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Full Length Research Paper

Phylogenetic analysis of multidrug resistant *E. coli* isolates from the urinary tract in Bushenyi district, Uganda using the new Clermont phylotyping method

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Due to the increasing rates of multidrug resistance (MDR) among the Enterobacteriaceae that cause urinary tract infections (UTIs), selection of antimicrobial agents for empirical therapy is becoming a major challenge. This study determined the antimicrobial resistance profiles, multidrug resistance profiles, multiple antibiotic resistance indices (MARI), factors associated with MDR UTIs and the phylogenetic groups of MDR *Escherichia coli* strains isolated from the urinary tract among patients attending hospitals in Bushenyi District, Uganda. In this cross-sectional study, a total of 86 bacterial uropathogens isolated from 267 study participants suspected to have UTIs were subjected to antimicrobial susceptibility tests using the Kirby Bauer Disk diffusion method. Data for the factors associated with MDR were obtained by the use of questionnaires. Phylogenetic groups of the MDR *E. coli* were determined using the new Clermont method for phylotyping *E. coli*. Descriptive and multiple logistic regression statistical tools were used to determine phylogenetic groups, and assess for statistically significant relationship between MDR UTIs and factors suspected to be associated with MDR UTIs respectively. The isolates assigned as group B2 9/12 (75.0%), B1 2/5 (40.0%) and A 2/7 (28.6%) by using the old Clermont method could not be phylotyped using the new Clermont method and were grouped as non-typeable strains of *E. coli*. Our study demonstrated high prevalence of the non-typeable strains of MDR *E. coli*, we therefore recommend the use of modern DNA sequencing-based approaches which is the gold standard for genotyping bacteria, that this current study could not afford.

Key words: Phylogenetic analysis, bacterial urinary tract infections, factors associated with, multidrug resistance, Bushenyi District, Uganda.

INTRODUCTION

Urinary tract infections are one of the most common infectious diseases, which damages different parts of the urinary system such as urethra, bladder, ureters and the kidney (Ullah et al., 2018). It is estimated that about 150 million people per year are diagnosed with UTIs costing greater than 6 billion US dollars used for health care (Stamm and Norrby, 2001; Gupta et al., 2001; Laupland et al., 2007; Ali et al., 2017). Urinary tract infections are commonly treated with β-lactam antibiotics and fluoroquinolones but the emergence of MDR strains among the leading uropathogens to commonly used antimicrobial agents is on the rise, resulting into impaired treatment of UTIs (Shabbir et al., 2018). According to Khawcharoenporn et al. (2013) and Hadifar et al. (2016), MDR was described as the non-susceptibility of bacteria to at least one antimicrobial agent in 3 or more antimicrobiaı classes. The development of the MDR strains in bacterial uropathogens has been attributed to several factors such as female gender, older age, history of UTIs, residence in the nursing home, hospitalization, prior exposure to antimicrobials, urinary catheterization and bacterial factors (Shabbir et al., 2018; Hasan et al., 2007).

Clermont et al. (2000) coined a triplex polymerase chain reaction (PCR) amplification technique based on the *chuA, yjA* and Tspe4.C2 genes' analysis to categorized *E. coli* into the following phylogenetic groups; A, B1, B2, or D (Clermont et al., 2000). Multilocus sequence type (MLST) data clearly indicated that 80-85% of the phylogenetic group allocations where actually correct (Iranpour et al., 2015) while a smaller fraction of *E. coli* strains with A0, D1 and D2 genotypes were not assigned into correct phylo-groups. Clermont et al. (2013) added *arpA* gene to the triplex PCR to make a quadruplex PCR. The addition of this new marker, *arpA*, improved the specificity and detection of new phylogenetic groups in *E. coli* including; A, B1, B2, C, D, E, F, and clade I (Clermont et al., 2013).

The increase in the rise of drug resistant bacteria resulted into MDR strains (Li and Webster, 2018). In Uganda, the policy of treatment of UTIs was put in place but susceptibility patterns of these bacteria seem to be changing (Kabugo et al., 2016). Most of the previous studies in Uganda by Mwaka et al. (2011), Odongo et al. (2013), Odoki et al. (2015), Katongole et al. (2015), Ampaire et al. (2015) and Kabugo et al. (2016) focused much on the prevalence and antimicrobial susceptibility patterns of bacterial uropathogens neglecting MDR factors associated with these bacteria and their phylogenetic groups. Khawcharoenporn et al. (2013) reported that, Infectious Diseases Society of America recent guidelines recommended that treatment of UTIs using antimicrobial therapy should be directly proportional to indigenous resistance profile of bacteria, drug availability and antimicrobial intolerance/allergy history of treated patients (Gupta et al., 2011). Due to that, indigenous epidemiological studies are vital in the choice of the most proper antimicrobials for empirical treatment, so as to combat the spread of MDR bacterial uropathogens within our communities and healthcare centers. To date there is no study focusing on the MDR bacterial uropathogens, factors associated with MDR UTIs and phylogenetic groups of the bacteria causing MDR UTIs in Bushenyi District, Uganda. Therefore, this study was designed to determine antimicrobial resistance profiles, multidrug resistance profiles, MARI, factors associated with MDR UTIs, and the phylogenetic groups of MDR *E. coli* strains isolated from the urinary tract among patients attending hospitals in Bushenyi District, Uganda.

MATERIALS AND METHODS

Study design

This was a cross-sectional health-point survey conducted from June, 2017 to January, 2018 on 86 bacterial uropathogens isolated previously from 267 study participants suspected to have UTIs that attended Kampala International University-Teaching Hospital (KIU-TH), Ishaka Adventist Hospital and Comboni Hospital Kyamuhunga by Odoki et al. (2019). This study used a survey formula previously reported by Kish (1965):

$n = z^2p\ (1\text{-}p)/d^2$

Where d=margin of error of setting a significance level of 0.05 (5%); z=level of significance (1.96) for confidence interval of 95%; p = prevalence of UTIs in Bushenyi District of Uganda, among patients receiving medical treatment at selected hospitals was 22.33% (Tibyangye et al., 2015). Patients of the following category were included in the study: symptomatic UTIs, suspected to have UTIs, and residents of Bushenyi District that are receiving medical treatment at KIU-TH, Comboni Hospital kyamuhunga and Ishaka Adventist Hospital. Patients of the following category were excluded from the study: critically ill, menstruating females, those unable to micturate and those who are currently on antibiotics or with history of antibiotic usage in a fortnight. Patients who met the selection criteria from each of the respective hospitals were recruited in the study using simple random sampling technique (Odoki et al., 2019).

Study variables

Questionnaires were administered to collect information from the study participants as regards sociodemographic data such as: age, gender, residence, marital status, level of education, circumcision and sexual intercourse. Data on the health status were obtained by the clinicians through clinical examinations and medical history of the study participants like: hypertension, genitourinary abnormalities, abortion, recurrent UTIs, previous hospital admission,

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family history of UTIs, previous UTIs, chronic respiratory disease, wrong prescription of antimicrobial agents, history of fluoroquinolone, cephalosporin and any antimicrobial use in the last 12 months. Data on selected factors suspected to be associated with MDR UTIs such as pregnancy, diabetes mellitus and human immunodeficiency virus (HIV), were obtained through laboratory investigations (Odoki et al., 2019).

Antimicrobial susceptibility testing

The antimicrobial susceptibility tests were done at Mbarara University of Science and Technology-Teaching Hospital (MUST-TH) microbiology laboratory on bacterial isolates previously isolated and characterized by Odoki et al. (2019). Antimicrobial susceptibility tests were performed on bacterial isolates from midstream urine (MSU) using antimicrobial discs, according to Clinical and Laboratory Standards Institute (CLSI) on Muller Hinton agar (CLSI, 2018). The prepared media was inoculated with bacterial suspension equivalent to 0.5 MacFarland turbidity. The commercially available antimicrobial discs containing the following antimicrobials: ampicillin (10 µg), nitrofurantoin (50 µg), cefoxitin (30 µg), co-trimoxazole (25 (1.25/23.75) µg), amikacin (30 µg), gentamicin (10 µg), imipenem (10 µg), and erythromycin (15 µg) (Himedia, India) were aseptically placed on the surfaces of the sensitivity agar plates with a sterile forceps and allowed to stand for 30 min. The plates were then incubated for 18-24 h at 37°C. Zones of inhibition after incubation were observed, measured and interpreted according to CLSI (2018). For each antimicrobial agent used, results were reported as sensitive or resistant. Isolates showing intermediate antimicrobial susceptibility were considered to be resistant. *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923) were used as quality control organisms for the antimicrobial susceptibility testing according to CLSI (2018). Multidrug resistant isolates were defined as isolates that demonstrated resistance to ≥ 3 of the following antimicrobial agent categories as previously reported by Khawcharoenporn et al. (2013) and Hadifar et al. (2016): (1) penicillin: ampicillin, (2) nitrofurans: nitrofurantoin, (3) cephalosporins: cefoxitin, (4) trimethoprime-sulphonamides: co-trimoxazole, (5) aminoglycosides: gentamicin and amikacin, (6) carbapenems: imipenem and, (7) macrolides: erythromycin.

Multiple antibiotic resistance indices

Calculation of MARI was done by dividing the number of antimicrobial agents that the bacterial uropathogen is resistant to, by the total number of antimicrobial agents to which the bacterial uropathogen was tested against (Ekwealor et al., 2016). Multiple antibiotic resistance index of ≥0.2 is an indication that this particular bacterial colony can be traced back to a habitat where several antimicrobial agents have been used or abused (Ehinmidu, 2003; Tambekar et al., 2006; Oli et al., 2013).

Phylogenetic analysis of the MDR *E. coli* isolates

The molecular biology tests to determine the phylogenetic groups of MDR *E. coli* strains using the new Clermont phylotyping method were done at Makerere University, College of Health Sciences, Molecular Biology Laboratory, Mulago, Kampala, Uganda. Deoxyribonucleic acids were extracted from pure colony of 31 different isolates of MDR *E. coli* by boiling method as previous described by Acaku et al. (2014). Simplex PCR for genotyping was done using primer flacking genomic regions in *chuA, yjaA,* TsPE4.C2 and *arpA* with slight modifications as previously described by Sáez-López et al. (2016). Polymerase chain reactions were done in a 20 µL Volume containing 2Xtaq master (NEB, USA) 10 pmol of forward and reverse primer as well as nuclease free water using these PCR conditions. Initial denaturation at 95°C for 5 min, denaturation at 94°C for 30 s, annealing at 52°C for 30 s for 30 cycles, extension at 6°C for 30 s for 30 cycles and final extension at 68°C for 10 min. Following amplification, the amplicons were analyzed by agarose electrophoresis using a 2% TBE- Agarose gel containing ethidium bromide. The gels were viewed and UVP gel imaging system-Bio DOC-It (CA, USA) to analyze an expected amplicon size (Table 1). Deoxyribonucleic acids were extracted from the control organism and were used as positive controls while PCR water was used for negative extraction as well as negative template controls. The *trpA* gene was used as an internal control on all isolates and several *E. coli* strains previously characterized and known to harbor; *chu*A*, yja*A*, arp*A*, trp*A genes and TspE4.C2 at Makerere University, College of Health Sciences, Molecular Biology Laboratory, Mulago, Kampala, Uganda were used as positive controls. The gene amplification results were considered to be positive, if the PCR products were similar to the expected amplicon size.

Data analysis

Data involving patients' socio-demographic characteristics, health conditions, MDR profiles and phylogenetic groups of MDR *E. coli* strains were entered in Microsoft Excel and exported to IBM SPSSv20 software for analysis. The MDR profiles and distribution of MDR among the bacterial uropathogens was determined using descriptive statistics. Descriptive statistics was also used to obtain the prevalence of phylogenetic groups of the MDR strains of *E. coli* according to the old and new Clermont phylotyping methods. The result of MDR UTIs was categorized into: presence or absence of MDR. Bivariate analysis was used to determine whether statistically significant relationship exists between MDR UTIs and factors suspected to be associated with MDR UTIs. Stepwise forward multiple logistic regression model was applied on all variables with *p* value ≤0.2 to assess the statistically significant relationship (p≤0.05) to eliminate confounding variables.

Ethical approval

Ethical clearance was obtained from MUST, Institutional Research and Ethics Committee (IREC) on Human Research (No. 01/01-17) and final clearance was sought from Uganda National Council for Science and Technology (UNCST) (No. HS 2232). The Helsinki declaration of 1964 revised in 2000 ethical principles was observed on all research protocols (WMADH, 2000).

RESULTS

Multidrug resistance profiles of the bacterial uropathogens

When the bacterial uropathogens were subjected to the following seven different categories of antibiotics; (1) penicillin: ampicillin, (2) nitrofurans: nitrofurantoin, (3) cephalosporins: cefoxitin, (4) trimethoprime-sulphonamides: co-trimoxazole, (5) aminoglycosides : gentamicin and amikacin, (6) carbapenems: imipenem and, (7) macrolides: erythromycin, the most resisted antimicrobial agents were erythromycin with 74/86 (86.0%) and co-trimoxazole 74/86 (86.0%), followed by ampicillin 69/86 (80.2%), and cefoxitin 37/83 (44.6%) (Table 2).

Table 1. Primer sequences, sizes of PCR products used in phylotyping MDR strains of *E.coli* using a simplex PCR method.

PCR reaction	Primer ID	Target	Primer sequences	PCR product (bp)	References
Simplex	chuA.1b chuA.2	*chuA*	5′-ATGGTACCGGACGAACCAAC-3′ 5′-TGCCGCCAGTACCAAAGACA-3′	288	Clermont et al. (2000)
Simplex	yjaA.1b yjaA.2b	*yjaA*	5′-CAAACGTGAAGTGTCAGGAG-3′ 5′-AATGCGTTCCTCAACCTGTG-3	211	Clermont et al. (2000)
Simplex	TspE4C2.1b TspE4C2.2b	TspE4.C2	5′-CACTATTCGTAAGGTCATCC-3′ 5′-AGTTTATCGCTGCGGGTCGC-3′	152	Clermont et al. (2000)
Simplex	AceK.f ArpA1.r	*arpA*	5′-AACGCTATTCGCCAGCTTGC-3′ 5-TCTCCCCATACCGTACGCTA-3	400	Clermont et al. (2004)
Group E	ArpAgpE.f ArpAgpE.r	*arpA*	5′-GATTCCATCTTGTCAAAATATGCC-3′ 5-GAAAAGAAAAAGAATTCCCAAGAG-3	301	Lescat et al. (2012)
Internal control	trpBA.f trpBA.r	*trpA*	5′-CGGCGATAAAGACATCTTCAC-3′ 5′-GCAACGCGGCCTGGCGGAAG-3′	489	Clermont et al. (2008)

Table 2. Antibiotic resistant profiles of the bacterial uropathogens.

Uropathogens	AMP	NI	CX	SXT	AK	GEN	IMI	E
E. coli n=36	29 (80.6)	5 (13.9)	13 (36.1)	35 (97.2)	0 (0.0)	3 (8.3)	3 (8.3)	36 (100.0)
K. pneumoniae=10	7 (70.0)	5 (50.0)	1 (10.0)	10 (100.0)	0 (0.0)	1 (10.0)	4 (40.0)	10 (100.0)
K. oxytoca=6	6 (100.0)	2 (33.3)	1 (16.7)	4 (66.7)	0 (0.0)	0 (0.0)	0 (0.0)	6(100.0)
P. mirabilis=3	3 (100.0)	2 (66.7)	3 (100.0)	3 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (100.0)
P. vulgaris=1	0 (0.0)	0 (0.0)	1 (100.0)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100.0)
S. aureus=27	22 (81.5)	2 (7.4)	18 (66.7)	19 (70.4)	9 (33.3)	10 (37.0)	2 (7.4)	15 (55.6)
E. faecalis=3	2 (66.7)	1 (33.3)	--	2 (66.7)	--	--	0 (0.0)	3 (100.0)
Total	69 (80.2)	17 (19.8)	37 (44.6)	74 (86.0)	9 (10.8)	14 (16.9)	9 (10.5)	74 (86.0)

AMP=ampicillin; NI=nitrofurantoin; CX=cefoxitin; SXT=co-trimoxazole; AK=amikacin; GEN=gentamicin; IMI=imipenem; E=erythromycin; "--"= not done.

Distribution of MDR and MARI

When the bacterial uropathogens were subjected to ampicillin, nitrofurantoin, cefoxitin, co-trimoxazole, gentamicin, amikacin, imipenem and erythromycin, selection of MDR strains were in the order: 6/6 (100.0%) *K. oxytoca*, 3/3 (100.0%) *P. mirabilis*, 1/1 (100.0%) *P. vulgaris* > 9/10 (90.0%) *K. pneumoniae* > 31/36 (86.1%) *E. coli* > 22/27 (81.5%) *S. aureus* > 1/3(33.3%) *E. faecalis* (Table 3). All the bacterial uropathogens tested showed MARI of ≥0.2 (Table 4).

Table 3. Distribution of MDR among the bacterial uropathogens.

Uropathogens	MDR strains n (%)
E. coli n=36	31 (86.1)
K. pneumoniae n=10	9 (90.0)
K. oxytoca n=6	6 (100.0)
P. mirabilis n=3	3 (100.0)
P. vulgaris n=1	1 (100.0)
S. aureus n=27	22 (81.5)
E. faecalis n=3	1(33.3)
Total	73 (84.9)

n=number; %=percentage.

Factors associated with MDR UTIs

When the predictor variables for MDR were subjected to bivariate analysis, they had the following logistic regression values: hospitalization (OR=3.616; 95% CI: 1.017-12.860; p<0.05) and previous use of any antimicrobial agent in the last 12 months (OR=5.175; 95% CI: 1.007-26.605; p<0.05) were found to be statistically significant (p<0.05) with MDR UTIs (Tables 5 and 6). When all the variables with a *p* value of 0.2 or less were entered into stepwise forward multiple logistic regression model, they had the following logistic regression values: hospitalization (OR=3.947; 95% CI: 1.050-14.831; p<0.05) and previous use of any antimicrobial agent (OR=6.004; 95% CI: 1.046-34.454; p<0.05) were found to have statistically significant relationships (p<0.05) with MDR UTIs (Table 7). However, age, gender, residence, marital status, level of education, sexual intercourse, pregnancy, hypertension, genitourinary tract abnormalities, diabetes mellitus, HIV, cephalosporin use in the last 12 months, fluoroquinolone use in the last 12 months, abortion, chronic respiratory disease, recurrent UTIs, previous hospital admission, family history of UTIs, previous UTIs and wrong prescription of antimicrobial agents were found to have no significant association with MDR UTIs (Tables 5 and 6).

