collection site/vegetation	Mound characteristics	Soldier characteristics	Possible genus identification
IDL,3DL, 4DL	Disturbed land	Even and uneven conoid Shaped mounds	Head capsule has a fontanelle, Labrum with glassy tip, pronotum saddle shaped, mandibles fully developed, mandibles black with sabre shape, 17 antennae segments, large body size, dark brown or light, brown in colour, distended head capsule round or oval shaped, soldiers very aggressive.	*Macrotermes*
21HG, 34HG	Herbaceous Grassland			
30FS,	Exotic Forest	Mounds Ventilated		
14SV, 32SV	Shrub Savanna	Inert termitaria playing host to other creatures		
7RFT,17RFT, 42 RFT	Rain fed Trees	Conoid mounds		
31HG,41HG	Herbaceous Grassland	Unventilated secured mounds		
11SV, 24SV, 26SV	Shrub Savanna	Mounds a bit elevated With at least some vents	Present a characteristic bad odour, yellow body with a brown tinge, left mandible has a tiny tooth, long slender mandibles with slightly incurved tips, semi rectangular head capsule, light brown head capsule with few bristles on the periphery, saddle shape pronotum, reddish brown labrum with hawk shaped, sickle shaped mandibles, the mesonotum and metanotum broader than the pronotum.	*Odontotermes*
28FS, 29FS	Bura Exotic Forest.	Colonies found in degrading humus, others build subterranean galleries on live trees (don't build mounds)	Snouted head capsule, Small in size with total body length of 5 mm, Single mandible	*Amitermes*

sequenced. Stop codons were hardly present in majority of the samples. This is congruent with all amplified sequences depicting the nature of a functioning mtDNA cytochrome oxidase II arrangement of nucleotides. The parts omitted in some sequences were automatically fixed with the MEGA program applied before testing. The third codon set was weighted with A+T sequences at an average content of 63%. Figure 3 shows the nucleotide constitution of varied termite species.

We worked out the two-by-two genetic ranges between the sequences using Kimura two parameters (Kimura, 1980) presented in Table 2. Distance within species of the first set containing specimens 1DL, 3DL, 4DL, 21HG, 14SV. 34HG and 32SV haplotypes was 0.003 while that of group two comprising specimens 7RFT, 17RFT, 20RFT, 42RFT 41HG and 8SV was 0.004. Sequence of sample 28FS clustered together with *Amitermes conformis.* Interspecific distances among the four different clusters of the termites ranged from 0.04 between first and second clusters to 0.23 between third and fourth clusters (Table 3). Within the same plant life, there was low genetic divergence measure between species which ranged between 0.00 to 0.01 for the DL, 0.00 to 0.01 for RFT, 0.03 to 0.14 for SV samples, 0.22 for FS specimens and 0.00 to 0.04 for HG specimens. This same divergence was higher for species from disparate plant life systems. An example is the genetic range linking forest species and those from the RFT vegetation which fell between 0.19 and 0.21 (Table 3). COII Intercluster divergences were approximately 10-fold higher than intracluster divergences.

Phylogenetic analysis

UPGMA formula of evaluation used for inferring the revolutionary account on MEGA 7.0.2 revealed three main clusters, representing three different genera (*Macrotermes, Odontotermes* and *Amistermes*). One cluster (supported by a bootstrap value of 84%) had three samples indicated as 1DL [KT845957], 17RFT [KT845959] and 21HG [KT845960] on the phylogenetic tree, plus a close association to representatives of genus *Macrotermes* (Figure 4). These members (including *Macrotermes* species, *Macrotermes subhyalinus* and *Macrotermes michaelseni*) had a close taxonomic affiliation with ≥98% sequence similarity amongst them. Another cluster was related to *Odontotermes* species. *Amitermes* representatives assembled separately in a group with a strong bootstrap rate of 80% together with sample 28FS [KT845963]. The two *Amitermes* spp. (*Amitermes conformis* and *Amitermes obeuntis*) had 91% sequence similarity with sample 28FS [KT845963].

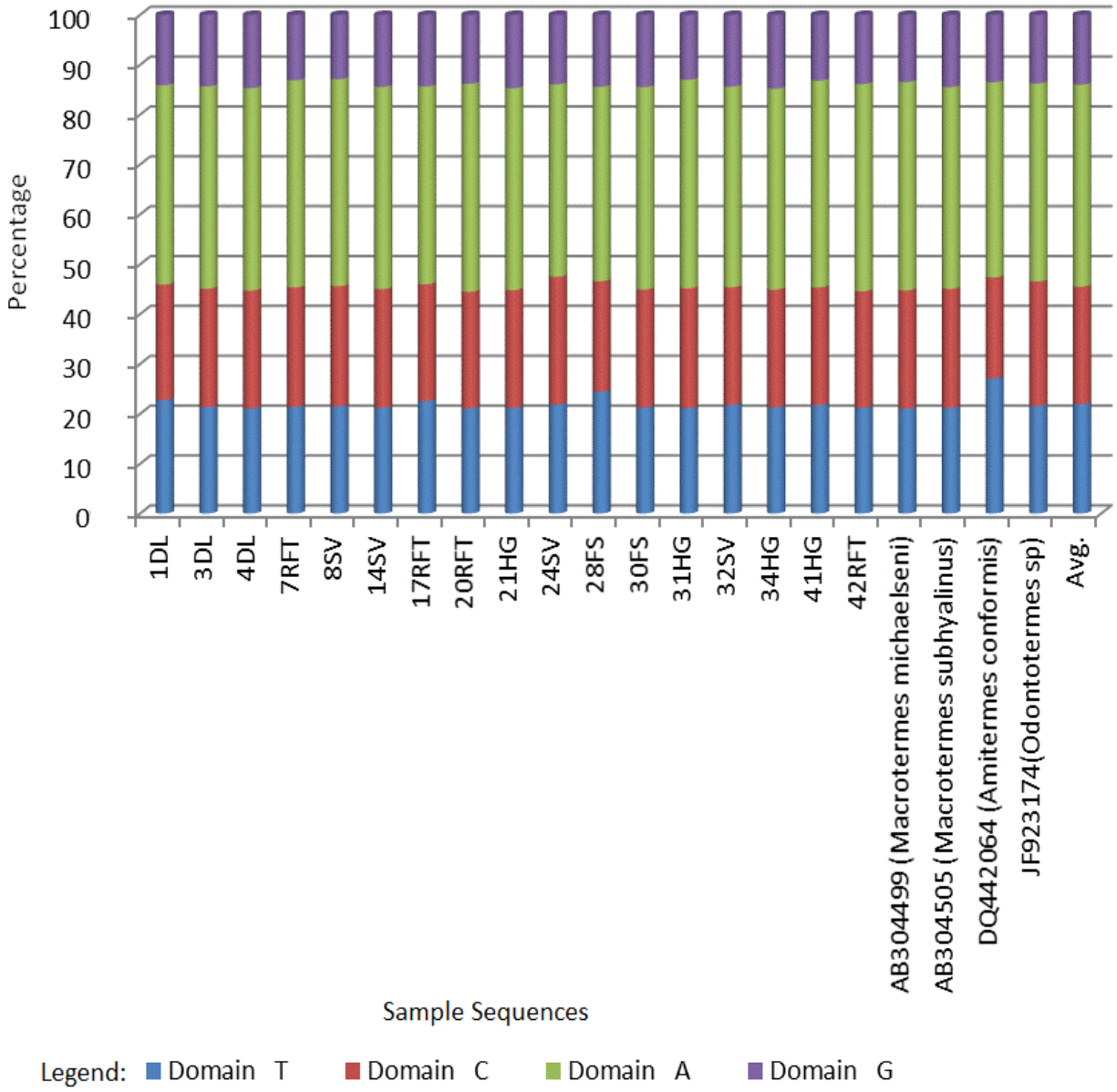

Figure 2. Nucleotide composition of COII gene in different species of termites collected from Taita Taveta County in 2013 and 2014. A= Adenine, C= Cytosine, T= Thymine and G= Guanine.

Termite taxa dispensation in the different vegetation types of the study site

Only a single termite species (*M. subhyalinus*) subjugated the fragmented land areas or DL. In the same way, the RFT land type comprised singly of the *M. michaelseni*. The herbaceous grassland vegetation type was inhabited by both *M. subhyalinus* and *M. michaelseni*. Notably, the shrub savannah vegetation type was rich species composition with three termite species; *M. subhyalinus, M. michaelseni* and an *Odontotermes* sp. Shockingly, the forest that is expected to be species-rich recorded only two termite species of genera; *Amitermes* and *Macrotermes.* The two species were *M. subhyalinus* and *A. conformis*. Overall, shrub savanna vegetation type was the richest termite species diversity (*M. subhyalinus, Odontotermes ceylonicus* and *M. michaelseni*) with a record of two different termite genera (*Microtermes* and *Odontotermes*). DL and RFT land systems were the lowest in species diversity. It was noted that termites of the genus *Macrotermes* had a high frequency of occurrence in all the land systems in the

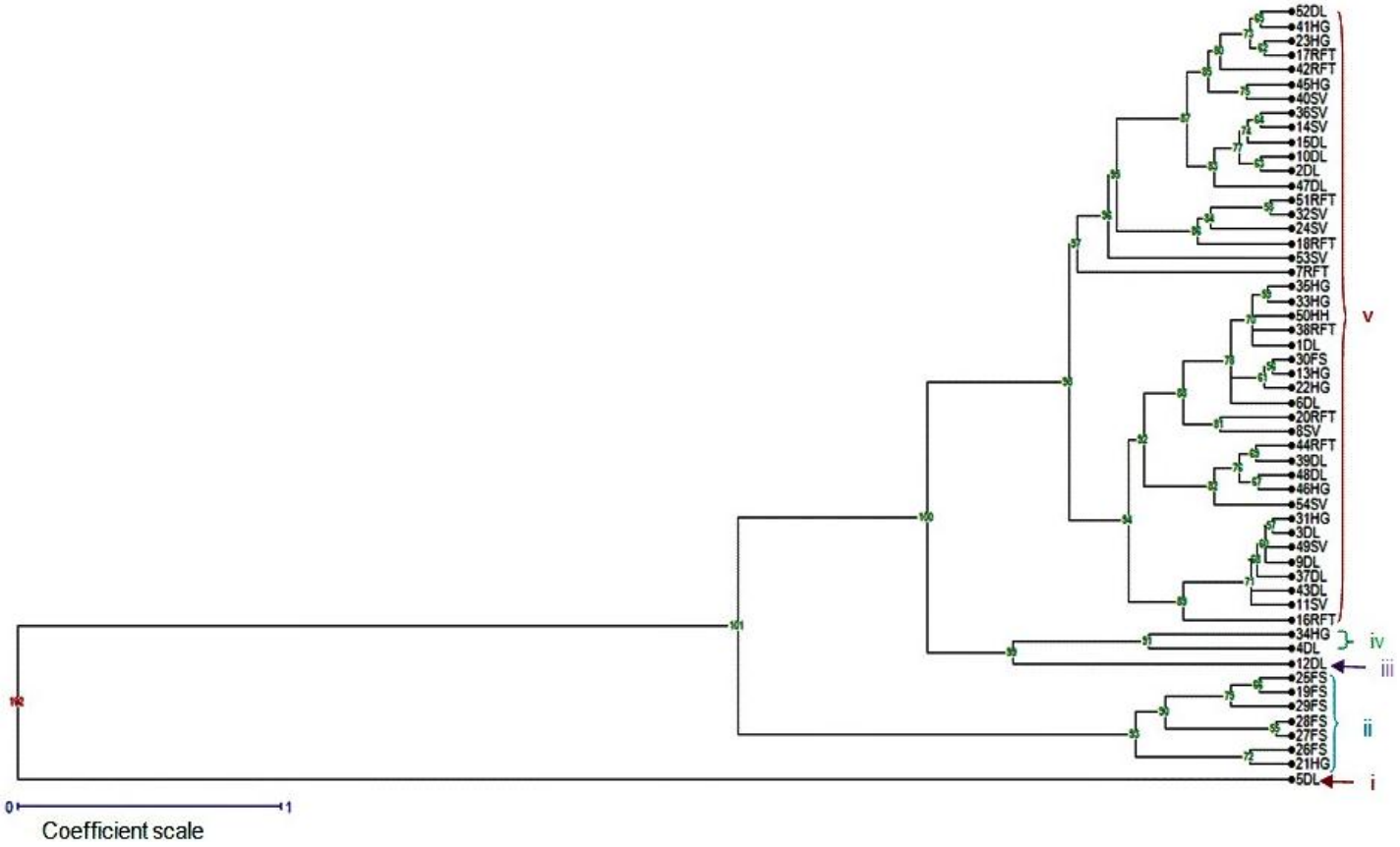

Figure 3. Cluster analysis based on morphological data with Euclidean distance measure. Numbers i-v marked in different colours are cluster grouping by morphology of the termite samples in 2013 and 2014.

study region while on the contrary, the other two genera *Odontotermes* and *Amitermes* were limited to SV and FS accordingly.

