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Table of Content

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Full Length Research Paper

ICU COVID-19 patients with bacterial and fungal super-infections in Saudi Arabia

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Bacterial and fungal infections in COVID-19 patients were reported all over the world. A retrospective study was conducted to determine the prevalence of bacterial and fungal superinfections in COVID-19 positive patients between April 2020 and December 2021. This research included patients admitted in a specialized medical center in Saudi Arabia. A total of 2643 patients with confirmed diagnosis of COVID-19 via reverse transcriptase-polymerase chain reaction assay (RT-PCR) conducted on nasopharyngeal swab and chest computed tomography. The age forty-nine years (SD 12·5) 2087 were men (79%). Patients with positive cultures for bacteria or fungi were included in the study, and patients with negative cultures were excluded. Total number of COVID-19 patients who acquired bacterial infection was 509 (19%) and two hundred forty-six (246) of COVID-19 patients were admitted to ICU and were under mechanical ventilation (9.3%). All mechanically ventilated cases developed bacterial ventilator associated pneumonia. Multidrug resistant organisms represent 97%. Extended spectrum b-lactamase comprise 35.1%, Methicillin resistant *Staph aureus* 32.2%, vancomycin resistant Enterococci 2.1%. Clostridium difficile was 1.7%. 46 patients under mechanical ventilation acquired fungal superinfection (18.6%) with long duration of mechanical ventilation more than 14 days (p value=0.019) and under dexamethasone treatment (p value=0.027). Candida is the most common type of fungi isolated (89%). *Candida auris* isolated from three cases (6.5%), one case developed invasive mucormycosis (2%) one acquired invasive Aspergillosis (2%). 5 patients died (11%). This research tries to provide factual evidence of types of bacterial and fungal superinfection in COVID-19 cases and implement prevention and control measures of infection.

Key words: Bacteria, fungal, COVID-19, infections, PCR.

INTRODUCTION

Viral pneumonia frequently leads to bacterial and fungal infections, particularly in critically unwell individuals. They increase mortality and the requirement for intensive care. There are many ways in which human fungi illnesses are fundamentally different from other infections. Fungi are eukaryotic pathogens that resemble their host cells, which hinder the synthesis of antifungal substances (Brachman, 2003). There are more immunosuppressed patients, and many of them are quite vulnerable to fungus infections. The so-called systemic mycoses, which are fungal invasive infections, have a significant negative impact on human health. Also emphasized by the Global

Action Fund for Fungal Infections (GAFFI) is the catastrophic effect that focused fungal illnesses have on people, many of whom have healthy immune systems (GAFFI, 2018). Invasive microbial co-infections can occur during hospitalization in COVID-19 patients who have been hospitalized to intensive care units (ICU), especially for a lengthy time. These co-infections have the potential to worsen patient outcomes (Arastehfar et al., 2021). The multi-resistant pathogen, *Candida auris*, which causes infections in immunocompromised people that are resistant to all main classes of antifungal medications, has just been identified as a serious global danger to human health (Clancy and Nguyen, 2017). Invasive fungal infections are a common disease in the fields of critical care and pulmonary medicine, and they commonly present diagnostic difficulties. Among the most typical are candidemia and invasive aspergillosis. Galactomannan (GM) and polymerase chain reaction (PCR) testing in both blood and bronchoalveolar lavage (BAL), as well as other laboratory methods for Aspergillus detection, have grown in importance in recent years for the diagnosis of this infection (Ramanan et al., 2017). An infection that is becoming more prevalent in the critical care setting and one that increases mortality and resource utilization in intensive care units (ICUs) is invasive candidiasis (IC), which most frequently manifests as candidemia (Magill et al., 2014). Only invasive sample or culture can provide a conclusive diagnosis of IC, but relying on these conventional techniques runs the risk of failing to identify this potentially fatal illness in a timely manner. Imaging and laboratory tests are used to detect invasive fungal infections (IFD). Smears, cultures, serological testing, and the G test are a few other tests. These tests have some restrictions even though they can detect IFD with accuracy. While fungal smears only take a few minutes but have significant false-negative rates, fungal cultures take two days. Additionally, serological testing, especially if just one test is used, cannot differentiate between present illnesses and previous infections. The antifungal antibody or antigen is also influenced by an individual's level of immunity. Although sensitive, polymerase chain reaction (PCR) testing is unable to detect the survival and proliferation of the fungus (Verma et al., 2019).

In a study that looked at the prevalence of bacterial superinfection in COVID-19 patients, Catano-Correa et al., 2021 found that 49.6% of the patients had sixteen bacterial isolates superinfected them. *Staphylococcus aureus* and *Klebsiella* (pneumoniae and Oxytoca) were the most prevalent. The prognosis and course of COVID-19 patients' bacterial superinfection are related. The admittance to intensive care units, the use of antibiotics, and death are all increased by this circumstance (Cataño-Correa et al., 2021). According to the recommendations of the European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG), invasive fungal disease (IFD) was identified (Donnelly et al., 2020). The following are the EORTC/inclusion MSG's criteria for patients with IFD: Fever, cough, shortness of breath, and other respiratory symptoms with a recent history of IFD diagnosis, treatment, radiotherapy, chemotherapy, or hormone use; a positive sputum fungal culture and/or sputum fungal smear; a positive G test; and the detection of elevated levels of interleukin-6, procalcitonin, or both. IFD is identified and diagnosed using the G test. The idea behind this procedure is that the (1,3)-D-glucan in the fungal cell wall can activate the G factor to catalyze the coagulation cascade in the Limulus plasma. A dynamic turbidimeter is then used to identify the subsequent conversion of fibrinogen into fibrin. It could be challenging to find a particular antibody for this use because (1,3)-D-glucan is a sugar with variable levels of polymerization. GBPs are naturally occurring proteins that bind to glucan and can be found in people, animals, plants, and even microorganisms. Compared to other GBP, the clotting factor G alpha subunit (GFSub) from Limulus polyphemus was more easily detectable (Verma et al., 2019)

In order to better treat concurrent COVID-19 infections in individuals with risk factors, it was discovered in our studies that isolated bacterial and fungal infections were common among COVID-19 positive patients. In addition, multidrug resistant bacteria and fungi isolated among these risk groups were identified to control its spread.

Bacteriological, mycological smear examination and cultivation, galactomannan (GM) detection test in addition to VITEK® 2 bacterial and yeast identification cards (YST) machine systems 7.01 and 8.01 software were used to differentiate most clinically significant bacterial, yeasts and yeast-like isolated from the COVID-19 patients.

MATERIALS AND METHODS

According to the Saudi Arabian Ministry of Health's regulations for medical research, the study was approved by the research committee of the specialized Medical Center hospital. The patients approved the use of their clinical data for study when they signed an informed permission form before being admitted to the hospital.

Study type and subjects studied

The study, which began in April 2020 and ended in December2021,

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was retrospective in nature. It was carried out on patients in a specialist medical facility in Saudi Arabia, one of the main facilities for treating SAR-CoV-2 patients, who ranged in age from 18 to 80. Reverse transcriptase-polymerase chain reaction (RT-PCR) testing on nasopharyngeal swab samples and chest computed tomography (CT) scans with characteristic symptoms were used to confirm the diagnosis of COVID-19 in a total of (2643) patients.

All COVID-19 positive patients that were admitted to either the ICU with or without the need for mechanical ventilation, patients that isolated in an isolation ward and patients under home isolation without need for hospital admission were eligible for follow up to detect any signs for bacterial or fungal superinfection.

People over 60, diabetes mellitus, cardiovascular disease (coronary artery disease or chronic heart failure), lung disease (chronic obstructive pulmonary disease, emphysema, asthma), immunosuppression (prednisone > 20 mg/day for more than 14 days, methotrexate > 0.4 mg/kg/week, or biologic therapy); or poor prognostic factors like lymphopenia were among the patients who were admitted to the hospital (basal consolidation, nodules, cavitation or pleural effusion) (Cataño-Correa et al., 2021).

Determination of bacterial and fungal superinfection

The following three diagnostic criteria had to be present 48 h or more after admission (represent as new-onset, not beginning or before of admission) in order to confirm that it was a superinfection. This was the basis for the diagnosis of bacterial and fungal superinfection in patients admitted with COVID-19 (that is, the emerging infection during the course of illness):

1. Clinical criteria include deterioration of ventilatory parameters, purulent sputum, prolonged fever (higher than 38.3°C), hemodynamic instability requiring vasopressor support, and purulent sputum.
2. Leukopenia or leukocytosis worsening as well as an increase in procalcitonin or C-reactive protein constitute paraclinical criteria.
3. Radiological requirements: Deterioration of the chest radiological pattern or a pattern that is not COVID-19-specific (basal consolidation, nodules, cavitation, or pleural effusion). When these three diagnostic criteria were met, sputum, tracheal aspirate, bronchoalveolar lavage if applicable were collected to identify the mycological and bacterial etiological agent responsible for the possible superinfection.

The bronchoscopic method known as bronchoalveolar lavage (BAL) was utilized to collect microbiological samples from 246 COVID-19-critically ill patients' lower respiratory tracts. Procedures were carried out using pressure-controlled breathing and intravenous sedation. In every instance, disposable scopes were utilized, and just a small team was present at the patient's bedside. Before the procedure, all necessary tools and supplies, including saline, syringes, mucoactive medications, microbiological recipients, connectors, and bronchoscopy system were prepared outside the patient room (scope and screen). N95 or FPP3 masks, goggles, double gloves, and a plastic protective gown with a head and neck cover were all utilized as level III personal protective equipment. Sputum samples were collected from 263 patients with COVID-19 that were not mechanically ventilated and able to provide sputum samples. The same safety precautions were followed during the collection. The sputum cultures were evaluated as follows if two sputum cultures were collected within 48 h of each other and provide identical results with radiographic evidence of pneumonia. This culture was considered as a single positive culture infection and the isolated organism counted and specified. On the other hand, the culture was regarded as colonization if organism(s) with of a change in the appearance of the infiltrate. The distinction normal oral flora or yeast were growing without radiographic signs between the diagnoses of infection and colonization was made based on a review of concurrent notes from the attending infectious diseases consultants, pulmonary critical care specialists, and/or internists/hospitalists. Hospital antibiotic orders also assisted in making this distinction.

In this study, the galactomannan (GM) detection test and sample examination by smear and mycological culture for fungal diagnosis was used in addition to VITEK® 2 bacterial and yeast identification card (YST) machine systems 7.01 and 8.01 software to differentiate most clinically significant yeasts and yeast-like. Patients with negative bacterial or fungal cultures were not included in the study; only those with positive cultures were. Patient demographics, comorbidities, clinical characteristics, laboratory results, microbiological analysis, hospital stay, length of mechanical breathing if necessary, immunosuppressive use, line days outcome, and antifungal and antibiotic therapy were all recorded in the data collection.

Once the microbiological isolation was determined, automated sensitivity tests were carried out using the VITEK-2® system (bioMerieux 8.01), adhering to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) version M100. The isolation of a single or predominant bacterium was considered positive for bacterial culture (31edition). Patients with negative bacterial or fungal cultures were not included in the study; only those with positive cultures were. Patient demographics, comorbidities, clinical characteristics, laboratory results, microbiological analysis, hospital stay, length of mechanical breathing if necessary, immuno-suppressive use, line days outcome, and antifungal and antibiotic therapy were all recorded in the data collection.