Determination of the phylogenetic groups of MDR *E. coli* strains

The results obtained in the phylotyping of the MDR *E. coli* strains isolated from urinary tract of patients attending hospitals in Bushenyi District, Uganda are as presented below. The phylotyping of *E. coli* based on the old Clermont method using *chuA, yjA* and Tspe4.C2 genes' analysis, revealed that these MDR strains of *E. coli* belongs to five phylo-groups namely: A, B1, B2, D and unknown (non-typeable) (Table 8 and Figures 1 to 3) while the new Clermont method showed that the MDR strains of *E. coli* belongs to seven phylo-groups namely: A, B1, B2, D, E, Clade1 and unknown (non-typeable) (Table 8 and Figures 1 to 4). The phylotyping of MDR *E. coli* strains based on the old Clermont method revealed that, most prevalent strains of MDR of *E. coli* belong to the phylo-group B2 with 12/31 (38.7%) followed by A 7/31 (22.6%), B1 5/31 (16.1%), D 5/31 (16.1%) and unknown 2/31 (6.5%) (Table 8 and Figures 1 to 4). The isolates assigned as group B2 9/12 (75.0%), B1 2/5 (40.0%) and A 2/7 (28.6%) by using the old Clermont method couldn't be phylotyped using the new Clermont method and were grouped as unknown or non-typeable strains of *E. coli* (Table 9). The contested results of phylogenetic groups; E or Clade 1, and D or E were screened with E specific primers (ArpAgpE) to obtain the exact *E. coli* phylogenetic group (Figure 5). All the isolates were subjected to the internal control specific primers (trpBA) for *E. coli* to ascertain species specificity of *E. coli* strains studied (Figure 6).

DISCUSSION

This study determined the antimicrobial resistance profiles, multidrug resistance profiles, multiple antibiotic resistance indices (MARI), factors associated with MDR UTIs, and the phylogenetic groups of MDR *E. coli* strains isolated from the urinary tract among patients attending hospitals in Bushenyi District, Uganda. This study provides an essential baseline information, that calls for continues monitoring of antimicrobial resistance (AMR) profiles among bacterial uropathogens, to provide reference guidelines to clinicians on the selection of the most suitable drugs in the treatment of UTIs. The increase of MDR among the bacterial pathogens in the world today poses a public health challenge in the management of infectious diseases (D'Andrea et al., 2011; Ullah et al., 2018).

Despite the fact that, some of the antimicrobial agents in the study demonstrated substantial sensitivities, some uropathogens showed extremely high level of AMR, more especially to erythromycin 74/86 (86.0%). The Gram negative bacteria demonstrated 56/56 (100.0%) resistance to erythromycin, this finding is more similar to previous reports by Kyabaggu et al. (2007); Kibret and Abera, (2011) and Adwan et al. (2014). The Gram positive bacteria resistance to erythromycin of 18/30 (60.0%) reported in this study is more comparable to previous report by Kabugo et al. (2016). The resistance of 74/86 (86.0%) to co-trimoxazole in this study is in agreement with previous reports of Odoki et al. (2015), 79/103 (76.7%); Ampaire et al. (2015), 12/14 (85.7%); Mwaka et al. (2011), 44/55 (80.0%) and Odongo et al. (2013), 60/82 (73.2%). The AMR of co-trimoxazole in this study is lower as compared to the finding of Katongole et al. (2015) who reported 51/53 (96.2%) and higher than

Table 4. Multiple Antibiotic Resistance Indices (MARI) of the bacterial uropathogens.

Uropathogens	MARI	Antibiotics to which the isolates are resistant
E. coli	0.9	AMP, NI, CX, SXT, GEN, IMI, E
K. pneumoniae	0.9	AMP, NI, CX, SXT, GEN, IMI, E
K. oxytoca	0.6	AMP, NI, CX, SXT, E
P. mirabilis	0.6	AMP, NI, CX, SXT, E
P. vulgaris	0.4	CX, SXT, E
S. aureus	1.0	AMP, NI, CX, SXT, AK, GEN, IMI, E
E. faecalis	0.8	AMP, NI, SXT, E

AMP=ampicillin; NI=nitrofurantoin; CX=cefoxitin; SXT=co-trimoxazole; AK= amikacin; GEN=gentamicin; IMI=imipenem; E=erythromycin.

Table 5. Bivariate analysis between socio-demographic variables and MDR UTIs.

Variable	Categories	Unadjusted OR	95% CI	p-value
Department	In-patients	3.616	1.017-12.860	0.047
	Out-patients	1		
Age	≤19 years	0.529	0.124-2.261	0.390
	≥20years	1		
Gender	Female	2.417	0.690-8.463	0.168
	Male	1		
Residence	Rural	1.463	0.415-5.160	0.554
	Sub-urban	0.564	0.065-4.894	0.604
	Urban	1		
Marital status	Married	1.964	0.516-7.471	0.322
	Single	1.226	0.124-12.120	0.861
	Others	1		
Level of education	No education	0.793	0.205-3.075	0.737
	Primary	0.939	0.180-4.900	0.941
	Secondary	0.527	0.061-4.591	0.560
	Tertiary	1		
Sexual intercourse	Yes	1.160	0.269-5.006	0.843
	No	1		

CI=confidence interval, p=probability, OR=odds ratio, p≤0.05 value is statistically significant under logistic regression.

the finding of Ali et al. (2017) who reported 236/351 (67.2%). Furthermore the AMR of 69/86 (80.2%) to ampicillin observed in this study is inconformity with other previous studies of Odoki et al. (2015); Ampaire et al. (2015) and Katongole et al. (2015) who reported 90/103 (87.4%), 10/14 (71.4%) and 7/8 (87.5%) respectively. The resistance of ampicillin demonstrated in our study is lower than 318.7/351 (90.8%) reported by Ali et al. (2017) and higher than 35/58 (60.3%) reported by Mwaka et al. (2011). This could be due to the nature of the studied participants such as diabetes, elderly, pregnant women, HIV and infants used in this study which were probably prone to recurrent UTI and subsequent therapeutic usage of this drug previously which might have led to a higher resistance.

Some antimicrobial agents' demonstrated high

Table 6. Bivariate analysis between health condition and MDR UTIs.

Variable	Categories	Unadjusted OR	95% CI	p-value
Pregnancy	Yes	0.459	0.103-2.060	0.310
	No	1		
Hypertension	Yes	1.082	0.212-5.516	0.924
	No	1		
Genitourinary abnormalities	Yes	0.747	0.229-2.440	0.629
	No	1		
Diabetes mellitus	Yes	1.082	0.212-5.516	0.924
	No	1		
HIV	Yes	1.905	0.223-16.292	0.556
	No	1		
Any antimicrobial agent use in the last 12 months	Yes	5.175	1.007-26.605	**0.049**
	No	1		
Cephalosporins in the last 12 months	Yes	2.619	0.778-8.813	0.120
	No	1		
Fluoroquinolones in the last 12 months	Yes	2.727	0.770-9.661	0.120
	No	1		
Abortion	Yes	1.043	0.111-9.800	0.971
	No	1		
Chronic respiratory disease	Yes	2.865	0.589-13.939	0.192
	No	1		
Recurrent UTI	Yes	0.747	0.229-2.440	0.629
	No	1		
Previous hospital admission	Yes	1.069	0.262-4.353	0.926
	No	1		
Family history of UTI	Yes	0.873	0.168-4.536	0.872
	No	1		
Previous UTI	Yes	0.758	0.226-2.537	0.653
	No	1		
Wrong prescription	Yes	2.800	0.552-14.191	0.214
	No	1		

CI=confidence interval, p=probability, OR=odds ratio, p≤0.05 value is statistically significant under logistic regression.

sensitivity against the bacterial uropathogens tested. Imipenem demonstrated 77/86 (89.5%) sensitivity in our study. This finding is comparable with findings of Katongole et al. (2015); Prakash and Saxena, (2013) and Jehan et al. (2015) who reported 17/19 (89.5%), 131/155 (84.5%) and 388/421 (92.2%) sensitivities respectively.

Table 7. Factors associated with MDR UTIs using stepwise forward multiple logistic regression analysis.

Variable	Categories	Adjusted OR	95% CI	p-value
Department	In-patients	3.947	1.050-14.831	0.042
	Out-patients	1		
Any antimicrobial agent use in the last 12 months	Yes	6.004	1.046-34.454	0.044
	No	1		

CI=confidence interval, p=probability, OR=odds ratio, p≤0.05 value is statistically significant under logistic regression.

Table 8. Phylo-groups in MDR strains of *E. coli* isolated from the urinary tract.

Phylo-groups	Old Clermont method	New Clermont method
B2	12 (38.7)	4 (12.9)
A	7 (22.6)	5 (16.1)
B1	5 (16.1)	3 (9.7)
Unknown	2 (6.5)	15 (48.4)
D	5 (16.1)	2 (6.6)
E	-	1 (3.2)
Clade I	-	1 (3.2)
Total	31 (100.0)	31 (100.0)

"-"=no phylo-group found.

The sensitivity shown by imipenem in our study is slightly lower than 319/351 (98.6%) reported by Ali et al. (2017). This study also found out a high sensitivity of bacterial uropathogens to amikacin 74/83(89.2%). The high sensitivity of amikacin in this study is similar to 324.7/351 (92.5%) sensitivity reported by Ali et al. (2017). The sensitivity shown by amikacin in this study is slightly higher than 273/421 (64.8%) and 106/155 (68.4%) previously reported by Jehan et al. (2015) and, Prakrash and Saxena, (2013) respectively. Furthermore, this study showed high sensitivity of bacterial uropathogens 69/83(83.1%) to gentamicin. The sensitivity of gentamicin demonstrated in this study is supported by previous study by Odongo et al. (2013). The sensitivity shown by gentamicin in this study is slightly lower than 242.5/351 (69.1%) and 11/18 (61.1%) previously reported by Ali et al. (2017) and Kabugo et al. (2016) respectively. Finally, this study demonstrated considerable sensitivity of 69/86 (80.2%) of nitrofurantoin to the bacterial uropathogens tested. This substantial sensitivity of nitrofurantoin shown in this study is in conformity with previous reports of Odoki et al. (2015) and Ekwealor et al. (2016) who reported 78/103 (75.7%) and 191/212 (90.1%) sensitivities respectively. The sensitivity shown by nitrofurantoin in our study is in agreement with study done by Kabugo et al. (2016) who reported 12/18 (66.7%) sensitivity of uropathogens to nitrofurantoin.

This study demonstrated that the prevalence of MDR strains observed in the *Klebsiella spp.* is higher than previous reports (Regmi et al., 2018; Mahato et al., 2018; Shabbir et al., 2018). This could be due to the high use of over the counter antibiotics, bought from unlicensed drug stores and in open markets and self-medication in Uganda (UNAS et al., 2015). The high prevalence of MDR strains in *Proteus spp.* is in agreement with previous study by Mahato et al. (2018). Lastly, the high prevalence of the MDR strains in *E. coli* and *S. aureus* in this study is in conformity with reports by Regmi et al. (2018) and Mahato et al. (2018) respectively. The high prevalence of MDR strains in *E. coli* in this study is of high concern, since *E. coli* is the most common cause of bacterial UTIs (Tibyangye et al., 2015; Odoki et al., 2019).

When the bacterial uropathogens were subjected to eight different antimicrobial agents, they all showed a MARI of ≥0.2. Multiple antibiotic resistance index is a method used to evaluate the extent to which a particular bacterial population have attained AMR (Ugwu et al., 2009; Chika et al., 2013). Multiple antibiotic resistance index value of ≥0.2 shows that this particular population of bacteria arose from an environment where various antimicrobial agents have been used and abused (Ehinmidu, 2003; Tambekar et al., 2006; Oli et al., 2013). This is an obvious indication that a large proportion of bacterial isolates from the urinary tract were exposed to several antimicrobial agents that have led to the significant increase in AMR. Related incidences of resistance have been reported elsewhere, though to

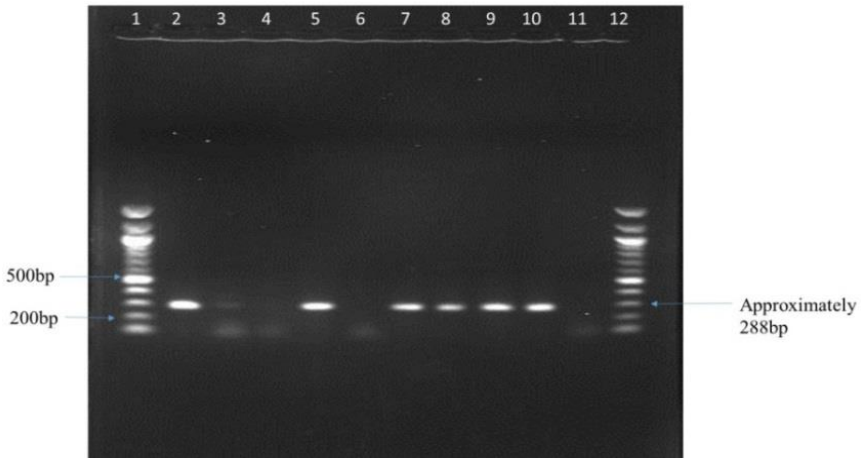

Figure 1. Gel images showing the *chuA* gene. Lanes 1 and 12 contain Markers of Molecular Weight (100 bp DNA ladder, New England Biolabs (NEB), USA). Lanes 5,7,8,9 and 10 are positive for the *chuA* gene. Lanes 3, 4 and 6 are negative for the *chuA* gene. Lanes 2 and 11 contain positive and negative controls respectively.

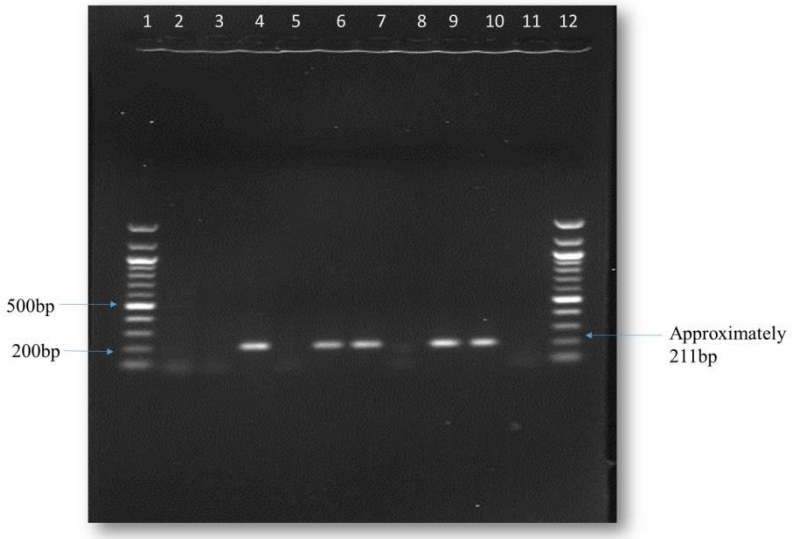

Figure 2. Gel images showing the *yjA* gene. Lanes 1 and 12 contain markers of molecular weight (100 bp DNA ladder, New England Biolabs (NEB), USA). Lanes 4, 6, 7 and 9 are positive for the *yjA* gene. Lanes 2, 3, 5 and 8 are negative for the *yjA* gene. Lanes 10 and 11 contain positive and negative controls respectively.

different sets of antimicrobial agents (Ehinmidu, 2003; Ekwealor et al., 2016). In addition, reports of bacterial uropathogens' resistance to frequently used antimicrobial agents have been documented (Prakash and Saxena, 2013).

This study demonstrated that hospitalization bears statistically significant association (OR=3.947; 95% CI: 1.050-14.831; p<0.05) with MDR UTIs. Our findings are in agreement with previous report by Tenney et al. (2017). Hospital environment is known to be a reservoir for MDR organisms and these antibiotic resistant organisms are found in high-touch surfaces in hospital wards or patients admission rooms, for example bedside tray tables, bed controls and call buttons (Dancer , 2014). These nosocomial MDR organisms includes: methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant enterococci (VRE), and resistant Gram-negative bacteria. The resistant Gram-negative bacteria that cause UTIs

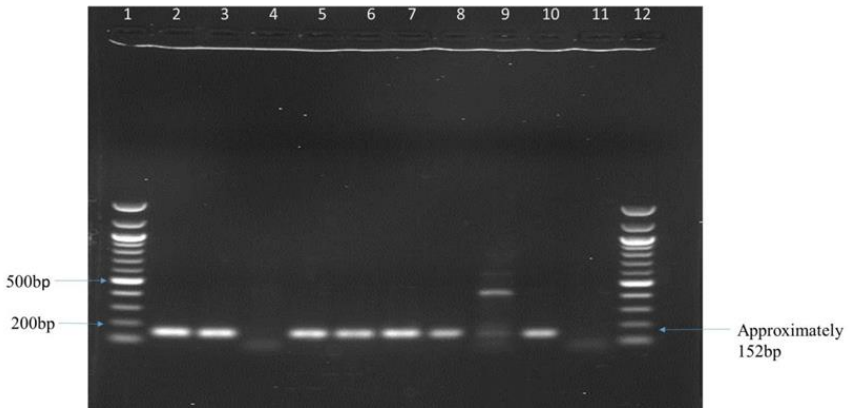

Figure 3. Gel images showing the Tspe4.C2. Lanes 1 and 12 contain markers of molecular weight (100bp DNA ladder, New England Biolabs (NEB), USA). Lanes 3,5,6,7,8 and 10 are positive for Tspe4.C2. Lanes 4 and 9 are negative for Tspe4.C2. Lanes 2 and 11 contain positive and negative controls respectively.

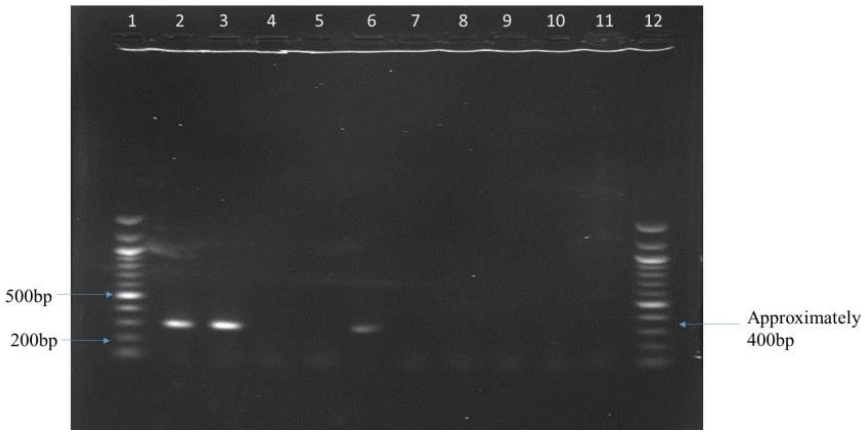

Figure 4. Gel images showing the *arpA* gene. Lanes 1 and 12 contain markers of molecular weight (100bp DNA ladder, New England Biolabs (NEB), USA). Lanes 3 and 6 are positive for *arpA* gene. Lanes 4,5,7,8,9 and 10 are negative for the *arpA* gene. Lanes 2 and 11 contain positive and negative controls respectively.

arise from the human gut flora (Sarowska et al., 2019; Kalluru et al., 2018). Patients and staffs harboring these MDR organisms potentially contaminates high-touch surfaces in hospital wards or patients' admission rooms, hence increasing the risk of infecting other patients, hospital staffs and visitors that may come into contact with these contaminated surfaces (Dancer , 2014; Nseir et al., 2011).

Furthermore, previous use of any antimicrobial agent in the last 12 months was found to have a statistical significant relationship (OR=6.004; 95% CI: 1.046-34.454; $p<0.05$) with MDR UTIs. This finding is supported by a previous study (Khawcharoenporn et al., 2017). The key factor involved in the development and spread of antimicrobial resistance is prior exposure to antimicrobial agents. There is a selection of a small fraction of cells from the wild type population that will be resistant to the antimicrobial agent being used during clinical treatment. This subpopulation of bacteria is selected to survive through spontaneous mutation that impedes antimicrobial action (Raymond, 2019; Cantón and Morosini, 2011).