DISCUSSION

The mound structures variations observed, points to the existence of different termite species inhabiting the studied area. Although we did not classify the mounds on the basis of how different termite species feed, it is general knowledge that most soil feeders and fungus-growers are capable of constructing mounds (Makonde et al*.,* 2015); thus suggesting their presence. We observed two types of mound structures; conoid mound with external openings being analogous to the species *M. subhaylinus* and closed dome shaped mounds being analogous to the species *M. michaelseni.* This was supported by the fact that we had only collected those species from the respective mounds. Studies by Vesala et al. (2017) and Ocko et al. (2017) had reported that a termite species of *M. subhyalinus* is capable of constructing vented termitaria and *M. michaleseni* to construct closed dome shaped mounds. Not long ago, a similar finding in the same region was reported on the mound structure of *M. subhyalinus* and *M. jeanneli* (Vesala et al*.,* 2019). Another study reported a scenario where it was difficult to ascertain the true mound builder as termites of two different termite genera both inhabited the mound (Makonde et al*.,* 2013). More recently, Paejaroen et al. (2021) found other species in the genus *Globitermes* and *Microcerotermes* to build large epigeal mounds in the tropics of Thailand which suggests more species could be involved in the ultimate morphology of a mound. Termite samples identified from the study by morphology were ultimately placed in three genera; *Macrotermes, Odontotermes* and *Amitermes* all of the family Termitidae. Coincidentally, the termite colonies that were identified to genus level through morphological characterization using soldiers were phylogenetically affiliated with the three genera indicating that morphological differences among genera are great enough for phylogenetic analysis of termites at the genus level. However, for species-level classification, this

Table 3. Pair-wise Kimura-2- parameter genetic distances based on COII gene fragment in termite species identified in Taita Taveta County in 2013 and 2014.

S/N	Sample/Spp	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	1DL																					
2	3DL	0.00																				
3	4DL	0.01	0.00																			
4	7LT	0.08	0.04	0.04																		
5	8SV	0.07	0.03	0.03	0.00																	
6	14SV	0.01	0.00	0.00	0.04	0.04																
7	17RFT	0.07	0.05	0.06	0.01	0.01	0.05															
8	20RFT	0.04	0.03	0.03	0.00	0.00	0.03	0.02														
9	21HG	0.01	0.00	0.00	0.04	0.03	0.00	0.05	0.03													
10	24SV	0.19	0.15	0.15	0.14	0.14	0.15	0.15	0.15	0.15												
11	28FS	0.21	0.19	0.21	0.20	0.19	0.20	0.21	0.19	0.20	0.22											
12	30FS	0.00	0.00	0.00	0.04	0.03	0.00	0.06	0.03	0.00	0.15	0.21										
13	31HG	0.07	0.03	0.03	0.00	0.00	0.04	0.01	0.00	0.03	0.14	0.19	0.03									
14	32SV	0.04	0.00	0.00	0.03	0.03	0.00	0.05	0.03	0.00	0.15	0.21	0.00	0.03								
15	34HG	0.01	0.00	0.00	0.04	0.03	0.00	0.05	0.03	0.00	0.15	0.21	0.00	0.03	0.00							
16	41HG	0.07	0.04	0.03	0.00	0.00	0.04	0.01	0.00	0.04	0.14	0.19	0.04	0.00	0.03	0.04						
17	42RFT	0.04	0.03	0.03	0.00	0.00	0.03	0.02	0.00	0.03	0.15	0.19	0.03	0.00	0.04	0.03	0.00					
18	AB304499 (Mm)	0.03	0.03	0.03	0.00	0.00	0.03	0.01	0.00	0.03	0.15	0.18	0.03	0.00	0.03	0.03	0.00	0.00				
19	AB304505 (Ms)	0.01	0.00	0.00	0.04	0.03	0.00	0.04	0.03	0.00	0.15	0.20	0.00	0.03	0.00	0.00	0.04	0.03	0.03			
20	DQ442064 (Ac)	0.26	0.22	0.22	0.22	0.22	0.21	0.24	0.21	0.22	0.23	0.09	0.22	0.22	0.22	0.22	0.22	0.21	0.22	0.22		
21	JF923174 (O.sp)	0.20	0.18	0.19	0.16	0.16	0.19	0.18	0.17	0.19	0.08	0.22	0.18	0.16	0.19	0.19	0.17	0.17	0.17	0.19	0.23	

Mm= *Macrotermes michaelseni*, Ms= *Macrotermes subhyalinus*, Ac= *Amitermes conformis*, O.sp= *Odontotermes species*

approach might take time and definitely need specialized knowledge of character differences (Janowiecki, 2015).

Molecular characterization results indicated that COII sequences guaranteed the ability to allocate the termites into molecularly well-defined species. There was accurate disparity of most sequences into different clusters. In comparison to their predetermined morphospecies, there was a high level of consistency. The nodes directly defining the clusters presented 99% nodal support. Furthermore, the three clusters remained definite to the fact that such groups comprised well-defined COII lineages instead of dissipated sequence dissimilarity (Hajibabaei et al., 2006). For our study, sequence separation in COX II mtDNA within distinct groups (intraspecific) were smaller than separation among groups (interspecific), although they prevailed within COII sequence divergences from Genbank. This portion of the mtDNA has a general characteristic of showing wide interspecies, yet little intraspecies separation, elucidating that species regularly create well distinct groups on phylogenetic tree. The empirical levels of sequence separation associated with species delimitation studies and in particular bar-coding (Hebert et al*.,* 2003) support the finding of this study.

High Adenine Thiamine content of 63% in the studied sequences did not deviate from what mtDNA usually presents in insects (Crozier and Crozier, 1993) because it is a common characteristic of Cytochrome Oxidase II mtDNA

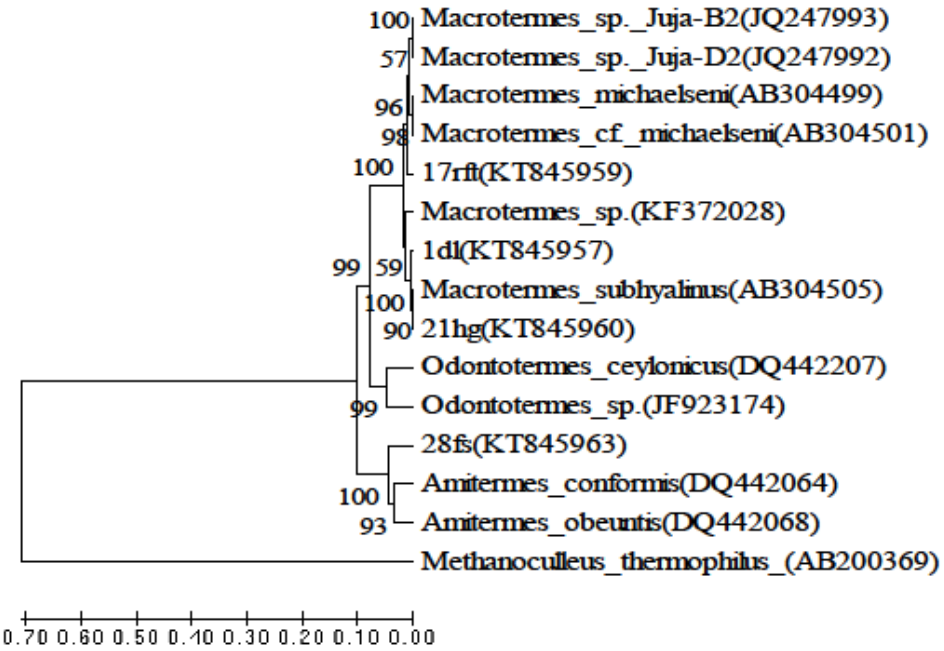

Figure 4. Phylogenetic analysis of COII gene in termites. Multiple sequence alignments were against a portion of the gene. A rooted phylogenetic tree was established with MEGA 7 by the UPGMA method with bootstrap values for 1,000 replicates shown at major nodes. The optimal tree with the sum of branch length = 1.77017127 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches.

region in arthropods. A+T partiality in such sequences is harmonious with information related to COII mitochondrial genes termite in the family Termitidae as well as some genera, that is, *Coptotermes* of the family Rhinotermitidae (Singla et al*.,* 2016; Yeap et al*.,* 2007). A report by Wahlberg et al. (2003) studying *Phyciodes*, family Nymphalidae, and order Lepidoptera also found A+T content from three varied gene portions in Nasuta subgroup of *Drosophila* in the range of 65-78%. In a different study by Hussin and Majid (2020), a lower value than that in our study was found (58.4%) and cited the use of incomplete sequences rather than complete COII gene region (which varies between insects in the range of 673 - 690 bp) as most probable reason for their findings. A study of genus *Coptotermes* in one of the Japanese forests (Tokuda et al*.,* 2012) also reported A+T bias and correlated it to the genetic structures and arrangement of cytochrome oxidase gene sequences. This propounds that AT alteration coercion as a consequence of preselection in order to incorporate amino acids encrypted by AT loaded lines accompanies sequence separation. The phylogenesis research using mtDNA COX II gene fragments therefore depicted the similarity between different termite species. The level of dissimilarity among them is very important in understanding their relatedness. There have however been congruous phylogenetic reconstructions in termites with single or combined mitochondrial markers and nuclear markers having no overlaps of intra- and intergroup sequence variability which avoids ambiguous definition of clusters (Hausberger et al*.,* 2011).

Our sequences from the DNA based characterisation are grouped into one major family Termitidae under the subfamilies *Macrotermitinae* and *Termitinae* and further to three main genera as in the morphological characterisation. From the genus *Macrotermes,* there was a clear separation into well supported two sub groups; *M. subhylinus* and *M. michaelseni* species. The grouping of these species was congruent with using conceptual approach for divergences occurring within the species and between species for termite delineation in *Termitidae*, going by the report of Osiemo et al*.* (2010). We did not however discover any obscure species as all the sequences clustering conjoined perfectly with morphospecies. The low sequence similarity between sample 28FS and Genebank samples may suggest a possibility of a new taxa, which still needs further detailed description using morphological, chemical and molecular properties. This is to say there was no sequences within the same morphospecies extensive branching nor clustering of distinct morphospecies. Other studies have analyzed morphospecies and found comparatively

greater diversity than in our studies e. g (Cassalla and Korb, 2019), hence it is completely hard to compare them with our findings. Osiemo et al. (2010), in their research on termite diversity assessment in Kakamega Forest (found in Kenya), had more or less similar findings. Their study however found that there was deep sequence separation in the phonologically unrecognized as well as identified morphologically different species in termites feeding on soil. It is for this reason that these were considered cryptical species and therefore placed together separately as groups of organisms with similar gene characteristics. The forest in question, unlike our study area, is large and diverse and is within the tropics hence their observation. Other authors who have successfully uncovered ambiguous convergence in overactive divergent groups like termites and nematodes have been reported (Bourguigno et al*.,* 2015).

