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Full Length Research Paper

Profiling of meropenem-resistant bacteria in a river receiving wastewater effluent from a pharmaceutical industrial unit

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The aim of the present study was to understand the seasonal occurrence and diversity of species of meropenem-resistant bacteria in the Gumuncheon river receiving effluents from a pharmaceutical industry in Seoul, Korea.Water samples were collected from the Gumuncheon river in Kyoung-gi province during winter (January), spring (April), summer (August), and fall (November) of 2018. Water samples were plated in triplicate on tryptic soy agar plates containing 16 mg/L meropenem. Meropenem-resistant bacteria were isolated and genetically identified using 16S rRNA analysis. The predominant bacterial genera identified were *Elizabethkingia*, *Pseudomonas*, *Chryseobacterium* and *Stenotrophomonas*. Among these; *Pseudomonas* species *Pseudomonas chengduensis* and *Pseudomonas taiwanesis* showed resistance against 15 antibiotics. To prevent the occurrence and spread of meropenem-resistant bacteria in rivers, it is necessary to implement methods that can simultaneously kill multi-drug resistant bacteria and remove antibioticsfrom pharmaceutical industry effluent discharge. Further, to stop the spread of meropenem-resistant bacteria in environment, effluent discharge water should be stringently assessed for their risk of being an environmental hazard.

Key words: Carbapenem, Meropenem, Multi-drug resistant, *Elizabethkingia, Pseudomonas, Chryseobacterium*, *Stenotrophomonas.*

INTRODUCTION

Carbapenem antibiotics, such as meropenem, imipenem, and doripenem, are the last-line of antibiotics used on bacteria resistant to β-lactam antibiotics. *In vitro* experiments have shown that carbapenem antibiotics have broader antibacterial activities than combinations of penicillin, cephalosporin, and β-lactam/β-lactamase inhibitors (Bassetti et al., 2009). Generally, imipenem, panipenem, and doripenem are effective antibiotics against Gram-positive bacteria (GPB), whereas carbapenem antibiotics, such as meropenem, biapenem,

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and ertapenem, are more effective against Gram-negative bacteria (GNB) (Bassetti et al., 2009).

Carbapenem antibiotics enter GNB through outer membrane proteins (OMPs) known as porins (Martinez-Marinez, 2008). Carbapenem antibiotics pass through the periplasmic space and subsequently inhibit peptide cross-linking by permanent acylation of penicillin-binding proteins (PBPs). PBPs facilitate the synthesis of peptidoglycans present in the bacterial cell wall (Hashizume et al., 1984). Cell wall synthesis is a dynamic three-dimensional process with synthesis and autolysis occurring simultaneously. As a result, inhibition of PBPs causes weakening of peptidoglycans, thereby resulting in cell rupture due to osmotic pressure (Van Dam et al., 2009).

Carbapenem resistance has been reported in many bacteria. For example, Enterobacteriaceae including *Escherichia coli*, *Klebsiella* spp., *Klebsiella pneumoniae*, and *Enterobacter* spp., and GPB, including *Stenotrophomonas* spp., *Streptococcus* spp., *Staphylococcus* spp., *Bacillus magaterium,* and *Bacillus licheniformis* show gradual resistance to carbapenem antibiotics often used in clinical practice (Papp-Wallace et al., 2011; Go et al., 2017; Hwang and Kim, 2018). In particular, carbapenem-resistant *Enterobacteriaceae* (CRE) are becoming important public health risks (Gupta et al., 2011).

The pharmaceutical industry produces effluent discharge which carries various types of antibiotics that are not degraded during the wastewater treatment process. Antibiotics that are discharged without being completely degraded can cause disturbances in aquatic ecosystems and will be hazardous for human life (Kim and Kim, 2016). Pharmaceutical industry wastewater treatment plants may also discharge wastewater directly into rivers if they satisfy the effluent standards for general metrics such as biochemical oxygen demand (BOD), chemical oxygen demand (COD), suspended solids (SS), total nitrogen (T-N), and total phosphorus (T-P), similar to non-pharmaceutical wastewater treatment plants (Hwang and Kim, 2018).

The purpose of this study was to identify the seasonal frequency of occurrence, species variety, and antibiotic resistance spectrum of meropenem-resistant bacteria in rivers receiving wastewater discharged from a pharmaceutical industrial park.

MATERIALS AND METHODS

Collection area and methods

Gumuncheon river; from where the study samples were collected, is a tributary of the Balancheon river. The Hyangnam Pharmaceutical Industrial Park, located in Hwaseong-si (Gyeonggi Province, South Korea), houses 10 pharmaceutical companies that discharge wastewater into the Gumuncheon river. We sampled 1L of river water at 37.097598N and 126.902025 E in January, April, August, and November of 2018. Samples were collected in sterile collection bottles and refrigerated during transportation to the laboratory (Hwang et al., 2018).

Water temperature, dissolved oxygen (DO), and the pH of the river water were measured using a DO/pH meter (DH-32P, Toa, Japan). Prior to analyzing T-N, T-P, and total organic carbon (TOC), a 0.45 μm pore filter (Advnatec, Japan) was used to remove any suspended solids and temperature, DO, and pH were measured at the sampling point. T-N and T-P were measured using Integral Futura continuous flow analyzer (Alliance, USA), while TOC was measured using TOC-L (Shimadzu, Japan). BOD and COD were analyzed in accordance with the water pollution standard method (NIER, 2018).

Isolation of heterotrophic bacteria

To measure the heterotrophic bacterial count within each sample, the samples were diluted 100-fold using sterilized saline solution and 1.0 ml from each dilution each was spread 3 times on 3 agar plates (pour plate agar, Difco, USA) and incubated at 35°C for 48 h.

Identification of meropenem-resistant bacteria and antibiotic resistance test

To identify the meropenem-resistant bacteria, samples were diluted by 10-fold using sterilized saline solution and 0.1 ml each was spread on three tryptic soy (TS) agar plates (Difco, USA) containing 16 mg/L of meropenem (Daewoong Pharmaceutical, Korea) and cultured at 35°C for 48 h. Meropenem-resistant bacteria were subjected to 16S rRNA analysis for species identification. Colony polymerase chain reaction (PCR) was used to amplify the 16S rRNA gene of resistant bacteria from pure colonies (Kim and Kim, 2016). To determine the antibiotic sensitivities of the identified bacteria, disk diffusion assays were performed with ampicillin, ceftizoxime, vancomycin, imipenem, clindamycin, gentamicin, erythromycin, ciprofloxacin, nitrofurans, rifampin, ampicillin/sulbactam, aztreonam, spectinomycin, trimethoprim, tetracycline, and chloramphenicol. The diameter of the inhibition zone was measured in mm and interpreted according to the Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI, 2013; Hwang et al., 2018).

RESULTS

The general quality metrics of the river samples are shown in Table 1. Temperature (7.5-28.5°C), pH (7.15-7.45), DO (6.1-11.0 mg/L), BOD (2.7-5.8mg/L), COD (8.2-15.1 mg/L), T-P 0.103-0.182 mg/L), T-N (8.056-12.566 mg/L), and TOC (5.3-9.2 mg/L) were measured.

The numbers of heterotrophic and meropenem-resistant bacteria identified are shown in Table 2. The counts of heterotrophic bacteria in the winter, spring, summer, and fall seasons were 3.4, 4.0, 5.6 and 5.1 ($\times 10^4$ CFU/ml respectively, while the numbers of meropenem-resistant bacteria were 14.0, 8.3, 1.2 and 9.0 ($\times 10^2$ CFU/ml) respectively.

The prevalence of each strain by season is shown in

Table 1. Quality of water sampled from the river receiving pharmaceutical industry discharge water.

Indicators	Sampling months in 2018			
	January	April	August	November
Temperature(°C)	7.5	20.1	28.5	18.2
pH	7.42	7.15	7.34	7.45
DO (mg/L)	11.0	10.0	6.1	8.4
BOD (mg/L)	5.8	4.0	2.7	4.3
COD (mg/L)	15.1	10.7	9.7	8.2
Total phosphorus (mg/L)	0.145	0.127	0.182	0.103
Total nitrogen (mg/L)	12.566	8.305	9.644	8.056
TOC (mg/L)	9.2	5.6	5.3	5.8

DO: dissolved oxygen, BOD: biological oxygen demand, COD: chemical oxygen demand, TOC: total organic carbon.

Table 2. Season-wise distribution of culturable bacteria and meropenem resistant bacteria.

Indicators	Sampling months in 2018			
	January	April	August	November
Heterotrophic bacteria(CFU/ml)	$3.4\times10^4 \pm 1.3\times10^3$	$4.8\times 10^4 \pm 2.6\times10^3$	$5.6\times10^4 \pm 1.5\times10^3$	$5.1\times10^4 \pm 2.8\times10^3$
Meropenem resistant Bacteria (CFU/ml)	$4.0\times10^2 \pm 2.0\times10^2$	$8.3\times10^2 \pm 2.1\times10^2$	$1.2\times10^3 \pm 4.0\times10^2$	$9.0\times10^2 \pm 1.2\times10^2$
Meropenem resistant bacteria (%)	1.18	1.73	2.14	1.82

Table 3. The *Elizabethkingia* genus was the dominant genus in the winter season, and was also partially identified in the spring and fall when the water temperature was low; however, it was not identified in summer. The *Pseudomonas* genus was not identified in the winter and summer but was identified during the spring and the fall. The *Chryseobacterium* genus was identified as the dominant genus in the spring, summer, and winter; the *Stenotrophomonas* genus was also identified in the same periods. The *Cupriavidus*, *Acinetobacter*, and *Pandoraea* genera were identified in the summer season only.

The results of the antibiotic resistance tests of the isolated meropenem-resistant bacteria are shown in Tables 4, 5, and 6. The *Elizabethkingia* genus commonly showed resistance to eight antibiotics (ampicillin, ceftizoxime, vancomycin, imipenem, meropenem, nitrofuratoin, aztreonam, and trimethoprim), while the *Pseudomonas* genus showed resistance to thirteen antibiotics (ampicillin, ceftizoxime, vancomycin, imipenem, meropenem, clindamycin, erythromycin, nitrofurantoin, rifampin, ampicillin/sulbactam, trimethoprim, tetracycline, and chloramphenicol). The *Chryseobacterium* genus, which was the dominant genus in the spring, summer, and fall, showed resistance to five antibiotics (colistin, ampicillin, ceftizoxime, ampicillin/sulbactam, and aztreonam). The *S. maltophilia*, which appeared in the spring, summer, and fall, showed resistance to ten antibiotics (ampicillin, ceftizoxime, vancomycin, imipenem, meropenem, clindamycin, ampicillin/sulbactam, spectinomycin, aztreonam, and trimethoprim). Among bacteria that appeared only in the summer season, *C. plantarum* showed resistance to seven antibiotics (ampicillin, vancomycin, imipenem, meropenem, clindamycin, gentamicin, and aztreonam), *A. junii* showed resistance to nine antibiotics (ampicillin, ceftizoxime, vancomycin, imipenem, meropenem, clindamycin, nitrofurantoin, rifampin, and trimethoprim), and *P. pnomenusa* showed resistance to eleven antibiotics (colistin, ampicillin, vancomycin, meropenem, clindamycin, gentamicin, erythromycin, nitrofurantoin, rifampin, spectinomycin, and aztreonam).

DISCUSSION

We evaluated the occurrence and season-wise prevalence of meropenem-resistant bacteria in Gumuncheon river receiving wastewater effluents from a pharmaceutical industrial unit. The average water temperature during the winter was 7.5°C, which was lower than the temperature of other sessions for the river.

Table 3. Number of meropenem-resistant bacteria detected by season.

Species	Sampling months in 2018				Total
	January	April	August	November	
Elizabethkingia amiricola	11	-	-	3	14
Elizabethkingia ameningoseptica	1	-	-	-	1
Elizabethkingia anophelis		1	-	-	1
Pseudomonas taiwanesus	-	3	-	-	3
Chryseobacterium indologenes	-	17	-	-	17
Pseudomonas chengduensis	-	2	-	2	4
Stenotrophomonas maltophilia	-	2	5	4	11
Chryseobacterium cucumeris	-	-	22	15	37
Cupriavidus plantarum	-	-	3	-	3
Acinetobacter junii	-	-	3	-	3
Pandoraea pnomenusa	-	-	3	-	3
Pseudomonas pseudoalcaligenes	-	-	-	3	3
Total	12	25	36	27	100

Table 4. Antibiotic resistance spectrum of meropenem-resistant *Elizabethkingia miricola, Elizabethkingia meningoseptica, Elizabethkingia anophelis,*and *Pseudomonas taiwanesis*.

Spectrum	Species			
	E.miricola **(n=14)** **R%**	***E. meningoseptica*** **(n=1)** **R%**	***E. anophelis*** **(n=1)** **R%**	***P. tawnensis*** **(n=3)** **R%**
Ampicillin	100	100	100	100
Ceftizoxime	100	100	100	100
Vancomycin	21	100	100	100
Imipenem	100	100	100	100
Meropenem	100	100	100	100
Clindamycin	57	0	0	100
Gentamicin	100	0	100	33
Erythromycin	79	0	100	100
Ciprofloxacin	57	0	100	100
Nitrofurantoin	100	100	100	100
Rifampin	0	0	0	100
Ampicillin/Sulbactam	100	0	100	100
Aztreonam	100	100	100	100
Spectinomycin	43	0	100	100
Trimethoprim	57	100	100	100
Tetracycline	0	0	0	100
Chloramphenicol	0	0	0	100

The increase in water temperature was assumed to be the result of an inflow of effluents from the sewage treatment plant. The concentration of T-P, a limiting nutrient for microbial growth on water surface, was found to be 0.103-0.182 mg/L, which was exceedingly higher than the concentration needed for microbial growth (100 µg T-P/L). It showed that the sampled river water satisfied the conditions for growth of antibiotic resistant bacteria (Correll, 1999). Our results clearly show that the present allowed limit of T-P concentration (4 mg/L) for pharmaceutical industrial wastewater treatment plant effluents should be lowered, considering the additional

Table 5. Antibiotic resistance spectrum of meropenem-resistant *Chryseobacterium indologenes, Pseudomonas chengduensis, S. maltophilia*-and *Chryseobacterium cucumeris.*

Spectrum	Species			
	C. indologenes **(n=17)** **R%**	***P. chengduensis*** **(n=4)** **R%**	***S. maltophilia*** **(n=11)** **R%**	***C. cucumeris*** **(n=37)** **R%**
Ampicillin	100	100	100	100
Ceftizoxime	100	100	100	100
Vancomycin	100	100	100	46
Imipenem	6	100	100	0
Meropenem	100	100	100	100
Clindamycin	0	100	100	0
Gentamicin	94	100	55	100
Erythromycin	0	100	73	0
Ciprofloxacin	0	100	0	0
Nitrofurantoin	77	100	82	19
Rifampin	0	100	82	0
Ampicillin/Sulbactam	100	100	100	100
Aztreonam	100	0	100	100
Spectinomycin	0	100	100	0
Trimethoprim	0	100	100	0
Tetracycline	0	100	0	0
Chloramphenicol	0	100	9	0

Table 6. Antibiotic resistance spectrum of meropenem-resistant *Chryseobacterium plantarum, A. junii, Pseudomonas. pnomenusa*, and *Pseudomonas pseudoalcaligenes.*

Spectrum	Species			
	C. plantarum **(n=3)** **R%**	***A. junii*** **(n=3)** **R%**	***P. pnomenusa*** **(n=3)** **R%**	***P. pseudoalcaligenes*** **(n=3)** **R%**
Ampicillin	100	100	100	100
Ceftizoxime	0	100	0	100
Vancomycin	100	100	100	100
Imipenem	100	100	0	100
Meropenem	100	100	100	100
Clindamycin	100	100	100	100
Gentamicin	100	0	100	0
Erythromycin	0	0	100	100
Ciprofloxacin	0	0	0	0
Nitrofurantoin	33	100	100	100
Rifampin	33	100	100	67
Ampicillin/Sulbactam	0	0	0	100
Aztreonam	100	0	100	100
Spectinomycin	67	0	100	0
Trimethoprim	0	100	33	100
Tetracycline	0	0	0	0
Chloramphenicol	0	33	0	100

amount of T-P that could be introduced into the river from nearby farmland or domestic sewage (Hwang and Kim, 2018).

The percentage of meropenem-resistant bacteria among the heterotrophic bacteria identified in the river samples was 1.18-2.14%, which was higher than the percentage measured in a similar river which did not receive the wastewater effluents (Kim and Kim, 2015). It is known that as the number of non-pathogenic bacteria increases, the number of antibiotic-resistant bacteria could also increase through gene transfer from pathogenic to non-pathogenic bacteria (Levy and Marshall, 2004).