The high prevalence of MDR strains in *E. coli* in this study is of high concern, therefore there is need to understand the genetic relatedness between the MDR strains in order to compare their genetic diversity with already documented strains. According to the old Clermont method, phylotyping of *E. coli* was based on the *chuA, yjA* and Tspe4.C2 genes' analysis and this revealed that, the high prevalence of the B2 phylo-group in UTIs is in agreement with previous report by Iranpour et al. (2015). Previous studies globally, have shown that pathogenic strains of invasive *E. coli* mainly are

Table 9. Number of *E. coli* isolates and the alterations/confirmations in phylo-groups designated by old and new Clermont methods.

Old Clermont genotypes		New Clermont genotypes			
Clermont genotypes	**Phylo-group**	**Clermont genotypes**	**Phylo-group**	**No. of isolates (%)**	**Alteration of old and new Clermont methods of genotypes**
- - -	A	+ - - -	A	5	0
- - -	A	- - - -	Unknown	2	A to unknown
+ - +	D	- + - +	B2	3	D to B2
+ - +	D	+ + - +	D	2	0
+ + +	B2	+ + + +	Unknown	9	B2 to unknown
+ + -	B2	+ + + -	Clade1	1	B2 to Clade1
+ + -	B2	+ + + -	E	1	B2 to E
+ + +	B2	- + + +	B2	1	0
- - +	B1	+ - - +	B1	3	0
- - +	B1	- - - +	Unknown	2	B1 to unknown
- + +	Unknown	+ - + +	Unknown	1	Unknown to unknown
- + +	Unknown	- - + +	Unknown	1	Unknown to unknown

"+"=positive for gene used in Clermont phylotyping; "-"=negative for gene used in Clermont phylotyping.

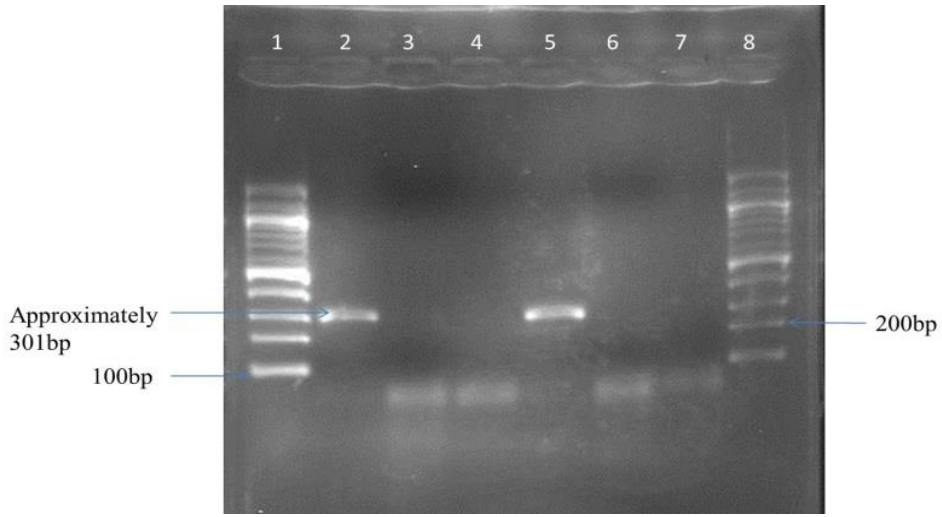

Figure 5. Gel images showing the *arpA* gene. Lanes 1 and 8 contain markers of molecular weight (100 bp DNA ladder, New England Biolabs (NEB), USA). Lane 5 is positive for the *arpA* gene. Lanes 3, 4 and 6 are negative for the *arpA* gene. Lanes 2 and 7 contain positive and negative controls respectively.

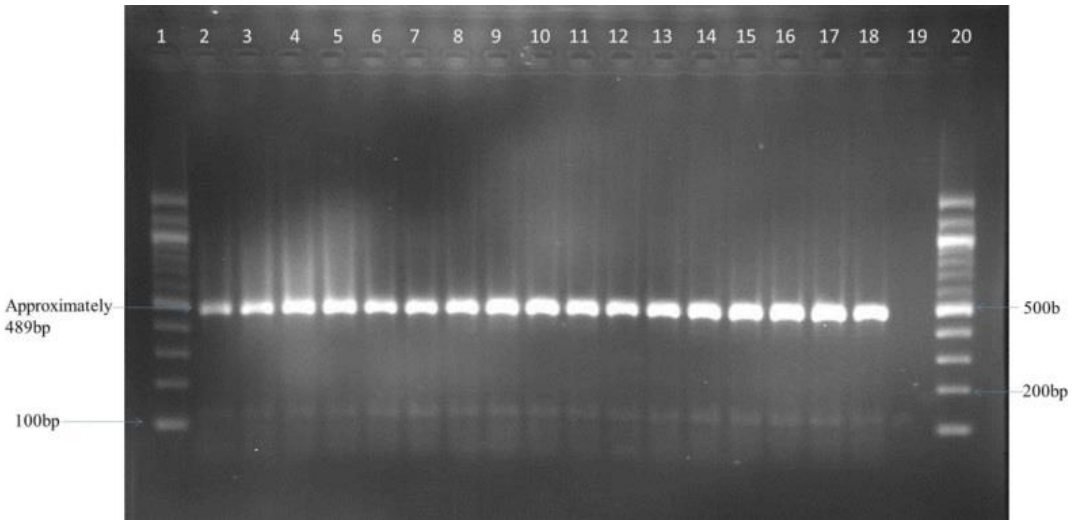

Figure 6. Gel images showing the *trpA* Internal control. Lanes 1 and 20 contain Markers of Molecular Weight (100bp DNA ladder, New England Biolabs (NEB), USA). Lanes 3-18 are positive for *trpA* gene. Lanes 2 and 19 contain positive and negative controls respectively.

composed of group B2 than group D. While group A or group B are mostly commensal strains (Basu et al., 2013; Ejrnaes, 2011). The new Clermont method revealed that the isolates assigned as phylo-group B2 9/12 (75.0%), B1 2/5 (40.0%) and A 2/7 (28.6%) by using the old Clermont method could not be phylotyped, consequently they were grouped as unknown or non- typeable strains of MDR *E. coli*. This kind of alteration of the phylogenetic groups of *E .coli* from old Clermont to the new Clermont method was also reported by Müştak et al. (2015), although to different sets of phylogenetic groups.

In 2013 Clermont and his colleagues were unable to detect these phylogenetic groups (- - - +, - - + +, + - + + and + + + +) using the new Clermont method, despite the enormous work they did to screen over 1000 *E. coli* strains (Clermont et al., 2013). In 2015 Iranpour and colleagues were able to detect two of these novel isolates belonging to phylogenetic groups (+ - + + and + + + +) in *E. coli* isolated from the urinary tract (Iranpour et al., 2015). Surprisingly, this study was able to find two more novel isolates belonging to the phylogenetic groups (- - - +, - - + +) which was not detected by Clermont and colleagues in 2013 using the new Clermont method (Clermont et al., 2013). The isolates designed as unknown or non-typeable strains of MDR *E. coli* are to be subjected to MLST as recommended by Clermont and colleagues in 2013 to delineate their phylogenetic group identities.

Our study had the following limitations: insufficient data on the previous patients' antimicrobial use as some of them did not have records of previous medications. Also, we did not distinguish, recurrent, uncomplicated and complicated UTIs. Therefore the resistance pattern in these subjects could not be attained. Finally, we were unable to afford a modern DNA sequencing-based approach which is the gold standard for genotyping bacteria.

Conclusion

Erythromycin, co-trimoxazole and ampicillin were the most resisted antimicrobial agents studied. The significant increase in the values of MARI and the high prevalence of MDR strains in *E. coli*, which is the leading cause of UTIs is of utmost concern. Multiple logistic regression revealed that hospitalization and previous use of any antimicrobial agent in the last 12 months bear statistically significant associations with MDR UTIs. The new Clermont method revealed that the isolates assigned as phylo-group B2 9/12 (75.0%), B1 2/5 (40.0%) and A 2/7 (28.6%) by using the old Clermont method couldn't be phylotyped, consequently they were grouped as unknown or non-typeable strains of MDR *E. coli*. To prevent the development of MDR among the bacterial uropathogens, this study therefore recommend antimicrobial susceptibility testing of the common bacterial uropathogens implicated in UTIs before antimicrobial agents prescription by clinicians among patients of the following category: hospitalized and patients with history of previous use of any antimicrobial agent in the last 12 months. For empiric treatment of UTIs in Bushenyi District, nitrofurantoin still remains the first line of choice. Although the Old and the new Clermont method is accepted for phylotyping of *E. coli,* this study recommends the use of modern DNA sequencing-based approaches which is the gold standard for genotyping bacteria, that this current study couldn't afford.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Prevalence of *Rickettsia typhi* in rodent fleas from areas with and without previous history of plague in Mbulu district, Tanzania

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Murine (endemic) typhus is a flea-borne infectious disease caused by *Rickettsia typhi.* The disease transmission cycle has similarities to that of *Yersinia pestis* causing plague. It is hypothesized that murine typhus is prevalent in areas with plague transmission. This study aims at detection of *R. typhi* in rodent fleas by conventional polymerase chain reaction (PCR). A cross sectional study was carried out in Mbulu district in villages with, and without previous history of plague from November 2018 to February 2019. Sherman® traps were set in forest and agricultural habitats while box traps were set inside houses. Captured rodents were anaesthetized using halothane and fleas were removed from the fur using a hard brush and preserved in 70% ethanol. PCR amplification of the targeted citrate synthase (*gltA*) gene of *R. typhi* was done using primers RpCS.877p and RpCS.1258n. 12 (24%) of the DNA from rodent fleas was positive for *R. typhi*. Of these, 5 (10%) and 2 (4%) were from farms and forests with previous plague history respectively, while 3 (6%) and 2 (4%) were from houses and farms with no previous plague history, respectively. This suggests the prevalence of murine typhus is independent of plague infections.

Key words: Polymerase chain reaction (PCR), plague, prevalence, *Rickettsia typhi*, rodents.

INTRODUCTION

Rickettsial diseases are worldwide emerging arthropod borne zoonoses that are caused by an obligate intracellular Gram-negative bacterium often found in vector fleas (Abdad et al., 2019; Noh et al., 2017). Rickettsioses are traditionally divided into the spotted fever, typhus, and the scrub typhus groups (Mcleod et al., 2004; Giulieri et al., 2012).

Murine typhus, also called endemic typhus or flea-borne typhus is caused by *Rickettsia typhi* (Civen and Ngo, 2008). The disease is transmitted by fleas *(Xenopsylla cheopis)* found on rodents (Eremeeva et al., 2008). People get murine typhus when rodent flea faeces containing the rickettsial agents contaminate the bite sites or other skin openings during feeding (La Scola et al., 2000). The conjunctiva can also be the port of entry for *R. typhi* (Noden et al., 2017). Similar to plague,

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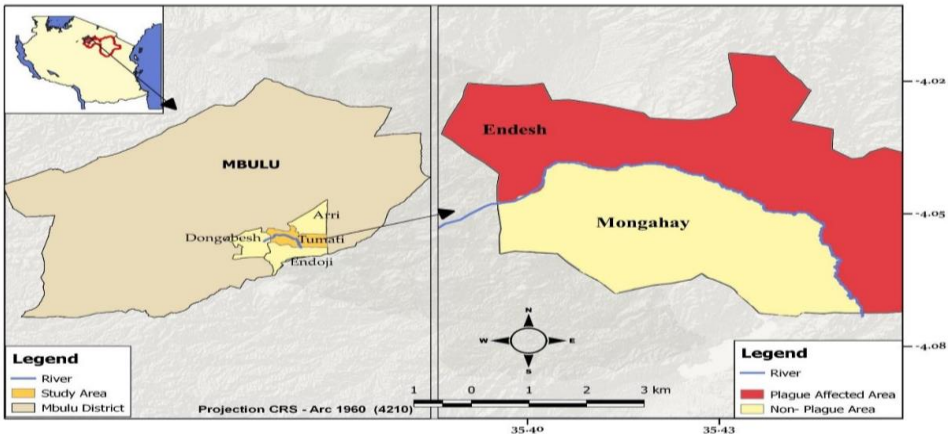

Figure 1. Map of Mbulu district showing villages with and without previous history of plague.

murine typhus would occur in areas where rodents and the fleas are abundant mostly in farms, forests and residential houses (Laudisoit et al., 2014).

Limited studies on murine typhus have been done in Tanzania, specifically in Mbeya and Moshi districts by Dill et al. (2013) and Prabhu et al. (2011). However, more extensive studies have been done in other countries, such as the serological evidence of exposure to *R. felis* and *R. typhi* in Australian veterinarians by Teoh et al. (2017) and the short report on murine typhus in Caldas Colombia (Hidalgo et al., 2008).

Apart from the limited information on murine typhus in Tanzania, little is known on its prevalence in the areas with previous history of *Yersinia pestis* plague, such as Mbulu district. Since murine typhus transmission cycle is similar to that of plague, there is the likelihood that murine typhus is prevalent in Tanzania in areas with previous history of plague. Mbulu District is known as one of the hotspots of plague in Tanzania (Makundi et al., 2008). The outbreak was revealed in some of villages including Tumati and Arri in the Division of Dongobesh in Mbulu, where 35 cases of plague were initially reported, with six deaths (Makundi et al., 2008). The victims had clinical symptoms of plague, including buboes, high fever, chills, headache, weakness, vomiting, nausea and prostration (Ben-Ari et al., 2011; Leirs et al., 2010). This study aimed at detecting *R. typhi* in rodent fleas by conventional polymerase chain reaction (PCR) from areas with and without previous history of plague in Mbulu District. This information will contribute to the design of disease control strategies of murine typhus and other rickettsial diseases in the future.

MATERIALS AND METHODS

Study area and design

A cross sectional study was carried out in Mbulu district (3°45'00.0"S 35°20'00.0"E) with the altitude ranging from 1930 to 2250 m above sea level (Ziwa et al., 2013). This involved two villages with and without previous history of plague, namely Endesh and Mongahay respectively (Figure 1). Samples were collected in three habitats, agricultural land, forest and inside houses in both villages.

Rodent trapping and sample collection

During rodent trapping four transect lines were set in agricultural and forest habitats in previously plague and non-plague affected villages. Each habitat had 20 Sherman® traps (Standard medium size LFA: 7.6 X 8.9 X 23 CM) in each line placed 10m apart from line to line and from trap to trap. Ten percent of the houses in the village were randomly selected making a total of 43 houses in both villages. Each of five Sherman® traps and box traps were randomly set inside each house. Trapping was conducted once per month from November 2018 to February 2019 for three consecutive nights, left overnight and inspected every morning. A total of 50 rodents were captured and anaesthetized using halothane. The following information was recorded rodent species, by looking their morphology, site of collection, trap used (whether box or Sherman® traps), sexual maturity and number of fleas collected. Total of 100 fleas, were removed from the fur of rodents using a hard brush and collected in a clean basin covered with a white paper. The fleas were then preserved in Eppendorf tubes with 70% ethanol before being transported to the laboratory for the molecular studies. Only 50 fleas were tested by PCR.

DNA extraction and storage

Fleas were rinsed twice in distilled water for 10 min and then dried on sterile filter paper. Handling was performed in a laminar flow biosafety cabinet. Fleas were individually crushed by sterile mortar and pestle then preserved in sterile Eppendorf tubes, as described by Leulmi et al. (2014). DNA was extracted from each flea using the kit (QUICK –gDNA™ Min prep USA) according to the manufacturer's instructions. The genomic DNA was stored at -20°C under sterile conditions until used in PCR assays.

PCR amplification of *R. typhi* DNA

The DNA extracted from each flea was tested by conventional PCR

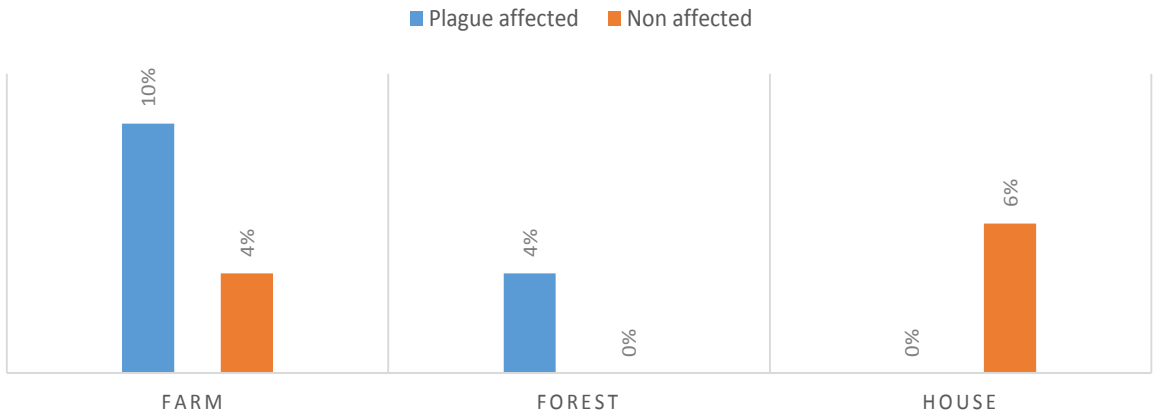

Figure 2. PCR results showing prevalence of *R. typhi* in rodent flea.

using forward and reverse primers RpCS.877p: GGGGGCCTGCTCACGGCGG RpCS.1258n: ATTGCAA AAAGTACAGTGAACA respectively (Macrogen Humanizing Genomics, Republic of Korea). This amplifies a 381 bp fragment of the citrate synthase encoding gene (*gltA*) for *R. typhi* (Regnery et al., 1991; Webb et al., 1990; Portillo et al., 2017). DNA amplification was done in a total volume of 17 µl using Taq polymerase (Cycler (Perkin-Elmer Cetus, Norwalk, Conn). Mixed samples were amplified for 40 repeated cycles, denaturation at 94°C for 30 s, annealed at 58°C for 30 s, and subjected to the sequence extension at 68°C for 1 min and 30 s for 50 samples as described by Webb et al. (1990).

Agarose gel electrophoresis of PCR product

A total of 1.5 g of agarose powder was weighed and dissolved into 1X dissolving buffer in a conical flask to make 1.5% agarose gel. Before loading the agarose gel was pre-stained with Gel Red (Biotium, Hayward, CA). A total of 17 µl of the PCR product was loaded onto the 1.5% agarose gels and electrophoresed at 100V for 40 min (Sousa et al., 2017). The amplified products were visualized against a ladder marker followed by appropriate examination of the band using UV trans illuminator. *R. typhi* targeted amplified DNA sequence by PCR in (*gltA*) is at 381 bp band on agarose gels upon electrophoresis (De Sousa et al., 2006). The positive control was kindly provided by the Molecular Biology Laboratory, University of Dar es Salaam (UDSM).