Morphological characterisation using soldier morphology of termites in the current study was possible only to the genus level whereby three genera were identified. Identification based on DNA approach was possible to the species level and termite samples into three genera and four different species. Notably, before construction of the phylogenetic tree, there was a specimen which was presumably placed as *Nasutititermes* species, because of the external features it conferred. However, after phylogenetic analysis, it became apparent that the sample had closer genetic association with *A. conformis* and very distinct from *Nasutitermes* genus and thus was classified as a species of the genus *Amitermes*. Termite characters sometimes develop in response to their immediate environment for the sake of adaptation and so would present as a certain species. This is what leads to such morphological ambivalence (Hausberger et al*.,* 2011). Using both techniques, we were able to resolve the termite identification problem. Overall, molecular genetics have nearly endless possibilities for their applications to the study of termites.

Although many mounds were recorded in the herbaceous grassland vegetation, only one termite species from the genus *Macrotemes* was identified. The only possible explanation we could give for this observation was that the same colony was reestablished in different parts of the vegetation cover due to increase in their population. The many numbers of inactive mounds in the disturbed land was attributed to increased human activities which trigger deliberate destruction often in sites where agricultural activities were ongoing and also for fear that termites would cause damage. Notably, in sites such as the Taita Taveta University and Kenyatta High School (areas without farming practices), a few active mounds were recorded. Reduced species abundance in relation to land fragmentation is not a unique occurrence as it has been widely reported in Africa and other parts of the world (e.g. Ivory Coast: Coulibaly et al*.,* 2016; Vietnam: Neoh et al*.,* 2015; Panama: Basset et al*.,* 2017). This supports the speculation that it is a worldwide trend. For instance, recording only a single termite species in the disturbed sites was a clear indication that agricultural activities were rampant hence termite migration and consequently inactive mounds. Egan et al. (2021) also reported similar findings. The shrub savanna vegetation type had the highest termite diversity, demonstrating high termite activities in the vegetation type. The possible explanation for this is that most members from the genera *Macrotermes* and *Odontotermes* are general wood and litter feeders, hence the high activities in such environment. Hypothetically, dryland savannah has lower diversity in comparison to wet savannah (Luke et al*.,* 2014). Two termite species belonging to different genera were recorded in the forested vegetation. These were *Amitermes* and *Macrotermes.* The former, (though it can be found in a range of habitats) was only restricted to the forest. The explanation to this may be the forests or undisturbed areas support even the species that are or could be endangered as there are limited human disturbance. This however is dependent on whether the forest is protected or not as unprotected ones are subject to disturbance (Schyra et al., 2018). Moreover, it has been reported that *Amitermes* species has been reported to survive in cool and wet environments (Davies et al., 2014). In a West African study of termite species richness in protected and disturbed land of both savannah and forest ecosystems using COII gene, species richness decline was found in forest than in disturbed land whereas it was not the case for the savannah. However, collectively, the forest had more termite encounter rate as well as species number (Schyra et al*.,* 2019). In both ecosystems, there was low composition of termites in the disturbed sites. Another study (Jamil et al*.,* 2017) on the contrary, reported low species composition in the protected forests areas of Samusan Wildlife Sanctuary when compared to other forest studies elsewhere and they accredited their findings to intensive and extensive logging activities and loss of primary forest. In general, higher altitudes decrease heterogeneity of species of termites. This possibly is because at high altitudes there are reduced temperatures associated with rates of metabolism.

Conclusion

The results have shown that mound building termites of the three genera reported above have dominance over the studied regions of Taita Taveta county. The study demonstrates that agricultural activities affect termite activities and consequently the species abundances. Further, the study confirms that termite species identification based on polyphasic approach is more

concise and informative than using the morphological characters alone. The knowledge gathered from this study using the dataset will be essential in other studies aiming to identify Kenyan species of termites in terms of ubiquity as well as their organization. Such identifications have been proven to be hard when done using only the morphology based approach. Thus, the study underlines the value of employing the two methods; morphology based and DNA-based characterization techniques. These contributions provide a strong foundation for future work that is needed to better understand this important economic pest.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Isolation, biochemical characterization and safety screening of potential probiotic lactic acid bacteria from spontaneously fermented cereal products from Western Kenya

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The global demand for non-dairy beverages has sky rocketed especially so during this Covid-19 pandemic for potential health benefits. Development of probiotic strains from fermented cereal and legumes with the ability to grow well and adapt to gastrointestinal conditions at the same time possess high therapeutic ability will be a great achievement. This study aimed at isolating and screening probiotic potential Lactic Acid Bacteria (LAB) involved in traditional fermentation of cereals (maize, sorghum and millet). A total of ten isolates were obtained from the cereals out of which five isolates that met preliminary attributes of probiotic bacteria were selected for further investigation. Two isolates SPU2 and FPU1 were found to survive a low pH which is a desirable attribute for the survival of probiotic bacteria in the gut. MPU1, FPU1 and SPU2 are possible thermophiles and can survive at low pH and moderate high salt concentration. The enzymes DNase and gelatinase used to test pathogenicity of a microorganism were not produced by all the isolates in this study. The isolates recorded a high susceptibility to the eight antibiotics. This study also revealed that the tested isolates have the ability to grow well even at the minimum tested pH of 1.0 for 1 and 2 h of incubation, respectively. Most isolates were resistant to 0.3% bile concentration with over 92% survival. FPU1 was more resistant at bile concentration of 1% than all the rest while MPU1 was most resistant at 2% bile salt. Traditionally fermented cereals are potential sources of safe bacteria that can be tried in the production of functional foods.

Key words: Probiotic potential LAB, bile concentration, sodium chloride, pH, cereals.

INTRODUCTION

The love for functional foods has increased as a result of factors such as health awareness and a lot of research information emphasizing the interconnection between food, health and diseases (Küster-Boluda and Vidal-Capilla, 2017). This knowledge is causing abrupt change from foods of animal origin to those originating from

plants (Brigitta and Ágoston, 2019). Whole grain cereals contain phytochemicals such as phytic acid, lignans and phenolic substances which are good for health and disease prevention (Călinoiu and Vodnar, 2018). To improve the nutritional properties of cereals several technologies have been employed but fermentation is the most outstanding (Nkhata et al., 2018). Fermentation improves the nutritional value of food, stability, safety and organoleptic properties (Kawaljit et al., 2017). This process therefore could be a potential technology for producing new bioactive compounds from natural food raw material. Malnutrition continues to be a great challenge and burden in our country today. To improve food security and nutritional value of food consumed among the Kenyan households, intentional efforts and appropriate strategies need to be adopted to guarantee affordable healthy meals. Development of non-dairy probiotic products from cereals could lead to commercialization of products of unique flavour, taste and of nutritional adequacy. Cereal grains are rich in carbohydrates, calories, proteins, vitamins and minerals and are therefore good substrates for the development of probiotics which can be used in probiotic foods (Achi and Asamudo, 2019). Cereal products may have a range of bioactive substances with potential health benefits. Fermentation of food raw material generally involves LAB (Kawaljit et al., 2017; Bintsis, 2018). The natural presence of LAB in cereals is of great interest in producing fermented cereal products (Tsafrakidou et al., 2020). Fermented cereal beverages are reported to be among the most active functional foods (Bansal et al., 2016). Continuous consumption of such functional foods can ensure overall good and prevent diseases. Probiotics are living microorganisms in foodstuffs which, when consumed at certain levels in nutrition, stabilizes the gastrointestinal tract microflora thereby conferring health benefits to the consumer (Markowiak and Śliżewska, 2017). Probiotics also carry active biological substances in reasonable quantity that influence good health (Terpou et al., 2019). The potential health benefit of a given probiotic depends on its profile characteristics (Shi et al., 2016). The most common probiotics in the market are *Lactobacillus* and *Bifidobacterium* (Tsafrakidou et al., 2020). In general, most probiotics are Gram-positive, usually catalase-negative, rods with rounded ends, and occur in pairs, short, or long chains (Peyer et al., 2016). They are non-flagellated, non-motile and non-spore-forming, and are intolerant to salt (Elshaghabee et al., 2017). Most probiotics LAB have optimum growth temperature at about 37°C while other strains at 30°C and pH optimum for initial growth is normally in the range from 6.5 to 7.0 (Peyer et al., 2016). The identification of specific microflora involved in indigenous cereal-legume fermentation is needed to amplify and control positive factors as well as to minimize or prevent negative factors such as growth and metabolism of pathogenic and toxicogenic bacteria (Enujiugha and Adebanjo, 2017). This study aimed at biochemically characterizing and safety screening of probiotic potential LAB isolated from spontaneously fermented cereal products from Western Kenya.

METHODOLOGY

Cereal (Sorghum, millet and maize) samples (500 g each) were obtained randomly from cereal store traders from Vihiga County, Western Kenya. A total of 25 samples were collected for each cereal. The samples were packaged in kaki bags and taken to the Food and Microbiology Laboratories, Technical University of Mombasa for analysis.

Fermentation of cereal products

Fifty grams of each cereal sample (maize, sorghum and finger millet) were weighed after thorough mixing, sorted and cleaned. A blender was used for grinding the cereal flours with sterilization of the blender after every cereal sample was ground with 70% ethanol. The sample flours were each transferred to fermenting bottles aseptically, two parts water added, mixed and sealed. Fermentation was done by incubating at 30°C for 48 to 72 h. 10 g was drawn from each fermentation aseptically for probiotic potential LAB isolation.

Isolation of LAB

LAB was isolated from traditionally fermented cereals (maize, sorghum and millet). The fermented samples were appropriately suspended and diluted in sterile saline. An initial dilution of (10^{-1}) was obtained after homogenizing 10 ml of each sample with 90 ml of 0.85% (w/v) sterile sodium chloride solution. Dilutions of up to 10^{-7} were serially made for every fermentation. From each of the 10^{-5} to 10^{-7} corresponding dilutions, 1 ml sample was plated out onto (De Man, Rogosa and Sharpe) MRS agar (De Man et al., 1960) supplemented with 0.05 g/L Cysteine-HCL (MRS-CysHcl) and M17 agar plates by spread plate technique in triplicate. Incubation of the inoculated plates was then followed at 37°C for 48 to 72 h anaerobic jars. The plates were keenly examined. The bacteria colonies with distinct morphologies such as color, form, margin, consistency and surface elevation were randomly selected. Purification of selected colonies was performed by sub-culturing twice on MRS agar plates through streaking. MRS broth with 30% glycerol (El-Soda et al., 2003) at +4°C (Patil et al., 2010) was used for maintenance of the pure cereal LAB isolates. Gram staining, cell morphology, catalase test (Sharpe, 1979), carbon dioxide production from glucose and antibiotic susceptibility test were carried out. Five isolates were selected for further screening.