The *Elizabethkingia* genus contains Gram-negative, obligate aerobic bacillus species, and is an emerging healthcare threat as it has been reported to be associated with various life-threatening infections including sepsis, neonatal meningitis, and nosocomial pneumonia. Moreover, improperly processed animal-derived food and companion animals are known reservoirs for this antibiotic resistant bacterial pathogen. *Elizabethkingia anophelis* isolated in clinical practice (Figueroa Castro et al., 2017; Lee et al., 2021) and *E. anophelis* isolated from horses (Johnson et al., 2018) were reportedly susceptible to ciprofloxacin; however, the *E. anophelis* isolated in the present study was ciprofloxacin-resistant. Further studies are required to determine whether this difference was due to mutation of one or more genes involved in bacterial DNA separation (Drlica and Zhao, 1997). Similarly, *E. meningoseptica* isolated from clinical samples has shown resistance to various β-lactams and colistin, but is reported to be susceptible to vancomycin (Ratnamani and Rao, 2013). However, the *E. meningoseptica* isolated in the present study was resistant to not only ampicillin, imipenem, meropenem, and the β-lactam/β-lactamase inhibitor ampicillin/Sulbactam, but also to colistin and vancomycin. On the other hand, the *E. meningoseptica* isolated in the present study showed greater susceptibility to tetracycline, clindamycin, erythromycin, and gentamicin than *E. meningoseptica* from hospital effluents described previously. Thus, more in-depth studies are requiredto determine whether this difference could be attributed to the difference in their source (NIER, 2013; Gullberg et al., 2011). *E. miricola*, which is an opportunistic oral pathogen, has exhibited resistance to many antibiotics, including imipenem, meropenem, carbapenem, colistin, and gentamicin (Zdziarski et al., 2017; Howard et al., 2020); the *E. miricola* isolated in the present study also showed resistance to those antibiotics.

There have been no reports of *Pseudomonas taiwanesis,* a known pathogen of soil microorganisms (Wang et al., 2010) and insects (Chen et al., 2014), acting as a clinical pathogen. However, the *P. taiwanesis* isolated in the present study showed resistance to sixteen antibiotics (ampicillin, ceftizoxime, vancomycin, imipenem, meropenem, clindamycin, erythromycin, ciprofloxacin, nitrofurantoin, rifampin, ampicillin/ sulbactam, spectinomycin, aztreonam, trimethoprim, tetracycline, and chloramphenicol). Similarly, *Pseudomonas chengduensis* has been reported in landfill leachate (Tao et al., 2014), but has not been identified in clinical samples. The *P. chengduensis* isolated in the present study showed resistance to ampicillin, ceftizoxime, vancomycin, imipenem, meropenem, clindamycin, gentamicin, erythromycin, ciprofloxacin, nitrofurantoin, rifampin, ampicillin/ sulbactam, spectinomycin, trimethoprim, tetracycline, and chloramphenicol. *Pseudomonas pseudoalcaligenes*, another member of the Pseudomonas genus, showed resistance to six antibiotics (ampicillin, ceftizoxime, vancomycin, clindamycin, erythromycin, and chloramphenicol), similar to *P. pseudoalcaligenes* strains isolated from hospital effluents.

Chryseobacterium, which was the dominant genus identified in sampled water during spring, summer, and fall, is a Gram-negative bacillus commonly found in environment. *Chryseobacterium indologenes* is an opportunistic pathogen that causes sepsis (McKew, 2014; Izaguirre-Anariba and Sivapalan, 2020). *C. indologenes* isolated from patients has been reported to be resistant to ampicillin, amoxicillin-clavulanate, cefepime, meropenem, gentamicin, and nitrofurantoin, however the *C. indologenes* isolated in the present study showed resistance to colistin, ampicillin, ampicillin-sulbactam, ceftizoxime, vancomycin, meropenem, gentamicin, and aztreonam. *Chryseobacterium cucumeris*, another member of the *Chryseobacterium* genus, was resistant to colistin, ampicillin, ampicillin-sulbactam, ceftizoxime, meropenem, gentamicin, and aztreonam.

Stenotrophomonas maltophilia, isolated in the spring, summer, and fall, is strongly associated with human respiratory infection. It is a multi-drug resistant bacterium commonly found in environment. Due to its low permeability, it is known to be resistant to cephem antibiotics, such as cefepime and ceftazidime, as well as to β-lactams. Moreover, presence of genes encoding β-lactamases, multi-drug resistant efflux pumps, and antibiotic-modifying enzymes confers it with resistance to various antibiotics (Brook, 2012; Adegoka et al., 2017). Similarly, the *S. maltophilia* isolated in the present study showed resistance to not only β-lactam and cephem antibiotics, such as ampicillin and ceftizoxime, but also to vancomycin, imipenem, meropenem, clindamycin, ampicillin/sulbactam, spectinomycin, aztreonam, and trimethoprim.

Cupriavidus plantarum has been isolated from the plant rhizosphere (Estrada-de Los Santos et al., 2014), but there are no known reports of this bacteria in clinical samples. The *C. plantarum* identified in the present study

showed resistance toampicillin, vancomycin, imipenem, meropenem, clindamycin, gentamicin, and aztreonam.

Acinetobacter junii is an opportunistic pathogen that has been reported to show resistance to ampicillin and ciprofloxacin (Cayô et al., 2011); in the present study, the *A. junii* showed resistance to a greater number of antibiotics (ampicillin, ceftizoxime, vancomycin, imipenem, meropenem, clindamycin, nitrofurantoin, rifampin, and trimethoprim). Similarly, *Pandoraea pnomenusa* isolated from patients with cystic fibrosis has been reported to show resistance to cephem antibiotics such as ceftizoxime andβ-lactam/β-lactamase inhibitors (Ambrose et al., 2016); however, the *P. pnomenusa* identified in the present study was resistant to colistin, ampicillin, vancomycin, meropenem, clindamycin, gentamicin, erythromycin, nitrofurantoin, rifampin, spectinomycin, and aztreonam, but not to cephem antibiotics orβ-lactam/β-lactamase inhibitors.

Resistance to carbapenem antibiotics is known to result from mutations that affect the production of β-lactamase and the functional expression of efflux pumps, porins, and PBPs (Drlica and Zhao, 1997; Giske et al., 2008; Hashizume et al., 1984). There is an imminent need to study the underlying mechanism of antimicrobial resistance observed in meropenem- resistant *Acinetobacter junii, C. cucumeris, C. indologenes*, *Cupriavidusplantarum*, *E. anopheles*, *Elizabethkingi ameningoseptica*, *Elizabethkingi amiricola*, *Pandoraea pnomenusa*, *Pseudomonas pseudoalcali genes*, *Pseudomonas chengduensis*, *Pseudomonas tawnensis*, and *Stenotrophomonas maltophilia*, isolated in the present study.

Biofilms, sewage effluent sediments, wastewater treatment plant effluents, sewage sludge, pharmaceutical manufacturing plants, liquid manure tanks, and manure-fertilized soil are known to act as hot spots for the occurrence and spread of antibiotic resistance (Berkner et al., 2014). In South Korea, the industrial effluents are generally checked for total coliforms and ecotoxicity according to the classification of effluent discharge zones. Our results show that there is a need to perform toxicity tests and environmental hazard assessments on effluents containing antibiotics or antimicrobial substances, such as pharmaceutical industry discharge water, to prevent the occurrence and spread of antibiotic resistance (Hernando et al., 2006; Escher et al., 2011). In Korea, the ecotoxicity testing is performed by using water fleas (Hwang and Kim, 2018). It is believed that the toxicity testing of the effluents that include trace amounts of antimicrobial substances, too should be performed using microorganisms. The pharmaceutical industrial park that discharges wastewater into the river sampled in the present study has a wastewater treatment facility; however, there is a high probability of occurrence of antibiotic resistance due to an inflow of processed water containing various clinical disinfectants and discharge from antibiotic-manufacturing pharmaceutical factories. In general, the concentrations of antibiotics present in pharmaceutical effluents are significantly lower than the ones used in clinical practice (NIER, 2013). These concentrations, though not high enough to kill bacteria, are sufficient to exert selective pressure on bacteria to develop antimicrobial resistance (Gullberg et al., 2011). Therefore, it is necessary to implement proper treatment methods, including membrane filtration, ozonation, and UV disinfection, to completely remove the antibiotics and antibiotic-resistant bacteria present in pharmaceutical effluents to reduce their flow into the environment (Pruden et al., 2013).

Conclusion

The present study identified the species, contamination level, seasonal distribution, and antibiotic-resistance spectrum of meropenem-resistant bacteria in a river receiving pharmaceutical industry discharge. Our study showed that the presence of multi-drug resistant bacteria in the river water poses a threat to human health due to wider reach and use of river water. The outcomes of our study highlight the need to implement methods that can simultaneously disinfect multi-drug resistant bacteria and remove antibiotics from effluent containing discharge water from pharmaceutical and industrial units.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

Adegoka AA, Stenstrom TA, Okoh AI (2017). Stenotrophomonas maltophilia as an Emerging Ubiquitous Pathogen: Looking Beyond Contemporary Antibiotic Therapy. Frontiers in Microbiology 8:2276.

Ambrose M, Malley RC, Warren SJC, Beggs SA, Swallow OFE, McEwan B, Stock D, Roddam LF (2016). *Pandoraeapnomenusa* Isolated from an Australian Patient with Cystic Fibrosis. https://www.frontiersin.org/articles/10.3389/fmicb.2016.00692/full. Accessed 14 June 2019.

Bassetti M, Nicolini L, Esposito S, Righi E, Viscoli C (2009). Current status of newer carbapenems. Current Medical Chemistry 16(5):564-575.

Berkner S, Konradi S, Schönfeld J (2014). Antibiotic resistance and the environment—there and back again. EMBO Reports 15(7):740-744.

Brook JS (2012). *Stenotrophomonasmaltophilia*: an Emerging Global Opportunistic Pathogen. Clinical Microbiology Reviews 25(1):2-41.
Cayô R, Yañez San Segundo L, Pérez del Molino Bernal IC, García de la Fuente C, Bermúdez Rodríguez MA, Calvo J, Martínez-Martínez L(2011). Bloodstream infection caused by *Acinetobacterjinii*in a patient with acute lymphoblastic leukaemia after allogenichaematopoietic cell transplantation. Journal of Medical Microbiology 60(3):375-377.
Chen WJ, Hsieh FC, Hsu FC, Tasy YF, Liu JR, Shih MC (2014). Characterization of an Insecticidal Toxin and Pathogenicity of *Pseudomonas taiwanensis* against Insects. PLoS Pathogens 10(8):e1004288.
Clinical Laboratory Standards Institute (2013). Performance standards for antimicrobial susceptibility testing; Twenty-third informational supplement. M100-S23. Wayne, PA: Clinical Laboratory Standards Institute.
Correll DL (1999). Phosphorus: A Rate Limiting Nutrient in Surface waters. Poultry Science 78(5):674-682.
Drlica K, Zhao X (1997). DNA gyrase, topoisomerase IV, and the 4-quinolones. Microbiology and Molecular Biology Reviews 61(3):377-392.
Escher BI, Baumgartner R, Koller M, Treyer K, Lienert J, McArdell CS (2011). Environmental toxicology and risk assessment of pharmaceuticals from hospital wastewater. Water Research 45(1): 75-92.
Estrada-de Los Santos P, Solano-Rodríguez R, Matsumura-Pa, LT, Vásquez-Murrieta MS, Martínez-Aguilar L (2014). *Cupriavidusplantarum* sp. nov., a plant-associated species. Archives of Microbiology 196(11): 811-817.
Figueroa Castro CE, Johnson C, Williams M, VanDerSlik A, Graham MB, Letzer D, Ledeboer N, Buchan BW, Block T, Borlaug G, Munoz-Price LS (2017). *Elizabethkingiaanophelis*: Clinical Experience of an Academic Health System in Southeastern Wisconsin. Open Forum Infectious Diseases 4(4):251.
Giske CG, Buar L, Sundsfjord A, Wretlind B (2008). Alterations of porin, pump, and Penicillin-binding proteins in carbapenem resistant clinical isolates of *Pseudomonas aeruginosa*. Microbial Drug Resistance 14(1):2-30.
Go E, Ju S, Yoo J, Jeon S (2017). Distribution of carbapenem-resistant Enterobacteriaceae (CRE) in Korea. http://www.cdc.go.kr/CDC/info/CdcKrInfo0301.jsp?menuIds=HOME006-MNU3003-MNU2950-MNU2951&cid=141732. Accessed 15 April 2019.
Gullberg E, Cao S, Berg OG, Ilbäck C, Sandegren L, Hughes D, Andersson DI (2011). Selection of Resistant Bacteria at Very Low Antibiotic Concentrations. PLoS Pathogens 7(7): e1002158. https://doi.org/10.1371/journal.ppat.1002158.
Gupta N, Limbago BM, Patel JB, Kallen AJ (2011). Carbapenem-resistant Enterobacteriaceae: epidemiology and prevention. Clinical Infectious Diseases 51(1): 60-67.
Hashizume T, Ishino F, Nakagawa J, Tamaki S, Matsuhashi M (1984). Studies on the mechanism of action of imipenem (N-formimidoylthienamycin) in vitro: binding to the penicillin-binding proteins (PBPs) in *Escherichia coli*and *Pseudomonas aeruginosa*, and inhibition of enzyme activities due to the PBPs in *E. coli*. The Journal of Antibiotics (Tokyo) 37(4):394-400.
Hernando MD, Mezcua M, Fernández-Alba AR, Barceló D (2006): Environmental risk assessment of pharmaceutical residues in wastewater effluents, surface waters and sediments. Talanta 69(2):334-342.
Howard JC, Chen K, Anderson T, Dalton SC (2020). *Elizabethkingia miricola* bacteraemia in a haemodialysis patient. Access Microbiology 2(2):1-3.
Hwang SH, Kim YJ. (2018). Meropenem-resistant bacteria in hospital effluents in Seoul, Korea. Environmental Monitoring and Assessment 190(11):673-680.
Izaguirre-Anariba DE, Sivapalan V (2020). Chryseobacterium indologenes, an Emerging Bacteria: a case report and review of literature. Cureus 12(1):e6720.
Johnson WL, Ramachandran A, Torres NJ, Nicholson AC, Whitney AM, Bell M, Villarma A, Humrighouse BW, Sheth M, Dowd SE, McQuiston JR, Gustafson JE (2018). The draft genomes of *Elizabethkingiaanophelis* of equine origin are genetically similar to three isolates from human clinical specimens. PLoS ONE 13(7):e0200731. https://doi.org/10.1371/journal.pone.0200731.
Kim JG, Kim YJ (2016). Study on Antibiotic Resistant Enterobacteria in Pharmaceutical Effluent. Journal of Environmental Health Sciences 42(1):34-40.
Kim YJ, Kim JO (2015). Study on Oxyteracycline Resistant Bacteria in the Surface Water. Journal of Environmental Health Sciences 41(1):40-48.
Lee YL, Liu KM, Chang HL, Lin JS, Kung FY, Ho CM, Lin KH, Chen YT (2021). A dominant strain of *Elizabethkingia anopheles* emerged from a hospital water system to cause a three-year outbreak in a respiratory care center. Journal of Hospital Infection 108:43-51.
Levy SB, Marshall B (2004). Antibacterial resistance worldwide: causes, challenges, responses. Nature Medicine 10(12):122-129.
Martinez-Marinez L (2008). Extended-spectrum β-lactamases and the permeability barrier. ClinicalMicrobiology and Infection 14(1): 82-89.
McKew G (2014). Severe Sepsis Due to *Chryseobacteriumindologenes* in an Immunocompetent Adventure Traveler. Journal of Clinical Microbiology 52(11):4100-4101.
National Institute of Environment Research (NIER) (2018). Water pollution test standards. https://qaqc.nier.go.kr/qaqcnew/standard/standardlist.do#n.Accessed 14 January 2018.
National Institute of Environment Research (NIER) (2013). http://www.ndsl.kr/ndsl/commons/util/ndslOriginalView.do?dbt=TRKO&cn=TRKO201300007867&rn=&url=&pageCode=PG18.Accessed 3 November 2018.
Papp-Wallace KM, Endimiani A, Taracila MA, Robert A, Bonomo RA (2011). Carbapenems: Past, Present, and Future. Antimicrobial Agents and Chemotherapy 55(11):4943-4960.
Pruden A, Larsson J, Amezquito A, Collignon P, Brandt K, Graham D, Lazorchak J, Suzuki S, Silley, P, Snape J, Topp E, Zhang T, Zhu Y (2013). Management Options For Reducing The Release of Antibiotics And Antibiotic Resistance Genes To The Environment. Environmental Health Perspectives 121(8):878-85.
Ratnamani MS, Rao R (2013). *Elizabethkingiameningoseptica*: Emerging pathogen in bedside hemodialysis patients. Indian Journal of Critical Care Medicine 17(5):304-307.
Tao Y, Zhou Y, He X, Hu X, Li D (2014). *Pseudomonas chengduensis* sp. nov., isolated from landfill leachate. International Journal of Systmatic and Evolutionary Microbiology 64(1):95-100.
Van Dam V, Olrichs N, Breukink E (2009). Specific labeling of peptidoglycan precursors as a tool for bacterial cell wall studies. Chembiochem 10(4):617-624.
Wang LT, Tai CJ, Wu YC, Chen YB, Lee FL, Wang SL (2010). *Pseudomonas taiwanensis* sp. nov., isolated from soil. International Journal of Systmatic and Evolutionary Microbiology 60(9):2094-2098.
Zdziarski P, Paściak M, Rogala K, Korzeniowska-Kowal A, Gamian A (2017). *Elizabethkingiamiricola* as an opportunistic oral pathogen associated with super infectious complications in humoral immunodeficiency: a case report. BMC Infectious Diseases 17(1):763-768.