RESULTS

A total of 50 fleas were sampled from 50 rodents in villages with previous history of plague (Endesh) and villages without previous history of plague (Mongahay). DNA from each flea was tested by conventional PCR. A 381 bp fragment of the citrate synthase encoding gene (*gltA*) for *Rickettsia* spp was amplified then visualized on agarose gels after electrophoresis using UV trans illuminator. Altogether 12 (24%) of the DNA from rodent fleas was positive for *R. typhi* (*gltA).* Of these 5 (10%) and 2 (4%) were from farms and forests of Endesh respectively, while 3 (6%) and 2 (4%) were from houses and farms in Mongahay (Figure 2). Chi square test showed that prevalence of *R. typhi* was not statistically different between villages with or without plague history ($\chi 2$ =50.62 df =49 p= 0.084). This entails that prevalence of *R. typhi* is not associated with plague outbreak in an area. Suggesting that *R. typhi* can be equally prevalent in plague affected as well as non-affected areas. Amplification of the gene (*gltA*) encoding for *Rickettsia* was done in six fleas from plague affected and non-plague affected villages of Mbulu district followed by agarose gel electrophoresis. For negative control PBS was used. 381 bp fragment was observed in samples 1, 23, 24, 25, 26 and 27 (Figure 3). The size of band was determined by the DNA ladder Marker (LD). For rodent species, total of seven species of rodents were identified to be positive for *R. typhi* in both plague and non plague affected areas as shown in (Figure 4).

DISCUSSION

The aim of this study was to determine the prevalence of *R. typhi* in rodent fleas from areas with and without previous history of plague in Mbulu district, Tanzania. Demonstration of *R. typhi* was done by detecting *R. typhi* DNA of rodent fleas by PCR. Results from rodent fleas showed the prevalence of *R. typhi* not to be significantly higher in a village with previous history of plague than the one without previous history of plague ($p>0.05$). This suggests that prevalence of *R. typhi* in fleas in Mbulu district may not be necessarily associated with plague infection in the studied areas.

Murine typhus and plague have similarities in their epidemiology (Drancourt and Raoult, 2016) however, they do not necessarily infect the host concomitantly (Fenollar and Mediannikov, 2018). The rather faint bands in the electrophoresis of some of the flea PCR products (Figure 3) was possibly due to reduced amount of DNA available. Therefore, it is recommended to pool more

Figure 3. Demonstration of citrate synthase gene *(gltA)* of 381 bp in rodent flea by agarose gel electrophoresis. LD= Ladder Marker; 1, 23, 24, 25, 26 and 27= Positive Samples. PC= Positive Control: NC= Negative Control: bp = Base Pairs.

fleas in order to get a clearer gel electrophoresis band (Phan et al., 2011). Also, instead of extracting the DNA from fleas one can use DNA from rodent serum and get similar results with a clear *gltA* band when the right primers are used (Giulieri et al., 2012).

Other diagnostic tools namely ELISA and IFA have been used to detect *R. typhi* infection *(*Portillo et al., 2017). However, long-term reliance on serological tests and microscopy has led to underdiagnosis, inappropriate therapy, and undocumented morbidity and mortality. Recent approaches therefore integrate molecular approaches in the diagnosis of murine typhus to enable early detection and appropriate treatment (Paris and Dumler, 2016). The PCR was used as a highly sensitive and specific test in studies carried out in Tanzania among pregnant women (Scola and Raoult, 1997). These studies found a prevalence of 28% in Dar es Salaam and 0.5 to 9.3% in the towns of Kilimanjaro and Mbeya respectively (Leulmi et al., 2014).

Generally, murine typhus is more prevalent in areas where rodents and fleas are abundant (Conlon, 2007). Similar to plague, murine typhus occurs in agricultural land, forests and in residential houses (Laudisoit et al., 2014). Some domestic animals are also known to harbor the plague pathogen (Nyirenda et al., 2018). Murine typhus has no dramatic clinical effects and it is often neglected however, it can damage body organs, lead to coma, and even death. Hence intervention has to be done as soon as possible in an affected or suspected area.

The rodent species *Mastomys natalensis* showed overall more positive sera followed by *Rattus rattus*. The other rodent species namely A*rvicantis* spp, *Lophuromys* spp, *Lemniscomys* spp, *Grammomys* spp gave lower numbers of positive sera possibly due to high abundance of *Mastomys natalensis* in both farms, forest and domestic house though more studies have to be conducted in the future to scientifically prove this assumption (Figure 4).

In conclusions the prevalence of *R. typhi* may not necessarily be associated with previous plague infection in the studied areas. Nevertheless, murine typhus is a potential health threat to communities in Mbulu and possibly other part of Tanzania. Further research and longitudinal surveillance of fleas, their rodent hosts and disease carrier status is needed to determine the geographical distribution, habitats, and prevalence of the pathogens. Isolation and characterization of the Rickettsia is essential in order to identify locally circulating strains and their potential threat to humans. The prevalence data reported herein provides a basis for the development of species-specific assays that will give a clearer picture of rickettsial diseases in Tanzanian communities in the future.

Study limitation

One among the shortcomings is that the number of villages were limited and therefore number of specimen collected were few.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

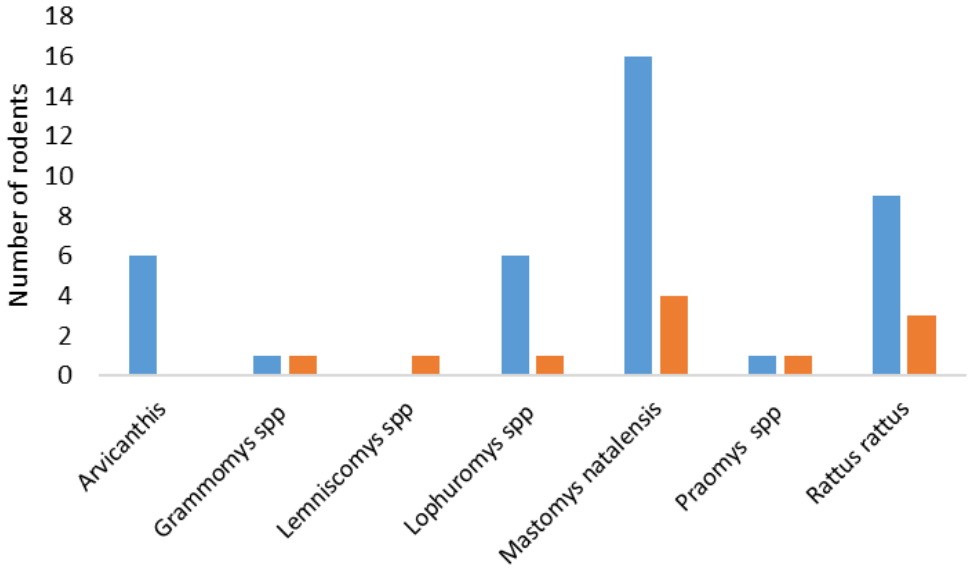

Figure 4. Rodent species carrying fleas positive for *R. typhi* in plague affected and non-affected villages.

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Full Length Research Paper

Biosurfactant production potential of bacillus obtained from dye effluent

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This study investigated the biosurfactant productions potentials of Bacillus isolated from dye effluent. Samples were collected under aseptic condition from three areas of Sokoto (Marina, Unguwar rogo and Minannata) in Nigeria and transported in an ice bag to microbiology laboratory of Usmanu Danfodiyo University, Sokoto. Enumeration, identification and characterization of the isolates were carried out using standard microbiological methods. The potential and ability to produce biosurfactants was determined using blood haemolytic tests, drop collapse and emulsification techniques. A total of nine organisms were isolated from these three locations sampled, and three were *Bacillus* species which are the predominant bacteria obtained from the three locations. Enumeration results revealed highest bacterial count at Unguwar rogo (17.33×10^5 cfu/ml). Haemolysis results revealed that all the isolated bacterial strains exhibited haemolytic activity. The result of drop collapse test showed that all the isolated organisms had good collapsing ability, and all the isolated organism had positive oil spreading and emulsification ability. This study showed *Bacillus* species are potential biosurfactants producers and should be studied in greater details as strains improvement may enhance the activity of biosurfactants.

Key words: Biosurfactants, drop collapse, emulsification, potential, dye effluent.

INTRODUCTION

Dye effluents are the liquid waste of dye. When the effluents are not properly managed, many pathogenic microorganism and chemicals in the effluents may predispose the inhabitants to serious health hazard (Ogbonna et al*.,* 2004). It may alter the physicochemical parameters of soil or water bodies thereby affecting the ecosystems (Tudunwada et al*.,* 2007). Another environmental consequence of discharging untreated dye

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effluents in the environment is that methanogens may produce excessive methane thus contributing to greenhouse effect and global warming (Faruk et al*.,* 2005). Surfactants are surface active compounds that can be chemically synthesized or biologically formed (biosurfactants). Chemically synthesized surfactants are toxic, non-degradable and may accumulate in living tissues leading to the development of cancer diseases (Seghal et al*.,* 2009; Lakshmipathy et al*.,* 2010). Biosurfactants are preferable to chemical surfactants due to the following characteristics: Low or no toxicity, biodegradability, better environmental compatibility, ability to act at wider range of temperature, pH values and salinity levels.

Furthermore, they may be produced from industrial waste and agriculture products which represent cheap substrates (Deleu and Paquot, 2004; Cho et al., 2005; Dehgan-Noudeh et al*.,* 2009).

Biosurfactants are amphiphilic biological compounds produced extra cellularly or as part of the cell membrane by a variety of bacteria, yeast and filamentous fungi from various substances including sugars, oil and wastes (Mata-sandoval et al*.,* 2000; Chen et al*.*, 2007). Biosurfactants are categorized mainly by their chemical composition and their microbial origin (Banat et al*.,* 2000; Anna et al*.,* 2001). In general, their structure includes hydrophilic moiety consisting of amino acids or peptides anions or cations; mono-, di-, or polysaccharides; and a hydrophobic moiety consisting of unsaturated, saturated, or fatty acid (Costa et al*.,* 2006).

Therefore, it is reasonable to expect diverse properties and physiological functions of biosurfactants such as increasing the surface area and bio-availability of hydrophobic water insoluble substrates, metal binding bacteria pathogenesis, quorum sensing and biofilm formation (Priya and Usharani, 2009). Unlike synthetic surfactants, microbial – produced compounds (i.e. biosurfactants) are easily degraded and particularly suited for environmental applications such as bioremediation and dispersion of oil spills (Mohan et al*.,* 2006).

Concerning biosurfactants, in order to reduce the production cost of biosurfactants, the yield and product accumulation must be increased through the development of economic engineering process and the use of cost effective substrate for the growth of microorganisms as biosurfactant-producers. The cost of the substrates will greatly influence the economical use of the biosurfactants. Interest in microbial surfactants has been steadily increasing in recent years (Woo and Park, 2004).

The search for biosurfactants producing microorganisms is still an important area of research because of the diversity of their molecules and wide variety of their application. The aim of this study was to isolate and identify biosurfactant producing bacteria from dye effluent, that is, from dye liquid waste.

MATERIALS AND METHODS

Sampling area

Dye effluents were collected from three (3) areas in Sokoto, Sokoto State, Nigeria. Sokoto is located to the extreme North West Nigeria between longitudes 4° 8'E and 6° 54'E and lattitude 12° N and 13° 58'N (Adamu et al*.,* 2015a).

Sample collection

Dye effluents were collected from three areas of Sokoto Township which are Marina, Unguwar rogo and Minannata areas of Sokoto. Samples were collected in sterile sample bottles and transported in ice box to microbiology laboratory, Usman Danfodiyo University, Sokoto. The triplicate dye effluent samples were collected by simple random sampling.

Media preparation

Mineral salt (Bushnell – Haas medium) (MSM)

Mineral salt medium of Isma'il et al. (2014) (composed of 1.2 g KH_2PO_4, 1.8 g K_2HPO_4, 4.0 g NH_4Cl, 0.2 g $MgSO_4.7H_2O$, 0.1 g Nacl, 0.01 g $FeSO_4.7H_2O$ and 20 g agar per liter at pH 7.4) were prepared and dispensed in three (3) flasks. To each of the flask, 2% $^{w}/_{v}$ of dye and glucose were added respectively (Seghal et al*.,* 2009). Nutrient agar, nutrient broth and blood agar medium were prepared and sterilized according to the manufacturer's instruction.

Microbiological analysis of dye contaminated soil effluent

Fivefold serial dilutions of the effluent suspension were carried out. Using spread plate technique, 1 mL aliquots of dilutions were inoculated in triplicates on nutrient agar plates for the enumeration of total aerobic heterotrophic bacteria. The nutrient plates were incubated at 37°C for 24 h; colonies which appear on nutrient agar plates were sub cultured into mineral salt media (MSM agar). Mineral salt media (MSM agar) with dye as carbon source were used for isolation of biosurfactant producing bacteria. Colonies which appeared on the plates were counted and expressed as colony forming units per milliliter (cfu/ml) of sample (Benson, 2001). Pure isolates were obtained by repeated sub culturing of fresh mineral salt media plates. The pure isolates were maintained on agar slants in a refrigerator (8°C); the isolates were identified by biochemical characterization using the schemes of Barrow and Feltham (1993) and Bergey's Manual identifications Plan.

Physicochemical analysis of dye contaminated soil effluent

The pH (hydrogen ion concentration), BOD (Biochemical Oxygen Demand), COD (Chemical Oxygen Demand), DO (Dissolved Oxygen), Temperature TS (Total Solid), TDS (Total Dissolved Solid), TSS (Total Suspended Solid), hardness, color chromium content were determined according to the methods described by Adamu et al. (2015b).

Identification and characterization of the bacterial Isolates

Pure cultures of the heterotrophic bacterial isolates were Identified by cultural, morphological (Gram staining) and biochemical

characteristics (urease activity, indole test, Citrate test, methyl red and Voge's proskauer test, triple sugar iron agar test) according to standard method of Cheesbrough (2000).

Screening of bacteria isolates for biosurfactant production

Four methods were used to screen the bacterial isolates for potential to produce biosurfactant. The methods were the blood hemolysis test, emulsification index, oil spreading, and drop collapse method as described by Thavasi et al. (2011) and Youssef et al. (2004). Isolates were grown in mineral salt medium (MSM) containing the dye as carbon source. The culture was incubated for 10 days at 30°C with regular shaking. After incubation period, the broth of each isolate was centrifuge at 6000 rpm for 10 min and the supernatants separated by filtration in order to obtain cell free supernatants. The supernatants were used for blood hemolysis, emulsification, drop collapse and oil spreading tests.

Blood hemolysis test

The bacterial isolates were inoculated on blood agar containing 5% ($^{v}/_{v}$) human blood. The plates were incubated at 30°C for 48 h (2 days Hemolytic activity was detected as the presence of a clear zone around a colony). The clear zone (Hemolytic activity) suggested the presence of biosurfactant (Youssef et al., 2004).

Drop collapse test

Drop collapse test was carried out according to the method described by Youssef et al. (2004). A drop of crude oil (Bonny light) was placed on a grease free slide and one drop of the free supernatant was placed at the center of the oil drop. Collapse of the drop was due to reduction of interfacial tension between the liquid drop (containing biosurfactant) and the hydrophobic surface of the oil. The time it took the oil drop to collapse was also recorded.

Oil spreading method

Oil spreading technique was carried out according to the method described by Youssef et al. (2004). 50 mL of distilled water was added to Petri – dished followed by addition of 100 uL of crude oil (Bonny light) to the surface of the water, then one drop of the supernatant was dropped on the crude oil surface. The diameter of the clear zone on oil surface was measured using a meter rule and the time taken to achieve the spread was noted.

Emulsification ability/index test

Emulsification activity was carried out using the method of Tabatabaee et al. (2005), and Techaoei et al. (2011). Four (4) mL of the crude oil was added to equal amount of cell free supernatant and vortexed at 500 revolutions per minute for 10 min. After 24 h, the height of the stable emulsion was measured using a meter rule. The emulsification index (E_{24}) was calculated as the rate of the height of the emulsion layer and the total height of liquid as given by the expression.

$$E`_{24} = \frac{\text{h emulsion}}{\text{h total}} \times 100$$

Where: E_{24} = emulsion index after 24 h; h emulsion = The height of emulsion layer;h total = The total height of the liquid

Statistical analysis

Data obtained from this research were analyzed using One-way analysis of variance (ANOVA) followed by Duncan Multiple Range Test.

RESULTS

Table 1 shows the physiochemical parameters of dye effluent analyzed. The color was dark-blue, odor was found to be partially agreeable. Temperature of Marina was 3°C, Unguwar rogo is 27°C, and Minannata is 30°C with Unguwar rogo having `significant difference from Marina and Minannata. pH of Marina is 10.50, Unguwar rogo is 10.40 and Minannata is 10.30 There are no significant difference p (<0.05) in the pH from the three sampled areas. COD of Marina is 5.5, Unguwar rogo is 5.3 and Minanata is 5.8. OD, TSS and chromium content all exceeded the limit for the discharge of effluent by the FEPA. BOD of Unguwar rogo and Minanata also exceeded the limit of FEPA for the discharge of effluent. There is significant difference p (<0.05) in the BOD of Marina with that of Unguwar rogo and Minannata.TH and TDS are also within the recommended limit of FEPA. Although the TH of Marina and Unguwar rogo differs significantly p (<0.05) from that of Minannata, the TDS of Marina is also significantly difference from that of Unguwar rogo and Minannata.

Table 2 shows the counts of bacteria from the dye effluent of the three sampled areas. The highest bacterial counts were obtained at Unguwar rogo (17.33×10^5 cfu/ml).

Result of blood hemolysis shown in Table 3 revealed that all the isolated bacterial strains exhibited hemolytic activity; it is always the first test to identify the potential of microorganism's ability to produce biosurfactant. The isolated bacterial strains from Marina show + (2) hemolysis, Unguwar rogo + (3) and Minannata + (2). The result of drop collapse in Table 3 shows that all the isolated organisms have good collapsing ability. Isolated organisms from Marina produced collapsing of drop within 5 s, those from Unguwar rogo produced collapsing of drop within 3 s and those from Minannata within 5 s. The result of oil spreading (oil displacement area) in Table 3 revealed that, isolates from Marina have spreading diameter of 5.7 cm, those from Unguwar rogo have 31.2 cm, and those from Minannata have 10.2 cm The result of Emulsification ability/index in Table 3 revealed that, isolated organisms from Marina have 4.6% Emulsification index/ability, those from Unguwar rogo have 5.3% and those from Minannata have 4.6%.

Table 4 shows the result of the biochemical tests carried out on isolate from dye effluent. The predominant bacterium isolated and identified was *Bacillus* spp.

Table 1. Physiochemical parameters of dye effluent.