Once the microbiological isolation was determined, automated sensitivity tests were carried out using the VITEK-2® system (bioMerieux 8.01), adhering to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) version M100. The isolation of a single or predominant bacterium was considered positive for bacterial culture (31edition) (Clinical Laboratory Standard Institute (CLSI), 2021)

To cultivate fungi, primary microbiological cultures were carried out on Columbia agar, chocolate agar, and thioglycolate broth (prepared culture media, Becton Dickinson, Sparks, MD, USA) (Oxoid Themo Fisher Scientific, Waltham, MA, USA). We subcultured Mucorales colonies on Sabouraud dextrose agar (Oxoid Thermo Fisher Scientific, Waltham, MA, USA). To distinguish between *C. albicans*, *C. tropicalis*, *C. krusei*, *C. glabrata* and Cryptococcus, chromagar Candida was used. Additionally, the most clinically relevant yeasts and yeast-like organisms were distinguished using the VITEK® 2 Yeast Identification Card (YST) machine systems 7.01 and 8.01 software (VITEK® 2 Systems 7.01 and 8.01 Software, 2018). Calcofluor or Blankophor were utilized to boost the sensitivity and specificity for recognizing *Aspergillus*-like features in direct microscopic inspection using the optical brightener methods for *Aspergillus* spp. conclusive confirmation by culture or non-culture approach, including; If the experiment was successful, Aspergillus morphological characteristics, including the distinctive acute angle branching septate hyphae of Aspergillus spp., were discovered under a microscope. Serum samples were used to carry out galactomannan (GM) detection (PlateliaTM Aspergillus Ag, Bio-Rad Laboratories, Munich, Germany) (PLATELIA™ ASPERGILLUS EIA). Agar plates with primary cultures were cultured for 48 h. *Aspergillus* spp. were subcultured on sabouraud-dextrose-agar (OxoidTM Thermo Fisher ScientificTM, Waltham, MA, United States of America) once growth of the species could be established. Before species identification, *Aspergillus* subcultures on sabouraud-dextrose-agar were cultured for 48 to 72 h for macroscopic and microscopic inspection. In accordance with Ministry of health of Saudi Arabia guidelines, all positive multidrug resistant bacteria strains and positive fungal infections in COVID -19 patients were sent for confirmation in the central regional laboratory.

Table 1. Prevalence of bacterial and fungal coinfections isolated from respiratory samples in ventilator admitted COVID-19 patients.

Item	Patients' numbers	Percentage	P value
Total number of positive COVID patient	2643		
Total number of patients with ventilator admitted ICU	246	9.3	p<0·0005
Total number of patients with ventilator associated bacterial infection	246	100	p<0·0001
Total number of patients with fungal superinfection	46	18.6	p<0·0005

Source: Authors

Statistical analysis

Both absolute (n) and relative percentage frequencies were used to characterize the variables. the overall prevalence of bacterial, fungal, and agent-specific superinfection were calculated. Using the Pearson Chi-square test (for nominal variables) or the Chi-square test for trend, if sociodemographic and clinical factors were related to bacterial and fungal superinfection (for ordinal variables) was determined. The prevalence ratios with a 95% confidence interval were calculated for the related sociodemographic and clinical parameters. A multivariate generalized linear model using the logarithm transformation and the binomial family was used to control confounding variables (log-binomial) (Thompson et al., 1998).

RESULTS

Between April 2020 and December 2021, 2643 patients with nasopharyngeal positive PCR COVID-19 cases [Mean age was 49.4 years (SD 12.5); 2087 (79%) were men) were enrolled. The total number of COVID-19 patients who acquired bacterial infection was 509 (19%). Two hundred and forty-six (246) patients were admitted to ICU and were under mechanical ventilation (9%). All mechanically ventilated patients acquired bacterial infections (100%) and forty-six of them acquired fungal superinfection (18.6%) (Table 1).

The 246 patients admitted to the ICU who were being ventilated mechanically and included in the statistical analysis had the following baseline characteristics: 194 (79%) of the patients were men, with a mean age of 62.4 years (12.5). Eighty-one percent of COVID-19 patients had diabetes (33), fifty percent had hypertension (123), and sixty-four percent had lymphopenia at baseline (157). The average time spent on mechanical ventilation was 27.1 days (19.8), and 41 out of 246 patients who had ventilator-associated pneumonia passed away in the intensive care unit (17%). A total of 46 patients (18.6%) had episodes of invasive fungal infections that were either confirmed or likely (pr/pb) (IFIs) (Table 2).

Out of the 246 cases admitted to the ICU and were under mechanical ventilation, forty-six (46) cases were superinfected with fungal infections (18.6%). All patients had episodes proven or probable (pr/pb) invasive fungal infections (IFIs). The patients were between 62 to 80 years. They were under dexamethasone treatment (p=0·027) and long duration of mechanical ventilation (>14 days; p=0·019). *Candida* other than *Candida auris* constituted the most common type of fungi isolate, forty-one (89%) out of the forty-six fungal superinfected cases were *Candida* other than *auris*. Three cases were *Candida auris* (6.5%), one case developed invasive *Mucormycosis* (2%) and one acquired invasive *Aspergillosis* (2%). The last five patients were died (11%) (Table 3).

Out of the 509 cases who acquired bacterial super infection in COVID-19 cases, two hundred and forty-six patients were admitted to the ICU and need mechanical ventilation (48%) while multidrug resistant organisms (MDRO) represent 97%. *Extended spectrum beta lactamases (ESBL)* comprise the most common organisms 35.1%, followed by *Methicillin resistant Staph aureus (MRSA)* 32.2%, then *Carbapenem resistant Enterobacteriaceae (CRE)* comprises 28.9% and *vancomycin resistant Enterococci (VRE)* 2.1%. The least was *Clostridium difficile (CDF*) was 1.7%. Using the VITEK-2® automated sensitivity system (bioMerieux 8.01) and adhering to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) version M100 (31 Edition), all MDRO and their drug susceptibilities were detected (Clinical Laboratory Standard Institute (CLSI), 2021) (Table 4).

263 patients out of the 509 COVID-19 cases that developed bacterial superinfection were not admitted to the ICU. 72% of the 263 infected cases, 190 cases had a genuine infection. The following were the main bacterial causal agents: *Staphylococcus* spp. (39.5%), *Escherichia coli* (21.1%), *Pseudomonas* pp. (18.4%), *Streptococcus* spp. (13.2%), *Klebsiella* spp. (7.9%). *MRSA* constitutes 26.7% of the isolated *Staph spp. and Extended spectrum beta lactamases E. coli were (27.5%) (*Table 5).

170 of the 246 COVID-19 patients admitted to the ICU were receiving Dexamethasone medication (69%) and 46 of them (27%) had a fungal infection. When patients were discharged from the intensive care unit, the mortality rate revealed that overall ICU mortality was significantly greater in patients with pulmonary Aspergilli and *Candida auris* linked with pr/pb COVID-19 than in those without (p 0.0001) (Table 6).

DISCUSSION

In China's clinical guidelines, a number of diagnostic and preventative strategies to address the issues in COVID-

Table 2. Demographic and clinical characteristics of patients with COVID-19 and bacterial-Fungal coinfections.

Item	Patients number	Percentage	P value
Age, years (n=246)	62.4 ±12		
Male	194	79	
Diabetes (n=246)	81	33	p<0·0001
Hypertension (n=246	123	50	p<0·0001
Chronic obstructive pulmonary disease (n=509)	34	13.8	p<0·005
Lymphopenia (n=503)	157	64	p<0·0001
Asthma (n=509)	14	5.7	p<0·005
LTC patients	51	20.7	p<0·005

LTCU (long terms care patients transfer to ICU).
Source: Authors

Table 3. Prevalence of fungal coinfections isolated from respiratory samples (BAL) in COVID-19 patients.

Item	Patients number	Percentage	P value
Total number of patient positive COVID	2643		
Total number of patients admitted to the ICU	246	9.3	
Total number of patients with ventilator admitted in ICU had fungal infection	46	18.6	
Invasive *aspergillosis*	1	2	p<0·0001
Candidemia	41	89	p<0·0001
Candida auris	3	6.5	p<0·0005
Invasive Mucormycosis	1	2	p<0·0001

Source: Authors.

Table 4. Prevalence of bacterial coinfections isolated from respiratory samples (BAL) in COVID-19 patients admitted to ICU

Item	Patients number	Percentage	P value
Total number of patient positive COVID-19	2643		
Total number of COVID-19 patients had bacterial infection	509	19	p<0·0005
Total number of patients with ventilator associated bacterial co-infection in COVID-19 ICU patients	246	9	p<0·005
MDRO	239	97	p<0·0001
ESBL	84	35.1	p<0·0005
MRSA	77	32.2	p<0·0005
CRE	69	28.9	p<0·0005
VRE	5	2.1	p<0·005
CDF	4	1.7	p<0·005

Source: Authors.

19 patients have been described. Secondary bacterial and fungal infections, however, have received less consideration, and a systematic diagnostic procedure is still lacking. In 2021, Garcia-Vidal and colleagues published a paper on their observations of co-infection and superinfection in COVID-19-positive hospitalized patients. 31/989 (3%) of the 989 COVID-19 patients admitted to a hospital in Barcelona, Spain had co-infections acquired in the community. *Streptococcus pneumoniae* and *Staphylococcus aureus* pneumonia made up the majority of these illnesses (Garcia-Vidal et al., 2021). 43/989 patients (4%) had a diagnosis of a hospital-acquired infection, with 25/44 (57%) of those cases occurring in critical care. Infections common to that 4% of patients included ventilator-associated pneumonia, hospital-acquired pneumonia, and bacteremia, with typical

Table 5. Prevalence of bacterial coinfections isolated from sputum samples in COVID-19 patients (Non ICU admitted).

Item	Patients number	Percentage	P value
Total number of patient positive COVID-19	2643		
Total number of COVID-19 patients had bacterial infection	509	19	p<0·0005
Total number of patients associated bacterial co-infection in COVID-19 non-ICU admitted patients	263	10	p<0·0005
True infection	190	72	P<0·005
Staphylococcus spp	75	39.5	p<0·0005
MRSA	20	26.7	p<0·0005
Streptococci spp.	25	13.2	P <0·005
Escherichia coli	40	21.1	P <0·005
ESBL (E. Coli)	11	27.5	P <0·005
Pseudomonas aeruginosa	35	18.4	P <0·005
Klebsiella spp.	15	7.9	P <0·005

Source: Authors.

Table 6. Patient characteristics, treatments, and secondary infections associated with death.

Item	Patients on ICU (n=246)	Percentage	P value
Duration of stay in ICU	32.7±24 days		
Duration of mechanical ventilator	27·1 days		
SOFA at admission	29	11.78	p<0·005
Dexamethasone	46	18.69	p<0·005
Death	11	4.47	p<0·005

SOFA: Sequential organ failure assessment.
Source: Authors.

nosocomial organisms predominating. These included Staphylococcus aureus, *Escherichia coli*, *Klebsiella* species and *Pseudomonas aeruginosa* (Garcia-Vidal et al., 2021). Coagulase-negative, the major of documented bloodstream infections (7/16; 44%) were caused by staphylococci. A co-infection with a fungus was found in 7/989 patients (0.7%). *Aspergillus fumigatus* tracheobronchitis was identified in three patients, while *Candida albicans* bloodstream infection, urinary tract infection, and intra-abdominal infection were identified in four patients.