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Short Communication

First report of *Fusarium oxysporum* causing root rot of garlic in China

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In the springs of 2020 and 2021, with a temperature of 15°C, root rot on garlic were widespread in Enshi, Hubei Province, China. Based on micro-morphological and cultural characteristics, the pathogen was identified as a *Fusarium* sp. Further, based on multilocus (ITS, *EF-1α*) phylogenic data, the strains were identified as *Fusarium oxysporum*. Koch's postulates were thus fulfilled by pathogenicity tests on garlic seedlings cultured in vitro.

Key words: Garlic, root rot, *Fusarium oxysporum.*

INTRODUCTION

Garlic (*Allium sativum* L.) is both flavorful and rich in nutrients, and has important medicinal value compared to other vegetables. It has bactericidal, anti-cancer, anti-corrosion and anti-aging properties, among others (Yayeh et al., 2021; Oosthuizen et al., 2018). Currently, the garlic planting area in the world exceeds 1.2 million hectares. China accounts for nearly 70% of the global garlic production (Seth et al., 2018). In recent years, root rot of garlic occurred more frequently in China, which leads to about 20-30% production loss rate and more than 50% in serious area (Xie et al., 2015). Garlic root rot symptoms are yellowing leaves, basal stem discoloration and rotten roots found in various provinces in China, caused by fungi such as *Pythium* sp. in Shandong (Zhang et al., 2021) and *Ceratobasidium* sp. in Jinxiang, Shandong, and Feng Counties, Jiangsu (Yin et al., 2020).

MATERIALS AND METHODS

The investigation of Local Agricultural Technology Extension Department indicated that the root rot incidence reached up to 35-40% in the garlic cultivation bases in Enshi Tujia Autonomous Prefecture, Hubei Province in China in the springs of 2020 and 2021. In order to isolate and identify the causal agent of this disease, symptomatic plants were collected and the infected roots were cut into small root segments. These root segments were washed thoroughly with 75% ethanol followed by surface sterilization with 0.1% $HgCl_2$ for 1 min and rinsed with sterilized distilled water three times. The sterilized root segments were put on potato dextrose agar (PDA) plates and penta chloro nitrobenzene agar medium (PPA) plates (Fusarium selective medium) (Nash

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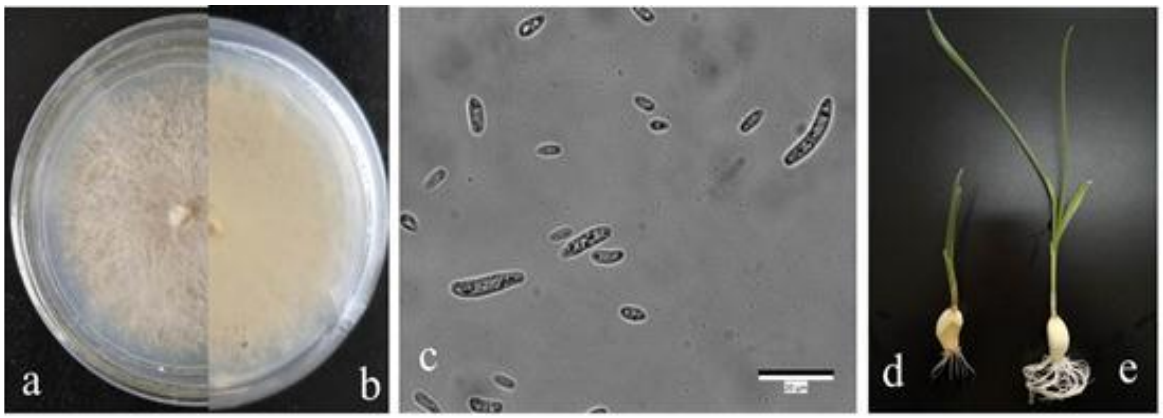

Figure 1. Symptoms and pathogen morphology of root rot on garlic caused by *Fusarium oxysporum. (*a & b) Front and back view of colony after 7 days at 25°C on PDA plates in the dark. (c) Conidiogenous cells and developing conidia scale bar=20 µm apparatus. (d) Lesion on garlic at 3 weeks after inoculation with conidial of *Fusarium oxysporum*. e) After inoculation with water as control.

and Snyder, 1962), at 25°C for 24 h in the dark. The isolated single-spore *Fusarium* colonies were inoculated on carnation leaf agar (CLA) medium and *Fusarium* species were identified through their morphological characteristics (Awere et al., 2021).

RESULTS

The colonies on potato dextrose agar medium (PDA) exhibited typical *Fusarium* characteristics, viz; *Fusarium*-like, floccose, and pale orange with aerial mycelia (Figures 1a and b). The colony had a diameter of 4.5 cm after 4-day culture at 25°C. The colony microconidia were in ovate or reniform shape, 0~1 septate, 4.8~10.1 µm×2.0~4.6 µm, whereas colony macroconidia were in falcate shape, 3~5 septate, and 20.2~36.6 µm × 3.3~4.5 µm (Figure 1c). Our observation of morphological characteristics was similar to those of *Fusarium oxysporum* (Leslie and Summerell, 2006). We obtained a total of 26 *Fusarium* isolates from 48 garlic rots collected in 2020 and 2021 in Hubei province. Since the morphological observation was consistent for all isolates, one isolated colony was selected for molecular identification. CTAB method was applied to extract pathogen genome DNA from isolate (GRR-1) (Wu et al., 2001). The primers EF1/EF2 were used to amplify the DNA sequence of translation elongation factor 1 alpha (*EF-1α*, a marker gene of *F. oxysporum*) (Geiser et al., 2004). The resultant *EF-1α* sequence (MW660368) of GRR-1 exhibited 99% identity with that of *F. oxysporum* (MK560296). Also, the results of ITS sequence (MW644753) analysis displayed a 99% match to one accession of *F. oxysporum* (MK560296) through BLAST against the NCBI nucleotide database (https://www.ncbi.nlm.nih.gov/). The phylogenetic tree of GRR-1 and the other *Fusarium* species in Figure 2 shows that GRR-1 and *F. oxysporum* comprise the same cluster.

Pathogenic examination of GRR-1 strain

The two-week-old garlic plants were used as materials for the pathogenicity tests. The root irrigation method was employed to inoculate the healthy garlic plants with 100 ml of conidial suspension (3.0×10^7 conidia/ml), and the same volume of sterile water served as a control. Three weeks after inoculation with conidial suspension, all the garlic leaves gradually turned yellow, which was consistent with the symptoms observed in the field. Four weeks after inoculation with conidial suspension, all the garlic root systems exhibited typical rotten symptoms (Figure 1d), followed by eventual plants withering and death, whereas the control plants stayed healthy (Figure 1e). We further isolated the same fungus colonies and confirmed Koch's hypothesis (Silva et al., 2013).

Conclusion

In this study, the root rot of garlic caused by *F. oxysporum* in Southern China was reported for the first time. Thus, the identification of *F. oxysporum* as the causal agent of the observed root rot on garlic is critical to the prevention and control of this disease in the future.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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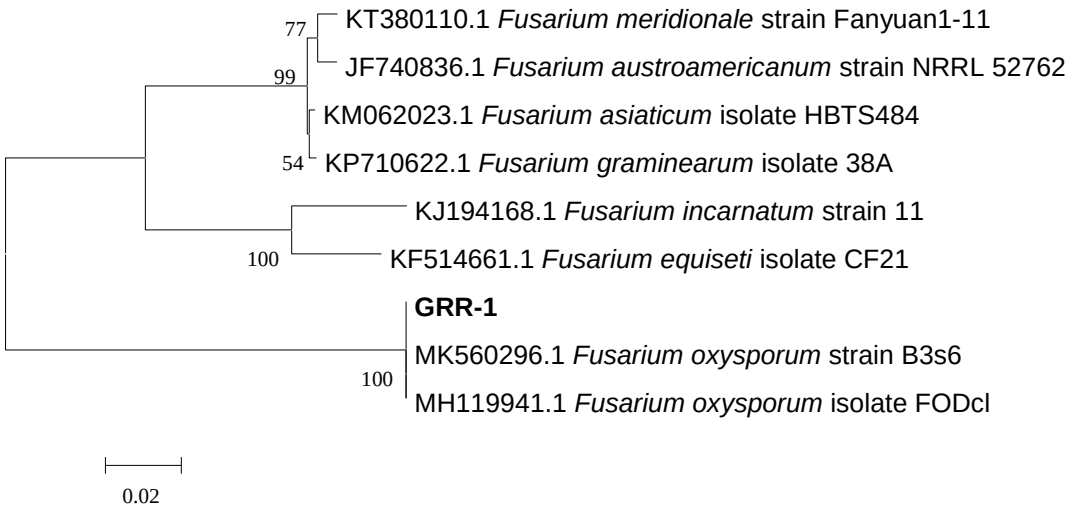

Figure 2. Phylogram generated from neighbor joining analysis based on alignment of ITS and *EF-1α* gene sequences. Values above the branches are parsimony bootstrap (equal or above 50%).

Vegetable Industry Technology System of China (CARS-24-G-14).

REFERENCES

Awere CA, Githae EW, Gichumbi JM (2021). Phytochemical analysis and antifungal activity of *Tithonia diversifolia* and *Kigelia africana* extracts against *Fusarium oxysporum* in tomato. African Journal of Agricultural Research 17(5):726-732.

Geiser DM, del Mar Jiménez-Gasco M, Kang S, Makalowska I, Veeraraghavan N, Ward TJ, Zhang N, Kuldau GA, O'donnell K (2004). FUSARIUM-ID v. 1.0: A DNA Sequence Database for Identifying *Fusarium*. European Journal of Plant Pathology 110(5):473-479.

Leslie JF, Summerell BA (2006). The *Fusarium* Laboratory Manual. Blackwell Publishing, Oxford, U.K

Nash SM, Snyder WC (1962). Quantitative estimations by plate counts of propagules of the bean root rot Fusarium in field soils. Phytopathology 52(6).

Oosthuizen CB, Reid A-M, Lall N (2018). Garlic (*Allium sativum*) and its associated molecules, as medicine. In: Medicinal Plants for Holistic Health and Well-Being. Elsevier 9:277-295

Seth T, Lyngdoh Y, Chattopadhyay A, Sharangi AB, Mishra G (2018). Export of onion, garlic and Chilli: three essential spices in daily kitchen. Springer 13:359-378.

Silva ADA, Pinho DB, Hora Junior B, Pereira OL (2013). First Report of Leaf Spot Caused by *Phyllosticta yuccae* on *Yucca filamentosa* in Brazil. Plant Disease 97:1257.

Xie YQ, Zhang LJ, Mao J (2015). Rhizosphere Soil Community Characteristics of Root Rot Diseased Garlic Plants in Xinjiang Area. Modern Agricultural Science and Technology 21:133-137.

Yayeh SG, Melkamu A, Amare H, Yigzaw D (2021). Assessment of small holder farmers garlic (*Allium sativum* L.) production practices under irrigated farming system in the Highlands of Ethiopia. African Journal of Agricultural Research 17(9):1172-1179.

Yin YS, Li JJ, Zhang FB, Zhang SQ, Gao M (2020). First Report of *Ceratobasidium* sp. Causing Root Rot of Garlic in China. Plant Disease 104(2): 569.

Zhang B, Zhang Y, Ma L Qi K, Wang P, Li CS, Qi JS (2021). Identification of Pythium species as pathogens of garlic root rot. Journal of Plant Pathology 103(1):259-267.

Wu ZH, Wang TH, Huang W, Qu YB (2001). Asimplified method for chromosome DNA preparation from filamentous Fungi. Mycosystema 20(4):575-577.

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Full Length Research Paper

Isolation and *in-vitro* assessment of antagonistic activity of *Trichoderma* spp. against *Magnaporthe oryzae* Longorola strain causing rice blast disease in Mali

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***Pyricularia oryzea (Magnaporthe oryzae)* causes blast diseases in rice (*Oryza sativa*) in Mali. The losses could reach 90% of production during rainy weather conditions. Isolation and characterization of *M. oryzae* and *Trichoderma* species were carried out to assess the importance and distribution of the pathogen and antagonist *Trichoderma* species in rice fields in Sikasso (Mali), and select, *in vitro, Trichoderma* species with high pathogen biocontrol activity. In the pathogen isolation, only one isolate of *M. oryzae* were obtained, while 12 *Trichoderma* isolates were obtained. In the fungal growth tests three isolates of *Trichoderma*: *Trichoderma harzianum* S31, *T. harzianum* S32, and *T. harzianum* S33 highly inhibited the growth of the pathogen with a coefficient of antagonism of 0.55, 0.71 and 0.78 respectively. These isolates were selected for further greenhouse and field tests.**

Keys words: Rice blast disease, *Trichoderma*, *Magnaporthe oryzae*, *Pyricularia oryzea*, antagonism, *Oryza sativa*, Mali.

INTRODUCTION

Rice (*Oryzae sativa*) is a staple food and cereal crop for more than half of the population in Mali where agriculture drives the national economy (ZEF, FARA, IER (2017)) Mali is one of the top rice producers in West Africa with a 3.19 million tons of rice produce in 2019 (FAOSTAT, 2019). Unfortunately, the country is also particularly vulnerable to agricultural diseases (Gurr et al., 2011), mainly rice blast diseases, which limit rice yields to below the global average, threatening smallholder farmers' livelihoods as well as food and economic security (USDA, 2012; Asibi et al., 2019; Soullier et al., 2020).

The fungal plant pathogen *Magnaporthe oryzae*, involved in causing serious blast diseases in rice in Mali, is very difficult to manage. At present, the losses caused

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by this pathogen could reach 30 to 90% of the rice production in the affected areas (Savary et al., 2000; Khemmuk, 2016). For that, chemicals, compounds are widely used to control blast disease pathogens (Dougoud et al., 2018). *Pyricularia oryzae (M. oryzea)* is the causal agent of this disease on wheat (Tembo et al., 2020), maize (Pordel et al., 2021) and *Panicum repens* (Well et al., 2005). It can cause blast disease on the three hosts. Pordel et al. (2021) in pathogenicity assays in greenhouse revealed that strains from maize can infect barnyard grass and conversely.