Parameter	Site			Recommended limit (FEPA)
	Marina	Unguwar rogo	Minannata	
Colour	Dark Blue	Dark Blue	Dark Blue	Nil
Odor	Partially Agreeble	Partially Agreeble	PartiallyAgreeble	Nil
Temperature (°C)	31.00±0.58[a]	27.00±1.15[b]	30.00±1.15[a]	<40
pH	10.50±0.50[a]	10.40±0.23[a]	10.3±0.09[a]	6.0-9.0
Total hardness (mg/L)	1.10±0.06[a]	1.05±0.03[a]	0.70±0.12[b]	125
Total dissolved solid (mg/L)	442.00±1.15[b]	493.33±8.82[a]	501.00±0.57[a]	500
Total suspended solid (mg/L)	306.00±3.46[c]	328.00± 2.31[a]	631.00±0.58[a]	<200
Dissolved oxygen (mg/L)	2.20±0 .12[c]	8.10±0.06[a]	3.80±0.17[b]	<2.0
Biochemical oxygen demand (mg/L)	10.90± 0.11[a]	30.50± 0.14[c]	16.4000±.23[b]	15
Chemical oxygen demand (mg/L)	5.50±0.29[a]	5.30±0.17[a]	5.80±0.23[a]	40
Chromium content (mg/L)	1.16±0.09[b]	1.29± 0.02[a]	1.44±0.02[a]	<1.0

Values are mean±SEM. Means with different superscript in a row are significantly different (p<0.05); One-way ANOVA Followed by Duncan Multiple Range Test. Mg/L, Miligram/ L, Liter; FEPA, Federal Environmental Protection Agency.

Table 2. Bacterial colony count of the dye effluent.

Sample area	Colony count (×10^5 cfu/mL)
Marina	8.00±01.0[b]
Unguwar rogo	17.33±4.63[a]
Minannata	12.33±3.9[b]

Values are mean±SEM. Means with different superscript in a row are significantly different (p<0.05); One-way ANOVA Followed by Duncan Multiple Range Test.

DISCUSSION

In this study of biosurfactant production potentials of *Bacillus* species obtained from dye effluent, physicochemical characterization/analysis of the dye effluent indicates high concentration of dissolved chemicals. This is in agreement with findings of Srinivasan et al. (2014) who reported that dye effluent is rich in various parameters/physicochemical properties. Higher bacterial colony counts were recorded from Unguwar rogo areas. This might be due to availability of nutrients, and favorable temperature of the effluent as well as the ability of the organisms to withstand, tolerate or adapt to the unfavorable condition of the effluent. This agrees with the findings of Adamu et al. (2015b) who suggested that difference in bacterial colony count could be due to availability of nutrients and favorable temperature of the effluent.

A total of nine bacteria were isolated and identified from this study. *Bacillus spp.* is the predominant from the three locations sampled (Marina, Unguwar rogo and Minannata). This might be due to the ability of *Bacillus* to survive in wide range of temperature, pH and having mechanistic enzymes dependent color removal strategy. This is in agreement with findings of Chen (2002) and Dave and Dave (2009) who reported that *Bacillus* has some enzymes system capable of color removal. The *Bacillus* identified in this study shows high hemolytic activity. This might be due extracellular secretions by catalytic enzymes. This agreed with the findings of Thavasi et al. (2011) and Elemba (2014) who suggested that the hemolytic ability could be attributed to extracellular secretions.

All the supernatant of three isolates were positive for drop collapse test, this is due to the reduction of surface-tension between the supernatant drop and hydrophobic oil surface this agreed with the findings of Tudunwada et al. (2007) who reported that drop collapses due to the reduction of surface-tension between supernatant drop and hydrophobic oil surface.

Also all the supernatant of the three isolate were positive for oil spreading test; this is due to the reduction of surface-tension between supernatant drop and hydrophobic oil surface. This also agreed with the findings of Tudunwada et al. (2007) who reported that oil spreading is due to the reduction of surface-tension

Table 3. Screening of biosurfactant producing organisms.

Isolate	Hemolysis	Emulsification index E_{24} (%)	Drop collapse		Oil spreading (oil displacement area)		
			Result	Time (s)	Result	Displacement (cm)	Time (min)
Bacillus lentus (MR)	+(2)	4.6	+	3	+	5.7	6
Bacillus brevies (UR)	+(3)	5.3	+	5	+	31.2	5
Bacillus lentus (MN)	+(2)	4.6	+	5	+	10.2	7

MR, Marina; MN, Minannata; UR, Unguwar Rogo.

Table 4. Morphological and biochemical characterization of isolates.

Coded isolate	Gram	MR	VP	H_2S	MOT	GLU	SUC	LAC	GAS	URA	CIT	Spore	IND	Organism
A1	G+rod and in chain	+	-	-	+	+	+	+	-	+	-	-	-	*B. spp.*
B3	G+rod and in chain	-	+	-	+	-	-	+	-	+	-	+	-	*B. spp.*
C1	G+rod and in chain	+	-	-	+	+	+	-	-	+	-	-	-	*B. spp.*

Gram, Gram reaction; MR, Methyl red; VP, voge'sproskauer; H_2S, Hydrogen sulphide production; MOT, Motility; GLU, Glucose; SUC, Sucrose; LAC, Lactose; GAS, Gas formation; URA, Ureas; CIT, Citrase; IND, Indole; B, Bacillus; P, *Pseudomonas;* E= *Escherichia.*

between supernatant drop and hydrophobic oil surface. Result of emulsification ability of the isolate revealed that all the three isolates have good emulsification ability. This is due to the stability of biosurfactant at different temperature and pH as agreed with the findings of Tabatabaee et al*.* (2005) and Techaoei et al*.* (2011) who reported that biosurfactant is stable at different temperature and pH.

Conclusion

This study indicates that *Bacillus* sp. isolated from Unguwar rogo could be a valuable source of biosurfactants. Although the composition was not determined, it can be suggested that the biosurfactants can be used in dye removal or decolorization of the effluents. Further studies need to be conducted in order to characterize the biosurfactants produced. Also molecular identification of the bacteria to the species level using 16s rRNA to know the type of species that produced this biosurfactant needs to be conducted

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Biofilm-inhibition activities of fractions of *Senna Siamea* (LAM) Irwin & Barneby leaf against *Escherichia coli*

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Escherichia coli **has carved its niche in the urinary tract with the formation of a formidable matrix called biofilm. This biofilm is not only recalcitrant to the body's immune system but also resistant to antibacterial agents*. Senna siamea* (Lam) Irwin and Barneby is a medicinal plant with established antibacterial effect against planktonic cells of many bacteria. An attempt was made herein to evaluate the effect of its leaf extract and fractions on biofilm of *E. coli* isolates. Crude extracts of leaf, stem bark and root of this plant were prepared using ethanol as the solvent for the cold extraction. Phytochemical screening was carried out on the three extracts. Two *E. coli* strains from different antenatal patients attending General Hospital, Kafanchan, Kaduna were donated to us by a researcher from Ahmadu Bello University, Zaria and the reference strain, *E. coli*, WDCM 00013 (from Germany) were tested for biofilm production using the Congo red method. Antimicrobial susceptibility testing of the crude extracts against the isolates was carried out using the agar diffusion method. The Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) were determined for the leaf extract of the plant using micro broth dilution and agar diffusion methods respectively. In order to establish the antibiofilm activities of the leaf extract of the plant, sub-inhibitory concentrations (sub-MIC) were used against the test isolates in the remaining assays in the work. Column chromatography backed by thin layer chromatography (TLC) was used to fractionate leaf extract (having the best antibacterial activity) of the plant, using different ratios of a combination of hexane, ethyl acetate and n-butanol as fractionating solvents. MIC and MBC of the leaf extract were and 50 mg/ml respectively. High values of percentage biofilm inhibition were observed against all the bacterial isolates from the antibiofilm assay. Combination of solvents in the increasing order of polarity enhanced the antibiofilm activity of the various fractions of the leaf extract of Senna siamea. In conclusion, further fractionation of Senna siamea leaf extract increases its antibiofilm activities.**

Key words: Biofilm, *Senna siamea,* column chromatography, thin layer chromatography, *Escherichia coli.*

INTRODUCTION

Biofilms are densely packed communities of microbial cells that grow on living or inert surfaces and surround themselves with secreted polymers. Many bacterial species form biofilms, and their study has revealed them to be complex and diverse. The structural and physiological complexity of biofilms have led to the idea

that they are coordinated and cooperative groups, analogous to multicellular organisms (Nadell et al., 2008). Researchers have estimated that 60-80% of microbial infections in the body are caused by bacteria growing as a biofilm, as opposed to planktonic (free-floating) bacteria. Some external biofilm, namely chronic wounds and dental plaque, can be manually removed. Because of their inaccessibility and heightened resistance to certain antibiotic combinations and dosages, internal biofilms are more difficult to eradicate (Anderson et al., 2003).

Escherichia coli bacteria in urinary tract infection present several virulence factors that allow them to colonize host mucosal uroepithelium, injure and invade host tissues, overcome host defence mechanisms, incite a host inflammatory response and eventually proceed from the lower urinary tract to the renal cavities and tissues. Several surface determinants involved in *E. coli* biofilms are flagella and motility, Fimbriae, Fimbriae, Autotransporter proteins, Curli, F conjugative pilus and Exopolysaccharide production

Senna siamea which belongs to the sub-family fabaceae (Caesalpinioideae) of family leguminosae has its leaf being used as vegetables in Thailand (Otimenyin et al., 2007). Also, Aliyu (2006) found that *S. siamea* is ethno medicinally used as laxative, blood cleaning agent, cure for digestive system, urinogenitory disorders, herpes and rhinitis.

However, despite a vast literature search, we are yet to come by a single published work on the antibiofilm activities of *S. siamea* plant which is the premise on which this work is undertaken. It is the aim of this research to determine the antibiofilm activities of *S. siamea* plant leaf, stem bark and root extracts against biofilm-forming *E. coli* clinical isolates while using the reference strain *E. coli* WDCM 00013 (obtained from Sigma Aldrich, Germany) as standard for measuring the activities of the clinical isolates. This will be done by comparing the minimum inhibitory concentrations (MICs) of the three extracts so as to pick the most active of the extracts. It is to be borne in mind that lower (than the MIC) concentrations are to be used for the antibiofilm assay since the target for disruption is the bacterial biofilms and not the bacteria themselves. It is also the aim of this research to further fractionate the most active extract for the in situ production of phytochemicals, tannin and flavonoids. The choice of these two metabolites is based on the fact that they are polar and that their standards are easily obtainable. The method to be employed is column chromatography backed with thin layer chromatography (TLC) while the use of standard tannin and flavonoids serves to locate the R_f values of the phytochemicals in the extract .The biofilms of the bacterial isolates will then be subjected to treatment with the *in situ* produced phytochemicals (Figure 1).

METHODOLOGY

Leaves, stem bark and root parts of *S. siamea* tree behind Ahmadu Bello University Fire Service were collected while the plant was identified at the herbarium section of the Biological Science Department, Ahmadu Bello University, Zaria, with the voucher number 613. They were shade dried, homogenised and extracted with ethanol through cold extraction for three days. After decantation, the filtrates were concentrated and dried in a water bath at temperature of 40°C. The dry extracts were subjected to phytochemical screening according to the standard methods of Trease and Evans (2009). The pellets of standard strain *E. coli* WDCM 00013 (from Sigma Aldrich, Germany) were propagated as provided by the American Type Culture Collection (2014). The test isolates, *E. coli* B27 and C1 from the urine of antenatal patients attending General Hospital, Kafanchan, Kaduna (donated by a co-researcher) were purified by regularly subculturing them on tryptic soy agar slant at 37°C for 24 h and subsequently subjected to congo red assay for detection of biofilm-forming capacity as described by Freeman et al. (1989).

The isolates were then subjected to antibacterial susceptibility testing using agar well diffusion method according to Clinical Laboratory Standards Institute (CLSI) guidelines. Broth dilution method was adopted to determine the minimum inhibitory concentrations (MIC) of the most active extract based on zones of inhibition of bacterial growth. Minimum bactericidal concentration (MBC) was also determined for the extract using the agar dilution method.

The antibiofilm activity of the most active plant extract was determined by the method described by Filoche et al. (2005). Overnight culture of all the test organisms was grown to provide pre-formed biofilms. One hundred microlitre (100 µl) of each of these cultures was put into a 96-well microtitre plate. The plate was then incubated for further 4 h at 37°C to allow cells' attachment. Next, 100 µl of the plant extract at sub-inhibitory concentrations (20, 10, 5.0, 2.5, 1.25 and 0.625 mg/ml) was added to the wells. Each test was provided in two wells as duplicate. Growth control (cells + broth), media control (only broth) and blank control (broth + extract) were included. The plate was then incubated at 37°C for further 24 h. Following 24 h incubation, the supernatant was removed and each well was rinsed with sterile saline three times. The modified crystal violet assay was then used to assess the biomass of the attached cells as follows: biofilms formed by adherent cells in plate were stained with 0.1% crystal violet and incubated at the room temperature for 20 min. Excess stain was rinsed off by thorough washing with deionized water and plates were fixed with 200 µl of 96% ethanol. Optical densities (OD) of stained adherent bacteria were measured at 630 nm using an ELISA microplate reader (Stepanovic et al., 2007). The percentage of biofilm inhibition was calculated using the following formula: [(OD growth control – OD sample) / OD growth control] × 100.

Fractionation of the most active extract of the plant

Twenty gram of ethanolic extract of the plant was subjected to column chromatography on silica gel (120 mesh) packed and eluted successively starting with 100% n-hexane, then ethyl acetate and then methanol. This was carried out by increasing the solvent

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Figure 1. *S. siamea* plant.

polarity in the following ratio of the solvents; 90:10, 80:20, 70:30, solvents were also tested. Several pools of elutes were collected in different beakers and some representative samples were subjected to thin layer chromatography (TLC). The TLC was developed in a TLC tank with silica gel 60 using n-hexane: ethyl acetate: methanol in ratio 2:7:1 as the developing solvent system and R_f value values were calculated using the formula: R_f value = Distance moved by the molecule/ Distance moved by the mobile phase

Visualisation was carried out by dipping the plate in vanillin sulphuric acid (1%). Elutes with similar R_f values were combined as a single fraction. Those with different R_f values were grouped. Fractions were then subjected to qualitative analysis with flavonoids and tannins being the two phytochemicals tested for. Fractions most positive for flavonoids were combined and a sample from this was run on TLC plates alongside standard flavonoids, quercetin to confirm the presence of flavonoids in the fraction. Similarly, the fractions most positive for tannins were run on TLC plates alongside a standard tannin to confirm the presence of tannin in the fraction.

Antibiofilm activities of the fractions

The flavonoid-rich fraction and the tannin-rich fraction were tested against the test bacterial isolates for their antibiofilm activities using the modified crystal violet method.

RESULTS

Biofilm production of the bacterial isolates

These are the images of the biofilms of *E. coli* C1, B27 and the standard strain *E. coli* WDCM 00013. Appearance of dark colonies in Figures 2 to 4 indicates the formation of strong biofilms by the isolates. Though all the three extracts of *S. siamea* plant did not show a good antibacterial activity, the leaf extract was chosen for the rest part of this study since it was the only extract with antibacterial activity in at least two concentrations or more in all the test isolates used in this study (Table 1).

Minimum inhibitory concentration (MIC) of the leaf extract was determined to be 25 mg/ml. The minimum bacteriocidal concentration (MBC) of the extract was found to be 100 mg/ml. Consequently, a sub-MIC concentration of 20, 10, 5, 2.5, 1.25 and 0.625 mg/ml were adopted for the extract in the biofilm inhibition assay (Tables 2 and 3).

Optical Densities of the Escherichia isolates biofilms

Escherichia coli isolates B27, C1 and WDCM 00013 had the following after being treated with Senna siamea leaf, stem bark and root extracts as shown in Appendix Table 1, 2 and 3 respectively.

The use of standard tannin and flavonoids (tannic acid and quercetin) during the thin layer chromatography confirmed the presence of tannin and flavonoid respectively in the extracts (Figure 5).

Antibiofilm activities of the phytoconstituents

Appendix Tables 4 and 5 show the optical densities (OD) obtained when the phytoconstituents were used against the Escherichia coli isolates. The percentage biofilm inhibition due to the anti-adhesive activity of the ethanolic extract of *S. siamea* leaf against test biofilm-forming *E. coli* isolates at the sub-inhibitory concentrations used is as shown in Tables 4 and 5.

Figure 2. *Escherichia coli* C1 isolate Biofilm.

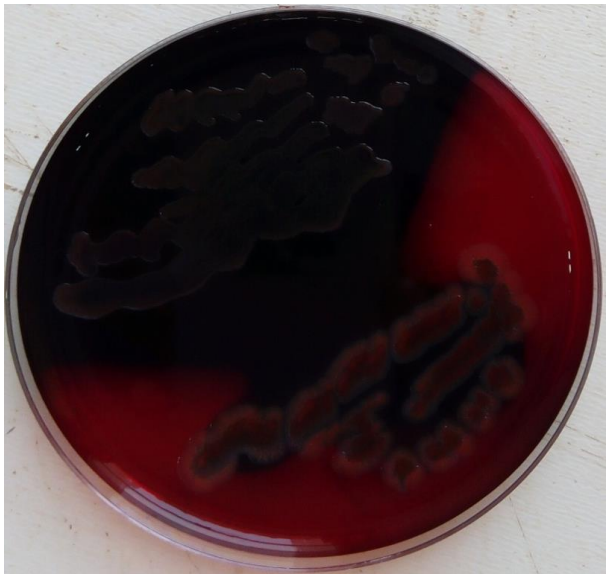

Figure 3. *E. coli* B27 isolate biofilm.

Figure 4. ***E. coli*** WDCM 00013 standard isolate biofilm.

DISCUSSION

S. siamea plant was reported to possess antimicrobial activities by Mohammed et al. (2013). However, in this study, *S. siamea* plant parts have not shown tangible antibacterial activities. Lack of significant antibacterial activity by the extracts of this plant may be explained by the fact the local usage of the plant for the treatment of typhoid is usually prolonged, perhaps to achieve the needed effective dosage level. This is in tandem with the study carried out by Doughari and Okafor (2008) in which the aqueous leaf extract of *S. siamea* only sparingly inhibited the growth of *Salmonella typhi* even at the highest concentration used in the study.

Interestingly, *S. siamea* leaf extract showed remarkable disruption of biofilms of the test organisms. This is evident in the high percentage biofilm inhibition observed when this extract was tested against strong biofilm-forming *E. coli* isolates; as high as 79.5% against the *E. coli* C1 in Table 2.

It is noteworthy to mention that in this work, the antibiofilm activities by the various extracts of *S. siamea* were best at lower concentrations as it can be seen in Table 2 that the *S. siamea* leaf extract had no activity at all against the standard *E. coli* WDCM 00013 at 20 mg/ml, only for this extract's activity to increase as the concentration decreases. This observation is similar to that of Gislene et al. (2000) where it was reported that plant extracts like *Syzygyum joabolanum* inhibited the growth of resistant bacteria at lower concentration. Supporting this fact is the work of Artini et al. (2018) where it was reported that different essential oils extracted from mediterranean plants were able to destabilize biofilm at very low concentration without impairing bacterial viability.

Furthermore, Lui (2003) reported that natural phytochemicals at the low levels present in fruit and vegetables offer health benefits, but these compounds may not be effective or safe when consumed at higher doses, even in a pure dietary supplement form. Though flavonoids and tannins were not isolated from the fractions of the plant's extract, the qualitative analysis conducted on the fractions showed that they contained these phytochemicals. This was further confirmed by the R_f values obtained from the thin layer chromatography as compared with those of the standards used. It can be

Table 1. Phytochemical constituents in the three extracts.

Sample Test	Ethanolic leaf	Ethanolic stem bark	Ethanolic root
Test for Carbohydrate	+	—	+
Test for anthraquinone	—	+	+
Test for unsat. steroid and tri terpenes	+	+	+
Test for cardiac glycoside	+	+	+
Test for saponin	+	+	-
Test for Tannins	+	+	+
Test for flavonoids	+	+	+
Test for alkaloids	+	+	+

: + = Present, — **=** Absent.