Biochemical characterization of the lactic acid bacteria isolates

In this study, 0.17 g/l bromothymol blue was added to MRS broth as

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pH indicator (pH 7). The MRS broth was filled in universal bottle with screw caps, each carrying 20 ml followed by autoclaving. An overnight culture isolate of each was used as the inoculum. The cells were spun down, normal saline (0.85%) used for re-suspension. From the suspension, a loopful of the mass was inoculated into each of the bottles. The following temperatures were assessed: 15, 37, 45 and 55, the NaCl concentration studied include: 2, 3, 6.5 and 10% (w/v), the following are pH evaluated 2, 3, 4 and 6 (Liong and Shah, 2004). The initial pH of MRS broth was modified using 1 M NaOH and 1 M phosphoric acid. Color change in any test tube implied growth.

Safety assessment of LAB

Antibiotic susceptibility

Zhang et al. (2016) method was followed with slight modification to determine antibiotic susceptibility of the cereal LAB isolates. Eight antibiotics include: Gentamicin, Ofloxacin, Nitrofurantoin, Cefaclor, Nalidixic Acid, Augmentin, Minocycline and Cefuroxime. Antibiotic-impregnated discs (Abtek Biological Ltd, England) were placed on plates streaked with isolates. Any sign of clearance or zone of inhibition along the tips after an overnight growth at 37°C was taken to mean the isolates were susceptibility to the antibiotics.

Test for hemolysis

The method of Linaje et al. (2004) was followed in determining hemolytic activity of the selected isolate. Fresh isolates grown for a night were spot inoculated on Blood Agar plates (HiMedia) and incubated for 48 h at 37°C.

Production of DNase enzyme by isolates

Gupta and Malik (2007) method was followed in this study. A clear pinkish zone around the colonies was considered to mean the isolates produced the enzyme DNase.

Gelatinase enzyme production

Evaluation of gelatinase enzyme production was done according to Harrigan and McCance (1990). MRS agar plates containing 3% gelatin were prepared then inoculated with an overnight culture of isolates by streaking. Clear zones around the colony against a dark base indicated a positive effect.

Probiotic potential attributes of the isolates

Tolerance to low acid conditions

Liong and Shah (2004) method was followed with slight changes. MRS broth with pH adjusted to 1, 2, 3 and 6.5 were used to study how the LAB isolates can tolerate low pH for 1, 2 and 3 h. The cells were enumerated, growth expressed as colony forming units per milliliter (log CFU/ml) and percentage survival calculated.

Bile salts tolerance of the Isolates

Gilliland and and Walker (1990) and Aswathy et al. (2008) methods were considered to assess the effect of bile salt concentration on isolates growth rate. MRS broth was supplemented with bile salt (0.3, 1 and 2%) for 8 h. An inoculum of 100 µl was drawn and plated onto MRS agar. Incubation was followed at 37°C for 24 h and survival rate calculated.

RESULTS

The results of this research indicate that probiotic potential LAB could be isolated from spontaneously fermented cereal products. From all samples, 10 probiotic potential LABs (3, 4 and 3 from maize, finger millet and sorghum, respectively) were isolated 7 of which were presumptive *Lactococcus* and 3 *Lactobacillus* species (Table 1) based on biochemical characterization; a total of 3, 4 and 3. Out of the ten LAB, three isolates were found to be hetero-fermentative. Homo-fermentative LAB ferments carbohydrates with the production of only lactic acid which can lower the pH of medium close to 4.0-4.5, hetero-fermentative LAB on the other hand produces carbon-dioxide and other organic compounds (acetic acid, alcohol, acetaldehyde, diacetyl) which can further lower the pH to about 3.5.

Table 1 shows the tolerance of the 10 LABS to different conditions of temperature, salt concentration and pH. The temperature of 37°C and salt concentration of 2% favored the growth of all the bacteria. The isolates surviving at 45°C are comparable to yoghurt starter culture organisms that have an optimum between 38 and 42°C. Isolates MPU1, FPU1, FPU2 and SPU2 survived a temperature of 45°C. Five (5) isolates were able to withstand salt concentration of 6.5% while only one at 10%. The isolates that grew at 45°C, salt concentration of 6.5 and 10% and low pH are special species that could be of use in systems where either temperature of growth medium is elevated or pH fall extremely. Two isolates SPU2 and FPU1 can survive a low pH which is a desirable attribute for the survival of probiotic bacteria in the gut. Still those surviving at pH 2 are potential probiotics. MPU1 (*Lactobacillus* spp.), FPU1 (*Lactococcus* spp.) and SPU2 (*Lactococcus* spp.) are possible thermophiles and could thrive at reduced pH and moderate high salt concentration.

Safety assessment of LAB

The LAB isolates in this study were found to be sensitive to nearly all antibiotic used (Table 2). Any resistance shown was not biased towards a particular antibiotic, apart from Cefuroxime where two isolates showed resistance. The isolates' safety is guaranteed from the high percentage sensitivity, meaning they may not present resistance towards antibiotic use.

The isolates did not hemolyze the blood since clear zones were not seen around colonies on blood agar. Pathogenicity factors demonstrated by the production of gelatinase and DNase enzymes were not observed in this study among all isolates. The clear pinkish color around the colonies to demonstrate DNase enzyme production was not observed.

Table 1. Phenotypic characteristics of probiotic potential LAB isolated from traditionally fermented Cereals from Vihiga.

Source	Gram's reaction & Cell shape	Cultural characteristics	Catalase test	CO_2 from Glucose	Growth at temperatures (°C)				Growth in NaCl concentration (%)				Growth at pH				Possible species
					15	37	45	50	2	3	6.5	10	2	3	4	6	
MPU1	+Cocci	Ppc	-	-(Homo)	-	+	+	-	+	-	-	-	-	+	+	+	*Lactococcus* spp.
MPU2	+Cocci	Ppc	-	+(Heter)	-	+	-	-	+	+	+	-	-	+	+	+	*Lactococcus* spp.
MPU3	+Rod	Ppc	-	-(Homo)	-	+	-	-	+	-	-	-	-	+	+	+	*Lactobacillus* spp.
FPU1	+Cocci	Ppc	-	+(Heter)	-	+	+	-	+	+	+	-	+	-	+	+	*Lactococcus* spp.
FPU2	+Cocci	Ppc	-	-(Homo)	-	+	+	-	-	-	+	+	_	+	+	+	*Lactococcus* spp.
FPU3	+Cocci	Ppc	-	-(Homo)	+	+	-	-	+	+	-	-	-	+	-	+	*Lactococcus* spp.
FPU4	+Rod	Ppc	-	-(Homo)	-	+	-	-	-	+	+	-	-	+	+	+	*Lactobacillus* spp.
SPU1	+Cocci	Ppc	-	+(Heter)	-	+	-	-	+	+	-	-	_	-	-	+	*Lactococcus* spp.
SPU2	+Cocci	Ppc		-(Homo)	-	+	+	-	+	+	+	-	+	+	+	+	*Lactococcus* spp.
SPU3	+Rod	Ppc	-	-(Homo)	-	+	-	-	+	+	-	-	_	-	+	+	*Lactobacillus* spp.

(+) Indicate growth, (-) no growth, (+/-) Gram positive/Negative, (Ppc) Pin Point Colony, Homo (homofermentative), Heter (Heterofermentative). (MPU1- MPU3): Maize isolates, (FPU1- FPU4): Finger millet isolates, (SPU1-SPU3), Sorghum isolate.

Table 2. Antibiotic sensitivity of the LAB isolates.

S/N	Antibiotic	Concentration (µg)	Sensitive/resistant									
			M_{PU1}	M_{PU2}	M_{PU3}	F_{PU1}	F_{PU2}	F_{PU3}	F_{PU4}	S_{PU1}	S_{PU2}	S_{PU3}
1	Gentamicin (GEN)	10	S	S	S	S	S	S	S	S	S	R
2	Ofloxacin (OFL)	30	S	S	S	S	S	S	S	S	S	S
3	Nitrofurantoin (NIT)	200	S	S	S	S	S	S	R	S	S	S
4	Cefaclor (CCL)	30	S	S	S	S	R	S	S	S	S	S
5	Nalidixic Acid (NAL)	30	S	S	S	S	S	S	S	S	S	S
6	Augmentin (AMC)	30	R	S	S	S	S	S	S	S	S	S
7	Minocycline (MIN)	30	S	S	S	S	S	S	S	S	S	S
8	Cefuroxime (CXM)	30	S	R	S	S	S	R	S	S	S	S
	% Sensitivity		87.5	87.5	100	100	87.5	87.5	87.5	100	100	87.5

S=Sensitive (Prevented isolates growth), R=Resistant (Growth noted).

Assessment of probiotic attributes

In this study (Table 3), the number of bacteria cells in the medium decreased below the pH of 3 due to loss of viability. At pH 1 in all the tested isolates no viable cells were seen after 2 h, implying most isolates were killed by severe low pH. A good probiotic potential LAB isolate must resist harsh conditions found in the gastrointestinal tract and also colonize intestinal epithelium. In this study SPU2 was able to grow at pH 1 (experienced on empty stomach) for 2 h while the

Table 3. Acid tolerance of probiotic potential LAB isolates from traditionally fermented cereals of Vihiga.

Organisms	pH	Incubation time (min)								
		Cell survival (Cfu/ml)					% Cell survival			
		0	60	120	180	Mean	60	120	180	Mean
M_{PU1}	1	8.5	6.2	0	0	3.7	67.8	0	0	22.6
	2	8.6	6.7	6.1	5.8	6.8	73.3	66	62.7	67.3
	3	9.1	9.03	8.7	8.4	8.8	98.7	91	90.8	93.5
	6.5(Control)	9.12	9.14	9.23	9.25	9.2	100	100	100	
	Mean	8.83	7.77	6.0	5.86		84.95	64.25	63.38	
M_{PU2}	1	9.7	7.8	0	0	4.7	77.6	0	0	25.9
	2	9.5	8.1	6.5	5.8	7.3	78.6	62.8	55.4	65.6
	3	9.9	9.3	8.4	7.6	8.8	90.3	81.2	72	81.2
	6.5(Control)	10.05	10.3	10.35	10.46	10.29	100	100	100	
	Mean	9.79	8.88	6.31	5.97		86.6	61	56.85	
F_{PU1}	1	9.7	7.8	0	0	4.4	75.9	0	0	25.3
	2	9.7	7.02	6.4	5.6	7.1	68.2	61.8	54.1	61.4
	3	10.1	8.3	7.8	7.5	8.4	80.7	75.3	72.4	76.1
	6.5(Control)	10.15	10.28	10.36	10.38	10.29	100	100	100	
	Mean	9.9	8.37	6.14	5.87		81.2	59.28	56.63	
F_{PU2}	1	10.2	8.1	0	0	9.1	79	0	0	26
	2	10.12	8.3	6.6	5.7	7.68	80.9	63.8	54.8	66.5
	3	10.17	9.01	7.72	6.2	8.23	87.9	74.12	59.6	73.9
	6.5(Control)	10.21	10.25	10.34	10.41	10.3	100	100	100	
	Mean	10.18	8.92	6.17	5.58		86.95	59.48	53.6	
S_{PU2}	1	10.13	8.1	3.4	0	5.4	79.5	33	0	37.5
	2	10.13	8.4	6.3	5.4	7.6	82.4	61.5	52	65.5
	3	10.08	9.1	7.4	6.1	8.2	89.3	72.3	58.9	73.5
	6.5(Control)	10.15	10.19	10.24	10.35	10.2	100	100	100	
	Mean	10.12	8.95	6.84	5.46		87.8	66.7	52.73	

*Log CFU/ml = Average mean from 3 experimental results. *% cell survival = (log CFU/ml for pH 1, 2, 3/ log CFU/ml pH at 6.5) × 100. (MPU1-MPU2): Maize isolates, (FPU1- FPU2): Finger millet isolates, (SPU1), Sorghum isolate.

rest survived up to about 1 h. This study shows the tested isolates have the ability to grow well even at the lowest pH of 1.0 for 1 and 2 h of incubation, respectively used in this study.