Biological control is a promising tool to maintain good level of agricultural production while reducing the release of polluting chemical pesticides to the environment. Many researches showed that soil microorganisms, mainly fungi species, are promising as biocontrol agents (Swain et al., 2018; Sood et al., 2020; Es-Soufi et al., 2020). Out of these microorganisms, *Trichoderma* species, filamentous fungi previously considered to be culture contaminants species, are common inhabitant of rhizosphere and contribute to control of many soil-borne plant-diseases caused by fungi (Chuaki et al., 2002; Bastakoti et al., 2017). *Trichoderma* spp., have been largely studied as biological control agents against plant pathogens (Rivera-Méndez et al., 2020; Alfiky and Weisskopf, 2021; Bastakoti et al., 2017). In recent years, considerable success has been achieved in the control of plant diseases by the use of *Trichoderma* isolates which have become commercially available as biocontrol agents (Woo et al., 2014; Es-Soufi et al., 2020). Chou et al. (2019) combined the use of *Trichoderma harzianum* and a resistant rice variety, considered as sustainable approaches to reduce yield losses and to cope with recent restrictions on fungicide use to manage blast disease, showed that *T. harzianum* reduced the incidence of leaf blast and neck blast on IR504 (susceptible strain), but its efficacy was not consistent and the magnitude of disease suppression by T. harzianum was higher for neck blast than for leaf blast. Also, Mouria et al. (2018) applying *T. harzianum* at a concentration of 10^8 spores/ml, in alternation with the mancozeb at 1000 ppm against rice blast and rice leaf spot and the pyrazophos at 750 ppm against blast; showed that the alternation of pyrazophos and *T. harzianum* reduced blast at a rate similar to that noted when pyrazophos is used alone (that is, respectively 90.5 and 89.1%). This percentage is better than that recorded following treatment by *T. harzianum* alone (78.4%). Mancozeb alternated with *T. harzianum* reduced blast at a rate of (83.49%) compared with the fungicide or the antagonist alone (77 and 78.4%). Nevertheless, essential knowledge concerning the distribution and efficacy of *Trichoderma* species as antagonists for their effective use to act against rice blast pathogens is lacking in Mali. That is why, the present research was undertaken to explore the possibility of isolating and selecting *Trichoderma* strains with high biocontrol activity against rice blast disease pathogen.

MATERIALS AND METHODS

Source of isolates and pathogen isolation

Samples of contaminated soil (5) from rice fields (lowland), leaves (15) and panicles (3) of diseased rice were collected at the Longorola research station of the ″Institut d'Economie Rurale (IER)″ at the ″Centre Regional de Recherche Agronomique (CRRA)″ of Sikasso. Rice plant samples were collected from both leaf infections. Besides rice, samples were also obtained soil samples from rice growing fields in Sikasso.

Isolation of pathogen

Blast lesions were surface sterilized with 0.1% mercuric chloride for 1 min, washed with sterilize distilled water and placed over clean glass slides kept in sterile Petri dishes padded with moist cotton. The Petri dishes were incubated for 48 h at room temperature (28 ± 2°C). Single spore method was used for purification (Noman et al., 2018; Zhang et al., 2013). For that, single conidia were identified from the sporulating lesions using a stereomicroscope and aseptically transferred to a petri dish (PDA media). The isolated and purified strain was identified mainly from its macroscopic characters of its colonies and microscopic characters of its mycelium on the basis of identification keys (Botton et al., 1990).

Media suitable for culturing *P. oryzae*

The determination of suitable media for culturing *P. oryzae* (*M. oryzea*) was done according to the method described by Vanaraj et al. (2013). The *M. oryzae* Longorola strain (*M. oryzae* LS) was grown on PDA for 10 days at room temperature. From the margin of actively growing fungus, 5 mm discs were plugged out. Sterile Petri dishes containing PDA, oat meal agar, rice agar, rice polish agar and malt extract agar were inoculated each with a single 5 mm disc of the fungus and incubated at room temperature for 30 days. Four replications were maintained for each medium. The fungal growth was measured at 5-day-intervals until 30 days. Further, the colony characters of the single isolate on different media were recorded on 30th day. All the 11 isolates were grown on PDA and their colony morphology was observed.

Spore induction on stem bits

As the fungus grows and sporulates slowly on rice, but sporulate more quickly on maize and *P. ripens* and we need more spores for the pathogenicity test; we tested the spore production ability of the fungus on these plants. For that, the stem bits from maize, rice (20-day old crop) and *P. repens* were collected from the field and cut into small pieces of 1 cm in length. 15 pieces were placed in 50 ml Erlenmeyer flasks and sterilized for 1 h and 30 min. Each flask was inoculated with two 5 mm diameter mycelial discs of the Longorola isolate and incubated for 15 days at room temperature (Vanaraj et al., 2013). Three stem pieces were sampled at 5, 10 and 15 days after inoculation (DAI). Each stem piece was placed in a test tube containing 1 ml of sterile water, shaken well to dislodge the spores and decanted. The spore concentration was assessed using a haemocytometer.

Measurement of spore size

After spore induction on bits tests shows that the fungus grows and sporulates more quickly on maize bits than on rice and *P. repens*. The *M. oryzae* LS isolate was multiplied on maize stem bits for 15 days and spores were collected by placing stem piece in a test tube containing 1 ml of sterile water, shaking well to dislodge the spores and decanting. The length and width of 10 spores were measured for each isolate using a micrometer as in Vanaraj et al. (2013).

Isolation and identification of *Trichoderma* sp.

One gram of the soil sample was taken and added to 1 ml of sterilized distilled water to make a dilution of 10^{-1}. This suspension was then subjected to serial dilutions and a dilution of 10^{-5} was attained. One milliliter of each dilution viz., 10^{-3} to 10^{-4} was poured on to *Trichoderma* Specific Medium (TSM) (Elad et al., 1981) and purified by single spore method (Zhang et al., 2013). The TSM medium is composed of the following constituents (g/L): $MgSO_4$ 9; 7 H_2O, 0.2; K_2HPO_4, 0.9; KCl, 0.15; NH_4NO_3, 1.0; glucose, 3.0; chloramphenicol 0.25; *p-dimethylaminobenzenediazo* sodium sulfonate 0.3; pentachloronitrobenzene 0.2; rose-bengal 0.15; agar 20 Isolates were identified on the basis of their morphological characters, according to conidiophore, shape of the phialides and emergence of phialophores and phialospores (Soesanto et al., 2011). The purified and identified cultures of *Trichoderma* strains were maintained on PDA medium and stored at 4°C for further use.

Antagonism characterization

Out of 12 isolates of Trichoderma sp. obtained from different soil samples, only 3 strains showed, in pre-test, an antagonistic activity against the rice blast pathogen isolated. These three isolates identified as *T. harzianum* were tested for antagonism effect against the isolated rice pathogen: *M. oryzae*. The antagonism studies were done by using dual culture techniques as developed by Rahman et al. (2009). The mycelial bits of 5 mm diameter of *Trichoderma* sp. strain and pathogen were placed opposite to each other (4 cm) on Petri plates containing PDA. The plates were run in triplicates with one control set maintained without inoculating the *Trichoderma sp.* isolates. The plates were incubated at 28 ± 2°C for one week, and the growth of the pathogen tested against the 3 isolates of *Trichoderma* which showed antagonism activity in the pre-test. The data were recorded regularly on the growth of the pathogen and *Trichoderma* sp. tested. Percentage of mycelial growth inhibition (MGI) was calculated according to the formula:

MGI% = (dc - dt) ×100/dc

Where, dc= fungal colony diameter in control sets, dt= fungal colony diameter in treatment sets.

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Plates were tested in triplicate, with a control set maintained without inoculating Trichoderma sp. The plates were incubated at 27+-1°C for one week, and pathogen growth was tested against the 3 Trichoderma isolates that showed antagonistic activity in the pre-test. Data was recorded regularly on the growth of the pathogen and Trichoderma sp. Tested. The percentage inhibition of mycelial growth was calculated according to the formula:

MGI%=(dc-dt)×100/dc

Where, dc=diameter of fungal colony in control sets, dt=diameter of fungal colony in treatment sets.

The inhibition exerted by the genus Trichoderma was estimated by calculating the antagonism coefficient (Morsy, 2005) according to the following formula:

$a=(C_{tem}-C_{trait})/C_{tem}$

where a is the antagonism coefficient, Ctem the mean radius of the control colonies (strains of phytopathogenic fungi growing in the absence of antagonist), C_{trait} the mean radius of the colonies in the presence of the antagonist.

The evaluation of myco-parasitic activity was carried out in accordance with the method of Dabire et al. (2016). This method consisted in carrying out cocultures in direct confrontation between the pathogen and the antagonist for 21 days. After the 21 days, mycelial fragments of the pathogen in the area of intersection were cultured at 25°C for 5 days to observe the viability of the pathogen. Sandwiched Petri plates, a setup described in Li et al. (2018), was employed to determine if the *Trichoderm*a isolates tested can produce volatile compounds, and how the volatile compounds affect the growth of *M. oryzae* LS. After inoculating *M. oryzae* LS and *Trichoderma* isolates on PDA plate, *M. oryzae* LS plate was placed on top of *Trichoderma* plate, sealed with three layers of Parafilm, and incubated at 25°C. Each plate of *M. oryzae* LS also was sandwiched with an un-inoculated PDA plate (control treatment). Colony diameter of *M. oryzae* LS was measured 5 days later. We evaluated the inhibitory effect of *M. oryzae* LS volatile compounds on *Trichoderma* in the same way except that 5-day-old (after the inoculation of culture plug) *M. oryzae* LS culture was used to ensure enough *M. oryzae* LS biomass. Colony diameter of *Trichoderma* was measured 36 h later. The duration of volatile compound exposure between the two experiments was different because *Trichoderma* grew much faster than *M. oryzae* LS. Each treatment included three biological replicates and was repeated three times.

RESULTS AND DISCUSSION

Virulent strains of *M. oryzae*

In this study, only one isolate of *M. oryzae* (Figure 1) was obtained from a sample of rice from the Longorola research station of the CRRA in Sikasso (IER), and named *M. oryzae* Longorola strain (*M. oryzae* LS).

Growth of *M. oryzae* was rapid on PDA followed by malt extract agar (Table 1 and Figure 1). At 5 days after inoculation (DAI), the colony diameter was 3.45 cm. The *M. oryzae* isolate grew 8.95 cm in diameter in the PDA, contained in 9.0 cm Petri dishes, at 10 DAI. On the same day, its growth on the rice agar was 6.95 cm. At 15 DAI, the colony diameter of *M. oryzae* was 9.0 cm in both media tested.

The spore density was greatest when *M. oryzae* was propagated on maize followed by the rice stalk at all observation intervals (Table 1). The production of conidia increased over time for both maize and rice stalks. For maize, it was respectively 395, 1630, 2900 at 5, 10 and 15 DAI. This *M. oryzae* strain produce very few spores on *P. repens*, a grass which was found support growth and bigger spores of *M. oryzae* than rice. As maize stalk shows better grow and more spore production, we chose to multiply *M. oryzae* on maize stalk to have more spore in a relatively short time for tests.

The length and width of *M. oryzae* conidia (Figure 1)

Figure 1. Mycelia and spores of *M. oryzae*.

Table 1. Assessment of spore density on maize and rice stalks.

Media	Number of spores/ml of water		
	Days after incubation (DAI)		
	5	10	15
Maize	395	1630	2900
Rice	90	320	600
Panicum repens (*P. repens*)	43	109	200

Table 2. Length and width of the strain of *Magnaporthe oryzae* LS, isolated from Longorola (Sikasso), Mali.

Isolate	Length		Width	
	Interval	Average	Interval	Average
M. oryzae LS	20 - 23	21.5	8.95- 10.25	9.6

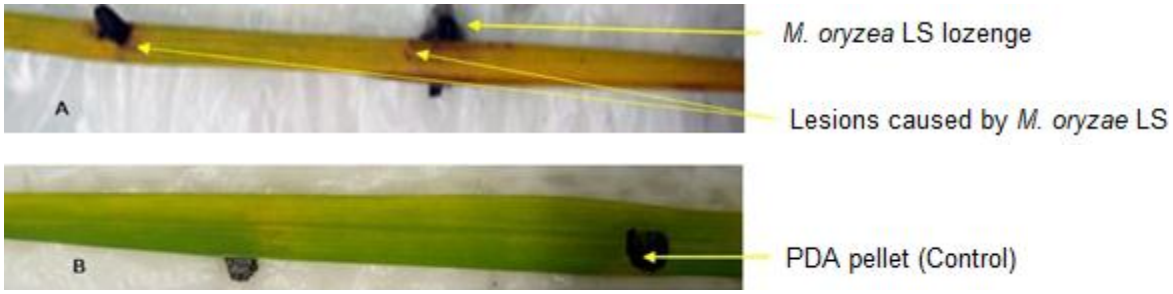

Figure 2. Lesions caused by *M. oryzae* LS pellets (A), while PDA pelletsdid not cause any damage (B).

were measured. Results of spore dimension measurement, showed that the mean spore length of the *M. oryzae* isolate was 21.5 µm, while the mean spore width was 9.6 µm (Table 2).

Virulence of *Magnaporthe oryzae* LS strain on rice

The strain of *M. oryzae* LS inoculated on the rice leaves, kept under humidity, showed lesions similar to those of blast (Figure 2A). The uninoculated control, meanwhile, showed no signs of pathology (Figure 2B).From the lesions of the inoculated rice leaves, we were able to re-isolate the *M. oryzae* LS strain. These results confirm the pathogenicity of the isolated *M. oryzae* LS strain.

Strains of *Trichoderma* sp. isolated and identified

Isolation of *M. oryzae* antagonist fungi from soil and rice

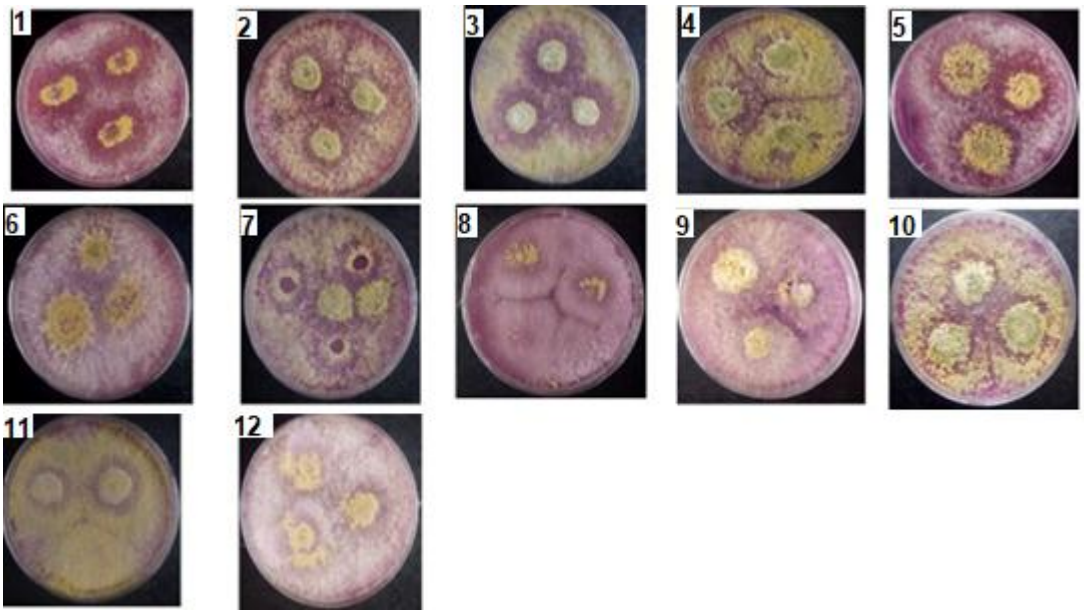

Figure 3. Macroscopic characteristics of the 12 *Trichoderma* isolates analyzed. These isolates are from analyzed soil samples of rice fields in Sikasso, Mali.

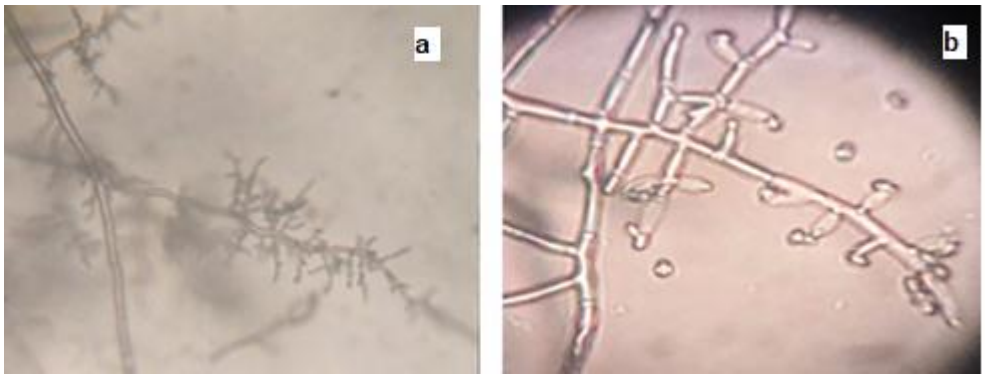

Figure 4. Conidiophore and conidia of *T. harzianum* isolates from soil 1 (a) and 3 (b).

plant samples yielded 12 colonies (Figure 3). These isolates gave colonies typical of *Trichoderma* sp. on *Trichoderma* selective medium. These isolates showed very rapid growth, good sporulation, and yellowish-green and green pigmentations on PDA medium. Three isolates from soil 3 (S31, S32 and S33) (Colonies number 1, 2 and 3 in Figure 3) showed very rapid growth and formed 2 concentric rings with green conidial production. The conidia production was denser in center then towards the margins on PDA medium. The mycelia of these 3 isolates are septated. For these 3 isolates, the conidiophore is sparsely branched and carries philaids (Figure 4). In turn, phialides carry sub-globular-shaped spores or conidia. On the basis of their cultural and morphological characters of these three *Trichoderma* isolates, were identified as *T. harzianum* (Gams and Bisset, 1998; Shah et al., 2012. These 3 strains, that showed, in a pre-test, an antagonistic activity against *M. oryzae* LS) are under molecular characterization to confirm the identity of each.