Table 2. Antibiofilm activity of *S. siamea* leaf extract on the bacterial isolates.

Concentration (mg/mL) / Isolate	Percentage biofilm inhibition (%)					
	20	**10**	**5**	**2.5**	**1.25**	**0.625**
B27	76.4	72.7	75.5	78.2	74.1	76.8
WDCM	0	18.6	39.5	61.8	68.2	65.5
C1	77.3	77.3	79.5	79.5	77.3	79.5

Table 3. Phytochemical compounds in various fractions.

Fraction type	Flavonoids	Tannins	Phenols
2:7:1 Hex-Eth.Acet-MeOH	**+++**	—	—
7:2:1 Hex-Eth.Acet-MeOH	—	**+++**	—

Hex-Eth.Acet-MeOH = Hexane-Ethyl acetate-Methanol; + = Detected, - = Not detected, ++ = more detected,+++ = very much detected **.**

Table 4. Antibiofilm activity of flavonoids-rich extract on the *E.coli* isolates.

Concentration (mg/mL) / Isolate	Percentage biofilm inhibition (%)					
	20	**10**	**5**	**2.5**	**1.25**	**0.625**
B27	83.3	77.7	66.7	50	66.7	66.7
C1	72.2	61.1	44	5.5	83.3	77.6
WDCM	77.7	72.2	72.2	72.2	77.7	44

seen that the flavonoids-containing fraction has higher antibiofilm activities than the tannin-containing fraction. According to Tsuchiya and Linuma (2000), antibacterial flavonoids might be having multiple cellular targets, rather than one specific site of action. One of their molecular actions is to form complex with proteins through nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation. This when compared with tannin's affinity for forming polymerization with extracting solvent, according to Naima et al. (2015), it will be understood why the flavonoid-rich fraction had more antibiofilm activities than

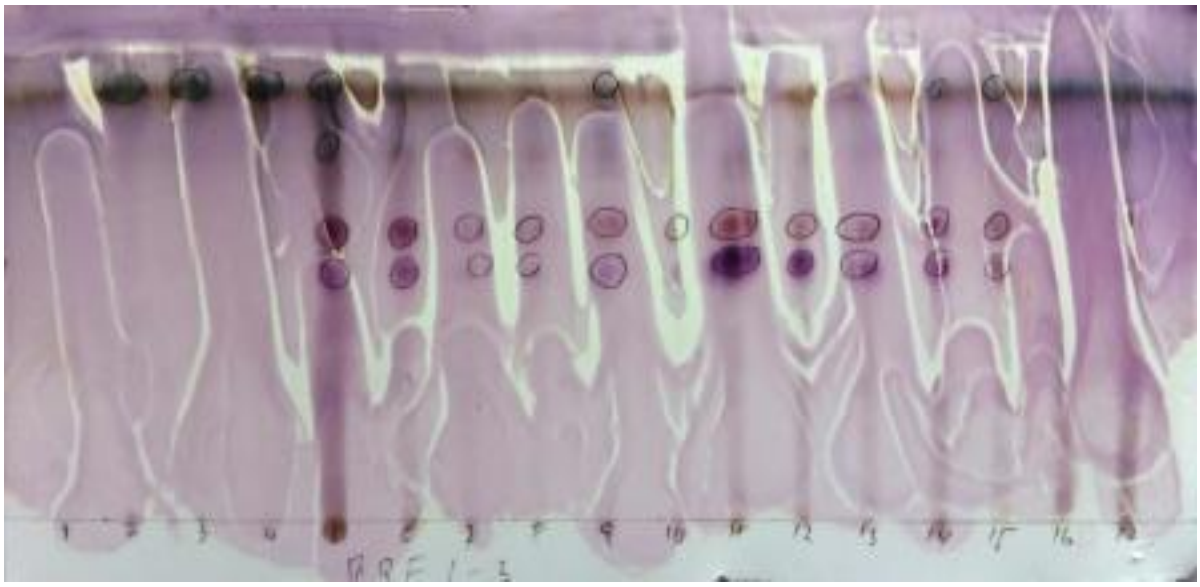

Figure 5. TLC plate showing the distance moved by tannin and flavonoids along with their standards from the baseline
NB: 5 and 6 are the starting points for tannin and its standard, tannic acid while 11 and 12 are the starting points for flavonoids and its standard, quercetin.

Table 5. Antibiofilm activity of tannin-rich extract on the *E. coli* isolates.

Concentration (mg/ml) / Isolate	Percentage biofilm inhibition (%)					
	20	**10**	**5**	**2.5**	**1.25**	**0.625**
B27	44.4	33.3	44.4	44.4	55.5	66.7
C1	22.2	11.1	44.4	55.5	88.9	88.9
WDCM	44.4	0	55.5	55.5	0	77.8

the tannin-rich fraction. Furthermore, the effect of mixing solvents in certain ratios for better extraction of plant's biomolecules has been demonstrated in this research as seen in the antibiofilm activities of 2:7:1 Hexane-Ethyl acetate-Metanol fraction.

In comparison, it could be seen that this work and our previous work, Usman et al. (2019) showed similar pattern of antibiofilm activities. However, it could be noted that while the minimum inhibitory concentration, MIC was 200 mg/ml in the previous work, it was 20 mg/ml in this work. Furthermore, the leaf extract of the plant in the previous work was not active enough for further assay whereas in this work, it was the best among the same set of extracts used in the previous work. This variation in activities of the same extracts of this plant might be due to change in time of collection of this plant's parts.

CONCLUSION

In this research, *S. siamea* leaf, ethanol extract had good antibiofilm activities against the *Escherichia coli* isolates used. Flavonoid-rich fraction had better biofilm disrupting activities against the test *Escherichia coli* isolates.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

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APPENDICES

Appendix Table 1. Optical densities (O.D) from microtitre plate reader of ethanol leaf extract against the *E. coli* isolates.

Concentration (mg/mL) / Isolate	Average O.D at					
	20	**10**	**5**	**2.5**	**1.25**	**0.625**
B27	0.083	0.073	0.071	0.096	0.093	0.079
WDCM	0.102	0.081	0.080	0.065	0.066	0.076
C1	0.065	0.068	0.063	0.075	0.064	0.055

Appendix Table 2. Optical densities (O.D) from microtitre plate reader of ethanol stem bark extract against the *E. coli* isolates.

Concentration (mg/ml) / Isolate	Average O.D at					
	20	**10**	**5**	**2.5**	**1.25**	**0.625**
B27	0.032	0.041	0.034	0.036	0.072	0.032
WDCM	0.74	0.045	0.049	0.055	0.063	0.071
C1	0.062	0.074	0.061	0.049	0.069	0.059

Appendix Table 3. Optical densities (O.D) from microtitre plate reader of ethanol root extract against the *E. coli* isolates.

Concentration (mg/ml) / Isolate	Average O.D at					
	20	**10**	**5**	**2.5**	**1.25**	**0.625**
B27	0.091	0.073	0.085	0.047	0.058	0.069
WDCM	0.069	0.061	0.058	0.075	0.092	0.046
C1	0.065	0.078	0.083	0.055	0.054	0.055

Appendix Table 4. Optical densities (O.D) from microtitre plate reader of flavonoid-rich extract against the *E. coli* isolates.

Concentration (mg/ml) / Isolates	Percentage biofilm inhibition (%)					
	20	**10**	**5**	**2.5**	**1.25**	**0.625**
B27	0.096	0.081	0.085	0.074	0.098	0.093
C1	0.125	0.114	0.096	0.108	0.109	0.111
WDCM	0.108	0.097	0.113	0.117	0.119	0.084

Appendix Table 5. Optical densities (O.D) from microtitre plate reader of tannin-rich extract against the *E. coli* isolates.

Concentration (mg/ml) / Isolate	Percentage biofilm inhibition (%)					
	20	**10**	**5**	**2.5**	**1.25**	**0.625**
B27	0.095	0.062	0.085	0.108	0.098	0.093
C1	0.125	0.111	0.066	0.069	0.091	0.101
WDCM	0.079	0.087	0.103	0.087	0.109	0.084

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Biological activity and production of metabolites from Amazon endophytic fungi

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Obtaining bioactive metabolites from endophytic microorganisms has become increasingly more interesting in the last few decades, since endophytes are known for their biotechnological potential. However, studies involving endophytic microbiota from tropical hosts are still scarce. In this study, the production of bioactive metabolites from endophytic fungi isolated from Amazonian plants were evaluated. Two fungi (*Talaromyces* sp. F15 and *Aspergillus* sp. F18) isolated from *Myrcia guianensis* (Myrtaceae) and one (*Penicillium* sp. F3) isolated from *Euterpe precatoria* (Arecaceae) were analysed. The fungi were cultivated in liquid medium and their metabolites were tested for antimicrobial, antioxidant and cytotoxic activity. Amylase, cellulase and lipase production, as well as biosurfactant production, were also evaluated. The metabolites of *Aspergillus* sp. F18 showed 69.4% antioxidant activity against DPPH free-radical molecules and cytotoxic activity against *A. salina*. *Penicillium* sp. F3 showed cytotoxic activity and stood out as the best amylase producer (31 U/ml). *Talaromyces* sp. F15 was the best lipase producer (4.5 U/ml) and the best biosurfactant source, with 33.3% emulsification index. These Amazonian host-associated fungi showed biotechnological potential, which are believed should be further investigated in order to elucidate the chemical structure of the metabolites responsible for the activities described here, as well as optimize their production.

Key words: Enzymes, lipase, cellulase, amylase, biosurfactant, antioxidant, antimicrobial, cytotoxic.

INTRODUCTION

Brazil is a country with vast biodiversity, and much of this wealth is concentrated in the Amazon region. As such, the Amazon region has numerous plant species from which many benefits have been discovered to date and,

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in turn, made use of, especially in the food and pharmaceutical industry (Banhos et al., 2014). People have been using natural compounds for the preparation of herbal teas and remedies for the prevention and treatment of various diseases for a long time and many of the current medicines available on the market have come from plants and microorganisms isolated from various traditional sources (Amedei and D'Elios, 2012; Fokou et al., 2016; Huang and Lin, 2017).

Among the microorganisms which are used as sources of biologically-active biomolecules are endophytes, and these are characterized by the fact that they inhabit plant tissues without causing apparent damage to their hosts. Nevertheless, endophytic microorganisms can be pathogens, commensals, or mutualists, depending on plant-endophyte interactions. Secondary metabolites produced by endophytic fungi have a broad spectrum of bioactivity, such as for antimicrobial, antioxidant, antitumor, enzymatic and surfactant compounds (Canuto et al., 2012; Pamphile et al., 2017; Silva et al., 2019; Toghueo, 2019).

Endophytes have the ability to interact with the plant at complex levels and, in some cases, maintain symbiosis relationships (Yan et al., 2019). Endophytic micro-organisms can even produce the same metabolites as their hosts (Strobel at al., 2004; Facundo et al., 2008), and thus stand out as an alternative for the preservation of several plant species, whose extracts are used in the production of some medicines (Mussi-Dias et al., 2012). However, endophytic fungi isolated from tropical plants are still little studied (Oliveira, 2010; Specian et al., 2014; Silva et al., 2019).

Metabolites produced by endophytic fungi have already been identified as being effective against tumors in human cervical cancer cell cultures (Wijesekara et al., 2013). These metabolites have shown antimicrobial activity against multi-drug resistant strains of bacteria, and demonstrate effective and promising action in this application (Arivudainambi et al., 2014). Also, these molecules present antioxidant activity, an important characteristic, since the production of free radicals and other reactive oxygen species (ROS) can damage biomolecules such as RNA and DNA, and result in several physiological disorders, such as cancer and premature aging (Huang et al., 2007; Morais et al., 2014; Caicedo et al., 2019).

Other important bioactive metabolites that are produced by fungi are enzymes. Hydrolytic enzymes catalyze hydrolysis reactions of different substrates and have a wide range of industrial uses. Lipases are responsible for catalyzing the hydrolysis of triacylglycerides formed by long chain fatty acids, and have widespread application in biocatalysis. Cellulases are involved in the degradation of cellulose, which are utilized for the hydrolysis of biomass for ethanol production, and the amylases act by breaking down starch molecules, for which there is a high demand from the food industry (Roveda et al., 2010; Gopinath et al., 2017; Bentil et al., 2018).

Biosurfactants are also metabolites of industrial interest, which are amphiphilic molecules that are capable of reducing the interfacial and surface tension of liquids and have properties and applications that involve actions such as, emulsification, lubrication, foaming, wetting, solubilization, detergent actions and phase dispersion (Nitschke and Pastore, 2002; Varjani and Upasani, 2017).

Since little is known about the biotechnological potential of metabolites produced by fungi isolated from Amazonian species, this study aimed to primarily evaluate the antioxidant, cytotoxic and antimicrobial activities, as well as the production of hydrolases and biosurfactants in metabolites obtained from submerged cultivation of endophytic fungi which had been isolated from the tropical species *Myrcia guianensis* and *Euterpe precatoria*.

MATERIALS AND METHODS

Microorganisms

The endophytic fungi used in this study were previously selected as producers of bioactive metabolites (Batista, 2018; Matias, 2018) and are held in the Chemistry Applied to Technology (QAT) research group's work collection at the Superior School of Technology (EST) at the Amazonas State University (UEA).

The fungi *Aspergillus* sp. F18 (Figure 1A) and *Talaromyces* sp. F15 (Figure 1B) were isolated from *Myrcia guianensis* stem and root, respectively. The vegetable material from *M. guianensis* were collected in Santarém, Pará State. Their identifications were carried out at the INPA Herbarium (National Institute of Amazon Research), and a voucher specimen was deposited under the registration number 181913 (Banhos et al., 2014).

The isolate *Penicillium* sp. F3 (Figure 1C) was obtained from the leaves of *Euterpe precatoria.* The leaves were collected in Manaus, Amazonas State. The plant was identified at the IFAM Herbarium (Amazonas Federal Institute of Education, Science and Technology), and a voucher specimen was deposited under the registration number 16782 (Batista et al., 2018).

The endophytic fungi were identified by their macroscopic characteristics (color, texture, topography, diffuse pigmentation, color, and topography of the back of the colony), and well as by their microscopic reproductive structures, via the microculture technique and comparison of the obtained results with taxonomic keys (Barnett and Hunter, 1972; Hanlin, 1996) (Figure 1).

The three isolates were stored in sterile distilled water (Castellani, 1939). Reactivation of the fungi occurred by inoculation from a stock culture onto potato dextrose agar (PDA) with subsequent incubation in a microbiological chamber (BOD) at 28ºC for approximately 5 days.

Production of fungal metabolites for biological assays

Under sterile conditions, three mycelial plugs of approximately 5x5 mm were inoculated into 125 ml Erlenmeyer flasks with 80 ml potato-dextrose liquid medium (PD) supplemented with 0.2% yeast extract. Cultures were produced in duplicate. The flasks were incubated in a shaker at 28°C and shaken at 120 rpm for 8 days. After this period, the metabolic fluid was filtered through a 0.45 µm

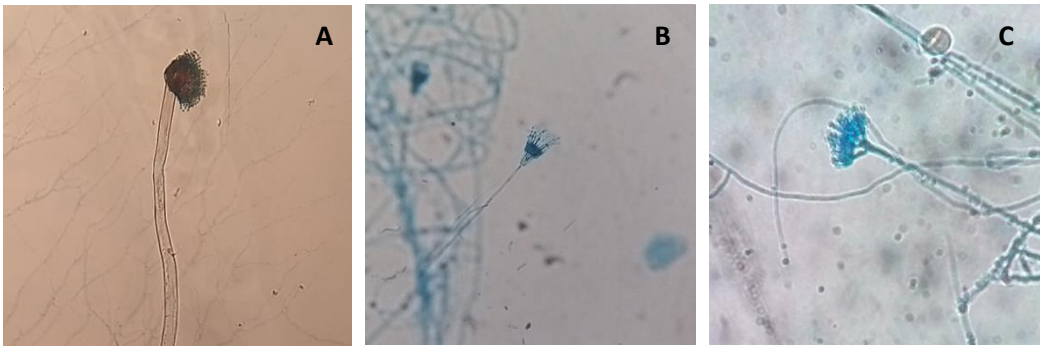

Figure 1. Microscopic images from Amazonian endophytic fungi. A: *Aspergillus* sp. F18 isolated from *Myrcia guianensis* stem. B: *Talaromyces* sp. F15 isolated from *Myrcia guianensis* root. C: *Penicillium* sp. F3 isolated from *Euterpe precatoria* leaves.

pore membrane filter (Souza et al., 2004). The filtered medium was stored at -18ºC for later use in biological activity assays.

Evaluation of antimicrobial activity

The microplate dilution technique was used (NCCLS, 2003) to verify the antimicrobial activity. The reducing of resazurin was used for antibacterial testing, and reducing of 2,3,5-triphenyltetrazoic chloride (TTC) for antifungal testing. The fungal metabolites were tested against strains of *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 35218), *Pseudomonas aeruginosa* (ATCC 9027) and *Candida albicans* (ATCC 12031).

The assay was performed using sterile 96-well microplates for each microorganism. 100 µl of the inoculum was pipetted at a concentration of 1.5 x 10^4 CFU/ml into the plate wells in triplicate. For sterility control and negative test control, we used the culture medium (Mueller Hinton broth for bacteria and potato dextrose broth for fungi). The positive control was Levofloxacin (15 mg/ml) for bacteria and Itraconazole (20 mg/ml) for fungi. Subsequently, the plates were incubated at 37ºC for 24 h. After this period, 20 µl of resazurin was added for the antibacterial activity assay and 20 µl of TTC for the antifungal activity assay. The plates were incubated again at 37 °C for approximately 2 h for reaction testing. Wells that remained colorless were considered as showing antimicrobial activity (Duarte, 2006).

Determination of antioxidant activity

Antioxidant activity was determined by the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH·) scavenging method. This assay is based on free radical reduction in the presence of an antioxidant (Molyneux, 2004). The DPPH solution was prepared at 0.06 mmol/l with methanol P.A., and protected from direct light exposure (Silva, 2012). The assay was performed by microplate spectrophotometry with 40 µl of the metabolic medium and the addition of 250 µl of the DPPH solution. For the negative control, 40 µl of methanol and 250 µl of DPPH solution were added (Duarte-Almeida et al., 2006). The microplate was protected from direct light exposure and after 10 min the absorbance readings were taken on a microplate spectrophotometer, at 517 nm. The flavonoid quercetin was used as standard. The percentage of DPPH radical scavenging was measured by the equation below using the absorbance decay values of the sample (Abs_{sample}) and of the control ($Abs_{control}$):

$$\boldsymbol{AA}\ (\%) = \frac{\left(\boldsymbol{Abs_{control} - Abs_{sample}}\right)}{\boldsymbol{Abs_{control}}}\ \boldsymbol{x\ 100}$$

Determination of cytotoxic activity

To evaluate the cytotoxic effect of the samples, the lethality test was performed with *Artemia salina* (Meyer et al., 1982; Mclaughlin, 1991). As a growth medium, a saline solution containing 20 g of synthetic sea salt in distilled water was used to prepare 1000 ml of 2% solution. For hatching, 10 mg of cysts of *A. salina* (Maramar) were used. Growth occurred at room temperature, with constant aeration under fluorescent lighting during 48 h. After hatching, the nauplii were transferred to 24-well plates, 10 nauplii distributed to each well and 1 ml of the test solutions added in duplicate. In the control, the test solution was replaced with the culture medium used for the fungal culture and, in another well, just the saline solution was used. 100 µl of Levofloxacin (15 mg/ml) was added to inhibit the proliferation of bacteria in the medium. After 24 h, the number of surviving larvae was analyzed in both control wells and treatments.