In this study, MRS broth was supplemented with bile salt concentration of 0.3, 1.0 and 2.0% and its effect on growth rate of isolate monitored. In the small intestines the physiological concentration of bile salts is in the range of 0.2 to 2.0%. Bile salt causes an increase in permeability of bacterial cell membranes composed mainly of lipids and fatty acids. The cultures were grown in 0.3, 1.0 and 2.0% of bile salt concentration and survivability monitored for 4 and 8 h, respectively. Among the isolate tested, MPU1 showed the highest survival of 99, 88 and 83% on 8-h incubation followed by FPU1 with 92, 86 and 77% for the same period of time (Table 4). The viable cell count of the cells decreased with increase in the concentration of bile salt to 2.0%. Most isolates are resistant to 0.3% bile concentration with over 92% survivals. FPU1 is more resistant at bile concentration of 1% than all the rest while MPU1 is most resistant at 2% bile salt.

DISCUSSION

Lactic Acid Bacteria are generally Gram-positive rods or coccobacilli occurring in chain. They are non-spore former, usually non motile, non-acid fast, non-respiring, devoid of cytochrome and catalase negative (Mokoena, 2017). They grow well under anaerobic conditions but may grow in microaerophilic as well as aerobic conditions.

Table 4. Bile salt tolerance of probiotic potential LAB isolates.

Organism	Time	Bile salt concentration (%)								
		Cell count (Cfu/Ml)					Cell survival rate			
		0	0.3	1	2	Mean	0.3	1	2	Mean
MPU1	4	10.3	10.06	9.2	8.8	9.6	99	88	85	91
	8	10.13	9.7	8.9	8.4	9.2	96	88	83	88
MPU2	4	10.04	9.3	8.85	7.7	8.97	92.6	88	76.7	85.8
	8	10.33	9.4	8.4	7.6	8.9	91	81.3	73.6	82
FPU1	4	10.3	10.1	9.5	8.8	9.7	98	92	85	92
	6	10.2	9.4	8.8	7.9	9.1	92	86	77	85
FPU2	4	10.32	10.04	9.2	8.6	9.5	97.3	89	83	89.8
	8	10.11	9.5	8.7	7.4	8.9	94	86	73.2	84.4
SPU2	4	10.2	9.7	8.8	8	9.5	95	86.3	78.4	86.6
	8	10.24	9.5	8.6	7.5	8.96	92.8	84	73	83.3

*Log CFU/ml: Average mean results of three experimental values. *% Survivability = (log CFU/ml Bile concentration (%). 3, 1, 2/log CFU/ml Bile concentration 0) × 100. (MPU1- MPU2): Maize isolates, (FPU1- FPU2): Finger millet isolates, (SPU1), Sorghum isolate.

They exhibit optimum growth at slightly lower acidic condition (pH 5.5 t- 6.0). They are strictly fermentative, with lactic acid as the major end product during sugar fermentation (Khalil and Nural, 2015). LAB can be classified on the basis of their morphology (cocci or rods, tetrad formation), mode of glucose fermentation, growth at different temperatures and salt concentrations, and configuration of the lactic acid production (D, L or both) (Markowiak and Śliżewska, 2017). Lactic acid bacteria have low acidification activity (Bintsis, 2018). The fast-acidifying LAB strains are found to be good for fermentation process as primary starter culture while poor acidification strains can be used as adjunct cultures depending on other properties. The proteolytic activity of probiotic culture is essential for the growth of the organisms and it is involved in the development of organoleptic properties of different fermented products (Amani et al., 2016). They have two different metabolic pathways for hexose fermentation. In homo fermentative pathway, lactic acid (more than 85%) is major end product whereas in heterofermentative pathway lactic acid, ethanol/acetone and CO_2 are the terminal products. These compounds impart characteristics flavour to the fermented foods. This study revealed that 70% of the LAB isolates are homofermentative, while 30% heterofermentative. The isolates growing at high salt concentration (6.5-10%) could be useful in the production of lactic acid where it is precipitated as lactate (Bonatsou et al., 2017). The antibiotic sensitivity property of LAB enables formulation of safe probiotic products for human consumption (Georgieva et al., 2015). In this study, the probiotic potential isolates were sensitive to the eight antibiotics. Probiotic LAB should be sensitive to antibiotics in order to avoid disseminating the resistance property to other pathogenic bacteria in the same niche, or the antibiotic resistance among them should be non-transferable (Jose et al., 2015). The lysis of red blood cells and the subsequent release of their contents into surrounding fluid is referred to as hemolysis (Deidda et al., 2020). Most pathogens are able to cause hemolysis *in vitro* and *in vivo*. Among the selection criterion for probiotic strains, is the absence of haemolytic and gelatinase activity an indicator that these bacteria are non-virulent (Rastogi et al., 2020). The isolates in these research findings are there not pathogenic. When selecting LAB for probiotic use, acid tolerance is one of the most important factors to consider. This will assist in determining whether they will be able to survive, grow and perform therapeutic activity in the gastrointestinal tract where pH is low (Terpou et al., 2019). The probiotic LAB strain must survive the acid conditions during gastrointestinal tract transit. The acidic pH in the gut normally range from 2 to 4 in normal conditions and during fasting it can reach up to pH 1. In this study, SPU2 was able to grow at pH 1 for 2 h while the rest survived up to about 1 h.

Conclusion

This research study focused on the potential of LAB isolated from fermented cereal-based products as probiotics for functional foods. The incorporation of such probiotic bacteria in foods have great potentials to improve the quality of life. The ability to survive low pH and resistance to bile concentration (0.3 and 1%) are

some of the desirable attributes of probiotic potential bacteria. The susceptibility to selected eight antibiotics, inability to produce gelatinase and DNase and non-hemolytic nature revealed the safety status of the isolates. The use of the tested isolates in production of functional foods requires further evaluation on the sensory aspect and acceptability to enhance the application of the strains in food industry.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Isolation and identification of phytopathogenic bacteria in vegetable crops in West Africa (Côte D'ivoire)

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Yield losses in food crops due to plant pathogenic bacteria are significant and increasing over the years. The increasing losses caused by bacterial plant pathology are explained by the emerging resistance of bacteria to the chemical agents used in plant protection. Moreover, these chemical agents harm the environment through residue accumulation leading to soil pollution and the perturbation of the soil's inner ecosystem. The most important bacteria causing plant pathology belong to the genera of *Pseudomonas, Ralstonia, Agrobacterium, Xanthomonas, Erwinia, Xylella, Pectobacterium,* and *Dickeya*. However, in Côte d'Ivoire, only the *Ralstonia* species have been identified. Therefore, this study aims to identify plant pathogenic bacteria present in market garden plants in Côte d'Ivoire. Three sites in the cities of Anyama, Abidjan, and Bingerville were selected for the sampling and the detection of *Pseudomonas syringea*, *Erwinia carotovora, Clavibacter michiganensis, Ralstonia solanacerum*, and *Xanthomonas campestris*. The samples consisted of healthy and affected plant leaves and soils. In brief, 70 bacterial strains were isolated and phenotypically identified in this study. Among them, we noticed that 20% were isolated from the leaves and 80% from the soil. Regarding the bacterial species, *C. michiganensis* (37.14%), *E. carotovora* (18.57%), *R. solanacerum* (15.71%), *X. campestris* (14.28%), and *P. syringae* (11.42%) were identified. The molecular identification has confirmed the identification of the 5 plant pathogenic bacteria within all the studied sites. To the researchers' knowledge, this study is the first to describe the identification of *P. syringea*, *E. carotovora, C. michiganensis,* and *X. campestris* isolated in plant crops in Côte d'Ivoire.

Key words: Phytobacteria, vegetable plants, PCR

INTRODUCTION

Vegetables contribute to more than 33% of world agricultural production and employ 800 million people (Kanda et al., 2014). Tomato *(Solanum lycopersicum)*, red pepper *(Capsicum spp.),* and, eggplant *(Solanum*

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melongena) are among the 40 most cultivated plant species in the world (FAO, 2008). In sub-Saharan Africa, market gardening represents an important sector of activity because of the nutritional material produced, but also because of the source of income that it provides (Djiéto-Lordon, 2007). In Côte d'Ivoire, the market gardening of vegetables represents an average of 27% of the gross national product (GDP). In addition, the demand for vegetables such as tomatoes, eggplants, and pepper is increasing due to population growth. Then, farmers are intensifying their production to cover the needs. However, this production faces many biotic constraints such as bacterial diseases causing high losses, especially in tropical climates (Lebeau, 2010). The plant pathogenic diseases led to the decrease in agricultural yield and the increase in the prices of the products on the market. Therefore, there is need to identify bacteria responsible for plant pathogenic diseases in Côte d'Ivoire to propose a solution to overcome the problem. According to their scientific and economic importance in the world, five bacterial species were highlighted (Mansfield et al., 2012). Firstly, *Ralsotonia solanacearum* is responsible for bacterial wilt in tomatoes, eggplants, and potatoes. The bacterium, initially, infects the roots and then, invades the vascular system of the plants (Nakahara et al., 2021). Next, *Pseudomonas syringae* an epiphytic bacterium that survives on weed roots, asymptomatic plants as well as seeds is known to cause symptoms of bacterial speckling (black dot surrounded by a yellow halo) (Canzoniere et al., 2021). *Xanthomonas campestris*, for its part, is responsible for black rot and causes vascular disease in some plants or leaf spots in others (Vicente and Holub, 2013). Following this, *Clavibacter michiganensis* spreads through plant vessels and causes symptoms such as wilting, stem canker, vascular discoloration, and cell habit (Yim et al., 2012). The contaminated seeds by *C. michiganensis* remain the main mean of bacterial propagation. Finally, *Erwinia carotovora* enters the plant through wounds. The plant walls are then degraded and the tissues are macerated by pectolytic enzymes causing soft rotting of stems and fruits (Boumaaza et al., 2018). Regarding the economical and nutritional implications of bacterial plant diseases, this study aimed to detect the presence of cited bacteria that can cause infections in tomato, eggplant, and chili pepper.

MATERIALS AND METHODS

Samples collection

The sampling sites chosen are fields where organic culture is practiced. Eggplant and pepper plant samples were collected from site 1, located in a forest of the city of Anyama and near a waterway. Tomato plant samples were collected from site 2, located in the city of Abidjan. Finally, eggplant and pepper plant samples were also collected on site 3 in the outskirt of Bingerville city (Figure 1). For each plant, samples of diseased and healthy leaves were collected and at the base of each plant, soil samples were also collected. All in all, for each site, 3 soil samples and 3 leaves samples were collected per plant. Soil samples (10 g) were taken at 15cm depth (Popoola et al., 2015). The leaves were cut off at the base of the petiole. Healthy and diseased leaves were collected at the rate of 10 leaves per plant (Gracein et al., 2012). Plant samples collected were carefully bagged, shipped to the laboratory, and processed immediately or stored at 4°C and processed within 48 h.

Pre-treatment

Small pieces of leaves were cut aseptically; the surface of the leaf was sterilized in 70% alcohol and washed in three series of sterile distilled water to remove traces of alcohol. The leaves were suspended in tubes containing 3 ml sterile distilled water for 15 to 20 min (Gracein et al., 2012; Nakahara et al., 2021). One gram of soil sample was weighed and added to a test tube containing 10 ml of physiological water (9% NaCl). Then, the tubes were shaken for 30 min, 50 rpm at room temperature to allow the separation of bacteria from the soil (Popoola et al., 2015 modified).