Antagonism tests

The average growth ranges of *M. oryzae* LS placed in cocultures in direct confrontation with the isolates of *T. harzianum* are given in Table 3. Analysis of data in Table 3 indicates that all of the *T. harzianum* isolates caused a significant reduction in the average growth radius of the *M. oryzae* LS strain tested. The highest coefficient of antagonism obtained was 0.78 obtained with the strain *T. harzianum* S33 (Table 3), followed by the strain *T.*

Table 3. Radial growth and coefficient of antagonism of 3 isolates of *T. harzianum* on *M. oryzae* LS.

Treatments	*M. oryzae*	
	Growth (cm)	Coefficient of antagonism
M. oryzae LS	4.5	-
M. oryzae LS + *T. harzianum S31*	2	0.55
M. oryzae LS + *T. harzianum S32*	1.3	0.71
M. oryzae LS + *T. harzianum S33*	1	0.78

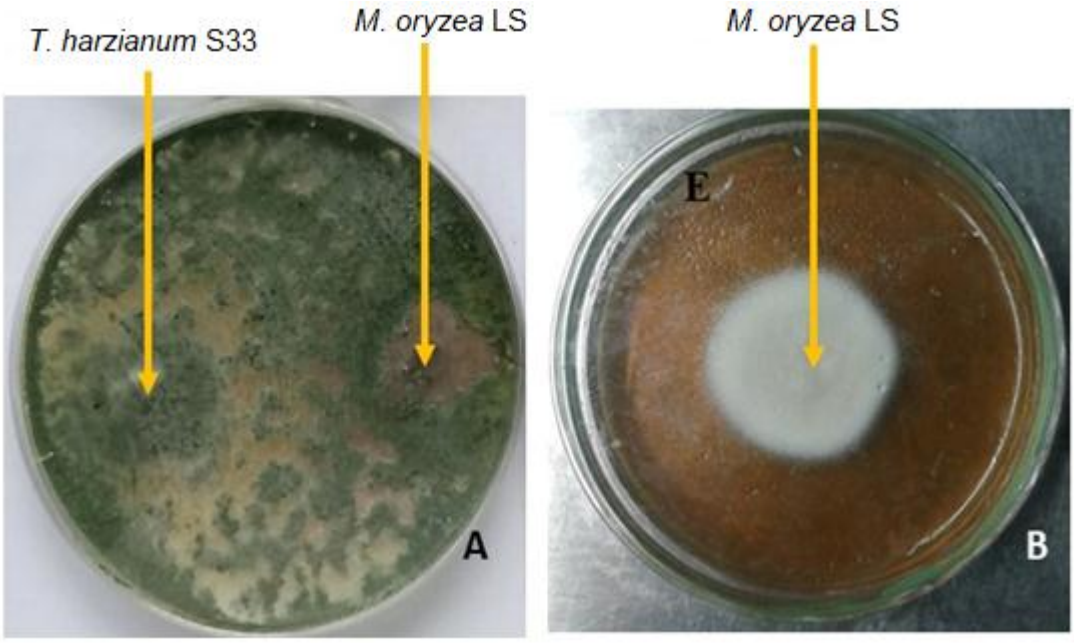

Figure 5. Effect of *T. harzianum S3*3 on the mycelium growth of *M. oryzae LS* (A) strain and the growth of the *M. oryzae* strain only (B).

harzianum S32 with a coefficient of antagonism of 0.71. The *T. harzianum* S31 strain gave the lowest coefficient of antagonism estimated at 0.55. The effect of *T. harzianum* S33 on the mycelium growth of *M. oryzae* LS is presented in Figure 5. The results on mycoparasitism presented showed that only the *T. harzianum* S33 demonstrates a clear mycoparasitic activity against *M. oryzae* LS (Table 4). None of the three *Trichoderma* strains tested were able to control the growth of the *M. oryzae* LS strain by producing volatile compounds.

DISCUSSION

Twelve different colonies of *Trichoderma* sp. were isolated from soil samples from the Longorola station and Sikasso rice fields. Küçük and Vanç (2003) isolated 19 strains of *Trichoderma* from 31 soil samples, while we isolated 12 from 5 sample from agricultural soil of Sikasso (Mali). Which is an indicator of the richness of these soils compared to those of the Turky? Growing the fungus on pieces of the host's stem is the easiest way to induce sporulation in order to study spores (Vanaraj et al., 2013). *M. oryzae* spores isolated from rice in this study were significantly smaller than those isolated from the rice fields of Baguineda. Gupta et al. (2020) states that the spore size of the fungus *M. oryzae* varies among isolates depending on environmental conditions. Measurement of the spore size of different isolates of *M. oryzae* from rice grown on PDA by Gayatonde et al. (2016) showed that the mean length ranged from 21.2 to 28.4 µm. In our study, the spore length of *M. oryzae* isolates grown on pieces of corn stalk was 21.5. Vanaraj et al. (2013) observed variations in the length and width of *M. oryzae* spores due to the effect of artificial media. They also noticed that temperature had no effect on the width of the spore while the length was affected.

The results obtained at the end of this study reveal that

Table 4. Mycoparasitic activity of *T. harzianum* S31, *T. harzianum* S32 and *T. harzianum* S33 strain on *M. oryzae* LS.

Pathogenic	Antagonists		
	***T. harzianum* S31**	***T. harzianum* S32**	***T. harzianum* S33**
M. oryzae LS - Exp 1*	**-***	**-**	**+**
M. oryzae LS - Exp 2	**-**	**-**	**+**
M. oryzae LS - Exp 3	**-**	**-**	**+**

Exp 1, Exp 2 and Exp 3 represent respectively Experiment 1, Experiment 2 and Experiment 3
*Result from 3 replicates.

out of all the *Trichoderma* isolated in Mali, only 3 have an inhibitory activity on the mycelial growth of *M. oryzae*, but to different degrees. Naravanasamv et al. (2015) demonstrated the inhibitory activity of *Trichoderma* species on the growth of *P. oryzae*. In this study, in addition to antifungal activity, only the *T. harzianum* S33 strain exhibited myco-parasitic activity on the *M. oryzae* isolate tested. In addition, an antagonism coefficient of 0.71 was obtained with this strain. These results are also in the same direction as those of Li et al. (2018) who showed that 5 isolates of *T. harzianum* tested have a strong inhibitory power against 3 pathogenic fungi, including *Fusarium oxysporum f*.sp. *cepae* (82.77%). However, not all of the 5 isolates exerted a myco-parasitic action on all the pathogens tested and the intensity of the inhibitions varied from one pathogen to another and from one antagonist to another but values antagonism coefficients greater than 0.75 were obtained with two of the isolates tested. The antagonism in the distance confrontation was much lower than in the direct confrontation. This could be explained by the fact that *T. harzianum* uses several modes of action and depending on the nature of the pathogen to exert their antagonistic power (Benitez et al., 2004; Sood et al., 2020). According to Benhamou and Chet (1997) fungi chitin is an essential constituent of the wall which surrounds and protects cells from the environment. In the same way, Latgé (2007) showed that the cell wall is essential for fungal growth and for the resistance of the fungus to external attacks. Its alteration linked to the action of *Trichoderma* would lead to an alteration of the mycelium which results in aggregation, retraction and vacuolation of the cytoplasm. The statements by Benhamou and Chet (1996) and Latgé (2007) were confirmed by Tapwal et al. (2015), who indicate that the inhibitory power of *Trichoderma* species is manifested by a significant lysis of the mycelial cells of the pathogens. Nusaibah and Musa (2019), reports that *Trichoderma* species have the ability to attack pathogens via different modes of action. They can use the antibiotic which results from the production of substances which act as "antibiotics" and which inhibit the growth of the pathogen. In some cases, the colony of *T. harzianum* grows on that of the pathogen. According to Sharma (2011), these types of interactions indicate competition and parasitism, respectively. The results obtained in remote confrontation mode indicate that the use of volatile compounds was not the main path that explains the observed antagonism.

Conclusion

Only one pathogenic isolate of *M. oryzae* was isolated from soil and diseased rice plant samples from Sikasso, Mali. Twelve isolates of *Trichoderma* were isolated from soils samples. Out of these isolates: *T. harzianum* S31, *T. harzianum* S32 and *T. harzianum* S33 showed high antagonism activity against *M. oryzae* LS isolated from Mali.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

Alfiky A, Weisskopf L (2021). Deciphering *Trichoderma*-plant-pathogen interactions for better development of biocontrol applications. Journal of Fungi 7(61):1-18.

Asibi AE, Chai Q, Coulter JA (2019). Rice Blast: A Disease with Implications for Global Food Security. Journals of Agronomy 9(8):1-14.

Bastakoti S, Belbase S, Manandhar S, Arjyal C (2017). *Trichoderma* species as Biocontrol Agent against Soil Borne Fungal Pathogens. Nepal Journal of Biotechnology 5(1):39-45.

Benhamou N, Chet I (1996). Parasitism of sclerotia od sclerotium Rolfsii by Trichoderma harzianum: ultrastuctural and cytochemical aspects of the interaction. Phytopath 86(4):405-416.

Benhamou N, Chet I (1997). Cellular and molecular mechanisms involved in the interaction between *Trichoderma harzianum* and

Pythium ultimum. Applied and Environmental Microbiology 63(5):2095-2099. doi: 10.1128/aem.63.5.2095-2099.1997.
Benitez T, Ana M, Rincón M, Carmen LA, Codón C (2004). Biocontrol mechanisms of *Trichoderma* strains. International Microbiology 7(4):249-260.
Botton BBA, Fevre M, Gauthir S, Guy PH, Larpent JP, Reymond P, Sanglier JJ, Vayssier Y, Veau P (1990). Beneficial and detrimental harvests of industrial importance. 2éme édition. masson collection biotechnologies pp. 34-42.
Chou C, Nancy CN, Hadi B, Tanaka T, Chiba S, Sato I (2019). Rice blast management in Cambodian rice fields using *Trichoderma harzianum* and a resistant variety. Crop Protection 135:104864. https://doi.org/10.1016/j.cropro.2019.104864
Chuaki T, Lavarde V, Lachaud L, Henneken C (2003). Invasive Infections Due to *Trichoderma* Species: Report of 2 Cases, Findings of In Vitro Susceptibility Testing, and Review of the Literature. Clinical Infectious Diseases 35(11):1360-1367.
Dabire TG, Bonzi S, Somda I, Legreve A (2016). Evaluation of the Potential of *Trichoderma harzianum* as a Plant Growth Promoter and Biocontrol Agent Against Fusarium Damping-off in Onion in Burkina Faso. Asian Journal of Plant Pathology 10(4):49-60.
Dougoud J, Clottey V, Bateman M, Wood A (2018). Study on crop protection in countries where the Green Innovation Centres for the Agri-Food Sector programme is active. Country report for the 'Green Innovation Centre' (GIC) in Mali P 113.
Elad Y, Chet I, Henis YA (1981). A selective medium for improving quantitative isolation of *Trichoderma* spp. from soil. Phytoparasitica 9(1):59-67.
Es-Soufi R, Tahiri, H, Azaroual L, El Oualkadi A, Martin P, Badoc A, Lamarti A (2020). Biocontrol Potential of *Bacillus amyloliquefaciens* Bc2 and *Trichoderma harzianum* TR against Strawberry Anthracnose under Laboratory and Field Conditions. Agricultural Sciences 11(3):260-277.
Food and Agriculture Organization Corporate Statistical Database (FAOSTAT) (2019). Statistical Database. Available online: http://www.fao.org (accessed on 10 April).
Gams W, Bissett J (1998). Morphology and Identification of Trichoderma. In: Trichoderma and Gliocladium: Basic Biology, Taxonomy and Genetics, Harman GE, Kubicek CP. Taylor and Francis, London, UK., ISBN-13: 9780203483558 1:3-34.
Gayatonde V, Mahadevu P, Vennela PJ (2016). Study of suitable culture media and other abiotic factors for the growth and sporulation of Magnaporthe Oryzae. Ecology, Environment and Conservation 22(2):297-301.
Gupta DR, Surovy MZ, Mahmud NU, Chakraborty M, Paul SK, Hossain MS, Bhattacharjee P, Mehebub MS, Rani K, Yeasmin R, Rahman M, Islam MT (2020). Suitable methods for isolation, culture, storage and identification of wheat blast fungus *Magnaporthe oryzae Triticum* pathotype. Phytopathology Research 2(1):1-13.
Gurr S, Samalova M, Fisher M (2011). The rise and rise of emerging infectious 643 fungi challenges food security and ecosystem health. Fungal Biology Reviews 25(4):181-188.
Khemmuk W (2016). Plant pathogenic Magnaporthales in Australia, with particular reference *to Pyricularia oryzae* on wild and cultivated rice. Master thesis presented at the University of Queensland, Austria 193 p.
Küçük C, Vanç M (2003). Isolation of *Trichoderma Spp.* and Determination of Their Antifungal, Biochemical and Physiological Features. Turkish Journal of Biology 27:247-253
Latgé JP (2007). The cell wall: A carbohydrate armour for the fungal cell. Molecular Microbiology 66(2):279-290
Li N, Alfiky A, Wang W, Islam M, Nourollahi K, Liu X, Kang S (2018). Volatile Compound-Mediated Recognition and Inhibition between Trichoderma Biocontrol Agents and *Fusarium oxysporum*. Frontiers in Microbiology 9(2614):1-16.
Morsy EM (2005). Role of growth promoting substances producing microorganisms on tomato plant and control of some root rot fungi. Ph.D. Thesis, Fac. Agric. Ain shams Univ., Cairo, Egypt.
Mouria A, Mouria B, Hmouni A, Touhami, AO, Douira A (2018). Integrated Control against Rice Blast and Leaf Spot by *Trichoderma harzianum* and Two Fungicides. Annual Research and Review in Biology 29(1):1-6.
Naravanasamv P, Thokala P, Muthkrishnan S, Kamil D (2015). Screening of different Trichoderma species against agriculturally important foliar plant pathogens. Journal of Environmental Biology 36(1):191-198.
Noman E, Al-Gheethi AA, Rahman NA, Talip B, Mohamed R, Nagao H, Kadir OA (2018). Single spore isolation as simple and efficient technique to obtain fungal pure culture, Conference series Earth Environmental Science 140(1):012155.
Nusaibah SA, Musa H (2019). A Review Report on the Mechanism of *Trichoderma* spp. as Biological Control Agent of the Basal Stem Rot (BSR) Disease of *Elaeis guineensis*, Trichoderma - The Most Widely Used Fungicide, Mohammad Manjur Shah, Umar Sharif and Tijjani Rufai Buhari, IntechOpen.
Pordel A, Ravel S, Charriat F, Gladieux P, Cros-Arteil S, Milazzo J, Henri AH, Javan-Nikkhah M, Mirzadi-Gohari A, Moumeni A, Tharreau D (2021). Tracing the Origin and Evolutionary History of *Pyricularia oryzae* Infecting Maize and Barnyard Grass. Phytopathology 111(1):128-136.
Rahman MA, Begum MF, Alam MF (2009). Screening of *Trichoderma* Isolates as a Biological Control Agent against *Ceratocystis paradoxa* Causing Pineapple Disease of Sugarcane. Mycobiology 37(4): 277-285.
Rivera-Méndez W, Obregón M, Morán-Diez ME, Hermosa R, Monte E (2020). *Trichoderma asperellum* Biocontrol Activity and Induction of Systemic Defenses against *Sclerotium cepivorum* in Onion Plants under Tropical Climate Conditions. Biological Control 141:104145.
Savary S, Willocquet L, Elazegui FA, Castilla NP, Teng PS (2000). Rice 706 pest constraints in tropical Asia: Quantification of yield losses due to rice pests in a range of 707 production situations. Plant Disease 84(3):357-369.
Shah S, Nasreen S, Sheikh PA (2012). Cultural and Morphological Characterization of *Trichoderma* spp. Associated with Green Mold Disease of *Pleurotus* spp. in Kashmir. Research Journal of Microbiology 7(2):139-144.
Sharma P (2011). Complexity of *Trichoderma-Fusarium* interaction and manifestation of biological control. Australian Journal of Crop Science 5(8):1027-1038.
Soesanto L, Utami DS, Rahayuniati RF (2011). Morphological characteristics of four *Trichoderma* isolates and two endophytic Fusarium isolates. Canadian Journal on Scientific and Industrial Research 2(8).
Sood M, Kapoor K, Kumar V, Sheteiwy MS, Ramakrishnan M, Landi M, Araniti F, Sharma A (2020). *Trichoderma*: The “Secrets” of a Multitalented Biocontrol Agent. Plants 9(6):762-787.
Soullier G, Demont M, Arouna A, Lançon F, Mendez del Villar P (2020). The state of rice value chain upgrading in West Africa. Global Food Security 25:100365.
Swain H, Adak T, Mukherje AK, Mukherjee PK, Bhattacharyya P, Behera S, Bagchi TB, Patro R, Khandual MK, Bag TK Dangar S, Jen LJ (2018). Novel Trichoderma strains. isolated from tree barks as potential biocontrol agents and biofertilizers for direct seeded rice. Microbiological Research 214:83-90.
Tapwal A, Tyagi A, Thakur G, Ghandra S (2015). In-vitro evaluation of *Trichoderma* species against seed borne pathogens. International Journal of Biological and Chemical Sciences 1(10):14-19.
Tembo B, Mulenga RM, Sichilima S, Kenneth K, Moses M, Patrick CC, Pawan KS, Xinyao H, Kerry FP, Gary LP, Ravi PS, Hans JB (2020). Detection and characterization of fungus (*Magnaporthe oryzae pathotype Triticum*) causing wheat blast disease on rain-fed grown wheat (*Triticum aestivum* L.) in Zambia. PLoS ONE 15(9):e0238724.
U.S. Department of Agriculture (USDA) (2012). National Agricultural Statistics Service, “Crop Production,” http://www.nass.usda.gov/Publications/Todays_Reports/reports/crop1012.pdf
Vanaraj P, Kandasamy S, Ambalavanan S, Ramalingam R, Sabariyappan R (2013) Variability in *Pyricularia oryzae* from different rice growing regions of Tamil Nadu, India. African Journal of Microbiology Research 7(26):3379-3388.
Woo SL, Ruocco M, Vinale F, Nigro M, Marra R, Lombardi N, Pascale A, Lanzuise S, Manganiello G, Lorito M (2014). *Trichoderma*-based Products and their Widespread Use in Agriculture. The Open Mycology Journal 8(Suppl-1, M4):71-126.