There have also been reports of low observed rates of bacterial and fungal infection in COVID-19 patients from other nations, including the UK. In 2020, Hughes and colleagues found 51/836 COVID-19 patients (6%) admitted to two London hospitals had bacterial infections. In this cohort, secondary bacterial infection was rare. Sixty positive blood cultures were examined, and 39/60 (65%) were found to be contaminants, with coagulase-negative Staphylococci making up the majority of these (Hughes et al., 2020). Although the incidence of bacterial and fungal infection in COVID-19 patients is similar in both investigations, their limitations must be taken into account. A limited amount of microbiological sampling was carried out following the diagnosis of COVID-19, according to Garcia-Vidal and colleagues. According to Hughes and colleagues, a considerably higher percentage of patients (77% versus 27%) had blood cultures tested on them. In both investigations, only a small percentage of patients (13 and 25%, respectively) received respiratory sampling. A significant percentage of patients also got empirical antibiotic therapy. These variables may have a considerable impact on the rate of infection detection in hospitalized COVID-19 patients, resulting in underreporting of infection rates (Rawson et al., 2020).

The difference between this study and previous studies may be attributed to the substantial number of COVID-19 cases included (2643) and the extended duration of the study. In this study, the total number of patients with ventilator admitted ICU was 246 (9.3%). Unfortunately, all acquired bacterial superinfection and 18.6% acquired fungal superinfection. This may be attributed to the age of the cases and their comorbidities as fifty (50%) of them were hypertensive, 33% were diabetic, 64% had lymphopenia, 5.7% were asthmatic and 20.7% were coming from long term care centers. Sixty-nine percent were under Dexamethasone treatment. These findings

are consistent with early Chinese reports that found secondary infections in up to 50% of COVID-19-dead patients but not in just 15% of COVID-19-survivors (Lu et al., 2020). In addition to the retrospective analysis of published research (n = 806 patients), only 8% of patients were reported to have bacterial and fungal coinfections, while >70% of patients received empiric antibiotic therapy (Rawson et al., 2020). In a similar vein, the comprehensive meta-analysis of 4,000 hospitalized COVID-19 patients, mostly from China, revealed that only 7% of patients had bacterial coinfections, rising to 14% in critically sick patients (Lansbury et al., 2020). This demonstrates that in COVID-19 cases, comorbidities and risk factors increase bacterial superinfection.

In the investigation, 64.8% of the total multidrug resistant organisms isolated from the ICU admitted patients were Gram negative organisms (ESBL and CRE), while 34% were resistant gram-positive organisms (MRSA, VRE). Out of all patients admitted to the ICU, the prevalence of fungal infections was 17% for *Candida* species other than *Candida* auris and 0.4% for *Aspergillus*. When Kubin et al. (2021) studied the incidence and antibiotic susceptibilities of bacterial and fungal infections in hospitalized patients with coronavirus, they discovered that gram-positive organisms accounted for 42% of bacteremias, followed by gram-negative organisms (35%) and fungi (22%). The majority of the organisms recovered from blood cultures (26%) belonged to the family Enterobacterales. But early in life, bacteremia brought on by *Staphylococci* and *Streptococci* was more common (Kubin et al., 2021). The slight difference in the percentage between the two studies may be attributed to the difference in the type of sample examined in this study. Sputum, tracheal aspirate, BAL if applicable was collected from the patients admitted to the Intensive care unit and were under invasive mechanical ventilation and BAL sample collection. The findings corroborated those of Alshrefy et al. (2022), who found a strong correlation between secondary bacterial infections and secondary fungal infections caused by invasive mechanical ventilation (p 0.001). Additionally, they stated that respiratory system infection had an incidence of 32.5% (n=102) and was the most prevalent secondary illness. Additionally, they stated that 102 (32.5%) patients, all of whom were mechanically ventilated in this study had secondary bacterial infections. The gram-negative organisms identified in this study were also the most often isolated bacterial pathogens, although their isolated organisms were as follows *Klebsiella pneumoniae* (n=17), *Pseudomonas aeruginosa* (n=34), and Acinetobacter baumannii (n=33) (Alshrefy et al., 2022) All patients in both studies were under steroids treatment. Both studies confirmed that antibiotic resistance is more prevalent with prolonged hospital stays and in patients under mechanical ventilation. This is in line with Nag and Cur (2021)'s findings, which claimed that COVID-19 patients with severe disease and those who need to spend a lot of time in intensive care units (ICUs) are more likely to get super-infected by nosocomial bacteria. Among COVID-19 patients, ventilator-associated pneumonia (VAP), bacteremia with sepsis, and urinary tract infections are the most often seen infection types (UTIs). Dexamethasone, a standard of care for COVID-19 patients who were critically ill, was also found to be substantially and independently related with superinfections, according to Sovic et al. (2022). Receiving dexamethasone was linked to superinfections (66% versus 32%, p0.0001) only patients receiving dexamethasone had invasive fungal infections [8/67 (12%) vs 0/88 (0%), p0.0001]. This is in comparison to this study; 170 out of 246 under dexamethasone treatment in the ICU (69%) all acquired bacterial infection (100%) and forty-six out them were superinfected with fungal infection (27%). Thus, there is an impact of dexamethasone therapy on the incidence of superinfections in hospitalized severely ill COVID-19 patients and more data studies are needed to establish the correlation (Søvik et al., 2022).

In this study, there were 263 COVID-19 patients who got bacterial superinfection and were not admitted to the ICU. But the true superinfection were190 cases (72%) that show bacterial culture with the same organism in two consecutive sputum cultures and in the presence of radiological findings of chest infection. Streptococcus spp. (13.2%), Staphylococcus spp. (39.5%), Escherichia coli (21.1%), Pseudomonas spp. (18.4%), and Klebsiella spp. (7.9%) were the predominant bacterial causal agents. 26.7% of the *Staph. aureus* isolates are MRSA. The findings are in line with a recent cohort study by Morovati et al. (2022) that discovered 72% of COVID-19 patients had positive sputum cultures. But he discovered that 63.2 and 8.8%, respectively, of secondary infections were caused by bacterial and fungal species respectively. *Streptococcus* spp. (21.5%), *Staphylococcus* spp. (16.7%), *Escherichia coli* (8.7%), *Pseudomonas* spp. (7.2%), Klebsiella spp. (4.7%), and Acinetobacter spp. (4.2%) were the main bacterial causal agents. Additionally, the two main fungal pathogens were *Aspergillus* spp. (2.1%) and *C. albicans* (6.7%) (Morovati et al., 2022).

CONCLUSION AND RECOMMENDATIONS

Superinfections with bacteria and fungi are very common in COVID-19 patients who require hospitalization; significantly among patients who have certain comorbidities, problems, a prolonged hospital stay, mechanical breathing, and steroid therapy. In order to identify priority clinical groups and improve care for these types of infections, which dramatically alter the development of cases with COVID-19 with the threat variables exposed in the population examined, it is crucial to use high indicator of dubitation and active surveillance.

Additionally, there is a constant need for prospective research to evaluate the effectiveness of specific biomarkers to exclude or include bacterial and fungal superinfection. If threat variables comparable to those established in this study are present, further research is required to determine whether early antifungal treatment or prophylactic is necessary. Additionally, more research is needed to define the timing of connected infections in order to distinguish between diseases tied to the population and infections linked to the healthcare system (HAI).

Additionally, academic groups and health authorities are urged to implement a workable, customized procedure for identifying bacterial and fungal illness in COVID-19 patients.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Molecular cloning and functional research in drug-resistance of transposase gene in *Burkholderia pseudomallei*

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Transposons are widely used in genetic engineering research and play an essential role in microbial drug resistance research. Transposase is the key to constructing efficient transposons, but no studies have reported transposons in *Burkholderia pseudomallei.* In this study, a new transposase gene was cloned and identified from HNBp001 isolated from Hainan province. Blast and phylogenetic tree analysis showed that the gene had high homology with IS21 transposase of Burkholderia strains from other regions. The transposase gene deletion and over-expression strains of HNBp001 were successfully constructed. The biofilm detection result shows that the over-expression strain's biofilm production was higher than the other two strains. Although there was no significant difference in the amount of biofilm production, the biofilm synthesis rate of the over-expression strain was significantly faster. Wild and deletion strains' results were the same in drug resistance, while over-expressed strains changed compared with the other two strains. Sodium Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis (SDS-PAGE) revealed that there was no difference whether the transposase gene was knocked out or not. Two different sizes of proteins of over-expressed strain were significantly lower than that in the normal and knock-out strain. These results indicate that the cloned transposase genes play an essential role in the biofilm formation and drug resistance in HNBp001, but the specific mechanism remains to be further studied.

Key words: *Burkholderia pseudomallei*, transposase, biofilm, drug-resistance.

INTRODUCTION

Burkholderia pseudomallei, a facultative intracellular bacterium widely distributed in the soil of Southeast Asia and Northern Australia that causes melioidosis, commonly exists in tropical and subtropical regions (Jayasinghearachchi et al., 2023; Limmathurotsakul et al., 2016; Seng et al, 2019; Dance and Limmathurotsakul, 2018). As early as 2008, the Center for Disease Control and Prevention of the United States (CDC) listed it as a

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Class I pathogen (Peacock et al., 2008). In China, melioidosis cases are mainly distributed in Hainan, Guangdong, Guangxi, and Taiwan. A significant number of melioidosis cases were reported in Hainan (Zheng et al., 2019; Chen et al., 2015). The bacterial can infect the body directly through trauma or in aerosol form through the respiratory tract. Patients infected with *B. pseudomallei* do not have typical symptoms. Their clinical manifestations are similar to many diseases, such as fever, tuberculosis, etc., which can easily lead to misdiagnosis (Tang et al., 2018; Hautbergue and Wilson, 2012; Jacob et al., 2012; Gassiep et al., 2020). *B. pseudomallei* are naturally resistant to a variety of commonly used antibiotics. In addition, due to the non-standard use of broad-spectrum antibiotics, the drug resistance rate to these antibiotics keeps rising (Wiersinga et al., 2018). Multiple drugs and pan-drug resistance *B. pseudomallei* can be easily isolated in clinical practice, and this kind of strain occupies an increasing proportion of hospital infections (Chen et al., 2016; Chou et al., 2007). Some clinical studies show that this pathogen is sensitive to imipenem (antibiotic sensitive rate 10-100%), resistant to penicillins, aminoglycosides, and cephalosporins in varying degrees (Fen et al., 2021; Bandeira et al., 2013). Carbapenems, β-lactam, and cephalosporins are still the preferred drugs for the treatment of melioidosis. However, the isolation rate of drug-resistant strains to these antibiotics is increasing.