Isolation and characterization of phytobacteria

Isolation

A serial decimal dilution of the bacterial suspensions obtained after pretreatment was carried out in 9 ml of sterile distilled water when the water contained in the tubes became slightly cloudy. Then, 1 ml of the diluted bacterial cell suspension was poured onto sterilized Petri plates containing nutrient agar King B or YPGA. The inoculated plates were incubated at 28° for 24 to 48 h. Depending on the appearance, coloring, and morphology of the bacterial colony; an isolated colony was picked and plated again on nutrient agar using a Pasteur pipette (Table 1). This step was repeated three times to allow purification of the isolated strain (Chartier, 2005; Amkraz et al., 2010; Gracein et al., 2012; Huynh et al., 2019).

Bacterial detection by polymerase chain reaction

The DNA extraction of isolated bacteria was performed using Qiagen® DNA extraction kit following the manufacturer's recommendations. Fives colonies of bacteria were diluted in 100µl water (Qiagen, 2003).

The PCR analysis was performed to amplify conserved genes for each phytobacterium. The reaction mix contained 1XFirepolMasterMix (SolisBiodyne), 16 µl molecular water, 5 µl of DNA extract, and 0.5 µM specific primers for each bacterium (Table 2). For amplification, initial denaturation was carried out at 95 °C for 10 min, followed by 32 cycles of denaturation at 95°C (30 s), annealing at 54°C (30 s), and elongation at 72°C (1 min). Finally, the elongation of 5 mn was carried out. The amplification program was conducted on Applied Biosystems (9700 *PCR* System thermocycler). *PCR* products were visualized using 1.5% (wt/vol) agarose gel electrophoresis and a 100-bp DNA ladder (Promega).

Bacterial activity tests

Bacterial growth in vitro

The bacterial count makes it possible to know the exponential growth time of the bacteria used. This count is done at different times (0, 2, 4, 6, 8, and 24h). A stock solution of fresh phytobacteria is serially diluted up to 10^{-8} and then plated on King B or LPGA

Figure 1. Sites of the sampling of the study.

Table 1. Isolation of bacteria.

Bacterial diseases	Bacteria causing the infection	Culture center	Temperature and incubation time	Aspects of the colonies	References
Bacterial wilt	*R. solanacearum*	King B	26°C, 48h	Bluish fluorescent	Huynh et al., 2019
Bacterial canker	*C. michiganensis*	LPGA	26°C, 24 - 48h	Cream to yellowish	Amkraz et al., 2010
Bacterial scib	*X. campestri*	LPGA	26°C, 24 - 72h	Pale or yellow	Gracein et al., 2012
Bacterial speck	*P. syringae*	King B	26°C, 24 - 48h	Round white and smooth	Amkraz et al., 2010
Soft rot	*E. carotovora*	King B	26°C, 24h	Convex and bluish	Chartier, 2005

Table 2. Oligonucleotides for molecular identification of bacteria.

Species	Sequences	Lenght (pb)	Target	References
R. solanacearum	ATTACGAGAGCAATCGAAAGATT TCGCTTGACCCTATAACGAGTA	91	16S-23S	Kumar et al., 2017
	GTCGCCGTCAACTCACTTTCC GTCGCCGTCAGCAATGCGGAATCG	280	16S-23S	
C. michiganensis	TCATTGGTCAATTCTGTCTCCC TACTGAGATGTTTCACTTCCCC	271	16S-23S	Yim et al., 2012
X. campestri	AGTTGCAGCAGCTGTTCT ATAGCACGTATTGGCAGGG	304	*rpfH*	Kiran et al.,2019
E. carotovora	TTACCGGACGCCGAGCTGTGGCGT CAGGAAGATCTCGTTATCGCGAGT	435	ARN 16S	Photchanachai et al., 2006
P. syringeae	ACGAGCTGAAGGAAGACA CAGCCTGGTTAGTCTGGTTA	525	HrpZpst	Zaccardell et al., 2005

Table 3. Distribution of isolated strains according to the sites.

Variable	Isolated strains				Positive PCR strains			
	Site 1	Site 2	Site 3	Total	Site 1	Site 2	Site3	Total
Ralstonia sp	4	2	4	10	1	1	2	4
Clavibacter sp	7	10	10	27	1	2	4	7
Pseudomonas sp	6	3	3	12	4	0	0	4
Erwinia sp	4	4	4	12	1	1	0	2
Xanthomonas sp	2	6	1	9	0	2	2	4

agar. The tests were repeated in triplicate. The dishes were incubated at 28°C for 24 h.

Bacterial pathogenicity test in vivo

One-month-old tomato plants were inoculated with a fresh culture of the following bacteria: *R. solanacerum, E. carotovora, X. campestris*, *C. michiganensis,* and *P. syringea*. The bacterial strains used are those that have been confirmed by PCR. Inoculation was done by adding 500µl of fresh bacterial culture to the leaf surface of each plant. The inoculated plants are observed until the appearance of the first symptoms (Table 1, Figure 9). The inoculated plants were re-isolated on agar and compared to the base strains as follows. Leaves from each plant were cut (3 leaves per plant) and placed in tubes containing 2ml of sterile distilled water for 1 hour. Dilutions in physiological water were then made and 10µl spots were deposited on the agar incubated at 28°C for 24 h. All the tests were repeated three times for each bacterium (Kumar et al., 2017 modified).

RESULTS

Collection of samples and bacterial identification

A total of 70 bacteria were isolated. The strains isolated from site 1 are 23 in number, 25 from site 2, and 22 from site 3. Among them, 20% were isolated from the leaves and 80% from the soil. According to the bacterial species, there were 38.57% of *Clavibacter sp*; 17.14% *Erwinia sp*, 14.728% *Ralstonia sp*; 12.85% *Xanthomonas sp,* and 14.42% *Pseudomonas sp.* 36% of the bacterial strains were isolated from site 2, while for sites 1 and 3 we had 33 and 31% of isolated bacteria respectively (Table 3), (Figures 2 and 3). The five investigated bacteria were recovered in the 3 sites.

Quantification of genomic DNA

Genomic DNA was extracted from 70 strains. The amount of nucleic acid was quantified using Nanodrop oneC instruments. Indeed, the highest amounts of DNA were identified in *X. campestris* followed by strains 26 and 15 of *C. michiganensis* then strains 10 and 8 of *Ralstonia*. The highest concentrations are 218, 246, 172, 165.5 and 165.1 µg/ml. The purity of the samples was between 1.4 and 2.1. The table shows the concentration and purity of the PCR-positive strains (Table 4).

Molecular characterization

Genomic amplification by PCR made it possible to obtain 271 bp fragments for *C. michiganensis* representing the 16S-23S gene. Products of 280 bp for *R. solanacearum* and 435 bp for *E. carotovora* were both obtained targeting the 16S-23S gene. *P. syringae* targets the *HrpZpst* gene with a fragment size of 525 bp, while the *X. campestris* (*hrp*) gene has a fragment size of 304 bp. For molecular characterization by PCR, we identified 28.57% positive phytobacteria. According to the bacterial species, there is 35% of *C. michiganensis*, 5% *E. carotovora*, 20% *R. solanacearum*, *X. campestris* and *P. syringae* (Figures 4 to 9).

Bacterial growth curve

The number of colony-forming units (CFU) was evaluated at different dilutions of isolates at different times. The isolates used are the PCR positive isolates. The number of colony-forming units (CFU) was assessed at different dilutions of isolates at different time points. The isolates used are the positive PCR isolates. The highest bacterial growth was observed in *E. carotovora*; followed by one of the *Ralstonia* strains. Two of the strains of *Clavibacter* had similar bacterial growth. They could be the same strain or a neighboring strain. *Clavibacter* strain 18 has much weaker growth than the previous two strains. All like the two strains of *Xanthomonas* and *Erwinia* which could come from distant strains (Figure 10).

Pathogenicity test

Pathogenicity tests showed that the selected strains were able to induce infection in tomato plants. The first symptoms appeared 14 days after inoculation for *R. solanacearum, E. carotovora,* and *X. campestris*. Early symptoms of *R* solanacearum were characterized by yellowing and wilting of leaves. Erwinia induces yellowing

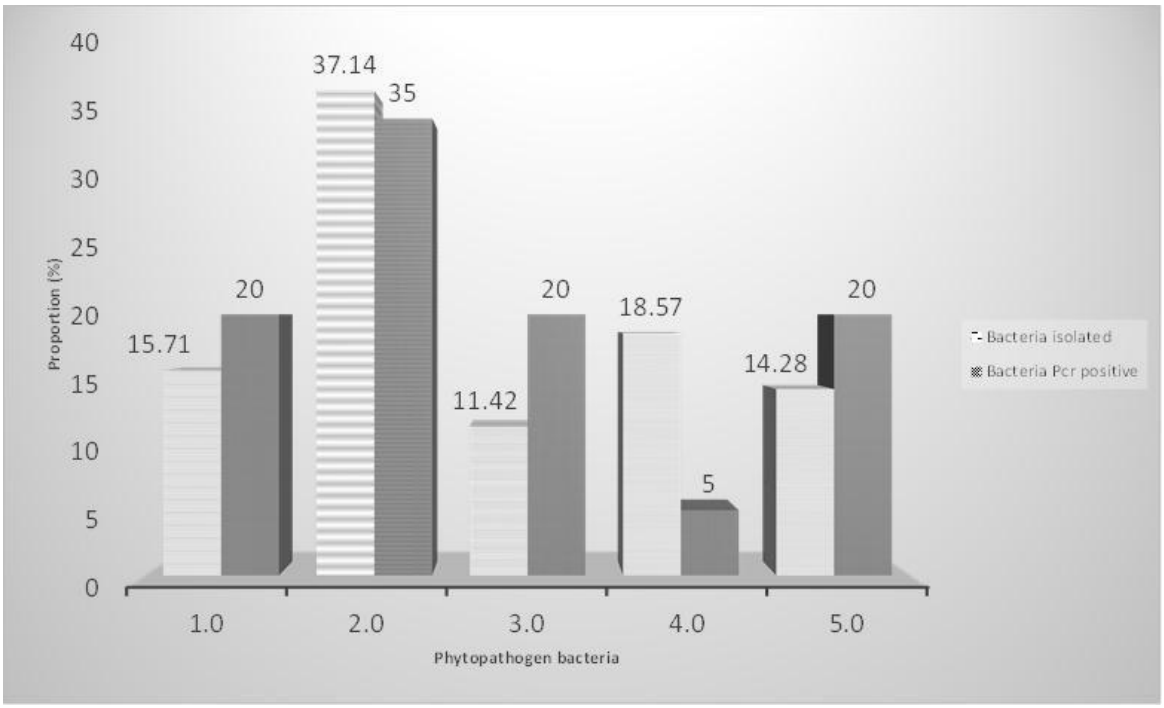

Figure 2. Distribution of bacteria strains.