ZEF, FARA, IER (2017). Country Dossier: Innovation for Sustainable Agricultural Growth in Mali. Program of accompanying research for agriculture innovation. Bonn and Accra. Centre for research, Forum for agricultural research in Africa and Council for scientific and industrial research.

Zhang K, Su Y-Y, Cai L (2013). An optimized protocol of single spore isolation for fungi. Mycologie 34(4):349-356.

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Full Length Research Paper

Isolation of potentially pathogenic bacteria from *Musca domestica* captured in hospitals and slaughterhouses, Khartoum state, Sudan

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This study aimed to isolate and identify bacterial and parasitic pathogens from houseflies captured in hospitals and slaughterhouses. The present study involved 300 houseflies, of which 150 houseflies were collected from hospitals and 150 from slaughterhouses. Two samples were obtained from each housefly; one sample was obtained from the surface of the housefly, and the second was extracted from the intestine of the fly. The bacteria were isolated using cystine–lactose–electrolyte-deficient agar (CLED) agar, while the parasites were studied using direct microscopic examination. Two hundred eighty-three bacteria were isolated from hospitals houseflies; 56.2% of them recovered from the surface of houseflies and 43.8% from the intestine of the flies. This result indicated that each housefly carried 1.9 bacteria. Three hundred sixty-six bacteria were isolated from slaughters houseflies; 53.8% of them recovered from the surface of houseflies and 46.2% from the intestine of the flies. This result showed that each housefly carried 2.44 bacteria. *Escherichia coli, Enterococci* spp and *Pseudomonas aeruginosa* were the most common bacteria isolated from the houseflies. This study identified high virulence bacteria such as *E. coli*, *Klebsiella pneumoniae* and *Shigella* spp. This finding reflects the level of hygiene in the studied area and arise alarm of consequent complications for human health.

Key words: Housefly, bacteria, parasite, culture, cystine–lactose–electrolyte-deficient agar (CLED).

INTRODUCTION

Housefly, *Musca domestica Linnaeus*, is an important medical insect and the most common fly in the world (Khamesipour et al., 2018; Zurek and Nayduch, 2016; Solà-Ginés et al., 2015) representing about 90% of flies in human habitation (Balla et al., 2014). Housefly is able to complete its entire lifecycle (larvae, pupae, adults) within human habitations and domestic animals (Khamesipour et al., 2018). During these developmental phases, the houseflies are strictly associate with microorganisms (Park et al., 2019) and due to their

breeding properties, saprophytic foraging behavior, and hematophagous playing an significant role in the transmission and spread of a wide variety of bacterial, fungal and viral pathogens (Stelder et al., 2021). Houseflies have been identified as mechanical vector and reservoirs for more than 100 pathogenic microorganisms (Reuben et al., 2020; Issa, 2019; Neupane et al., 2019; Nazari et al., 2017); they carry a variable and complex prokaryotic microbiota (Park et al., 2019). Some of the bacteria isolated from houseflies were highly virulent species such as *Pseudomonas*, *Escherichia coli, Klebsiella* spp., Vibrio cholera, *Bacillus anthracis*, *Streptococci*, *Enterococci, Staphylococci* and *Clostridium* spp (Khamesipour et al., 2018; Zurek and Nayduch, 2016). Recently houseflies were identified as a potential carrier of the bird flu virus which is a serious threat to human health, and livestock (Zurek and Nayduch, 2016; Davari et al., 2010). Furthermore, severe acute respiratory syndrome coronaviruses (SARS-CoV) have been reported to be mechanically transmitted by insects, particularly CoV-19 can survive on the faeces and surfaces for elongated periods, likely no report has linked the human transmission of CoV-19 with insects (Reuben et al., 2020) .

Houseflies have the capability to act as vector expanded by its ability to move several kilometers in a couple of days (Park et al., 2019). Some microorganisms live in or on the body of houseflies for up to 35 days (Ranjbar et al., 2016), the transfer of pathogenic agents occur by several means like; attaching them to their mouth or body surface or through regurgitation of vomitus and passage through the alimentary tract (Neupane et al., 2019; Ranjbar et al., 2016).

Houseflies lives closely with humans and are often found in abundance in areas of human activities such as food centers, restaurants, food markets, hospitals, livestock and slaughterhouses causing serious health problems (Reuben et al., 2020; Issa, 2019; Khamesipour et al., 2018). One of the major challenges facing the developing countries is control of communicable diseases in which housefly play significant role. Therefore, the present study aimed to determine the frequency and type of bacteria and parasites in the houseflies collected from hospitals and slaughterhouses environments in Khartoum, Sudan.

MATERIALS AND METHODS

This study was cross sectional study, conducted in Khartoum state, Sudan. *Musca domestica* flies represented the population in this study.

Sample collection and identification of houseflies

Samples were obtained via simple random technique. Three hundred houseflies were collected; 150 captured in hospitals (Al-Nao hospital and Al-Boluk pediatric hospital) and 150 captured in slaughterhouses and surrounding area (Omdurman, Kartoum State). From each housefly two samples were obtained, one sample obtained from the body surface of the fly and the second sample was extracted from the intestine of the fly. The houseflies were captured individually using sterile entomological nets and immediately transported to medical parasitology and entomology department, University of Science and Technology, Sudan. Houseflies were identified through observation of the morphological features, macroscopically and microscopically properties as mentioned in fly management handbook (Kirby, 2008).

Preparation of housefly body surface samples

To detect the microorganisms (bacteria and parasites) attached to the surfaces of the a housefly, each fly was immersed in 3 ml sterile peptone water buffer for 2 min and then the fly was kept into sterile Petri-dish. The peptone water buffer was incubated at 37°C for 4 h to encourage the growth of bacteria. A loop full of well mixed peptone water buffer was inoculated into sterile CLED agar and incubated aerobically at 37°C for 48 h, the remaining peptone water buffer was concentrated by centrifugation at 5000 rpm for 5 min. The deposit was examined microscopically using 10X and 40X objective lenses to detect the attached parasites

Preparation of housefly intestinal samples

Each housefly was disinfected by dipping into 70% Ethanol 'two times', sterile distilled water 'three times', 0.05% sodium hypochlorite 'two times' and sterile distilled water 'three times' and finally placed into sterile filter paper. The housefly was dissected under aseptic condition, the intestine was extracted and suspended in sterile 3 ml peptone water buffer, mixed thoroughly to allow bacterial release, and then the medium was incubated at 37°C for 4 h to encourage the growth of bacteria. A loop full of well mixed peptone water buffer was inoculated into sterile CLED agar and incubated aerobically at 37°C for 48 h.

Identification of detected microorganisms

The identification of isolated bacteria was done based on Chessborough's scheme (Cheesborough, 2006). Briefly it relies on colonial morphology, Gram stain reaction and biochemical tests. The biochemical tests for Gram positive cocci were catalase test, coagulase test, esculin hydrolysis, DNase test, whereas the biochemical reaction used for Gram negative rod were oxidase test, motility test, indole test, urease test, citrate utilization test and triple sugar iron. The analytical profile index 20 E identification system for *Enterobacteriacea*e and other non-fastidious Gram negative rods a long with RapiDEC *Staph* identification of frequently isolating *staphylococcus* were used to confirm the identification. The parasites had been identified using Arora scheme for identification of intestinal helminthes and protozoa depending on the morphology

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Table 1. Type and frequency of bacteria isolated from hospitals houseflies.

Isolate	Surface		Intestine		Total	
	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage
E.coli	23	14.5	21	17	44	15.5
K.pneumoniae	16	10	25	20.2	41	14.5
P.aeruginosa	16	10	8	6.5	24	8.5
P.stuartii	3	1.9	12	9.7	15	5.3
P.vulgaris	5	3.1	8	6.5	13	4.6
K.oxytoca	6	3.8	5	4	11	3.9
Y.enterocolytica	6	3.8	4	3.2	10	3.5
Shigella spp	8	5	1	0.8	9	3.2
S.paratyphi B	5	3.1	2	1.6	7	2.5
P.mirabilis	3	1.9	4	3.2	7	2.5
S.paratyphi A	2	1.3	2	1.6	4	1.4
S.typhimurium	2	1.3	3	2.4	5	1.8
K.aerogens	2	1.3	--	--	2	0.7
C.freudii	13	8.1	4	3.2	17	6
E.caloacae	6	3.8	--	--	6	2.1
C.davisae	1	0.7	1	0.8	2	0.7
Enterococci spp	20	12.6	20	16.1	40	14.1
S.aureus	15	9.4	3	2.4	18	6.4
Coagulase–ve Staphylococci	7	4.4	1	0.8	8	2.8
Total	159	100	124	100	283	100

of the parasite (Arora and Arora, 2010).

Data analysis

The bacteria per housefly was calculated by dividing the summation of isolated bacteria to total number of houseflies, data was analyzed using Microsoft excel 2007.

RESULTS

Two hundred eighty three bacteria were isolated from hospital's houseflies; 159 (56.2%) of them were isolated from the surface of the flies and 124 (43.8%) from the intestine of the flies. This result indicated that each housefly carried 1.9 bacteria. Sixty six (23.3%) of isolated bacteria were Gram positive cocci and (76.7%) were Gram negative rods. The identification of isolates revealed 19 different species, the most frequent isolate was *E. coli* 44 (15.5%), followed by *Klebsiella pneumoniae* 41 (14.5%) and *Enterococci* spp. 40 (14.1%). The most dominant bacteria isolated from the surface of houseflies was *E. coli* 23 (14.5%) followed by *Enterococci spp* 20 (12.6%), whereas the most dominant bacteria isolated from the intestine was *K. pneumoniae* 25 (20.2%) followed by *E. coli* 21 (17.0%), Table 1.

Three hundred and sixty six bacteria were isolated from slaughterhouses houseflies; 197 (53.8%) of them were isolated from the surface of the flies whereas 169 (46.2%) bacteria were isolated from the intestine of the flies. These results showed that the housefly carried 2.44 bacteria. The majority of isolates 249 (68.0%) were Gram negative rods, 117 (32.0%) bacteria were Gram positive Cocci. The biochemical tests showed 17 different species. The most frequent isolate was *E. coli* 73 (19.9%) followed by *Enterococci spp.* 66 (18.0%) and *P.aeruginosa* 53 14.5%), the most frequent bacteria isolated from the surface was *P. aeruginosa* 38 (19.3%) followed by *E. coli* 33 (16.8%) *and Enterococci* 29 (14.7%), whereas the most dominant bacteria isolated from the intestine was *E. coli* 40 (23.7%) followed by *Enterococci* spp. 37 (21.9%) and *K. pneumoniae* 20 (11.8%) (Table 2). Thirty six parasites were detected in hospital houseflies; the most dominant parasite18 (50%) was *G.lamblia* followed by 9 (25%) *E.histolytica*. The samples collected from slaughters houseflies revealed 21 parasites; the most dominant parasite was *G. lamblia* 9 (42.9%) followed by *E*. *histolytica* 7(33.3%). Only eggs of helimenth (*H.nana*) were detected in hospital houseflies (Table 3).

DISCUSSION

Houseflies significantly increase the risk of exposure to a

Table 2. Type and frequency of bacteria isolated from slaughters houseflies.

Isolate	Surface		Intestine		Total	
	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage
E.coli	33	16.8	40	23.7	73	19.9
K.pneumoniae	9	4.6	20	11.8	29	7.9
P.aeruginosa	38	19.3	15	8.9	53	14.5
P. vulgaris	4	2.0	10	5.9	14	3.8
K. oxytoca	1	0.5	3	1.8	4	1.1
Y. pseudotuberclosis	2	1.0	1	0.6	3	0.82
Shigella spp	5	2.5	1	0.6	6	1.6
S.paratyphi B	3	1.5	13	7.7	16	4.4
P. marabilis			3	1.8	3	0.82
S. typhi A	6	3.0	2	1.2	8	2.2
S. typhimurium	8	4.0	6	3.6	14	3.8
C.freudii	8	4.0	6	3.4	14	3.8
E.cloacae	6	3.0	5	3.0	11	3.0
S. marcescence	1	1.0			1	0.27
Enterococci spp	29	14.7	37	21.9	66	18.0
S.aureus	26	13.2	6	3.6	32	8.70
Coagulase negative *Staphulococc*i	18	9.1	1	0.6	19	5.2
Total	197	100	169	100	366	100

Table 3. Type and frequency of parasites in houseflies collected from hospitals and slaughterhouses.

Parasite	Hospitals		Slaughterhouses	
	Frequency	Percentage	Frequency	Percentage
G.lamblia	18	50.0	9	42.9
E.histolytica	9	25.0	7	33.3
Entamoeba coli	5	13.9	5	23.8
Egg of H.nana	4	11.1	0	00
Total	36	100	21	100

wide range of foodborne pathogens due to their uncontrolled movements, ability to fly long or short distances, and attraction to cooked and uncooked food material (Ghalehnoo, 2015). Moreover, houseflies easily access to hospitals setting in developing countries and play critical role in transmission of nosocomial infections.

In this study, CLED agar was the medium of choice for bacterial isolation to recover all Enterobacteriaceae, most pathogenic bacteria because it inhibits swarming of *proteus* and related genera due to deficiency of electrolytes and enable the differentiation between lactose fermenting from non-lactose fermenting bacteria due to presence of bromothymol blue indicator (Collee et al., 1996).

The result of present study showed that the houseflies collected from hospitals environment were less contaminated, where each housefly carried 1.9 bacteria, compared to that obtained from slaughterhouses in which the housefly carried 2.44 bacteria. This result is relatively higher than that reported by Zurek and Nayduch (2016), who carried out study to investigate the bacteria on houseflies collected from hospital environment and stated 1.4 bacteria per housefly. Zhang et al. (2017) reported 1 bacteria per housefly. Our finding was contradictory to Zurek and Nayduch (2016), report who stated that houseflies collected from hospital were more contaminated. However, the number and type of bacteria is a function of place where these flies are captured (Nazari et al., 2017). The differences in the rate of isolation also related to the techniques used in the isolation of the microorganisms (Acevedo et al., 2009). The high rate of isolation in the present study may be

owing to the low level of hygiene services in studied environments. The majority of isolates in our study belong to the family *Enterobacteriaceae* (Gram negative rod), which could be interpreted by the fact that *Enterobacteriaceae* is the main family that inhabits the gastrointestinal tract of human and animal and it is excreted in their stool, which is an excellent source of nutrition for the housefly.