The acquisition of drug-resistance genes, mainly delivered by the transposase, is one of the leading causes of bacterial drug resistance. Transposons can jump freely in DNA and transfer resistance genes among bacterial chromosomes, plasmids, and phages, resulting in the diversification of resistance (Klein and O'Neill, 2018; Kelly et al., 2009). The research of Kelly and Beuzon found that the transposon Tn1721 of tetracycline resistance gene *(tetA*) and the transposon complex Tn3-△Tn1721 containing β-lactams antibiotic resistance gene (blatem-1) existed in salmonella (Pasquali et al., 2005; Pezzella et al., 2004).

Transposons are widely found in bacteria, yeast, and higher plants and animals and were first discovered in maize by McClintock, an American geneticist, in the 1940s (McCLINTOCK, 1950). Transposons are widely used in genetic engineering, including mutant library construction, transgene, gene function analysis, etc (Mougiakos and Beisel, 2021; Dance and Limmathurotsakul, 2018; Delaurière et al., 2009; Ivics et al., 2009). Transposons can be divided into retrotransposons and DNA transposons, in which retrotransposons can increase the copy number of the inserted sequences, while DNA transposons cannot (Pradhan and Ramakrishna, 2022; Feschotte and Pritham, 2007; Schulman, 2013). DNA transposons, which require the involvement of transposase which can combine with the specific sequence of IR region to open the double strand of IR region and insert into the new genome, are widely studied (Gorbunova et al., 2021; Trubitsyna et al., 2015). Frequent transposition will threaten the existence of the species. Typically, the activity of transposase is deficient (Surm and Moran, 2021; Vigil-Stenman et al., 2017). Highly active transposons generally need to be artificially optimized. In this process, the acquisition of high catalytic activity transposase is indispensable. Although the transposase is essential in constructing highly active transposon, some transposase genes were reported in *B. pseudomallei*, but no further functional research was conducted. In this study, a new transposase gene was cloned, and the transposase detected strain and over-expression strain of BPHN001 was also successfully constructed. Moreover, the influence of the transposase on biofilm formation, antibiotic resistance, and protein expression was detected by different assays. The results will provide valuable information for the transposase to dissect other molecular mechanisms and functional studies.

MATERIALS AND METHODS

HNBP001 strain was isolated from Hainan. The expression vector PET-42a-c(+) and knock-out vector PK 18mobsacb (TPR) were preserved by our laboratory. *Escherichia coli* BL21 (DE3) and S17-12PIR strains were purchased from Biyuntian Biotechnology Co., LTD.

Cloning and recombination vector construction of NovelGene_03572 transpoase gene

Total DNA was extracted according to the instructions of the bacterial Genomic DNA Extraction Kit (TianGen, China). According to the sequence of RNA libraries, design specific amplification primer, 03572 - OE - F: 5 '- CGCGGATCCATGATCAAG GACGTTCTAC- 3' (BamH I); 03572 - OE - R: 5 '- CCCAAGCTTTCAATCTCCAGCGAGCTTT - 3' (Hind III), total bacterial DNA as a template to amplify the NovelGene_03572 open reading frame (ORF). PCR amplification system: 2xTaqMaster Mix 12.5 μL, upstream primer 1 μL (10 μM), downstream primer 1 μL(10 μM), genomic DNA 1 uL (100 ng/μL), ddH_2O 9.5 μL. Amplification procedure: 94°C for 5 min; 94°C 30 s, 58°C 30 s, 72°C 1 min, 35 cycles; 72°C for 10 min. All amplified products were purified using a bulk agarose gel DNA Recovery Kit (TianGen, China). The DNA fragments were constructed into the prokaryotic expression vector PET42a-c (+) and sequenced by Invitrogen (Pudong, Shanghai, China). The verified sequence was constructed to PK 18mobsacb (TPR). The details of primers are shown in Table 1. The online tool ProtParam was used to obtain the sequence of 03572 transposase protein amino acid (http://web.expasy.org/protparam/). The amino acid sequences of 03572 transposase protein and other microorganism transposase proteins were aligned using CLUSTAL_X, and the phylogenetic tree was generated with the MEGA4.0 program using the NJ algorithm with 1000 bootstrap trials (Xiu et al., 2016).

The obtained of 03572 transposase gene overexpression and deletion strains

The recombinant plasmids PET42A-C(+)-03572 and PK18

Table 1. List of primers used for knock-out NovelGene_03572 transposase gene.

Primer name	Primer sequence (5' to 3')	Product length (bp)
3572-up-F	CGCGGATCCCATACATTCCCGTCTTCGGT（BamH I）	499
3572-up-R	TCCCCCGGGCGTCGAGCCTGGCTTGATTC（Sma I）	
3572-down-F	TCCCCCGGGACGATGGAAGACTGGTTAGGC（Sma I）	691
3572-down-R	CCCAAGCTTGCGGGACGATGTGGAGAT（Hind III）	

Source: Authors

MobSACb(TPR)-03572 were transformed into *E. coli* S17-12pir receptor cells according to standard transformation steps, respectively. It was cultured in solid LB medium (containing 50 mg/L kanamycin) at 37°C overnight. Positive colonies were screened by colony PCR. Then the s17-12pir colony containing pet42a-c(+)-03572 or PK18mobsacb (TPR)-03572 recombinant plasmid was co-cultured with HNBp001 strain, respectively. The overexpression strain was screened on the LB sodium medium containing Gm and Kan. The deletion strain was screened on LB sodium medium containing 15% sucrose and Gm and finally verified by PCR (The primers of overexpressed strain were 03572-OE-F, 03572-OE-R; the primers of deletion strain were: 3572-up-F, 3572-down-R).

The detection of biofilm formation

Single colonies cultured in LB liquid medium at 37°C, 200 rpm/min overnight were selected from HNBp001 wild strain, deletion strain, and overexpression strain. The bacteria were transferred to the new LB liquid medium at a ratio of 2 to 10%, respectively, cultured with the condition of 37°C, 200 rpm/min, until OD_{600}=0.5-1. 200 µL of LB liquid medium was added without antibiotics into a sterile 96-well flat bottom culture plate for each well, 4 µL bacterial liquid was added into each well containing 200 µL LB liquid medium, incubated aerobically in the incubator at 37°C for 24, 48, and 72 h. At least three multiple wells were set for each strain. After incubation, the culture medium was gently poured out and gently rinsed twice with 200 µL phosphate buffer saline (PBS) per well. The medium was placed upside down in a cool, ventilated place for drying and fixation. 200 µL methanol was added, fixed for 15 to 30 min, methanol was discarded and dried thoroughly. After being dyed with 150 to 200 µL 1% crystal violet solution in each well for 5 min, it was gently rinsed with PBS until no obvious color can be seen in the blank dyeing well, then placed in a cool and ventilated place upside down for drying. The dye was fully dissolved with 200 µL 95% ethanol per well. The absorbance value was measured at 590 nm with a microplate reader.

The detection of the minimal inhibitory concentration (MIC)

Single colonies were selected from HNBp001 wild strain, deletion strain, and overexpression strain, and added into a diluent, respectively, then thoroughly mixed. Then 50 µl suspension was transferred into a 9 ml LB liquid medium and mixed thoroughly. LB liquid medium containing different strain was transfused into the 96-well drug-sensitive culture plate, each well added 100 ul, then the plate was sealed and incubated at 37°C (Fahim et al., 2022). The results were observed within 24 h. If the bacteria still grow well, even though, cultured in the highest drug concentration, the result was defined as "Resistance". In the event that the bacteria did not grow in all drug concentrations including the minimum concentration, the result was defined as "Sensitive". In case the bacteria did not grow in a drug concentration, but when the concentration was lower than it, then the germ survive and the number of the concentration was recorded as the MIC for the antibiotic.

Protein expression analysis by SDS-PAGE

Single colonies were selected from HNBp001 wild strain, deletion strain, and overexpression strain, cultured in LB liquid medium at 37°C. When the OD_{600}=0.6 was reached, 500 µl of the bacterial solution was taken from each strain. The supernatant was discarded after centrifugation at 12000 rpm. The bacteria were suspended with 80 µL sterile PBS, and a 5x protein loading buffer was added. SDS-PAGE was performed to observe the protein expression in different strains.

RESULTS AND DISCUSSION

Cloning and phylogeny

A gene named NovelGene_03572 was selected from the RNA library. The specific primers were designed using the HNBp001 DNA as a template, and specific amplification of the NovelGene_03572 gene was carried out. The agarose gel electrophoresis result showed a specific band between 250 and 500 bp, which was basically the same as the target fragment (384 bp). The target fragment was verified by sequencing.

The NovelGene_03572 transposase protein was blasted with 18 transposases from other microbial species, including *Caballeronia arationis, B. pseudomallei*, *Caballeronia* species *dw*_276, *Paraburkholderia atlantica*, *Trinickia soli*, *Burkholderia vietnamiensis*, *Caballeronia* spp. NCTM1, *Paraburkholderia caribensis*, *Paraburkholderia phymatum*, *Paraburkholderia steynii*, *Burkholderia thailandensis* E264, *Burkholderia* species SFA1, *Caballeronia* spp. GAFFF2 and *Cupriavidus* species AcVe19-1a. The results showed that their homology was 83% (Figure 1).

MEGA 4.0 software was used to construct phylogenetic trees by the neighbor-joining method. NovelGene_03572 transposase protein was highly similar to transposase subunit from *B. thailandensis E264*, IS21 family transposase from *B. pseudomallei.* The black squares represent the new genes cloned (Figure 2).

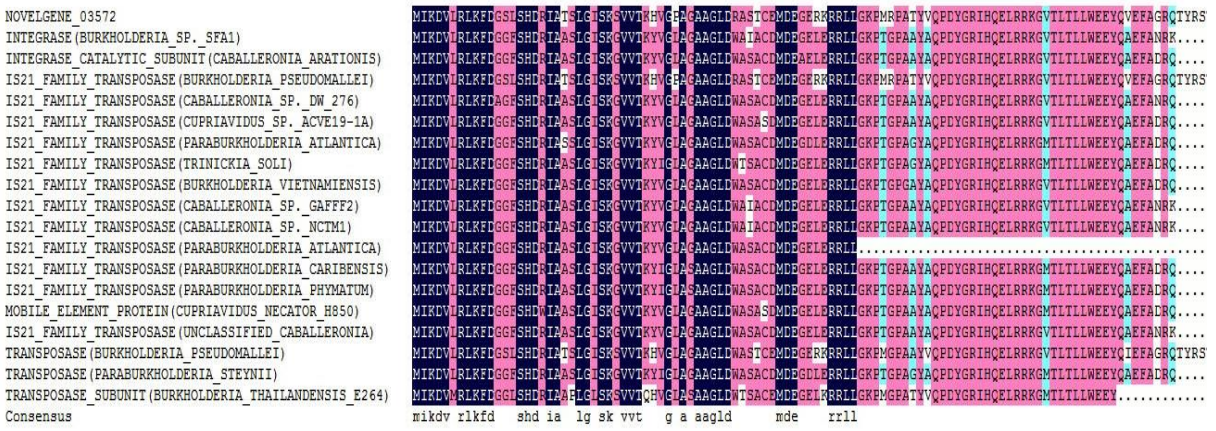

Figure 1. Protein comparison of NovelGene_03572 with 18 transposases from other microbial species.
Source: Authors

The construction of novel Gene_03572 transposase gene deletion and overexpression strains

The recombinant vector of PET42a-c(+)-03572 and PK 18mobsacb(TPR)-03572 were transformed into HNBp001, respectively. The overexpressed strains were screened by LB solid medium containing Kan (250 µg/mL) and Gm (100 µg/mL). Four colonies were selected from the plate for PCR with PET42a-c(+) universal primers. The results showed that No. 3 and No. 4 colonies had a specific band between 750 and 1000 bp. The amplification products' size was consistent with the target fragment (Figure 3a). The deletion strains were screened by LB solid medium containing Gm (100 µg/mL) and 15% sucrose. Colony PCR was performed by using 3572-up-F and 3572-down-R as primers. The length of the products were 1843 bp (wild strain) and 1190 bp (detected strain) (Figure 3b). The agarose gel electrophoresis result indicated that the PCR products met our expectations. We got the novelGene_03572 transposase gene deletion and overexpression strain in HNBp001.