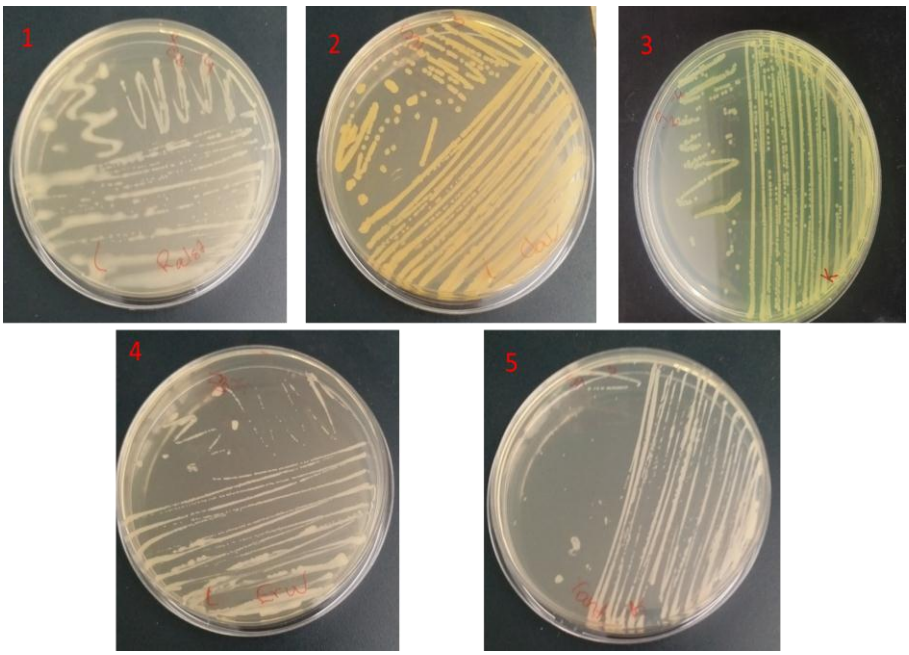

Figure 3. Bacteriological identication of phytopathogen bacteria. 1. *Ralstonia Solanacearum*; 2. *Clavibacter michiganensis*; 3. *Pseudomonas syringeae*; 4. *Erwinia carotovora*; 5. *Xanthomonas campestris*

of the leaves while small yellow to brown spots have been observed on the leaves of plants infected with *X. campestris. C. michiganensis* and *Pseudomonas* appear one month after inoculation in the plant. Symptoms of *C. michiganensis* were manifested by the appearance of a burning color on some leaves as well as their wilting. One

Table 4. ADN concentration.

Isolate	Concentration (ng/µl)	Purity (260/280)	Isolate	Concentration (ng/µl)	Purity (260/280)
Isolate 1	56.6	1.97	Isolate10	165.1	1.94
Isolate 2	163.7	1.79	Isolate11	135.1	2.11
Isolate 3	109.7	1.92	Isolate12	165.5	2
Isolate 4	151.4	1.24	Isolate13	117.7	1.96
Isolate 5	246.7	2.02	Isolate14	33	1.82
Isolate 6	21.4	2	Isolate15	80.2	1.34
Isolate 7	36.3	1.99	Isolate16	218	1.62
Isolate 8	123.7	2.1	Isolate17	16.3	2.09
Isolate 9	68.1	1.96	Isolate18	120.8	2.05

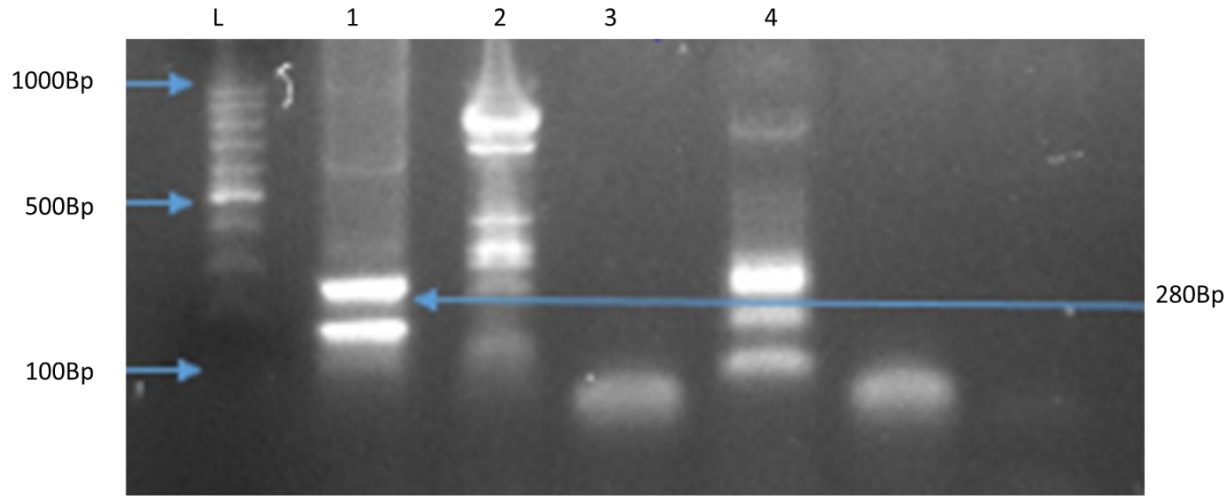

Figure 4. Molecular identification of *Ralstonia solanacerum.* L: Ladder, 1,2,3,4: Sample of tomato.

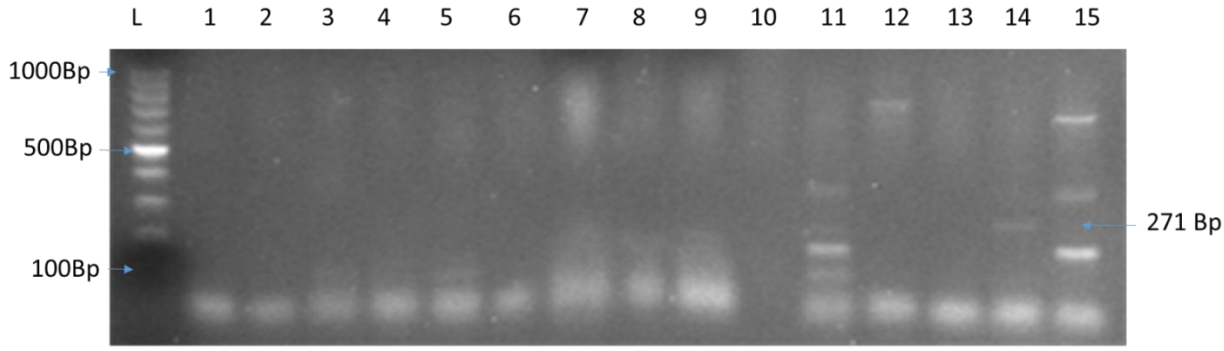

Figure 5. Molecular identification of *Clavibacter michiganensis*. L: Ladder, 1,2,3,4,5,6,7: Sample of eggplant; 8,9, 10,11, 12, 13, 14, 15: Sample of tomato.

appearance of leaves infected by *Pseudomonas* was characterized by the appearance of yellowish to brown spots on the leaves. The bacterial count of each strain was assessed weekly after inoculation. The bacteria concentrations obtained were listed in Figures 10 and 11. The figure showed that from 0 to 4 days, the bacterial

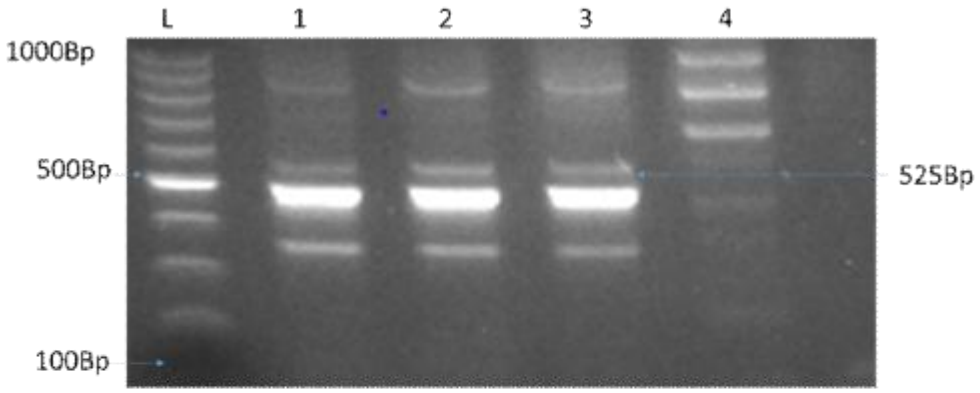

Figure 6. Molecular identification of *Pseudomonas syringea*. L: Ladder; 1.2.3.4: Sample of tomato.

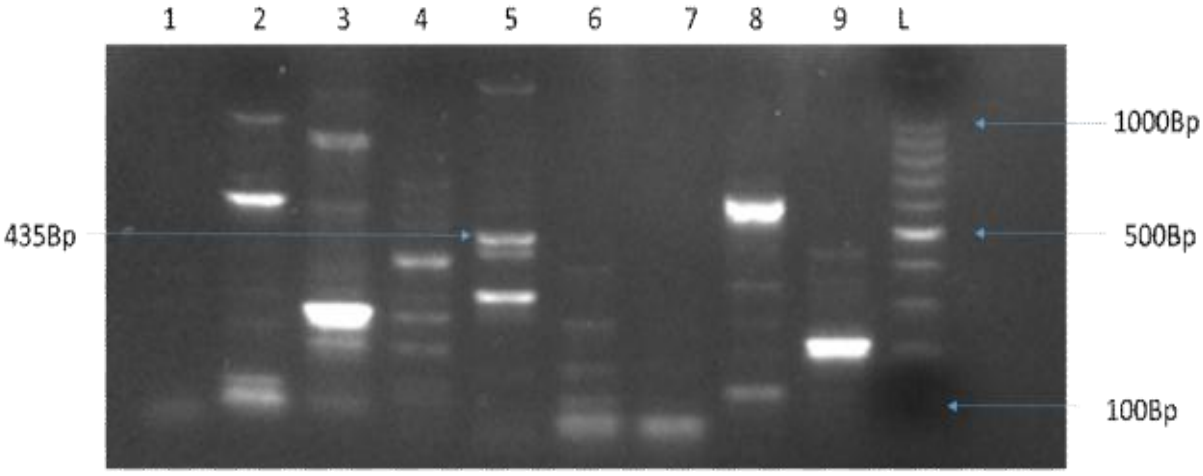

Figure 7. Molecular identification of *Erwinia carotovora*. L: Ladder (Promega 100pb); 1.2.3.4: Bacteria sample of tomato from site 1.2.3.

concentration of all bacteria was almost zero. Between 14 and 10 days, the concentration of bacteria increased slightly. From day 10, bacterial growth of *Clavibacter; Xanthomonas,* and *Erwinia* on leaves increased exponentially. As for *Ralstonia* and *Pseudomonas*, growth remained low throughout the experiment.

DISCUSSION

This study confirms the presence of phytopathogenic bacteria in leaf and soil samples from the sites. A collection of 70 bacterial strains was isolated. The bacteria isolated from site 1 are 33%. The results corroborate those of Kumar et al, 2017 in their study which shows that the rhizospheric bacteria were a mixture of antagonists and neutral and mutualistic pathogens. Site 1's cultivation system was organic and chemical-free. The constituent elements of the rhizosphere have therefore been preserved. And there is a close connection between subsoil microbes and aboveground components of the plant ecosystem (Kumar et al., 2017). Previously used as a household waste dump 36% of the phytobacteria were isolated from site 2. Indeed, this waste was the growth biotope of several microorganisms of bacterial, viral, and parasitic origin. And Cissé's study demonstrated that a biotope where contamination is high presents a high risk for cultivated plants (Cissé, 1997). *C. michiganensis* responsible for bacterial canker, was the most isolated phytobacteria on the three sites with 38.57% confirmation. The results coincide with those of Ftayeh and Nandi who state that *Clavibacter* is a devastating emerging disease for crops. It is a serious disease in the world and new epidemics have recently been reported in several countries (Ftayeh et al., 2011) hence its strong presence in these soils has

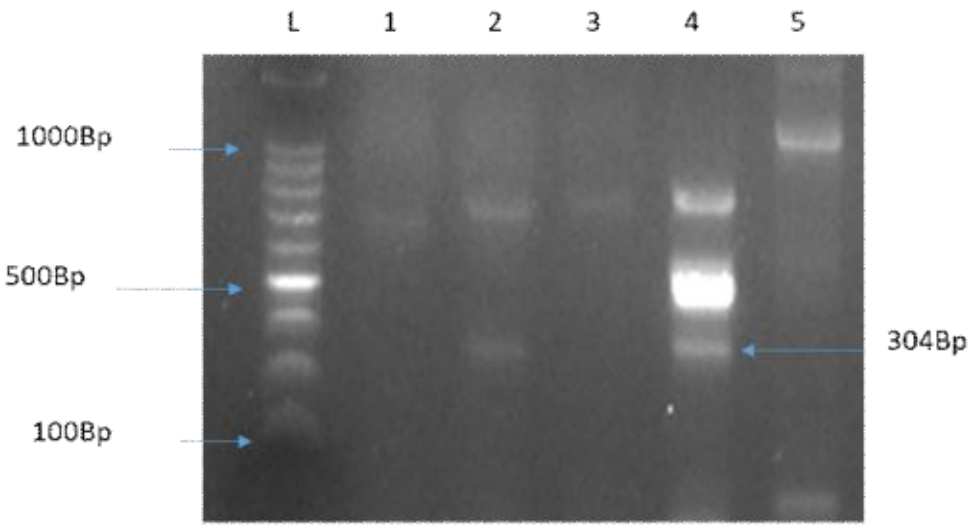

Figure 8. Molecular identification of *Xanthomonas campestris*. L: Ladder; 1.2: Sample of eggplant; 3.4: Sample of tomato.