The present study revealed that the most dominant bacteria isolated from the hospitals houseflies were *E. coli* (15.6%), followed by *K. pneumoniae* (14.5%) and *Enterococci spp* (14.1%). These bacteria "*E. coli*, *K. pneumoniae*, *Enterococci*" are among most important pathogens causing different diseases ranging from urinary tract to pneumonia infections and septicemia, and have the ability to acquire and transfer antibiotic resistance genes (Park et al., 2019; Zurek and Nayduch, 2016; Ahmad et al., 2011). Ranjbar et al. (2016) showed that houseflies are potential vectors of antibiotic resistant *K. pneumoniae* and Ahmad et al. (2011) stated that *Enterococci* are considered a reservoir of antibiotic resistance genes to a wide range of antibiotics.

The presence of houseflies in slaughterhouses reduces the meat hygiene standards and can transfer a variety of pathogenic organisms (Songe et al., 2016). The present study showed that the most common bacteria isolated from slaughterhouses was *E. coli* 73 (19.9%), followed by *Enterococci spp* 66 (18.0%) and *P. aeruginosa* 53 (14.5%). These findings were relatively in alignment with different reports in which *E. coli*, *Klebsiella* spp and *Pseudomonas* spp., were the most dominant bacteria isolated from hospitals and slaughters environments (Songe et al., 2016; Davari et al., 2010; Cheesborough, 2006). Other reports showed *Bacillus* spp, *Staphylococcus* spp. (Zurek and Nayduch, 2016), *Providencia stuartii* (Zhang et al., 2017) and *Proteus mirabilis* (Davari et al., 2010) as common bacteria.

Results of the study indicated that the bacteria were isolated more frequently from the body surfaces than gut of the housefly. These findings agreed with Issa (2019); and Khamesipour et al. (2018) reports and disagreed with other reports that stated the amount of pathogens present in the intestine is generally higher than the quantity present on the body surfaces (Boiocchi et al., 2019; Davari et al., 2010). Houseflies have the ability to transfer the foodborne bacteria to their eggs and newly emerged generation adults (Pava-Ripoll et al., 2015), this process may provide a plausible environment for emerging bacterial strains with new properties involving acquired virulence and antibiotic resistance genes (Akhtar et al., 2009).

Very few studies reported parasites from the housefly (Khamesipour et al., 2018). Our study showed that *G. lamblia* and *E. histolytica* were most the dominant parasites detected in houseflies, similarly Manandhar and Gokhale (2017) mentioned that the *G. lamblia* is the most common human protozoan entero-pathogen worldwide.

Conclusions

Our study showed high load of bacteria per housefly and identified high virulence bacteria such as *E. coli*, *K. pneumoniae* and *Shigella* spp. This findings reflect the level of hygiene in the studied area and arise the alarm of consequent complications.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Acevedo GR, Zapater M, Toloza AC (2009). Insecticide resistance of house fly, *Musca domestica* (L.) from Argentina. Parasitology Research 105(2):489-493.

Ahmad A, Ghosh A, Schal C, Zurek L (2011). Insects in confined swine operations carry a large antibiotic resistant and potentially virulent enterococcal community. BMC Microbiology 11(1):23.

Akhtar M, Hirt H, Zurek L (2009). Horizontal transfer of the tetracycline resistance gene tetM mediated by pCF10 among Enterococcus faecalis in the house fly (*Musca domestica* L.) alimentary canal. Microbial Ecology 58(3):509-518.

Arora DR, Arora BB (2010). Medical parasitology/ 3rd ed. 234-238. New Delhi: CBS Publishers and Distributors.

Balla HJ, Usman Y, Muhammad A (2014). The role of housefly (*musca domestica*) in mechanical transmission of intestinal parasites in Nigeria. Journal of Natural Science Research 4(8):60-65.

Boiocchi F, Davies MP, Hilton AC (2019). An Examination of Flying Insects in Seven Hospitals in the United Kingdom and Carriage of Bacteria by True Flies (Diptera: Calliphoridae, Dolichopodidae, Fanniidae, Muscidae, Phoridae, Psychodidae, Sphaeroceridae). Journal of Medical Entomology 56(6):1684-1697.

Cheesborough M (2006). District laboratory practice in tropical countries, part 2, Cambridge Edinburgh: Tropical Health Technology.

Collee JG, Fraser AG, Marmion BP, Simmons A (1996). Mackie & Mccartney Practical Medical Microbiology. 14th ed. Elsevier.

Davari B, Kalantar E, Zahirnia A, Moosa-Kazemi Sh (2010). Frequency of resistance and susceptible bacteria isolated from houseflies. Iranian Journal of Arthropod-Borne Diseases 4(2):50-55.

GhalehnooMR (2015). Housefly (*Musca domestica*) as Carrier of Enterotoxigenic Staphylococcus aureus in Broiler Farms in Iran: Is it Important for Public Health. International Journal of Enteric Pathogens 3(3):e25688.

Issa R (2019). *Musca domestica* acts as transport vector hosts. Bulletin of the National Research Centre 43:73.

Khamesipour F, Lankarani KB, Honarvar B, Kwenti TE (2018). A systematic review of human pathogens carried by the housefly (*Musca domestica* L.). BMC Public Health 18(1):1049.

Kirby SC (2008). Fly management handbook, a guide to biology, dispersal and management of the house fly and related flies. New Haven.

Manandhar R, Gokhale S (2017). Are Houseflies Still Important Vector of Gastrointestinal Infections? Journal of Bacteriology and Parasitology 8:4.

Nazari M, Mehrabi T, Hosseini SM, Alikhani MY (2017). Bacterial contamination of adult house flies (*musca domestica*) and sensitivity of these bacteria to various antibiotics, captured from hamadan city, Iran. Journal of Clinical and Diagnostic Research 11(4):DC04-DC07.

Neupane S, Nayduch D, Zurek L (2019). House Flies (*Musca*

domestica) pose a risk of carriage and transmission of bacterial pathogens associated with bovine respiratory disease (BRD). Insects 10(10):358.

Park R, Dzialo M C, Spaepen S, Nsabimana D, Gielens K, Devriese H, Crauwels S, Tito RY, Raes J, Lievens B, Verstrepen KJ (2019). Microbial communities of the house fly Musca domestica vary with geographical location and habitat. Microbiome 7(1):147.

Pava-Ripoll M, Pearson RE, Miller AK, Tall BD, Keys CE, Ziobro GC (2015). Ingested Salmonella enterica, Cronobacter sakazakii, Escherichia coli O157:H7 and Listeria monocytogenes: transmission dynamics from adult house flies to their eggs and first filial (F1) generation adults. BMC Microbiology 15:150.

Ranjbar R, Izadi M, Hafshejani TT, Khamesipour F (2016). Molecular detection and antimicrobial resistance of Klebsiella pneumoniae from house flies (*Musca domestica*) in kitchens, farms, hospitals and slaughterhouses. Journal of Infection and Public Health 9(4):499-505.

Reuben RC, Gyar SD, Danladi MMA (2020). COVID-19: Probable involvement of insects in the mechanical transmission of novel coronavirus (2019-nCoV). Microbes and Infectious Diseases 1(3):111-117.

Solà-Ginés M, González-López JJ, Cameron-Veas K, Piedra-Carrasco N, Cerdà-Cuéllar M, Migura-Garcia L (2015). Houseflies (*Musca domestica*) as vectors for Extended-Spectrum β-Lactamase-Producing Escherichia coli on Spanish Broiler Farms. Applied and Environmental Microbiology 81(11):3604-3611.

Songe MM, Hang'ombe BM, Knight-Jones TJ, Grace D (2016). Antimicrobial resistant Enteropathogenic *Escherichia coli* and *Salmonella* spp. in Houseflies infesting fish in food markets in Zambia. International Journal of Environmental Research and Public Health 14(1):21.

Stelder JJ, Kjær LJ, Jensen LB, Boklund AE, Denwood M, Carlsen M, Bødker R (2021). Livestock-associated MRSA survival on house flies (*Musca domestica*) and stable flies (*Stomoxys calcitrans*) after removal from a Danish pig farm. Scientific Reports 11(1):3527.

Zhang J, Wang J, Chen L, Yassin AK, Kelly P, Butaye P, Li J, Gong J, Cattley R, Qi K, Wang C (2017). Housefly (*Musca domestica*) and Blow Fly (Protophormia terraenovae) as Vectors of Bacteria Carrying Colistin Resistance Genes. Applied and Environmental Microbiology 15; 84(1):e01736-17.

Zurek K, Nayduch D (2016). Bacterial Associations across house fly life history: evidence for transstadial carriage from managed manure. Journal of Insect Science 16(1):2.

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Full Length Research Paper

Cellulolytic activity of bacteria from the gut of termites (*Macrotermes michaelseni)* from Eldoret and Kakamega

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Cellulose degrading bacteria in termites are important plant degraders as they play an essential role in digesting cellulose for termites. This study aimed to identify cellulose degrading bacteria in termites (*Macrotermites michaelseni)* collected from Kakamega and Kapsabet region. Six termites were aseptically crushed in an Eppendorf tube containing distilled water. To distinguish cellulose degrading bacteria from non-cellulose degrading isolates, the homogenates were inoculated on nutrient agar and carboxymethyl cellulose media. As a result, 14 isolates from Kakamega termites and 3 isolates from Kapsabet showed cellulolytic activity on carboxymethyl cellulose media based on the existence of a clear zone around their colony out of 24 obtained from both termites. The highest cellulolytic index obtained was 5.8, while the lowest cellulolytic index obtained was 1.5. These findings suggest that termites harbor cellulose-degrading bacteria that can be used in cellulose degradation.

Key words: Cellulose, cellulose degrading bacteria, termites, *Macrotermes*.

INTRODUCTION

Cellulose is the most prevalent component of plant cell walls and the world's most abundant renewable bio-resource (Behera et al., 2017). Cellulose is a glucose-based polysaccharide containing glycosidic connections (Kameshwar and Qin, 2016) It accounts for 35-50% of the plant's dry weight, while hemicelluloses and lignin account for 20-35% and 5-30% of the plant's dry weight, respectively, (Behera et al., 2017). Cellulase is a high-capacity enzyme that works in tandem with three other cellulases to hydrolyze cellulose into glucose. Endo-1,4-glucanase, exoglucanase, and -D-glucosidase are the three forms of cellulose (Sakolvaree and Deevong, 2016). To digest cellulose, bacteria produce the enzyme cellulase during their development on cellulose (Sreena et al., 2015). Endo-glucanases, cellobiohydrolases (or exo-glucanases), and -glucosidases all work together to break down cellulose completely into glucose (Sharma et al., 2015; Sreena et al., 2015). The endoglucanses attack the various interior sites of the cellulose fiber amphorous area at random. This frees up potential attack sites for

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the exoglucanase in the future. By removing mono and dimers from the reducing and non-reducing ends of the glucose chain, cellobiose and oligosaccharides are produced. Finally, -glucosidase breaks down the cellobiose into glucose monomers. Glucose is transported across the membrane to engage in energy-generating metabolic processes. Carboxymethyl cellulose (CMC) has been utilized extensively in the investigation of gut microbes that produce endo-1,4-glucanase. Due to the rise of cellulase applications in various sectors, cellulase is currently preferred as the third enzyme for industrial demand in the world, and it is predicted to become the largest amount of industrial enzyme in the future (Sakolvaree and Deevong, 2016).

Bacteria in soils, mammals, and termites have all been demonstrated to be capable of degrading complex cellulose in extensive investigations (Kameshwar and Qin, 2016; Sreeremya et al., 2016). Termite bacteria, on the other hand, are the most efficient cellulose degraders (Kudo, 2009; Mikaelyan et al., 2015), as evidenced by their ability to devour wood that is difficult to breakdown in nature (Auer et al., 2017). Macrotermitinae is a subfamily of the termites knows as fungus-growers (Vesala et al., 2017). They are among the most numerous and dominant insects in Asia's tropical and subtropical habitats, as well as the African rainforest (Ali et al., 2019; Femi-Ola and Oyebamiji, 2019). In several areas of Africa's savanna, they are the main decomposers of plant biomass (Vesala et al., 2017). Macrotermitinae are distinguished by their massive mound structures and underground gallery networks (Dangerfield et al., 1998; Sileshi et al., 2009). They build mounds to keep moisture, humidity, gas exchange, and temperature conditions stable, while the underground gallery system is used to gain access to foraging areas (Dangerfield et al., 1998; Vesala et al., 2017). They build mounds to maintain moisture, humidity, gas exchange, and temperature condition while the underground gallery system is used for accessing foraging areas (Dangerfield et al., 1998; Vesala et al., 2017). Remarkably is the termite's unique symbiotic relationship with the fungus termitomyces (Femi-Ola and Oyebamiji, 2019; Nobre, 2010). The symbiotic fungus grows on a fungus comb made by the termite.

The accumulation and underutilization of plant biomass in the ecosystem has been a global issue, prompting many scientists to perform research that will contribute to the reuse of this plentiful and renewable resource into valuable goods that will lead to the world's sustainable development. Researchers observed that the symbiotic micro-organisms in the intestines of termites assist in digesting termites.

Although effective isolation and identification with cellulolytic activity of these microbes were attempted, some species remained uncultured and therefore uninvestigated. As a result, there is a need to research efficient cellulolytic bacteria for the conversion of cellulose into glucose.

The purpose of this work was to isolate and detect active cellulolytic bacteria in the complete body of *Macrotermes michaelseni* that can be exploited in plant cellulose breakdown.

MATERIALS AND METHODS

Sample collection and processing

The termite samples were collected in January, 2018 in Vihiga that lies between 0.0768N latitude and 34.7078E longitude in the eastern part of Kakamega forest that is 1500-1600 m above sea level. The other termite samples were collected from the termite mound surrounding Kimondi River in Kapsabet. Kapsabet is a town in Nandi County located 40 km southwest of Eldoret on the way to Chavakali. It lies between latitude 0034N and longitude 34045E. The termite hills in Vihiga and Kapsabet were dug with a hoe and a piece of termite mound was selected. Six mound fragments were placed in separate boxes and transported to the laboratory for further examination. The boxes were kept in a cabinet at 25°C in the laboratory. Soldier castes were stored in 70% ethanol and utilized for morphology-based termite identification, while worker castes were employed for bacterial isolation within 24 h after sampling (Sakolvaree and Deevong, 2016).

Six worker termite were randomly chosen from the six mound fragments and sterilized with 70% ethanol for ten minutes and rinsed in sterile distilled water for another minute (Femi-Ola and Oyebamiji, 2019). Each termite were crushed with a glass rod in an Eppendorf tube with 1.5 µl microliters of distilled water to create a paste for bacteria isolation. The tubes were shaken for 24 h at 37°C at a speed of 150 rpm on a shaker (Kavitha et al., 2014).

Isolation and purification of bacteria

Each homogenous solution were plated on nutrient agar composed of (peptone, beef extract, sodium chloride, agar and distilled water) for the cultivation of bacteria and incubated at 37°C for seventy-two hours. Single distinct colonies that appeared on the plate were picked and re- grown on a new nutrient agar plate for twenty-four hours until pure isolates were obtained (Kavitha et al., 2014).