Effect of the novelGene_03572 transposase on biofilm formation in HNBp001

Biofilm formation experiment results show that, with the extension of incubation time, biofilm formation was gradually increased in all strains, including wild strain, deletion stain, and overexpression strain. The biofilm formation rate of the overexpression strain was significantly faster than the wild strain and deletion strain, and the biofilm formation quantity was higher than the other two strains after being cultured for 72 h (Figure 4). The results indicated that the novelGene_03572 transposase could affect the biofilm formation of HNBp001 to a certain degree.

Effect of NovelGene_03572 transpoase on drug resistance in HNBp001

The results showed no difference in drug sensitivity between wild and deletion strains. However, compared with wild and deletion strains, the minimal inhibitory concentration (MIC) of the overexpression strain against cefuroxime changed from resistance to 16 mg/L, the MIC against cefepime changed from 8 mg/L to resistance, the MIC against ciprofloxacin changed from 0.5 to 1 mg/L. MIC against meropenem changed from sensitive to 4 mg/L, and MIC against amikacin changed from 16 mg/L to resistance. Detailed results are shown in Table 2. In conclusion, the overexpression of the NovelGene_03572 transposase gene plays a vital role in developing drug resistance in the HNBp001 strain.

Protein expression analysis

SDS-PAGE result showed no difference in protein expression between the HNBp001 wild strain and transposase deletion strain. Interestingly, the protein expression in transposase overexpression strain had changed, two proteins were down-regulated, and the size of the two proteins was almost 37 and 130 kD, respectively (Figure 5). Dependent on the result, we suppose that the 03572 transposase genes do not express or the activity of the 03572 transposase protein is not high in normal conditions. Combined with the result of drug resistance, we have reason to believe the 03572 transposase protein can affect the drug resistance in the HNBp001 strain by down-regulating the two proteins, which were 37 and 130 KD, respectively.

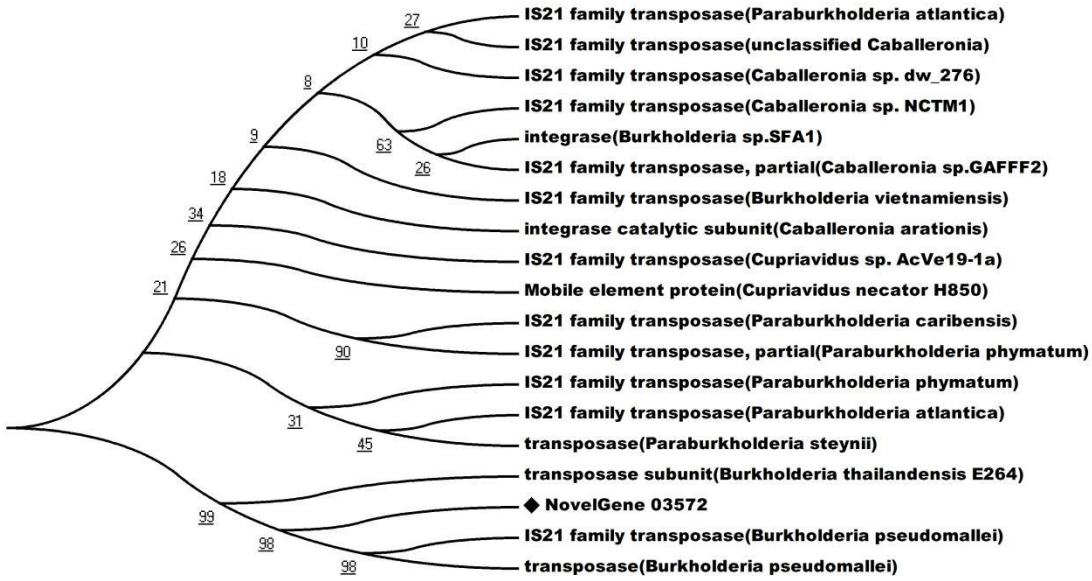

Figure 2. Phylogenetic relationships among the NovelGene_03572 transposase protein and other members of the transposases were estimated based on their amino acid sequences. Transposases amino acid sequences of the following proteins were used to make the tree: integrase [*Burkholderia sp*. SFA1] (BBQ02449.1), integrase catalytic subunit [*Caballeronia arationis*] (SAL06529.1), IS21 family transposase [*Burkholderia pseudomallei*] (WP_004536630.1), IS21 family transposase [*Caballeronia sp*. dw_276] (WP_213781838.1), IS21 family transposase [*Cupriavidus sp*. AcVe19-1a] (WP_209777234.1), IS21 family transposase [*Paraburkholderia atlantica*] (MPW09758.1), IS21 family transposase [Trinickia soli] (WP_102612997.1), IS21 family transposase [*Burkholderia vietnamiensis*] (WP_226103266.1), IS21 family transposase [*Caballeronia sp*. GAFFF2] (WP_250469771.1), IS21 family transposase [*Caballeronia sp*. NCTM1] (WP_250520593.1), IS21 family transposase [*Paraburkholderia atlantica*] (WP_227749239.1), IS21 family transposase [*Paraburkholderia caribensis*] (WP_252671094.1), IS21 family transposase [*Paraburkholderia phymatum*] (WP_244257842.1), Mobile element protein [*Cupriavidus necator* H850] (KAI3608382.1), IS21 family transposase, partial [unclassified *Caballeronia*] (WP_250520681), transposase [*Burkholderia pseudomallei*] (RAQ91269.1), transposase [*Paraburkholderia steynii*] (TCG03114.1), transposase subunit [*Burkholderia thailandensis* E264] (ABC34012.1).The neighbor-joining phylogenetic tree was constructed using the bootstrap method of MEGA 4.0 with 1000 replications, and the respective plant species of the above proteins are shown in the tree. The black square symbols indicate the NovelGene_03572 transposase protein.
Source: Authors

DISCUSSION

Antibiotics, one of the most significant medical discoveries, are vital in treating bacterial infections. Early antibiotics were mainly extracted from metabolic products by microbial fermentation. It becomes increasingly challenging to obtain antibiotics with development value through this method (Landecker, 2016). Researchers mainly focus on modifying existing known antibiotics to obtain new antibacterial drugs (Hutchings et al., 2019). At the same time, with the abuse of antibiotics in clinical practice, the effective concentration of antibiotics is getting higher and higher. Bacteria are becoming more and more resistant to certain antibiotics.

Moreover, some antibiotics even lose their effectiveness when targeting certain bacteria (Lee, 2019). Studies have shown that worldwide, almost 700,000 people died of infection related to drug resistance annually (Nicolaou and Rigol, 2018). This number is expected to reach 10 million by the middle of this century (O'Neill, 2014). It is urgent to develop new antibacterial drugs, clear the bacterial resistance mechanism, and find new antibacterial strategies. This study aims to clear the drug-resistance mechanism of B.p by finding a target protein of the 03572 transposase. Finally, two proteins which were down-regulated while the 03572 transposase over-expressed were found.

Drug resistance refers to the phenomenon that pathogenic bacteria are no longer sensitive to antibiotics or antibacterial drugs. The generation of drug resistance is one of the main reasons for the failure of clinical bacterial anti-infection treatment. Thoroughly studying the mechanism of bacterial resistance is the premise of developing new antibacterial drugs and optimizing clinical treatment plans. At present, the known mechanisms of bacterial drug resistance mainly include the following

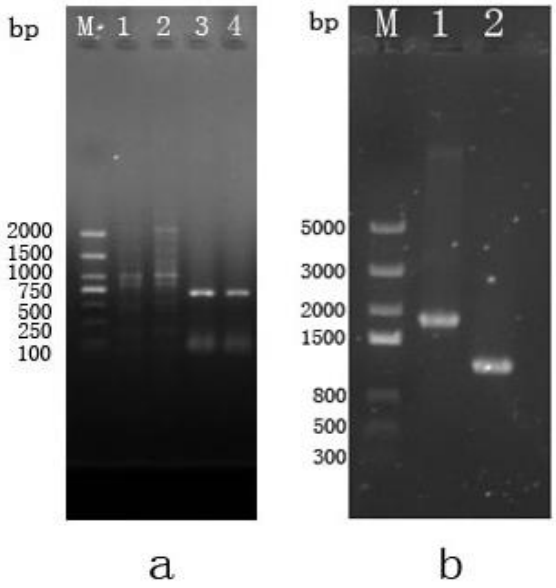

Figure 3. (a) Screening the 03572 transposase gene over expression strain by colony PCR. M: Marker (2000 bp DNA ladder); lane 1-4: Four single colony; (b) Screening the 03572 transposase gene deletion strain by colony PCR. M: Marker (5000 bp DNA ladder); lane 1: Wild strain colony (1843 bp); lane 2: Deletion strain colony (1190 bp).
Source: Authors

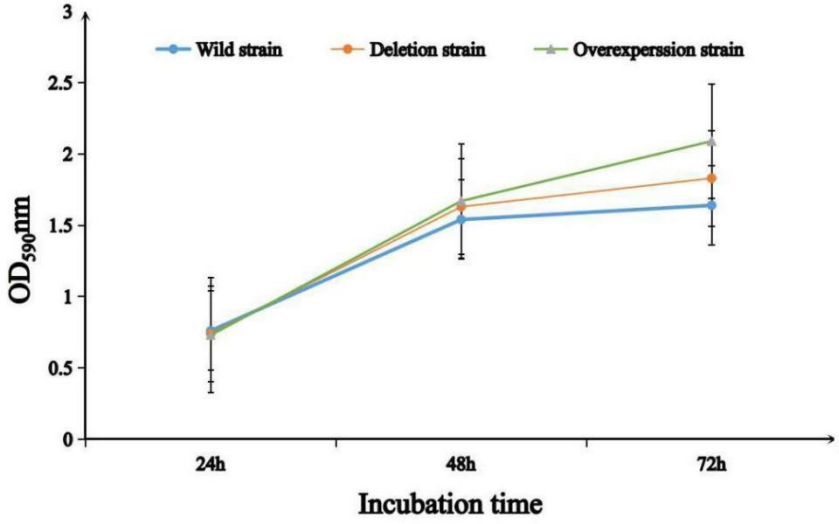

Figure 4. Detection of biofilm formation with the extension of incubation time in different strains.
Source: Authors

aspects: (1) Transmit the drug resistance gene to generations by the genome, plasmid, or transposase; (2) Reduce or inhibit drug activity by inhibiting the receptor affinity; (3) Produce hydrolytic enzymes to inactivate antibacterial drugs, for example, carbapenemase, erythromycin inactivation enzyme; (4) Change the structure of cell wall or cell membrane, decrease the number of antibacterial drugs into the cell, resulting in

Table 2. Drug resistance analysis in different strains.