Figure 9. Tomato plants inoculated with phytobacteria. A1-A2: Plant without bacteria (negative culture); B1: Tomato plants inoculated with *Xanthomonas campestris* after 3 days; B2: Tomato plants inoculated with *Ralstonia solanacerum* after 3 days; C1: Tomato plants inoculated with Xanthomonas campestris after 15 days; C2: Tomato plants inoculated with Ralstonia solanacerum after 15 days; E: *Erwinia carotovora;* R: *Ralstonia solanacerum;* C: *Clavibacter michiganensis;* X: *Xanthomonas campestris;* Cfu: Colony forming unity.

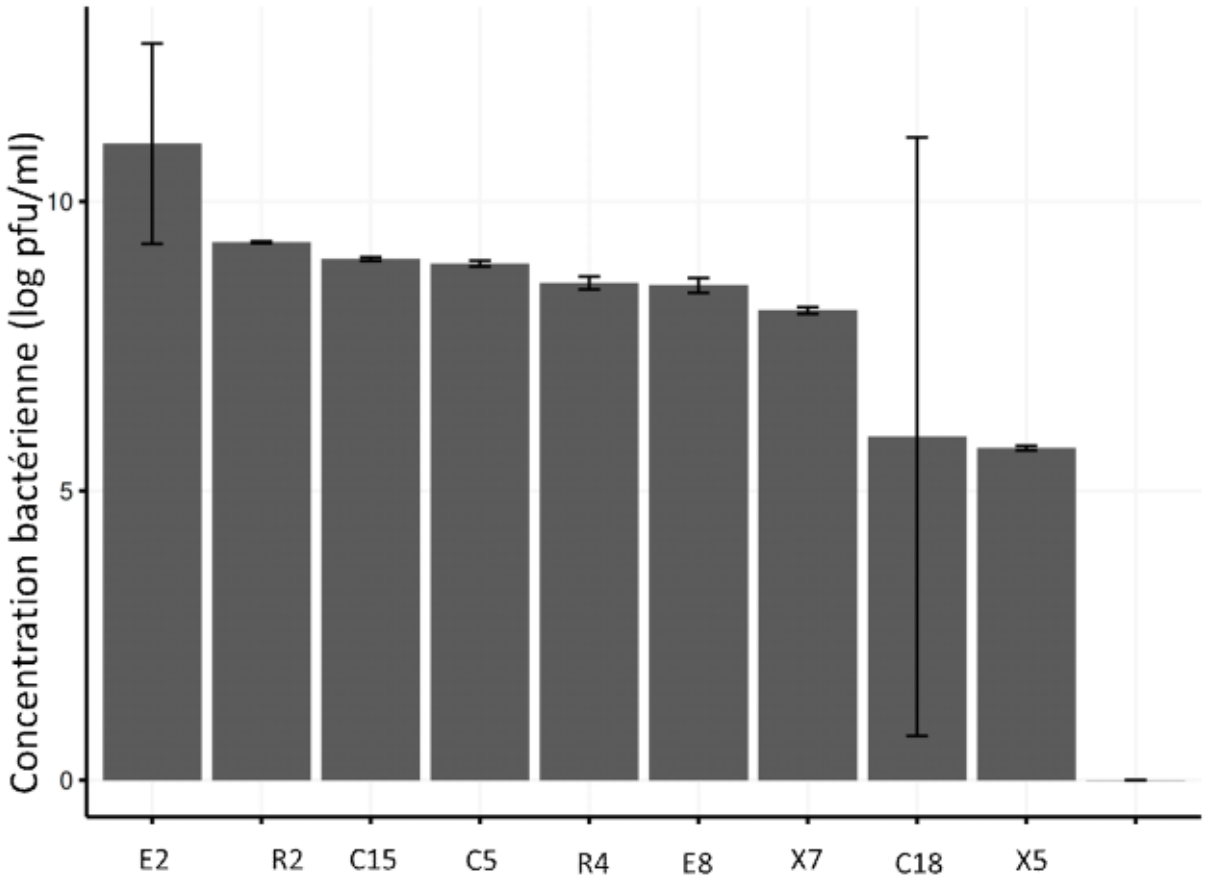

Figure 10. Bacterial concentration of plant pathogens.

caused significant crop losses. It is also considered one of the most devastating plant diseases of crops (Nandi et al., 2018). It is followed by *E carotovora* preferentially isolated on site 2 at 17.14%. The results are contrary to the results of a previous study by Benada which showed that *Erwinia sp* is a bacterium that preferentially infects fruits and potatoes (Benada et al., 2019). Damaged fruits and potatoes deposited on the plot of site 2 formerly used as a dump could be the cause of the appearance of this bacterium. And the insect vectors par excellence have allowed the spread of this bacterium in plants (MHMED, 2019).

One of the most important bacteria in peppers and crucifers is *Xanthomonas campestris*. It is seed-borne and can survive in weeds and plant debris for months. These matrices are a biotope for surviving *Xanthomonas* (Laala et al., 2021). *Xanthomonas campestris* was isolated with a percentage of 12.85% in tomatoes. A previous study isolated xanthomas in rice and it was *Xanthomonas oryzae pv. Oryzicole* in Ivory Coast (Diallo et al., 2010). However, strains of *Xanthomonas campestris* have just been isolated for the first time in Côte d'Ivoire. At the level of molecular characterization, 50% of *Pseudomonas* isolates were PCR positive. Although the hrp gene is unknown in virulence and pathogenicity, this region is essential for the production of symptoms in the host (Zaccardelli et al., 2005). It is considered a stable gene using 16S-DNA. In Côte d'Ivoire in seven agroforestry regions, the study by Guessan (Guessan et al., 2012) described a high prevalence of 87% for *Ralstonia* phylotype I. In this study, 36% of *Ralstonia solanacerum* were confirmed by PCR.

The isolates of *C. michiganensis* (21.42%) are Pcr positive. ITS region sought for molecular confirmation of Clavibacter is a relatively variable area. Yim et al. (2012) showed a difference of more than 50% between the ITS regions of tomato and pepper crops. And a diversity of genetic variation exists between Clavibacter populations in several countries (De Leon et al., 2009). Our pathogenicity test results showed that *R. solanacerum*; *E. carotovora* and *X. campestris* induce symptoms on tomato plants. The first symptoms appeared after 14 days. And according to the literature the first symptoms appear after an infection time of 14 days (Iiyama et al., 2021). These bacteria are preferentially present in the rhizosphere. They, therefore, infect the plant through the roots and invade the vascular system. The bacteria grow and produce extracellular polysaccharides which cause

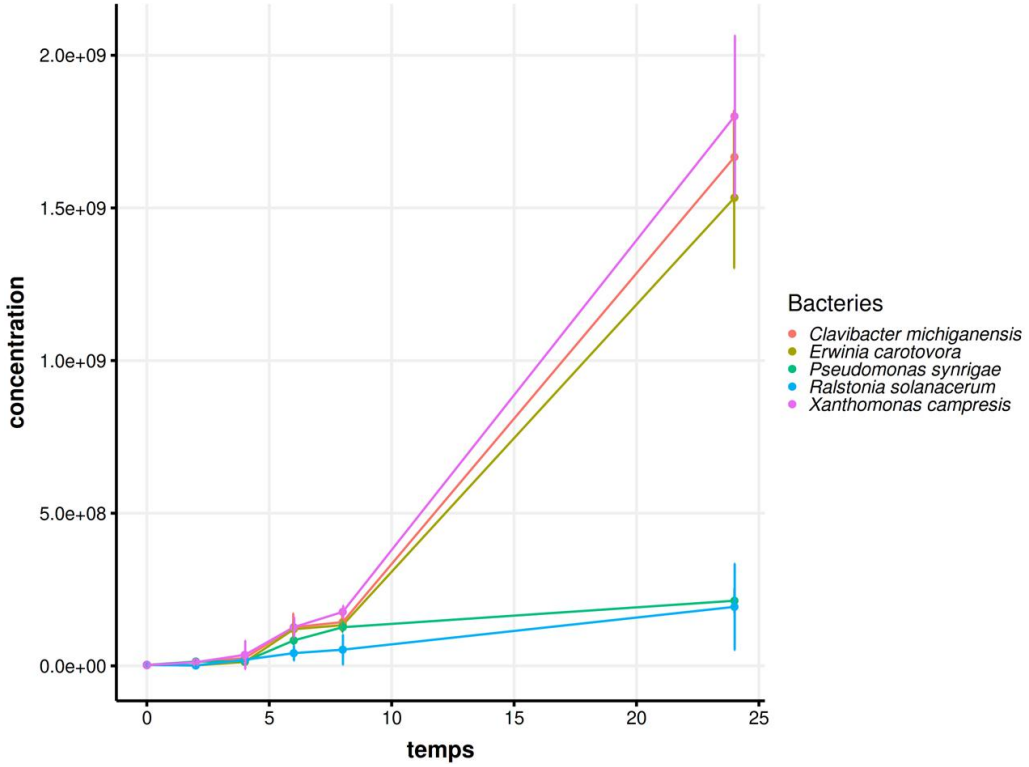

Figure 11. Concentration of phytobacteria.

obstruction of the first symptoms on the plant and the death of the latter (Nakahara et al., 2021). The *Ralstonia* strains used induced infection in tomato plants. It could therefore be race 3. Indeed, according to a study by (Kumar et al., 2017), the strains capable of infecting Solanaceae are part of race 3.

After one month of infection, all plants infected with *C. michiganensis* and *P. syringea* showed symptoms. These results are consistent with those of Yim et al. (2012) which show 25 days after inoculation, the plants show symptoms and 80% of the plants die. This study confirmed the presence of phytobacteria in plants. 70 strains of phytopathogenic bacteria were isolated in three sites (rural, semi-rural, and urban sites). And 20 strains have been confirmed by molecular diagnosis. *E. carotovora* strains *X. campestris C. michiganensis* and *P. syringea* were isolated for the first time in this study. *C. michiganensis* is the most predominant strain in the tomato and eggplant plant. *E. carotovora* and *R. solanacerum* are abundant at all sites. These strains induced in vivo infection tests with major leaf symptoms. Isolated bacteria are responsible for various infections resulting in huge production losses. To compensate for these losses, the use of biopesticides such as agriphages is a godsend, especially since their isolation is inexpensive. The isolation of agriphages would then be a considerable alternative to reduce production losses due to phytopathogens.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests

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