Preparation of spore suspension for the production of hydrolytic enzymes and biosurfactants

To produce the spore suspension, the fungi were cultivated in PDA in inclined test tubes (Gomes and Pena, 2016). 4 ml of sterile distilled water were added to each test tube. The tubes were shaken to float the spores present in the mycelium. An aliquot of 1000 µl was taken from each tube and from this tube the spore count was performed in a Neubauer chamber to adjust the inoculum to a concentration of 10^6 spores/ml for enzyme production and 10^8 spores/ml for biosurfactant production.

Amylase production

The fungi were grown in a liquid medium as described by Hegde et al. (2011), and composed of $NaNO_3$ (3.0 g/l), $MgSO_4.7H_2O$ (0.5 g/l), KCl (5.0 g/l), KH_2PO_4 (1.0 g/l), $FeSO_4.7H_2O$ (0.01 g/l), $CaCl_2$ (0.1 g/l) and starch (15 g/l), pH 7.0. 100 µl of spore suspension were added (10^6 spores/ml) and incubated in a shaker at 30°C, under constant stirring 120 rpm, for 7 days. Every 24 h, 1 ml aliquots were taken and filtered for subsequent measurement of enzymatic activity. Cultivations were performed in triplicate. Commercial amylase (Novozymes) was used as a standard for purposes of

comparison.

Determination of amylolytic activity

To measure amylase activity, a standard glucose curve was constructed according to the methodology of Vasconcelos et al. (2013). The measurement of enzymatic activity was carried out as described by Miller (1959), with some modifications, to determine the amount of reducing sugars formed during the incubation of the enzyme extract with the substrate using 3,5-dinitrosalicylic acid (DNS).

The reaction mixture was composed of 50 µl substrate (1% starch) diluted in 1 M sodium acetate buffer, pH 6 (m/v) and 50 µl enzyme extract. After incubation at 50°C for 30 min, 100 µl of DNS was added and the mixture was placed in a water bath for 5 min. 800 µl of distilled water were added and then the absorbance was read in a spectrophotometer at 540 nm. One unit of enzymatic activity (U) was defined as the amount of enzyme capable of releasing 1 µmol of reducing sugar per minute under the assay conditions.

Cellulase production

The fungi were grown in a liquid medium as described by Zanchetta (2012), composed of KH_2PO_4 (2.0 g/l), $(NH_4)_2SO_4$ (1.4 g/l), urea (0.3 g/l), $MgSO_4.7H_2O$ (0.3 g/l), $CaCl_2$ (0.1 g/l), $FeSO_4.7H_2O$ (5.0 mg/l), $MnSO_4.H_2O$ (1.6 mg/l), $ZnSO_4.7H_2O$ (1.4 mg/l), $CoCl_2$. H_2O (1.6 mg/l) and carboxymethylcellulose - CMC (10 g/l), pH 5.0. 100 µl of spore suspension was added (10^6 spores/ml) and incubated in a shaker at 28°C, 120 rpm during 7 days. Every 24 h, 1 ml aliquots were taken and filtered for subsequent measurement of enzyme activity. Cultivations were performed in triplicate. The commercial enzyme produced by *Aspergillus niger* (Sigma Aldrich) was used as a standard for comparative purposes.

Determination of cellulolytic activity

The standard curve was constructed with glucose. Enzyme activity was measured according to the methodology described by Miller (1959) with modifications. The reaction mixture was composed of 50 µl of enzyme extract and 50 µl of 1% CMC solution in sodium citrate buffer, 0.05 M pH 5, incubated at 50°C for 30 min. After incubation, 100 µl of DNS was added and then the mixture was placed in a water bath for 5 min at 100°C, followed by the addition of 800 µl of distilled water. The absorbances were read in a spectrophotometer at 540 nm against the blank which was composed of all the reaction components collected at time point zero of the experiment. One unit of enzymatic activity (U) was defined as the amount of enzyme capable of releasing 1 µmol of reducing sugar per minute under the assay conditions.

Lipase production

The fungi were grown in Erlenmeyer flasks containing 100 ml of liquid medium, as described by Nascimento et al. (2014). The medium was composed of NH_2NO_3 (1.0 g/l), $MgSO_4.7H_2O$ (0.6 g/l), KH_2PO_4 (1.0 g/l), peptone (20 g/l) and olive oil (1% and 0.065%), pH 6. 100 µl of spore suspension was inoculated (10^6 spores/ml) under sterile conditions and incubated in a shaker at 28°C, 160 rpm for 7 days. Every 24 h, 1 ml aliquots were taken and filtered for subsequent measurement of enzyme activity. Cultivations were performed in triplicate. The commercial enzyme produced by *Candida rugosa* (Sigma Aldrich) was used as a standard for comparative purposes.

Determination of lipolytic activity

The quantification of lipolytic activity was performed according to Winkler and Stuckmann (1979). An emulsion of *p*-nitrophenyl palmitate (pNPP) was prepared by the dropwise addition of 1 ml of solution A (30 mg of pNPP dissolved in 10 ml of isopropanol) in 9 ml of solution B (0.4 g of Triton X-100; 0.1 g of gum arabic and 90 ml of Tris HCl 50 mM pH 7.0) under intense agitation. The emulsion obtained and the aliquots taken from the fungal culture medium were stabilized for 5 min at 37°C. 0.1 ml of the supernatant was added to 0.9 ml of the substrate emulsion and the mixture was incubated for 15 min at 40°C. The absorbance of the mixtures was measured by spectrophotometer at 410 nm. One unit (U) of enzymatic activity was defined as the amount of enzyme required to release 1.0 µmol of *p*-nitrophenol per minute under these conditions (Pereira et al., 2015; Tombini, 2015). Enzyme activity was obtained by the relationship between the absorbance of the sample and the molar extinction coefficient of *p*-nitrophenol (ε = 12276).

Biosurfactant production

The liquid medium described by Jacobucci (2000) composed of $MgSO_4$ (0.5 g/l), Na_2HPO_4 (3.0 g/l), KH_2PO_4 (1.0 g/l) and yeast extract (1.3 g/l) was used. After autoclaving, 0.5 g/l of soybean oil was added to the medium and then homogenized. Afterwards, 1.0 ml of the spore suspension (10^8 spores/ml) of endophytic fungi (*Penicillium* sp. F3, *Talaromyces* sp. F15 and *Aspergillus* sp. F18) was inoculated into the medium. The fungi were grown in a shaker type incubator at 28 °C under constant stirring at 170 rpm for 8 days. At the end of the experiment, the metabolic medium was filtered to separate the mycelium with 0.45 µm filtering membrane and assisted by a vacuum pump. Afterwards, the cell free cultivation broth was used for the determination of the emulsification index E_{24} (%) and surface tension measurements. Cultures were performed in triplicate.

Determination of the Emulsification index

A mixture of 6 ml of the solvent (kerosene) and 4 ml of solution of the synthetic surfactant (control) or culture medium containing biosurfactant was mixed in a vortex-type stirrer for 2 min. The emulsifying activity was investigated after 24 h and the emulsification index E_{24} (%) was calculated by dividing the height measurement of the emulsion layer by the total height of the mixture, multiplying by 100 (Pornsunthorntawee et al., 2008). The assays were performed in triplicate. For positive control, 1% SDS solution was used and for negative control the culture medium without microorganism was used.

Surface tension measurements

Surface tension was measured by the ring method using a ring tensiometer (Krüss) at room temperature (25°C) (Du Nouy, 1925).

RESULTS AND DISCUSSION

Biological activities

The metabolites produced by the fungus *Aspergillus* sp. F18 isolated from *M. guianensis* showed antioxidant activity of 69.4%, while quercetin showed 90%. The other fungi tested showed no ability to sequester the DPPH free radical. Zhao et al. (2014) tested extracts obtained

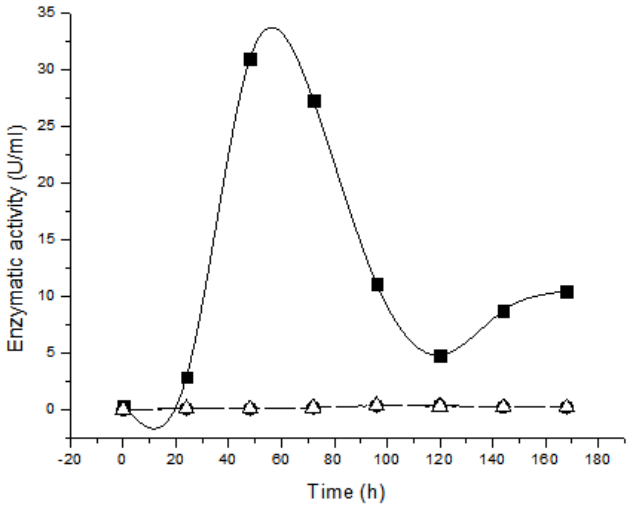

Figure 2. Amylase enzyme activity for the endophytic fungi *Penicillium* sp. F3 (■), *Talaromyces* sp. F15 (●) and *Aspergillus* sp. F18 (△), during the cultivation period.

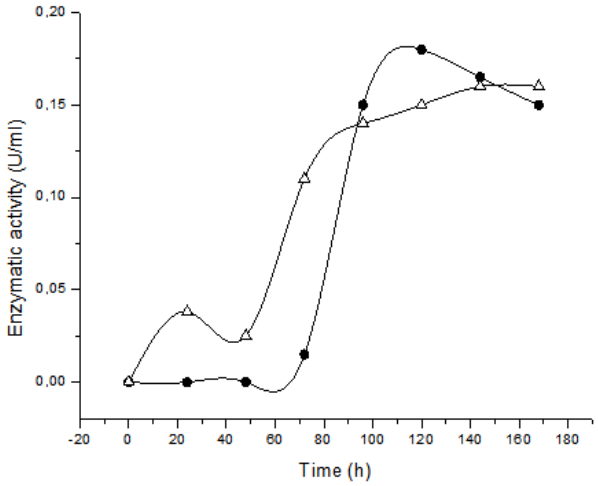

Figure 3. Cellulase enzyme activity for the endophytic fungi *Talaromyces* sp. F15 (●) and *Aspergillus* sp. F18 (△), during the cultivation period.

from the metabolites of the endophytic fungus *Aspergillus fumigatus* and observed that the ethyl acetoacetate extract was the most promising one, with considerable antioxidant activity (AA = 95%; CE_{50} = 38.64 µg/ml), which indicates the potential of endophytes as sources of antioxidant compounds.

Antimicrobial activity was determined by the microdilution technique. The fungal metabolites tested here showed no activity against the evaluated microorganisms. According to Furmanek et al. (2019), the microdilution method assesses the effectiveness of concentrations of used substances . In this study, the crude aqueous extract to access the antimicrobial activity was used, and the substances that could present the inhibition of microbial growth were probably not in an effective concentration (not detected by this method), thus it is necessary to concentrate the metabolites in order to access this biological activity. Proper interpretation of the results is also hampered by the dosages employed since, according to the Clinical and Laboratory Standards Institute's (CLSI) recommendations, a very major mistake in the interpretation of compound activity can be made in demonstrating the resistance according to this method (false sensitivity) (CLSI, 2015).

The metabolites of the fungi *Aspergillus* sp. F18 (isolated from *M. guianensis*) and *Penicillium* sp. F3 (from *E. precatoria*) were cytotoxic in the tests performed against *A. salina*, and lead to the death of all nauplii within 24 h. Miao et al. (2012) found that the substance 6.8-di-O-methylaverufin, produced by an endophytic fungus, showed significant toxicity for *A. salina*, with CL_{50} from 0.5 µg/mL. The lethality of simple organisms such as *A. salina* has been used for rapid and relatively simple monitoring of biological response (Meyer et al., 1982). The lethality assay allows the evaluation of general toxicity and is therefore considered essential as a preliminary bioassay in the study of compounds with potential biological activity (Colegate and Molyneux, 1993). According to Uzma et al. (2018), endophytic fungi represent a rich source of bioactive metabolites that can be manipulated to produce novel analogs for chemotherapy, such as taxol, podophyllotoxin, camptothecin, and vinca alkaloids. Therefore, the fungal metabolites tested here should be evaluated in further studies for their cytotoxic activity using cell assays.

It is worth mentioning that the secondary metabolites produced by endophytic fungi can suffer alterations when grown in the laboratory. Temperature, composition of the culture medium and aeration can interfere with the quantity and type of compounds that are produced (Strobel et al., 2004). Thus, it is important to optimize the cultivation conditions in order to increase the metabolite yields.

Enzyme production

Maximum amylase production (31 U/ml) was observed after 72 h of cultivation of the fungus *Penicillium* sp. F3, isolated from the *E. precatoria* leaf (Figure 2). The fungus *Talaromyces* sp. F15, isolated from the *M. guianensis* stem, however, showed low enzymatic activity (0.37 U/ml), as did the fungus *Aspergillus* sp. F18, isolated from the *M. guianensis* root (0.34 U/ml). The obtained result was compared with the commercial enzyme, which obtained activity of 38.5 U/ml, indicating the potential of *Penicillium* sp. F3 as a source of amylolytic enzymes.

The fungi *Talaromyces* sp. F15 and *Aspergillus* sp. F18 showed low cellulolytic activities after 120 h of cultivation of 0.18 and 0.16 U/ml, respectively, as shown in Figure 3.

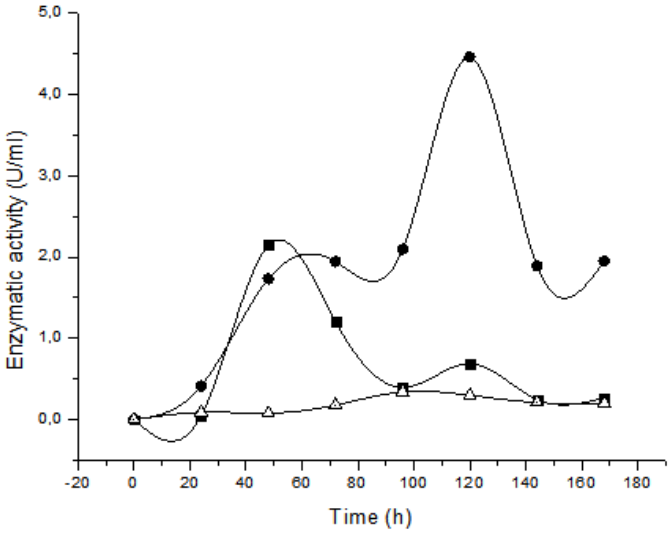

Figure 4. Lipase enzyme activity for the endophytic fungi *Penicillium* sp. F3 (■), *Talaromyces* sp. F15 (●) and *Aspergillus* sp. F18 (△), during the cultivation period.

However, the fungus *Penicillium* sp. F3 did not produce cellulase under the evaluated cultivation conditions. Reddy et al. (2015) obtained, under similar cultivation conditions used in this study, a cellulolytic activity of 2.48 U/ml for *A. niger*.

The evaluated commercial enzyme presented activity of 30.9 U/ml. Therefore, it is worth noting that the endophytic fungi evaluated in this study must be subjected to new culture conditions in order to increase cellulase production.

The maximum lipase production was observed after 120 h of cultivation of the fungus *Talaromyces* sp. F15 (Figure 4), and obtained an activity level of 4.45 U/ml. The fungus *Penicillium* sp. F3 showed a maximum activity level of 2.14 U/ml in just 48 h.

However, the fungus *Aspergillus* sp. F18 showed an activity level of 1.04 U/ml. Romdhane et al. (2013) obtained enzymatic activity of 9.8 U/ml for the purified lipase produced by the fungus *T. thermophilus*. Thus, the endophytic fungus *Talaromyces* sp. F15 isolated from *M. guianensis* is shown to be a promising lipase producer. The evaluated commercial enzyme presented activity of 24.4 U/ml.

Biosurfactant production

The 3 endophytic fungi were evaluated for emulsification index levels and reduction of surface tension for biosurfactant production. Table 1 shows the results obtained for emulsification index tests (E_{24}) in the presence of kerosene.

It is noted that the fungal culture media *Penicillium* sp. F3 and *Aspergillus* sp. F18 showed no emulsion formation in the presence of organic solvent. For the fungus *Talaromyces* sp. F15 emulsion formation was obtained, with E_{24} of 33.3%. SDS synthetic surfactant has higher emulsification capacity than fungal metabolites, which was expected since SDS is a pure substance.

Table 2 shows the reduction in surface tension of the cell-free culture medium for the 3 evaluated fungi. Note that for the fungus *Talaromyces* sp. F15, surface tension reduction was 33%, from 56.5 mN/m to 40.5 mN/m.

According to Haba et al. (2000), microorganisms that are good biosurfactant producers are able to reduce the surface tension of the medium to 40 mN/m or less, which indicates the potential of the endophytic isolate as a source of surfactant molecules. According to Ron and Rosenberg (2002), Bach et al. (2003) and Hamme et al. (2006), low molecular weight biosurfactants reduce surface tension more efficiently, while those with high molecular weight are characterized by the formation of more stable oil/water emulsions. Therefore, it can be inferred that the biosurfactant produced by the Amazonian endophytic fungus *Talaromyces* sp. F15 is a low molecular weight molecule.

Conclusion

The results showed that the endophytic fungi isolated from tropical species *M. guianensis* and *E. precatoria* are promising in the search for new bioactive compounds of industrial interest, mainly in the production of amylase and biosurfactants. The activities tested indicated significant results, which should be improved by further

Table 1. Results of the emulsification index tests (E_{24}) of the endophytic fungi cultivation broth and the 1% Sodium Dodecyl Sulfate (SDS) surfactant.

Endophytic fungi	E_{24} (%)
Penicillium sp. F3	*
Talaromyces sp. F15	33.3
Aspergillus sp. F18	*
SDS 1%	88.0

*Not detected.

Table 2. Reduction in the surface tension (ST) of the metabolic media after 8 days of cultivation of the endophytic fungi.

Endophytic fungi	Initial ST (mN/m)	Final ST (mN/m)	Reduction in ST (%)
Penicillium sp. F3	56.5	52.53 ± 1.5	5
Talaromyces sp. F15	56.5	40.5 ± 0.5	33
Aspergillus sp. F18	56.5	49.97 ± 2.5	10

studies of optimization of cultivation conditions.

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CONFLICT OF INTERESTS

The authors declare no conflict of interests.