Antibiotics	Minimum inhibitory concentration (mg/L)		
	Wild strain	Deletion strain	Overexpression strain
Cefuroxime	R	R	16
Cefepime	8	8	R
Ciprofloxacin	0.5	0.5	1
Meropenem	S	S	4
Amikacin	16	16	R

R: Resistance; S: Sensitive.
Source: Authors

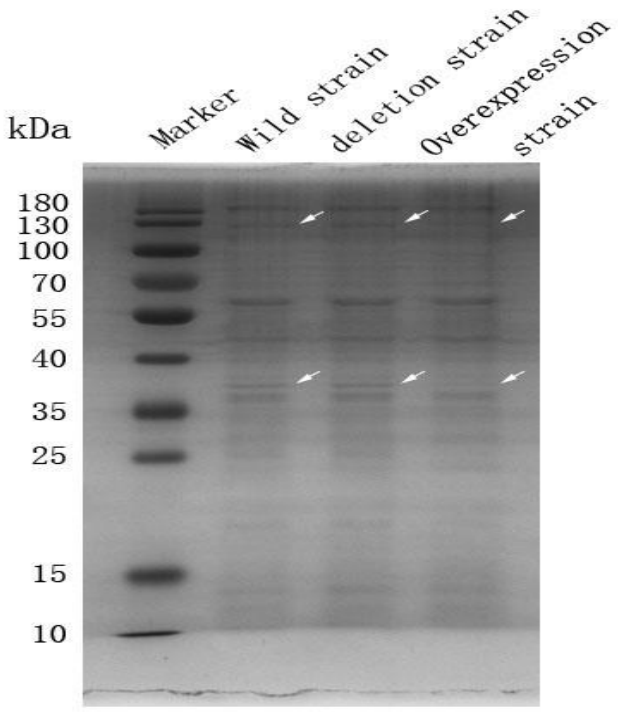

Figure 5. Protein expression analysis in different strains by SDS-PAGE. M: Marker (180 kDa protein ladder); lane 1: Total protein of wild strain; lane 2: Total protein of deletion strain; lane 3: Total protein of overexpression strain. Arrows: down-regulated proteins.
Source: Authors

drug resistance, such as the formation of biofilm (Al-Bayati and Samarasinghe, 2022); (5) Use the efflux pump to transport antibacterial agents to the cells to reduce the damage to the cells (Ogawara, 2019).

The formation of biofilm is one of the primary methods for the establishment of microbial resistance (De Silva and Heo, 2022; Rather et al., 2021; Dai et al., 2021; Pibalpakdee et al., 2012). The exopolysaccharides (EPS), a critical matrix component of biofilms, is negatively charged and can combine with the positively charged amino to prevent hydrophilic antibiotics from entering cells (Ma et al., 2022). In addition, some specific genes are expressed in the biofilm formation process. For example, pvrR and tolA genes are highly expressed in the biofilm formation process in *Pseudomonas aeruginosa* (Drenkard and Ausubel, 2002; Whiteley et al., 2001). Previous studies have shown that the amount of biofilm can affect the drug resistance of bacteria. In this study, we found that the overexpression of 03572 transposase could not significantly up-regulate the amount of biofilm in the HNBp001 strain. Still, its synthesis rate was substantially faster than wild and

deletion strains. We believe that the quicker the biofilm is formed, the earlier the osmotic barrier can be established which enhances bacterial resistance.

Drug sensitivity assay results showed that the deletion of the 03572 transposase gene did not affect the resistance of the HNBp001 strain. Still, its overexpression could affect the MIC of 5 antibiotics: cefuroxime, cefepime, ciprofloxacin, meropenem, and amikacin. SDS-PAGE also showed that the overexpression of the 03572 transposase gene could affect the protein expression of HNBp001, which significantly down-regulated the expression of two proteins (37 and 130 KD). Still, there is no difference in the protein expression between wild and deletion strains. Therefore, we speculated that the 03572 transposase gene was not expressed or the 03572 transposase protein was inactive in normal conditions. When certain conditions or substances stimulate the strain, the transposase expression is activated, the transposase's content reaches a certain level, and the transposition occurs, which makes the genes related to drug resistance up-regulated or down-regulated, thus resulting in the generation of drug resistance. Here, we only know that two proteins are down-regulated, and the drug resistance changed after 03572 transposase gene over-expression. No evidence exists to prove whether these two proteins are drug resistance or biofilm formation-related proteins; thus, further studies are needed.

Conclusion

The NovelGene_03572 gene, cloned from the HNBP001 strain, was identified as a transposase gene by Blust and phylogeny analysis. Deleting the NovelGene_03572 gene did not affect biofilm formation and drug resistance. However, the overexpression of the NovelGene_03572 gene can accelerate the generation of biofilm and affect the resistance to cefuroxime, cefepime, ciprofloxacin, meropenem, and amikacin, and down-regulate the expression of two proteins (37 and 130 KD). According to the results obtained, it was shown that the new transposase gene plays a very important role in drug resistance in *B. pseudomallei*. This study laid a foundation for in-depth studies on drug resistance in *B. pseudomallei* and provided a new idea for constructing a transposon suitable for *B. pseudomallei*.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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Aflatoxins contamination in raw and roasted cashew nuts in Mtwara, Tanzania

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The aim of this work was to determine the contamination levels of raw and roasted cashew nuts sold in Masasi and Newala districts of Mtwara region in Tanzania. A total of 60 samples including 40 roasted (24 samples from Newala and 16 from Masasi) and 20 raw samples (12 from Newala and 8 from Masasi) were collected. Determination of total aflatoxins levels in raw and roasted cashew nuts samples was carried out by immune affinity high performance liquid chromatography (HPLC). The levels of contamination ranged from not detected (less than limit of quantification) to 3.29 µg/kg for both aflatoxin B1 and total aflatoxin in the cashew nuts samples. None of the samples had total aflatoxins contamination greater than the recommended maximum residues of 4 µg/kg set by European Commission (2010) or 10 µg/kg set by FAO and WHO (1995). About a quarter (38%) of the samples had total aflatoxins less than limit of quantification. All roasted cashew nut samples were found to have total aflatoxins less than 3 µg/kg while about 86% of raw cashew nut samples had total aflatoxins less than 3 µg/kg.

Key words: Aflatoxin, cashew nuts, contamination, Mtwara, Tanzania.

INTRODUCTION

Cashew nuts (*Anacardium occidentale L*.) is derived from South American Countries (Bolivia, Brazil, Ecuador, and Peru) and it is one of the extremely important tropical fruit crops. A cashew fruit consists of an apple that bears fruit in which the kernel is embedded. It is widely cultivated in tropical regions all over the world and it is mainly centralized in third world countries like India, Tanzania, Mozambique, Nigeria, Guinea-Bissau and Kenya (Gong et al., 2016).

Tanzania is among the World's largest producer of raw cashew nuts, whereby in 2017/18, total production of raw cashew nuts was 313,826 metric tons where by 90% of cashew nuts were exported in raw form due to the country's low processing capacity (TIC, 2019). Cashews are grown mainly in Mtwara, Lindi, Ruvuma and Pwani, which occupies a total plantation area of about 695,683 Ha. About 90% of the area planted with cashew nuts is found in three regions of Mtwara, Lindi and Pwani (Tanzania Investment Centre (TIC), 2019). Most of cashew nuts processing in Tanzania is done manually mainly by small scale processors (CBT, 2018). The domestic consumption of cashew nuts had increased due

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to improved distribution systems through street vendors, shops, mini markets and supermarkets (CBT, 2018). Much of the production is concentrated in a few districts such as Tandahimba, Newala, Masasi, Mtwara, Mkuranga and Nachingwea. It provides an important source of income for 250,000 smallholder farmers in Tanzania, their production accounts for 80-90% of Tanzania's marketed cashew nuts crop. The average smallholder cashew farmer occupies about one to two hectares of cashew nut trees; sometimes intercropped with food crops, mainly cassava, grain staples and legumes (FARMER & CASES, Smallholder Cashew Business Model in Tanzania: Lessons from the Tandahimba Newala Cooperative Union (TANECU) Ltd).

Cashew nuts can be vulnerable to pre and/or post-harvest molds attack due to its high nutritional content but may be accelerated by inappropriate marketing and storage conditions (El-Samawaty et al., 2013). Also environmental factors like humidity and temperature during storage influence the infestation by fungi and aflatoxins production (Hedawoo and Bijwe, 2018). Some type of mold produces highly toxic secondary metabolites known as aflatoxins, which can occur in both industrialized and developing countries when the environmental, social and economic conditions combine with humidity and temperature favor the growth of moulds (Ashraf, 2012). Cashew nuts infection by toxigenic fungi has been reported in a number of studies and revealed a high risk due to contamination with mycotoxins (Alhussaini, 2012; Ashraf, 2012; Adetunji et al., 2018; El-Samawaty, 2013). The mold that attacks cashew nuts are *Aspergillus* species, which produces secondary metabolites known as aflatoxins and it has carcinogenic, estrogenic, immunosuppressive and teratogenic effects in humans and farm animals (Adetunji et al., 2019). There are about eighteen types of aflatoxins that have been identified, but the naturally occurring and well-known forms are AFB_1, AFB_2, AFG_1 and AFG_2 (Adetunji et al., 2018).

The Food and Agricultural Organization (FAO) reported 25% of foodstuffs available worldwide are contaminated with mycotoxins, with aflatoxins being identified as the most toxic of these mycotoxins (Eskola et al., 2020). Several countries have been conducting researches on the occurrence of aflatoxins contamination in cashew nuts including Saud Arabia, Nigeria, South Africa and Brazil with findings revealing levels above but some below the Codex Alimentarius and European Union (EU) limits (El-Samawaty et al., 2013; Adetunji et al., 2018; Adetunji et al., 2019). A study conducted in Nigeria on microbiological quality and risk assessment for Aflatoxins in groundnuts and roasted cashew nuts meant for human consumption showed consumers were at a risk of exposure to foodborne diseases and aflatoxins contamination (Adetunji et al., 2018). Another study showed that cashew nuts were susceptible to fungal deterioration and possibly aflatoxins contamination especially during storage, AFB1 was found in 92.3% of cashew nut samples (Ashraf, 2012). A study comparing the fungal metabolite profile of cashew nuts from two African countries (Nigeria and South Africa) showed total aflatoxins of 0.03 to 0.77 µg/kg and 0.01 to 0.28 µg/kg (Adetunji et al., 2019).

In Tanzania different studies have been conducted on the level of aflatoxins such as in maize, cereal based complimentary flour, groundnuts, cereal flours and milk (Nyangi et al., 2016; Rushunju et al., 2013, Mohammed et al., 2016). Although cashew nut is one of the major cash crops in South East and Northern Coastal belt of Tanzania (Annual Agriculture Sample Survey crop and Livestock report, 2016/17) currently, there is limited documented information on the status of aflatoxins contamination. This research aimed at determining the levels of aflatoxins contamination in raw and roasted cashew nuts from Mtwara region, which had the highest production of about 191,025 tonnes, which is 49.2% of all the production in Tanzania (Annual Agriculture Sample Survey crop and Livestock report, 2016/17). The results of this study will provide information on levels of cashew nuts contamination by aflatoxins and contribute to raise awareness and efforts of food control authorities in developing strategies to ensure public safety.