Morphological characteristics of bacterial isolates

Morphological characteristics of the pure isolates' colonies, such as form, surface, margin, and color, of the colonies on the plate were observed (Sharma et al., 2015). Gram staining was also done to see whether the twenty-four isolates were Gram-negative or Gram-positive (Ayitso and Onyango, 2016). This process was performed by the collection of a portion of the colony using an inoculating loop under aseptic conditions, which was transferred to a water-containing slide to form a thin coating. The slide was then passed through the Bunsen burner to fix the bacteria. The slide cooled in the air and then poured crystal purple stain for a minute, followed by a wash through flowing tap water. Following that, the slides were drenched with Gram iodine for a minute before being rinsed with water. To prevent the cells from bleaching, the decolorizer was poured for a few seconds and promptly washed with water. Finally, the saffron stain was put on the slide to stain the Gram-negative bacteria for two minutes, rinsed with water, and dried. The slides were viewed under a microscope by applying oil immersion and analyzed under 100x objectives lens to determine whether or not the bacteria were Gram-positive (purple or blue) or Gram-negative

Table 1. Morphological characteristics of the bacterial isolates

S/N	Isolate	Termite	Shape	Surface	Colour	Shape of bacteria	Gram stain
1	KG11	Macrotermes	Irregular	Raised	Cream	Bacillus	Negative
2	KG12	Macrotermes	Spherical	Raised	Cream	Coccus	Positive
3	KG13	Macrotermes	Spherical	Raised	Orange	Coccus	Negative
4	KG14	Macrotermes	Irregular	Raised	Brownish	Coccus	Negative
5	KG15	Macrotermes	Irregular	Raised	Cream	Bacillus	Positive
6	KG16	Macrotermes	Spherical	Raised	Red	Bacillus	Negative
7	KG21	Macrotermes	Spherical	Raised	Yellow	Coccus	Positive
8	KG22	Macrotermes	Spherical	Raised	Cream	Coccus	Negative
9	KG23	Macrotermes	Spherical	Raised	Cream	Coccus	Negative
10	KG24	Macrotermes	Spherical	Raised	Greyish	Coccus	Negative
11	KG25	Macrotermes	Spherical	Raised	Greyish	Bacillus	Positive
12	KG26	Macrotermes	Spherical	Raised	Red	Bacillus	Negative
13	KG31	Macrotermes	Spherical	Raised	Yellow	Bacillus	Positive
14	KG32	Macrotermes	Spherical	Raised	Orange	Bacillus	Positive
15	KG33	Macrotermes	Spherical	Raised	White	Coccus	Positive
16	KG34	Macrotermes	Spherical	Raised	Cream	Coccus	Positive
17	KG35	Macrotermes	Irregular	Raised	Yellow	Coccus	Negative
18	KG36	Macrotermes	Irregular	Raised	Cream	Coccus	Negative
19	EG11	Macrotermes	Spherical	Raised	Cream	Bacillus	Positive
20	EG12	Macrotermes	Spherical	Raised	Cream	Bacillus	Positive
21	EG13	Macrotermes	Spherical	Raised	Orange	Coccus	Positive
22	EG21	Macrotermes	Oval	Flat	Greyish	Bacillus	Positive
23	EG31	Macrotermes	Filamentous	Flat	Orange	Coccus	Positive
24	EG32	Macrotermes	Spherical	Raised	Orange	Coccus	Positive

(red) and cocci or bacillus.

Screening for carboxymethyl cellulose hydrolyzing bacteria

The isolates obtained were grown on the agar medium, added by 1% level CMC at 37°C for 48 h. Thereafter, a gram's iodine solution was poured onto the plates for 15 min. Afterward, the solution was poured out and observed in the presence of a clear zone around the colonies. A clear zone around colonies suggested the bacterial synthesis of extracellular cellulase. The cellulolytic potential of the positive isolates was assessed using the cellulolytic index (CI), which is defined as the ratio of the diameter of the zone of hydrolysis to the diameter of the colony mentioned by Saini et al. (2017).

RESULTS

Sample collection and processing

Termites used in this study were collected from two different locations in Kakamega and Kapsabet. In both sites, termites were found in mounds that were stronger than normal soil. In Kakamega, the termites mound contained fungus combs in them with and the mound was constructed in with plant stems as opposed to the Kapsabet termite's mound, which was made up of networks linked to each other. The termites collected were identified as *Macrotermes michaelseni* by an entomologist at the University of Nairobi.

Isolation and purification of bacteria

A total of twenty four isolates were obtained from Kakamega and Kapsabet termite after successful purification of the isolates. The isolates were denoted as KG from Kakamega and EG from Kapsabet (Table 1).

Morphological characteristics of bacterial isolates

Colony characteristics of each isolates showing different characteristics in shape, elevation margin and color are presented in Table 1. 17 of the twenty-four isolates had circular shapes and elevated surfaces, 5 were irregular, and the other two had oval and filamentous shapes, respectively. The gram staining procedure revealed that the majority of the isolates stained gram positive, while 9 isolates stained gram negative (Table 1). On the other

Table 2. Cellulolytic activity of the seventeen isolates.

Isolate	Location	Diameter of clear zone (mm)	Diameter of colony (mm)	Cellulolytic index
KG12	Kakamega	7	5	0.15
KG13	Kakamega	8	6.9	0.40
KG14	Kakamega	29	16	0.81
KG15	Kakamega	29	23	0.26
KG16	Kakamega	79	45	0.76
KG21	Kakamega	18	5	2.60
KG23	Kakamega	15	3	4.00
KG24	Kakamega	24	18	0.33
KG25	Kakamega	32	15	1.13
KG26	Kakamega	67	41	0.63
KG31	Kakamega	20	10	1.00
KG32	Kakamega	10	5	1.00
KG33	Kakamega	17	7	1.43
KG35	Kakamega	16	5	2.20
EG11	Kapsabet	31	6	4.17
EG12	Kapsabet	41	6	5.83
EG21	Kapsabet	22	17	0.29

hand, morphological presence on the microscope showed that 10 isolates were rods and 14 isolates were spherical.

Screening for carboxymethyl cellulose hydrolyzing bacteria

To assess cellulolytic activity, all twenty-four isolates were subjected to a carboxymethyl cellulase activity. As a result, 17 isolates had a clear zone surrounding their colony, suggesting carboxymethy cellulose activity. The cellulolytic activity of the isolates after staining with gram's iodine is shown in Table 2.

All the twenty four isolates were screened for carboxymethyl cellulase activity to determine their cellulolytic activity. As a result, 17 isolates showed presence of clear zone around their colony thus indicating carboxymethy cellulose activity. Among 17 isolates that showed clear zone, 14 isolates came from termites in Kakamega while 3 isolates were from Kapsabet. Based on cellulolytic index among Kakamega termites, isolate KG23 showed the highest cellulolytic index of 4.00 while the lowest was KG12 with cellulolytic index of 0.16 respectively. In contrast, isolate EG 12 from Kapsabet termite had the highest cellulolytic index (5.83), while isolate EG21 had the lowest (0.29).

DISCUSSION

Sample area

This study successfully isolated cellulose degrading bacteria in *Macrotermites michaelseni* collected from Kakamega and Kapsabet. This study supports the idea that termites harbour celluloytic bacteria inside their gut (Gupta et al., 2012; Kakkar et al., 2015). After culturing on nutrient agar, a total of twenty-four isolates were collected. Kakamega termites had a higher bacterial population than Kapsabet termites. This research backs up previous findings showing the presence of bacteria in termites (Ferbiyanto et al., 2015; Pourramezan et al., 2012). While it is known that one termite has a large number of microbial species in its gut, only a few were culturable during this research. One possible explanation for this may be the difficulty in simulating natural environments in the gut atmospheres.

Isolation and purification of bacteria

The morphological characteristics of the bacterial isolates obtained in this study varied and differed between the two sites. When opposed to Kapsabet termites, Kakamega termites had a higher diversity of culturable bacteria. These findings are consistent with those of Kavitha et al. (2014) and Ntabo et al. (2010), who isolated diverse bacteria in soil feeding termites and surrounding soil from Juja and Kakamega forest termites in Chennai, respectively.

Some of the isolates grew faster and were visible after twenty four hours while other became clearly visible after forty eight hours of incubating. The majority of the cells were able to maintain violet stain, suggesting gram positive organisms, whereas fewer cells were able to keep primary stain, indicating gram negative organisms.

However, these findings contradict those of Ayitso and Onyango (2016), who found that gram negative microbes outnumber gram positive ones. Microorganisms may be endemic to certain geographical regions due to differences in soil composition, food type, rainfall received, and agricultural activity carried out in the area, all of which may influence the sort of bacteria found in termites (Ayitso and Onyango, 2016).

As previously observed in other termites, the majority of the isolates collected in this study were found to be rods, indicating that they belonged to the genus *Bacilli*. Other isolates were cocci in form. These findings are consistent with previous research that has consistently found *bacillus* to be the dominant isolate. For example, the frequency of *Bacillus* in the *M. michaelseni* worker and soldier was observed by Ayitso and Onyango (2016). *Bacillus* in subterranean *Psammotermes hypostoma Desneux* has also been discovered in another study by Ali et al. (2019).

Screening for carboxymethyl cellulose hydrolyzing bacteria

Cellulolytic bacteria produce cellulases enzymes that break down the glycosidic bonds between cellulose microfibrils, releasing oligosaccharides and improving cellulose digestion (Hidayat, 2021). This enzyme aids in the breakdown of complex cellulose compounds into smaller molecules, allowing bacteria to digest them (Peristiwati and Herlini, 2018). All plates were screened using 1% carboxymethyl cellulose media to test the isolates' cellulolytic capability, as described above. The presence of endoglucanase was demonstrated by the creation of a clear zone after pouring gram's iodine (Hidayat, 2021).

Only seventeen isolates had clean zones surrounding their colonies, despite the fact that 24 isolates developed effectively on carboxymethyl cellulose media. The cellulose in the media was hydrolyzed as a result of the bacteria's cellulolytic enzymes. As a result of the binding of gram's iodine with polysaccharide during the hydrolysis process, clear zones formed around the colony, forming a clear zone (Gohel et al., 2014).

Cellulolytic index was used to test the cellulolytic potential of the positive isolates. This was achieved by dividing the diameter of the clear zone by the diameter of the colony. According to Hidayat (2021), isolates with cellulolytic index greater than 1.50 are regarded as potential cellulose producers. The cellulolytic index of the 17 isolates that formed clear zone around their colony varied from one organism to another. The highest cellulolytic index obtained was 5.8, while the lowest cellulolytic index is 1.5 as mentioned above. These findings are in agreement with those obtained by Kakkar et al. (2015), who determined cellulolytic activity in *Odontotermes parvidens* guts. However, the highest cellulolytic index (3.50) value reported in their study was lower than the one obtained from this study. Another study by Hidayat (2021) obtained similarly cellulolytic index results ranging from 1.16 to 4.89 as obtained from this study.

Conclusion

This study indicated the existence of cellulose degrading bacteria in termites to break down carboxymethyl cellulose media, indicating their potential in cellulose degradation. The cellulolytic bacteria obtained in this study can be employed in plant waste, production biofuel thus leading to sustainable development.

Recommendation

To better understand the mechanism of plant cellulose degradation, more research should be done to evaluate the efficacy of these isolates in degrading plant biomass in order to improve its long-term use in biotechnology, biofuel, and bio products.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

Ali HR, Hemeda NF, Abdelaliem YF (2019). Symbiotic cellulolytic bacteria from the gut of the subterranean termite *Psammotermes hypostoma Desneux* and their role in cellulose digestion. AMB Express 9(1).

Auer L, Lazuka A, Sillam-Dussès D, Miambi E, O'Donohue M, Hernandez-Raquet G (2017). Uncovering the Potential of Termite Gut Microbiome for Lignocellulose Bioconversion in Anaerobic Batch Bioreactors. Frontiers in Microbiology 8:2623.

Ayitso AS, Onyango DM (2016). Isolation and Identification by Morphological and Biochemical Methods of Antibiotic Producing Microorganisms from the gut of *Macrotermes michaelseni* in Maseno, Kenya. Journal of Applied Biology and Biotechnology 4(01).

Behera BC, Sethi BK, Mishra RR, Dutta SK, Thatoi HN (2017). Microbial cellulases – Diversity & biotechnology with reference to mangrove environment: A review. Journal of Genetic Engineering and Biotechnology 15(1):197-210.

Dangerfield JM, Mccarthy TS, Ellery WN (1998). The mound-building termite *Macrotermes michaelseni* as an ecosystem engineer. Journal of Tropical Ecology 14(4):507-520.

Femi-Ola TO, Oyebamiji BA (2019). Molecular Characterization and Cellulolytic Activities of Bacterial Isolates from the Hindgut of Wood-

feeding Termites *Amitermes evuncifer Silvestri*. Journal of Advances in Microbiology 14(2):1-10.
Ferbiyanto A, Rusmana I, Raffiudin R (2015). Characterization and Identification of Cellulolytic Bacteria from gut of Worker *Macrotermes gilvus*. HAYATI Journal of Biosciences 22(4):197-200.
Gohel HR, Contractor CN, Ghosh SK, Braganza VJ (2014). A comparative study of various staining techniques for determination of extra cellular cellulase activity on Carboxy Methyl Cellulose (CMC) agar plates. International Journal of Current Microbiology and Applied Sciences 3(5):261-266.
Gupta P, Samant K, Sahu A (2012). Isolation of Cellulose-Degrading Bacteria and Determination of Their Cellulolytic Potential. International Journal of Microbiology, pp. 1-5.
Hidayat MR (2021). Isolation and Identification of Cellulolytic Bacteria Symbiont from Various Termites on Different Nest Type in Bukit Baka Bukit Raya National Park, West Kalimantan, Indonesia. Walailak Journal of Science and Technology (WJST) 18(14):12708-12.
Kakkar N, Sanjeev KG, Baljeet SS (2015). Studies on cellulolytic activity and structure of symbiotic bacterial community in Odontotermes parvidens Guts. International Journal of Current Microbiology and Applied Sciences 4(10):310-315.
Kameshwar A, kumar S, Qin W (2016). Recent Developments in Using Advanced Sequencing Technologies for the Genomic Studies of Lignin and Cellulose Degrading Microorganisms. International Journal of Biological Sciences 12(2):156-171.
Kavitha D, Vijayarani K, Kumanan K (2014). *16S rRNA* Typing of cellulolytic bacteria from the termite *Odontotermes formosanus*. Indian Journal of Veterinary and Animal Sciences Research 43(5):359-368.
Kudo T (2009). Termite-Microbe Symbiotic System and Its Efficient Degradation of Lignocellulose. Bioscience, Biotechnology and Biochemistry 73(12):2561-2567.
Mikaelyan A, Dietrich C, Köhler T, Poulsen M, Sillam-Dussès D, Brune A (2015). Diet is the primary determinant of bacterial community structure in the guts of higher termites. Molecular Ecology 24(20):5284-5295.
Nobre T (2010). Dispersion and colonisation by fungus-growing termites. Communicative and Integrative Biology 3(3):248-250.
Ntabo R, Boga H, Muigai A, Mwirichia R (2010) Isolation and characterization of bacteria isolates from soil feeding termites and soil from Juja and Kakamega forest in Kenya. In: scientific technological and industrialization conference, Jomo Kenyatta University of Agriculture and Technology 2010. Digital Repository.
Peristiwati NYS, Herlini H (2018). Isolation and identification of cellulolytic bacteria from termites gut (*Cryptotermes sp.).* Journal of Physics: Conference Series 1013:012173.
Pourramezan Z, Ghezelbash GR, Romani B, Ziaei S, Hedayatkhah A (2012). Screening and identification of newly isolated cellulose-degrading bacteria from the gut of xylophagous termite *Microcerotermes diversus* (Silvestri). Microbiology 81(6):736-742.
Saini A, Aggarwal NK, Yadav A (2017). Isolation and Screening of Cellulose Hydrolyzing Bacteria from Different Ecological Niches. Bioengineering and Bioscience 5(1):7-13.
Sakolvaree J, Deevong P (2016). Isolation and Characterization of Cellulase Producing Bacteria from the Gut of a Higher Termite, *Termes propinquus*.
Sharma D, Joshi B, Bhatt MR, Joshi J, Malla R, Bhattarai T, Sreerama L (2015). Isolation of Cellulolytic Organisms from the Gut Contents of Termites Native to Nepal and Their Utility in Saccharification and Fermentation of Lignocellulosic Biomass. Journal of Biomass to Biofuel 2:11-20.
Sileshi GW, Nyeko P, Nkunika POY, Sekematte BM, Akinnifesi FK, Ajayi OC (2009). Integrating Ethno-Ecological and Scientific Knowledge of Termites for Sustainable Termite Management and Human Welfare in Africa. Ecology and Society 14(1).
Sreena C, Resna N, Sebastian D (2015). Isolation and Characterization of Cellulase Producing Bacteria from the Gut of Termites (*Odontotermes* and *Heterotermes* Species). British Biotechnology Journal 9(1):1-10.
Sreeremya S, Nishaa S, Rajiv P (2016). Optimization of Conditions and Production of Carboxy Methyl Cellulase by Bacteria Isolated from Higher Termite Soil. Journal of Bioprocessing and Biotechniques 6(2).
Vesala R, Niskanen T, Liimatainen K, Boga H, Pellikka P, Rikkinen J (2017). Diversity of fungus-growing termites (*Macrotermes*) and their fungal symbionts (*Termitomyces*) in the semiarid Tsavo Ecosystem, Kenya. Biotropica 49(3):402-412.

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