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Full Length Research Paper

MALDI-TOF identification of *Campylobacter* isolated from patients consulted in private laboratories in France

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Campylobacter **is a major agent of gastroenteritis worldwide. The incidence and prevalence of campylobacteriosis have been increasing in both developed and developing countries over the last decade. In this study, 197 strains of successive *Campylobacter*-like were identified in French laboratories in September 2018. Bacterial isolates from clinical samples were identified with a mass spectrometer (Ultraflex III TOF/TOF and the BIOTYPER database from Bruker Daltonics). Of the 197 isolates tested, 143 were identified as *Campylobacter jejuni* (72.59%), 28 as *Campylobacter coli* (14.21%), 2 as *Arcobacter butzleri* (1.02%), 1 as *Campylobacter fetus* and 1 as *Campylobacter lari* with (0.51% each) by the MALDI-TOF mass spectrometry. Isolation rate of *Campylobacter* was highest in the 0 - 9 age group (22%). The proportion of male and female patients was 59.4% (CI 95% = 52.2-66.3) and 40.6% (CI 95% = 33.7-42.8) respectively. Sixty strains (30.5%) were resistant to tetracycline and 52 (26.4%) resistant to ampicillin. This study showed that the MALDI-TOF mass spectrometry is a rapid and accurate identification method of *Campylobater* spp in patients treated in private French laboratories.**

Key words: *Campylobacter*, identification, MALDI-TOF, patients, France.

INTRODUCTION

Campylobacter enteritis was first identified by Butzer in the early 1970s. This pathogen is considered as one of the leading bacterial species of foodborne diseases in humans around the world (Abdi-Hachesoo et al., 2014; CNRCH, 2018). As a result, campylobacteriosis is a major public health concern in many developed countries (Wardak et al., 2007) and in developing countries infection has strikingly increased in recent years (Ewnetu

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and Mihret, 2010; Gwimi et al., 2015). Human campylobacteriosis has been linked to mishandling and consumption of contaminated poultry (Humphrey et al., 2007). Among the pathogenic species, *Campylobacter jejuni* and *Campylobacter coli* are leading causes of foodborne gastroenteritis and enteritis in humans worldwide (Friedman et al., 2000; Reddy and Zishiri 2007). In most European countries, the majority of *Campylobacter* infections are domestically acquired (EFSA, 2010). Thus, in 2017 several species of *Campylobacter* with a dramatic increase of *C*. *jejuni* were isolated in many blood cultures of patients. Nevertheless, *C*. *jejuni* along with *C*. *coli* and *C*. *fetus*, mostly isolated in stool, remain the common *Camplylobacter* of human pathogens (CNRCH, 2018). Similarly, other *Campylobacter* spp. such as *Campylobacter lari* and *Campylobacter upsaliensis* have been implicated in human gastrointestinal infections (Obeng et al., 2012; CDC, 2013).

Most *Campylobacter* enteric infections are self-limited and do not require antimicrobial drug treatment. However, because of severe or long-lasting *Campylobacter* infections their treatment may require antimicrobial drug therapy (Gallay et al., 2007). Therefore, macrolides as first-line therapy and fluoroquinolones as alternative therapy are recommended (Nachamkin and Blaser, 2000; Gallay et al., 2007). However, the resistance of *Campylobacter* to antimicrobial agents has substantially increased during the past two decades and become a matter of concern in severe human *Campylobacter* infections (Lucey et al., 2002; Nachamkin et al., 2002). The objectives of the present study were therefore 1) to identify *Campylobacter* species in patients consulted in private laboratories in France, using the MALDI-TOF method and 2) to test the bacteria's susceptibility to antimicrobials. With the low identification rates and continuous resistance of pathogenic bacteria species to antibiotics, an accurate and efficient analytical method such as MALDI-TOF MS will be a robust tool for controlling the extend of bacterial infections in both developed and developing countries.

MATERIALS AND METHODS

Bacterial strains

A total of 197 *Campylobacter*-like strains were collected in September 2018 by CNR. Each strain was identified after being subcultured on a trypticase soy blood agar plate (bioMérieux, Marcy l'Etoile, France) and incubated overnight in a microaerobic atmosphere at 37°C. A typical *Campylobacter* colonies obtained were used for mass spectrometry identification.

Mass spectrometry identification

Sample preparation

A part of a colony of each isolate, taken directly from the agar plate after 18-24 h of incubation to obtain fresh bacteria, was deposited on a microtitre 384 target plate ground steel T F, (Bruker Daltonics, Bremen, Germany) in a single spot and allowed to dry at room temperature. One microlitre of matrix solution (saturated solution of a cyano-4-hydroxycinnamic acid in 50% acetonitrile) was added to the sample and was then crystallized through air-drying at room temperature for 5 min.

MALDI-TOF mass spectrometry measurements

MALDI-TOF mass spectrometry measurements were performed with an Ultraflex III TOF/TOF mass spectrometer (Bruker Daltonics) equipped with a 200-Hz smartbeam 1 laser. The parameter settings were as follows: delay, 80 ns; ion source, 1 volt, 25 kV; ion source, 2 volts, 23.4 kV; lens voltage, 6 kV; and mass range, 0-20 137 kDa. Each run included an *Escherichia coli* control sample provided by Bruker Daltonics where the presence of eight specific proteins insured that the spectrometer was set properly. Raw spectra of the strains were analysed by MALDI BIOTYPER 2.0 software (Bruker Daltonics) using the default settings (all of the settings were potentially adjustable). The whole process from MALDI-TOF mass spectrometry measurement to identification was performed automatically without any user intervention. Briefly, the software generated a list of peaks up to 100. The threshold for peak acceptance was a signal-to-noise ratio of 3. After alignment, peaks with a mass-to-charge ratio difference of <250 ppm were considered to be identical. The peak list generated was used for matches against the reference library, by directly using the integrated pattern-matching algorithms of the software. All parameters were the same regardless of the presumptive bacterial species analysed. Concerning *Campylobacter* and related species, the BIOTYPER 2.0 database was composed of four *Arcobacter butzleri*, two *A. cibarius*, two *A. cryaerophilus*, one *A. halophilus*, one *A. nitrofigilis*, two *A. skirrowii*, three *Campylobacter coli*, five *C*. *fetus*, four *C. helveticus*, two *C. hyointestinalis*, six *C*. *jejuni*, four *C. lari*, one *C. sputorum* and four *C. upsaliensis*. The spectra were obtained in the positive linear mode after 1000 shots (size, 61 794 points; delay, 232 points). A score was attributed to each identification. When this score was >2.00, the identification was considered correct at the species level; between 1.7 and 1.999, the identification was considered correct at the genus level; and <1.7, the identification was not similar enough to a spectrum to draw a conclusion.

Antimicrobial susceptibility test

The antimicrobial susceptibility was performed by the agar diffusion method according to the criteria proposed by the CA-SFM and harmonized according to the criteria proposed by EUCAST (EUCAST, 2018): MH-F medium (Mueller-Hinton + 5% defibrinated horse blood and 20 mg/L β-NAD) (bioMérieux, Marcy l'Etoile, France) was used with an inoculum corresponding to 0.5 McFarland. Six antibiotics belonging to five families were tested: Ampicillin, Amoxicillin- clavulanic acid (beta-lactams), ciprofloxacin (quinolones), erythromycin (macrolides), tetracycline (cyclines) and gentamicin (aminosides). The plates were incubated in microaerobic atmosphere conditions at 35 ± 2°C, 24 h in microaerobic jar conditions (generation of atmosphere using a Anoxomat (Smart)). The reading at 24 h (or 48 h) was performed using the SIRScan system (I2A, Montpellier, France) then visual verification of the diameters read on the camera. A biologist always checks the values read. At the validation, any discrepancy with the result reported by the correspondent was verified and if necessary indicated on the final report. *Campylobacter jejuni* ATCC 33560 was used as quality control.

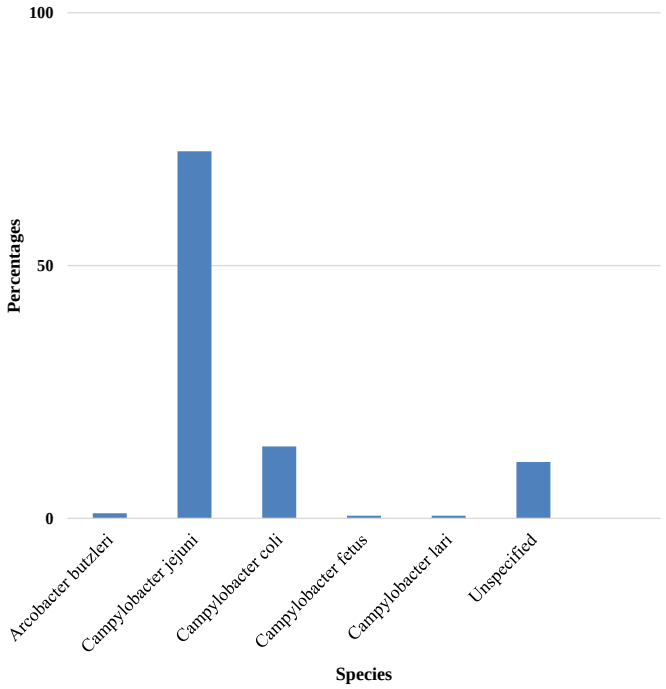

Figure 1. Isolated *Campylobacter* species in patients.

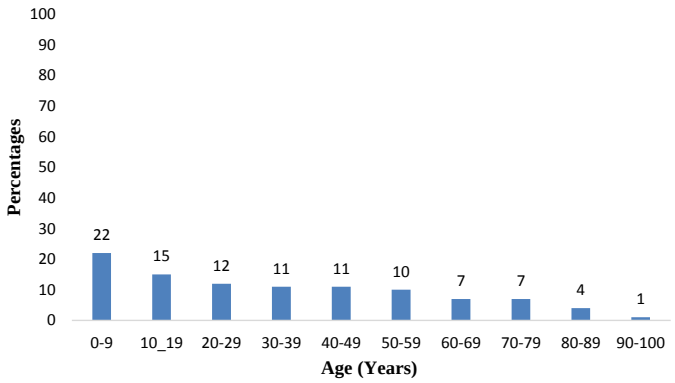

Figure 2. Prevalence of *Campylobacter* infections by age group.

Data processing

Data were analyzed using the software package Epi Info 7.1.2.0 (Centers for Disease Control and Prevention [CDC], Atlanta). Multivariable logistic regression was used to estimate odds ratios (ORs) with 95% confidence intervals (95% CI) also calculated. The statistical significance was evaluated using the Fischer exact 2-tailed p value, and a $p \leq 0.05$ was considered significant.

RESULTS

Campylobacter isolated in patients

Of the 197 isolates tested, 143 were identified as *Campylobacter jejuni* (72.59%), 28 as *C. coli* (14.21%), 2 as *Arcobacter butzleri* (1.02%), 1 as *C. fetus* and 1 as *C. lari* with (0.51% each). Twenty-two (11.2 %) of the isolates were undefined species (Figure 1). At least, 175 (88.8%) were culture positive and 22 (11.2%) were culture negative.

Age and sex distribution of *Campylobacter* infections

The isolation rate of *Campylobacter* was highest in the 0 - 9 (22%) age group, followed by 10-19 (15%), 20 - 29 (12%), 30 - 39 and 40-49 (10% each). Age group above 50 recorded the least isolation rate (≤10%) (Figure 2). The proportion of male and female patients was 59.4% (CI 95% = 52.2-66.3) and 40.6% (CI 95% = 33.7-42.8) respectively. Table 1 shows a breakdown of the different species isolated according to the sex of the patient.

Antibiotic resistance profiles of *Campylobacter* species

The resistance rates were 26.4, 1, 1, 30.5, 0.5 and 1% to ampicillin, amoxicilin- clavulanic acid, erythromycin, tetracycline, gentamicin and ciprofloxacin, respectively (Table 2).

DISCUSSION

The identification of *Campylobacter* species and related organisms at the species level has always been difficult using phenotypic methods because of their low metabolic activity, whereas molecular methods are more reliable but time-consuming (Bessède et al., 2011). The development of MALDI-TOF MS, a rapid and cost effective analytical method, has profoundly improved the bacterial identification process (Mandrell et al., 2005; Kolinska et al., 2008; Alispahic et al., 2010). In overall, we isolated about 88.8% of a combined species of *Campylobacter.* This is consistent with reported percentages of *Campylobacter* isolated in Nigeria (62.7%; Ewnetu and Mihret, 2010) and in Ethiopia (72.7%; Gwimi et al., 2015). The relative high percentage of *Campylobacter's* identification may be attributed to the use of mass spectrometry by the CNR. Several studies, (e.g. 20%, Coker et al., 2002; 5.8 - 9%, Girgis et al., 2014; 17.3%, Karikari et al., 2017), have also reported much lower identification rates of *Campylobacter* than those recorded in this study. The incidence of *Campylobacter*-associated food poisoning has gradually increased, and this organism is now considered as the leading cause of bacterial gastroenteritis worldwide (Bessède et al., 2011). A study has shown that campylobacteriosis incidences have been globally in rise in the past decade. Thus, the numbers of campylobacteriosis incidences have increased in North America, Europe and Australia (Kaakoush et al., 2015).

Table 1. The different species of *Campylobacter* identified according to the sex of the patients.

Sex	Species N (%)						Total N (%)
	US	*A. butzleri*	*C. jejuni*	*C. coli*	*C. fetus*	*C. lari*	
M	16 (13.7)	2 (1.7)	84 (71.8)	13 (11.1)	1 (0.8)	1 (0.8)	117 (59.4)
F	6 (7.5)	0 (0)	59 (73.7)	15 (18.7)	0 (0)	0 (0)	80 (40.6)
Total	22 (11.2)	2 (1.0)	143 (72.6)	28 (14.2)	1 (0.5)	1 (0.5)	197 (100)

Legend: *A* = *Arcobacter*, *C* = *Campylobacter*, M = male, F = female, US = Undefined species.

Table 2. Antibiotic resistance profile of *Campylobacter* isolates.

Antibiotics	Species N (%)							Total N (%)
	-	US	*A. butzleri*	*C. jejuni*	*C. coli*	*C. fetus*	*C. lari*	
AMP	-	0 (0)	2 (3,8)	45 (86,5)	5 (9,6)	0 (0%)	0 (0%)	52 (26,4)
AMC	-	0 (0)	2 (100)	0 (0)	0 (0)	0 (0)	0 (0)	2 (1,0)
GMC	-	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0,5)
ERY	-	0 (0)	0 (0)	0 (0)	2 (100)	0 (0)	0 (0)	2 (1,0)
CIP	R/S	0 (0)	0 (0)	2 (100)	0 (0)	0 (0)	0 (0)	2 (1,0)
TET	R/S	0 (0)	0 (0)	60 (30)	0 (0)	0 (0)	0 (0)	60 (30,5)

Legend: AMP = Ampicillin, AMC = Amoxicillin/clavulanic acid, GMC = Gentamicin, ERY = Erythromycin, CIP = Ciprofloxacin, TET = Tetracycline, *A* = *Arcobacter, C* = *Campylobacter*, US = Undefined species, R = Resistant, S = Sensible.

Incidences and number of cases may substantially vary among countries or regions or within a given country (Kubota et al., 2011; Sadkowska-Todys and Kucharczyk 2012). These variations are attributable to several factors such as sensitivity of detection methodologies, geographic locations, target population, differences in the standard and stringency of biocontrol protocols, surveillance bias, food practices as well as the availability of natural reservoirs of *Campylobacter* species (Kaakoush et al., 2015).

In contrast, the identified species were *C. jejuni* (72.59%), *C. coli* (14.21%), *Arcobacter butzleri* (1.02%), *C. fetus* and *C. lari* (0.51% each). These results are similar to those previously reported in France: *C. jejuni* (78%), *C. coli* (14%) and *C. fetus* (4%) (Bessède et al., 2011). Because of the low identification rates of the analytical methods, incidences of *C. jejumi* and *C. coli* infections are likely to be underestimated (Wagenaar et al., 2013). In most industrialized countries, *Campylobacter* organisms are, along with *Salmonella*, the most common cause of foodborne bacterial gastroenteritis (Allos and Blaser, 1995; Frost, 2001). Furthermore, consumption of undercooked (raw, rare, or “pink”) chicken and beef was the most important food-specific risk factor for *Campylobacter* infection in France (Gallay et al., 2008; Berthenet et al., 2019). In the present study, high rates (22%) of *Campylobacter* were identified in children under 10 years old. This result was consistent with those reported in 2017 by French National Reference Centre for Campylobacter and by developing countries (Coker et al., 2002). While infection with *C. jejuni* or *C. coli* can occur in patients of all ages, a recent study in Denmark showed that infection is more prevalent in toddlers (1 to 4 years) and young adults (15 to 24 years) than in other age groups (Nielsen et al., 2013).

Our study also showed that *Campylobacter* infections were more prevalent in male (59.4%) than in female (40.6%) patients. This is corroborated by previous studies, which reported high campylobacter prevalent in male patients compared to female patients (Friedman et al., 2000; Fitzgerald et al., 2011). However, some studies found that females have higher risk of getting infected by *Campylobacter* than males (Gillespie et al., 2006; Karikari et al., 2017).The isolated strains in our study showed considerable resistance to ampicillin (26.4%), to tetracycline (30.5%) whereas the resistances to ciprofloxacin, erythromycin and gentamicin were relatively low (0.5-1%). These results are in agreement with 30.7% resistance to tetracycline, 26.9% to ciprofloxacin, 1.7% to erythromycin and 0.9% to gentamicin previously reported in France (Gallay et al., 2008). A similar resistance to tetracycline (22%) was documented in Ethiopia (Ewnetu and Mihret, 2010). Furthermore, our results are lower than 48.0% resistant to ampicillin reported by Gallay et al. (2008). High resistance rates to tetracycline have been described in Ghana (92.3 - 100%, Karikari et al., 2017), in Spain (72%; Prats et al., 2000) in human isolates. At the global scale, the tetracyclines are a heavily used class of antibiotics both in human and in veterinary medicine (Iovine, 2013). The discovery in 1950 that the addition of

antibiotics to animal feed at sub therapeutic levels could lead to increased growth rates of these animals. This results in research into methods to improve or stabilize meat supplies to the consumer (Kaakoush et al., 2015). For instance, by the turn of the 20th century, the majority of antibiotic used in the United States was for agricultural purposes. This approach has led to a dramatic increase in antibiotic resistance in several human pathogens that originate from domesticated animals, including *Campylobacter* species (Barton, 2014). Since the beginning of the 1990s, the resistance of *Campylobacter* organisms to antibiotics has increased (Gallay et al., 2008). Thus, a strong relationship has been observed between the amounts of fluoroquinolone in animal feed and the presence of pathogen strains of *Campylobacter* in humans (Kaakoush et al., 2015). Although the *Campylobacter* infection is self-limiting, the extra-intestinal infection or septicaemia may occur, and thereby requiring treatment using appropriate antibiotics.

Conclusion

The identification by MALDI-TOF mass spectrometry is particularly efficient for the identification of campylobacters (should be campylobacters, low cas for c) and makes it possible to identify genera and species difficult to access by traditional identification tests (phenotypic and molecular methods): *C. lari*, *C. upsaliensis*, *C.* "anaerobic" "(*C. ureolyticus* in particular), Arcobacters and enterohepatic Helicobacters, some of whose pathogenesis is close to *Campylobacter* (especially *Helicobacter pullorum*, *Helicobacter cinaedi*). The systematic identification by mass spectrometry, a quick and inexpensive method, of several colonies (including of atypical aspect) pushing on the selective media increases the rate of detection. Therefore, it seems that the results using mass spectrometry correlates with the profiles of the control strains using mass spectrometry but further confirmation is needed.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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