MATERIALS AND METHODS

Sample collection

A total of 60 samples of roasted and raw cashew nuts were collected in January 2021, in two districts of Mtwara region; Masasi and Newala. Therefore, 20 raw cashew nut samples and 40 roasted cashew nuts were sampled due to the fact that roasted cashew nuts are mostly consumed. Same as Newala which is divided into 16 wards, sampling was carried out in different wards due to the availability of cashew nuts processors as compared to other wards (Mpita, 2014). The collected samples were packaged in a clean 200 g zipped plastic bags and transported to the Tanzania Bureau of Standards food laboratory in Dar es Salaam for analysis.

Sample preparation

Each cashew nuts sample (150 g) was ground using a mechanical homogenizer (Hsiangtai grinding machine model SM-450L, serial number 080684) and sub divided to obtain a representative sub-sample for analysis. Then aflatoxins were extracted from 25±0.1 g for each grounded by adding 100 ml of 70:30 methanols:water into the Erlenmeyer flask containing the sample. The flask was covered by aluminium foil then the mixture was shaken by using orbital shaker (SSL1) for 30 min at 250 rpm. The extract was filtered using a filter paper (Whatman 1 circles 125 mm ø). Then 4 ml of the extract was diluted by eight milliliter of distilled water (MillQue, distillate, Elix technology model) into the Teflon tube then vortex for 30 s by using vortex (Tabloys Advanced vortex mixer).

Immunoaffinity chromatogram

Clean up stage followed whereby the diluted extract was allowed to pass through the immunoaffinity columns (RomerLab, Austria)

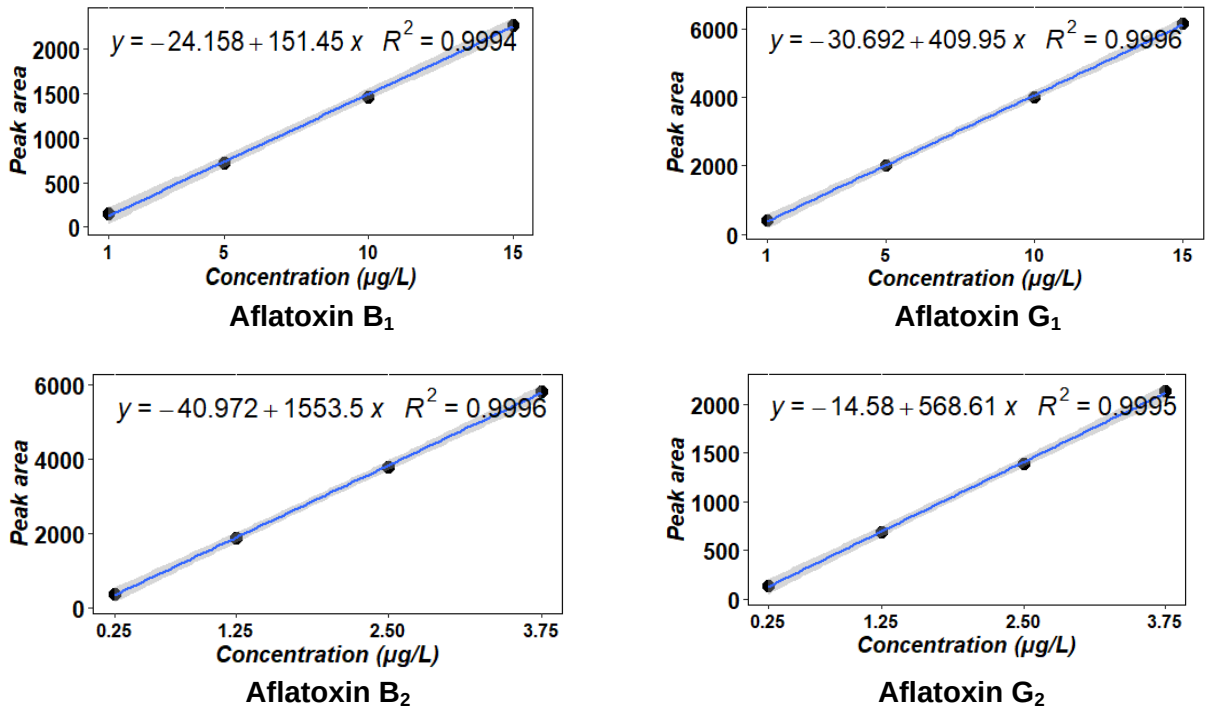

Figure 1. The calibration curves for aflatoxin standard curves.
Source: Authors

which are attached to the closed adapter by gravity, then the column was rinsed twice with distilled water the second rinse by using vacuum pressure at the end of the cleanup stage the column was removed from the adapter.

The vials were placed under the column for collection of eluent. A 0.5 ml × 2 ml of ethanol HPLC grade was used to elute the bonded aflatoxins. Then 0.3 ml of eluate was mixed with 0.6 ml of water and 0.1 ml acetonitrile and the mixture was vortexed for 30 s by Talboys advanced vortex mixture set at the speed of 2500 rpm. The sample was injected into the HPLC for quantitative determination of Aflatoxin B_1, B_2, G_1 and G_2.

Method validation

The HPLC method used was validated by evaluating its linearity, accuracy and sensitivity. The accuracy of the method was determined by spiking of cashew nut sample which was free from aflatoxins contamination and calculating the percentage recovery. About 25 g of aflatoxins free cashew nut samples were spiked with AFB_1 standard at 5 µg/kg. The sensitivity of the methodology or system used was evaluated by limit of detection (LOD) and limit of quantification (LOQ). The limits of detection (LODs) were calculated as concentrations whose peaks were three times the peaks of signal to noise (S/N) ratio, whereas the corresponding limits of quantification (LOQs) were calculated as concentrations using the peaks which were ten times the peaks of signal to noise (S/N) ratios (Saadati et al., 2013).

Quality control

The evaluation of the reliability of results, in spite of using validated methods was conducted. The method was found to have a very good separation in different aflatoxins as shown in Figure 2. The recovery of aflatoxins ranged from 92.6 to 102.4% which indicated that the method was suitable for aflatoxins analysis. This recovery is within the acceptable recovery range of 90 to 110% (SANTE, 2020).

The limit of detection and quantification for determination of aflatoxins in cashew nuts method by HPLC ranged from 0.13 to 0.16 and 0.16 to 0.29 respectively. All samples that were found to have aflatoxins levels below the detection limit were termed as not detected results.

The linearity of the method was obtained by plotting the instrument response (peak areas) against concentration (µg/L) from four known concentration of aflatoxins standards. The results shows that all calibration curves had strong linear relationship (>0.999) between peak area and concentration as shown in Figure 1. This linear relationship was higher than the minimum acceptable level of 0.998 (Christian, 2007).

HPLC analysis of aflatoxins in cashew nuts

A mixture of aflatoxins standard solution B1, B2, G1, G2 of the following concentration 2.02, 2.01, 0.5 and 0.503 µg/ml respectively was used for calibration (Biopure lot number 16192N, Romer Labs, Austria). The diluent was the same as the mobile phase (Water 6: methanol 3: acetonitrile 1). The concentration used was 0.25, 1.25, 2.5 and 3.75 µg/L for B_2 and G2; 1, 5, 10 and 15 µg/L for B_1 and G_1. HPLC coupled with fluorescence detector (serial number: DE60558333, model: G1321A), Pump (serial number: DE62976952, Model: G1311A), Auto sampler (serial number: DE647710, model: G1329A), column oven (serial number: JP94178283, model: G1322A) all from Agilent technology, series 1200, 5301 Stevens Creek Blvd, Santa Clara, CA 95051, USA) were used to analyze the standards and the extracted samples.

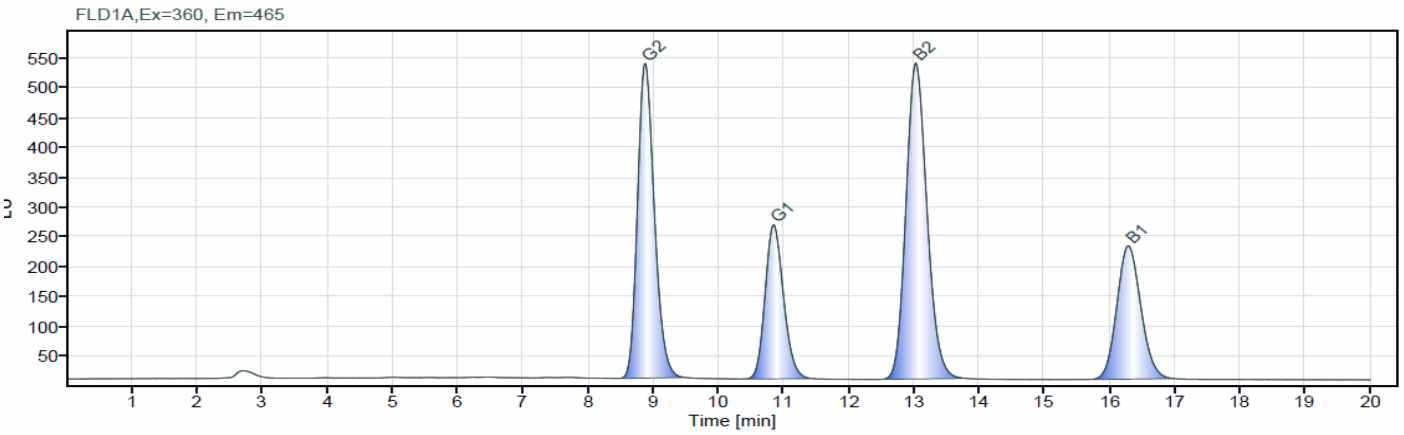

Figure 2. Chromatogram of different aflatoxins from standard (10 µg/L for B1 and G1; 2.5 µg/L for B2 and G2.
Source: Authors

Table 1. Demographic, general information and awareness of aflatoxins in small scale cashew nuts dealers in two districts of Mtwara region.

Category	Sub-category	Number of observation (%)	Aflatoxin knowledge (%)
Gender	Male	36	9
	Female	64	18
District	Newala	49	10
	Masasi	51	20
Education level	Primary	58	6
	Secondary	39	28
	University/tertiary	3	33
Type of respondent	Processor	1	1
	Consumer	43	0
	Both	56	25
Type of end product	Raw	3	50
	Roased	6	0
	Both raw and roasted	91	15

Source: Authors

The column C18, ZORBAX Rx- C18 4.6 × 250 mm, 5 µm was used to separate groups of AF B_1, AFB_2, AFG_1 and AFG_2 at the column temperature of 300°C and flow rate of 1.2 ml/min. The injection volume of the extracted samples and standard solution was 50 µL. Derivatization of AFG_1 and AF B_1 was conducted after separation with UVA photo ion to allow their detection with fluorescence detector at an emission wavelength of 465 nm and an excitation wavelength of 360 nm.

Readings from the HPLC machine were transformed into peaks and from the peaks to data through computer programmed with LC/MSD Chemstation software Revision B. 04.02 SP1 (212) connected to the HPLC machine.

Quality control was done by running quality control material aflatoxins in corn with the concentration of Aflatoxin B_1: 8.8± 3.1 µg/kg, B_2: < 1 µg/kg, G_1: < 1 µg/kg, G_2: < 1 µg/kg. A blank sample was prepared using distilled water which was prepared using Evoqua Water Technologies PTE LTD Farrernberg-Germany. The method was found to have a very good separation in different aflatoxins. The recovery of aflatoxins ranged from 92.6 to 102.4% which is within the acceptable recovery range of 90 to 110% (SANTE, 2020). The limit of detection and quantification of aflatoxins in cashew nuts by HPLC ranged from 0.13 to 0.16 and 0.16 to 0.29 respectively. All samples that were found to have aflatoxins levels below the detection limit were termed as not detected results.

Statistical analysis

Statistical Package for Social Sciences (IBM SPSS® Version 25

(2017) was used for calculating frequencies and descriptive summaries on data for awareness of aflatoxins contamination. Data on levels of aflatoxins contamination in cashew nuts were analyzed with using R- version 4.0.3 (2020). Analysis of variance (ANOVA) was used to test for significant differences on aflatoxins in raw and roasted cashew nuts from different districts. Mean separation test was done by Turkey HSD multiple rank test with agricolae package.

RESULTS AND DISCUSSION

The demographic characteristics of cashew nuts processors and cashew nuts consumers on awareness, handling practices and factors associated with aflatoxins contamination in cashew nuts were investigated and the levels of contamination were evaluated.

Awareness on aflatoxins and factors associated with aflatoxins contamination in cashew nuts

Demographic characteristics of cashew nuts dealers showed that most of them were female (64%) and education level ranging from primary school (58%), secondary school (39%) and few had tertiary/university education (2.5%). A study in India revealed that the cashew industry provides employment to a large number of poor women workers from rural areas (Pattanayak, 2020). Most of them were both consumers and processors of both raw and roasted cashew nuts as shown in Table 1.

Generally, very few respondents (<20%) in either category had heard aflatoxins in their lifetime. Almost 30% of all respondents who heard the word aflatoxins, heard it during different trainings. For example, more than 50% of all respondent had primary school education, only 6% had heard the word aflatoxins whereas 28% of respondents who had secondary school education had heard about aflatoxins. Similar findings in a study on the awareness of mycotoxins infections in Kilosa district of Tanzania found out that respondents with low level of education (below secondary level) were 1.805 times more likely to have low level of awareness and knowledge than those who had higher education (Magembe et al., 2016).

In addition to these respondents who were aware of aflatoxins, all were also aware that aflatoxins is caused by fungi, cashew nuts can be contaminated by aflatoxins and poor storage might be a cause of fungal growth and thus contaminated with aflatoxins and eating contaminated cashew nuts can cause illness or death. One cashew nuts respondent who was aware of aflatoxins responded that poor air circulation in storage conditions can results to fungal growth while the remaining 16 dealers mentioned that high moisture content during storage of cashew nuts can results to fungal growth. More than 60% of the processors produce both roasted and raw cashew nuts (Table 1) but roasted cashew nuts are highly consumed than raw (more than 50%) (Table 2). Most of the cashew nuts processors are also good consumers of the cashew nuts (>50%).

Storage practices of cashew nuts

The cashews are stored in the form of shelled cashew nuts, processing is done batch wise and especially when one receives an order for processed cashew nuts. Processed cashew nuts are not kept for more than six month before they are sold; more than 40% processors from Newala and more than 50% processors from Masasi were storing the processed cashew for not more than three month (Table 3).

Almost all the processors owned a farm where their raw materials come from, only few (<20%) obtain their raw materials from other farms. A study done by Azam-Ali and Judge (2001) showed an estimation of 280,000 households, covering an area of 400,000 ha, is involved in cashew production and the government is actively supporting them in improving the condition of the trees and maximizes agronomic potential. The processed cashew nuts are kept in either plastic buckets or in plastic bags ready to be sold. All the respondents were storing the shelled cashew nuts in jute bags.

Most of the products from street vendors were found packed in transparent nylon bags with or without labels and some of them were found not packed at all. On the other hand, the local processors were found keeping the processed cashew nuts in plastic buckets prior to packing in zipped plastic bags of different sizes ready for selling; details were on the label of some of the packages whereas other packages lack the details. A study done by Ramadhani et al. *(*2014) found similar scenario where plastic buckets (87.5%) and other materials such as paper boxes (12.5%) but for street, vendors' plastic films (polyethylene bags) were the main packaging materials used (97.5%).

A study done by Ramadhani et al. (2014) on the physicochemical quality of street vended roasted cashew nuts in Tanzania also found that immediately after roasting, cashew nuts were stored in plastic buckets (87.5%) and other materials such as paper boxes (12.5%). A study to evaluate the effect of packaging materials on moisture and microbiological quality of roasted cashew nuts revealed that plastic and glass bottles had counts within the acceptable limits (Oladapo et al., 2014).

About a quarter (38%) of the samples had total aflatoxins less than limit of quantification (0.16 µg/kg) while all roasted cashew nuts from both districts had total aflatoxin less than 3 µg/kg (Table 4). All roasted cashew nut samples were found to have total aflatoxins less than 3 µg/kg while about 86% of raw cashew nut samples had total aflatoxins less than 3 µg/kg. None of the samples had aflatoxins contamination greater than recommended maximum residues of 4 µg/kg set by European Commission (2010) or 10 µg/kg set by FAO and WHO

Table 2. Eating practices for cashew nuts dealers in Mtwara region.

Category	Response	Newala (%)	Masasi (%)
Frequency	Rarely	12	7
	Sometimes	29	30
	Daily	59	63
Amount eaten (g)	100	25	38
	200	68	43
	More than 300	7	19
Type eaten	Raw	0	2
	Roasted	54	62
	Backed	12	10
	Any type	34	26

Source: Authors

Table 3. Storage information for cashew nuts dealers in Mtwara region.

Category	Sub-category	District	
		Newala (%)	Masasi (%)
Storage time (months)	1-3	49	73
	3-6	49	16
	More than 6	2	11
Source of raw materials	Other farms	23	13
	Own farm	60	68
	Own farm and others	17	19
Storage area	Bare ground	0	3
	Jute bag	100	97
Storage type	Plastic bags	47	47
	Plastic backets	53	26

Source: Authors

(1995) for similar products such as pistachio and almond. In both districts, raw cashew nut samples had high levels of total aflatoxins than roasted cashew nut samples indicating that roasting reduces the levels of contamination.

Aflatoxins contamination within the districts

The levels of aflatoxins in cashew nuts (raw and processed) are expressed in Table 5. Statistical difference was observed in aflatoxin B_1 and total aflatoxins. Raw cashew nuts were found to have statistically higher values of total aflatoxins compared to roasted cashew nuts in both districts. For those samples where aflatoxins were detectable, aflatoxin B_1 was generally the major contributor to total aflatoxins.

The levels of aflatoxins in this study was found to be higher than the levels found in the studies done in Nigeria and South Africa (Adetunji et al., 2019) that found total aflatoxins in cashew nuts to be between 0.28 and 0.77 µg/kg respectively. High levels of aflatoxins in cashew nuts (31.50 µg/kg) were detected in a study done in north eastern Brazil in 2010 (Milhome et al., 2014).

Another study done in Vietnam on multi-mycotoxin (18 toxins) in cashew nuts showed a high level of contamination of up to 32.1 µg/kg for aflatoxin B_1 (Le et al., 2021). Effect of roasting on degradation of aflatoxins have been observed in different crops such as pistachio (Yazdanpanah et al., 2005), soybeans (Hamada and Megalla, 1982), peanut (Martins et al., 2017) and other crops that have been reviewed by Emadi et al. (2021).

Table 4. Aflatoxin (µg/kg) contamination in Masasi and Newala districts in raw and roasted cashew nuts.

District	Process	Range B_1	Range TAF	TAF <LOQ	TAF <3	3<TAF<5
				%		
Masasi	Raw	ND - 3.29	ND - 3.29	25	88	12
	Roasted	ND - 2.17	ND - 2.36	63	100	0
Newala	Raw	0.96 - 3.18	1.17 - 3.24	0	83	17
	Roasted	ND - 2.59	ND - 2.78	46	100	0

Source: Authors

Table 5. Aflatoxin contamination (µg/kg) in raw and roasted cashew nuts from Masasi and Newala districts.

District	Process	Aflatoxin G_2	Aflatoxin G_1	Aflatoxin B_2	Aflatoxin B_1	Total
Masasi	Raw	0.05 ± 0.02^a	0.01 ± 0.02^a	0.02 ± 0.01^a	1.71 ± 0.43^a	1.79 ± 0.43^a
	Roasted	0.04 ± 0.01^a	0.01 ± 0.02^a	0.02 ± 0.01^a	0.83 ± 0.12^b	0.90 ± 0.12^b
Newala	Raw	0.04 ± 0.02^a	0.03 ± 0.02^a	0.03 ± 0.01^a	2.02 ± 0.23^a	2.12 ± 0.22^a
	Roasted	0.04 ± 0.01^a	0.02 ± 0.01^a	0.03 ± 0.01^a	0.87 ± 0.13^b	0.97 ± 0.14^b

Means with different letters within the column are statistically significant ($p<0.05$).
Source: Authors

The lower value of aflatoxins in roasted cashew nuts can be accounted for by application of heat during heating that can degrade the toxins or enhance the reactions between the aflatoxins and other compounds of the cashew nuts and might modify the structures of the toxins (Farahmandfar and Tirgarian, 2020).

The higher value of aflatoxins in raw cashew nuts is linked principally to water activity (a_w). This observation is attributable to improper drying which predisposes stored produce to growth of mycotoxigenic fungi such as *Aspergillus* species which is conjectured to also increase with storage time (Temba et al., 2017).

Conclusion

Determination of the levels of aflatoxins contamination in the two districts of Mtwara region in Tanzania showed that in all samples, AFB_1 and total aflatoxins levels were below the maximum limit recommended by the European Commission regulations (4 µg/kg) as well as for the Tanzania Bureau of Standards (10 µg/kg). The study revealed that raw cashew nuts were more contaminated than roasted cashew nuts in both districts suggesting that roasting is one way of reducing contamination. Aflatoxicosis is still one of the main public health concerns in Tanzania that lead to health hazards in the population. There is a need to reduce the contamination by controlling aflatoxins contamination through Good Agriculture Practices (GAP) at farm level as well as improved storage conditions. It is important that farmers, processors and everyone who are involved in cashew nuts value chain to be educated on the potential carcinogenic nature of the aflatoxins in human health.

Recommendations

The small scale cashew nuts processors do not have any instrument for moisture content determination and the removal of the outer and inner coat of the nuts is being done by bare hands, therefore the authorities should take the lead in the efforts to establish mandatory regulations in cashew nuts farming, processing and storage to decrease contamination risks to toxigenic fungi. Also strict hygienic measures should be implemented during storage, drying and packing so as to minimize contamination, this will enhance international trade efforts and improved public health.

CONFLICT OF INTERESTS

The authors disclose no conflicts of interest.

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