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Full Length Research Paper

A survey of the aflatoxin level and molecular identification of fungal contaminants in poultry feed mills from different geopolitical zones of Nigeria

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Aflatoxin contamination of poultry feeds in Nigeria is a common problem in most feed mills. A survey on the distribution of aflatoxin in feed mills from different parts of Nigeria was carried out. The aflatoxin concentration in most feed mills from the North-western part of Nigeria was low compared to the concentration in the feed mills from the South-eastern part. The total aflatoxin level in three feed mills (B, C and D) out of the four feed mills sampled in North-West ranged from 8.4 ± 2.7-13 ± 4.2 µg/kg. However, feed mill A, had higher concentration of aflatoxin 120 ± 38 µg/kg compared to the others. Three of the North-west feed mills with low aflatoxin concentration were below the 20 µg/kg recommended by United States Food and Drug Administration (USFDA) but the concentrations were higher than the 10 µg/kg recommended by the European commission (EU). The total aflatoxin in the three feed mills sampled from the South-eastern part of Nigeria ranged from 30 ± 9.6 µg/kg in feed mill G to 34 ± 11 µg/kg in feed mill E. The results from the South-eastern part are comparable with the data from the South-western part of the country. AFG2 was very low in the feed mills sampled in the studied geopolitical regions while AFB1 appeared to be relatively high in all the feed mills in these regions. Screening of the contaminated feeds for aflatoxigenic fungi showed that *Aspergillus* species were the most common fungal contaminants, with *Aspergillus amstelodami* and *Aspergillus niger* being the most isolated fungi. The study has provided a comparative data on aflatoxin distribution in poultry feeds across some geopolitical zones in Nigeria. The obtained data could be useful in aflatoxin mapping in the studied geopolitical zones. Major fungal contaminants of the feed samples from all the geopolitical zones were also identified in this study.

Key words: Aflatoxin, *Aspergillus*, fungi, mycotoxin, Nigeria, feed mills, poultry feed.

INTRODUCTION

A typical poultry ration is made up of several grain sources, each of which may be contaminated with one or several mycotoxins. Contamination of forages and cereals by mycotoxigenic fungi often occur in the field, or

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during processing, transportation and storage when conditions for growth such as temperature and relative humidity are available. Temperature and relative humidity of above 30°C and 80-100% respectively are favorable for fungal growth (Blaha and Lohnisky, 1990). Other conditions that favor fungal growth on feed or feed materials include nutrient availability (Njobeh et al., 2003) and oxygen supply (Filtenborg et al., 2000). Occurrence of mycotoxins in animal feed is most likely to occur in countries where the environment is hot and humid (Paterson and Lima, 2011). Mycotoxigenic fungi invade only a minor fraction of feed particles with appropriate condition for growth. Several mycotoxins occur in poultry feed and their ingredients, and these include aflatoxins, ochratoxins, fumonisins, cyclopiazonic acid, deoxynivalenol (DON), zearalenone (ZEN), nivalenol and ergot alkaloids (Zollner et al., 1999; Dalcero et al., 2002; Biselli and Hummert, 2005; Oliviera et al., 2008; Spanjer et al., 2008). Among these mycotoxins, aflatoxins are the most spread, most dangerous and most studied in the tropics. Aflatoxins are structurally related hepatocarcinogenic fungal metabolites produced on nuts and cereals by fungi of the *Aspergillus* species like *A. flavus*, *A. parasiticus* and *A. nomius* (Bressac et al., 1991; Groopman and Kensler, 1996). Aflatoxin contamination of animal feeds in the tropics is common due to the ubiquitous nature of the aflatoxin producing fungi. The concern for food safety due to aflatoxin contamination is more common in Sub-Saharan Africa, as compared to the temperate regions, where staple foods like maize and groundnut are less prone to aflatoxin contamination (Bankole and Adebanjo, 2003; Bankole et al., 2006). Aflatoxin-contamination of poultry feeds results in increased mortality of birds, decreased blood cell count, lower egg production, lower feed consumption rate, impaired resistance to infectious diseases, reduced vaccination efficiency and induced pathological damage to the liver and other organs (Mgbeahuruieke et al., 2018; Kamalavenkatesh et al., 2005). Approaches for reducing aflatoxin concentration in poultry feeds include, drying grains before storage to reduce the moisture content of the feed; and biological methods (Schaller, 2009). The use of microbial products which absorb mycotoxins from contaminated feeds has also been practiced (Xiao et al., 1991). Additionally, feed additives such as adsorbents have also been recommended as good detoxifying agents of contaminated feeds (Oguz et al., 2000; Kana et al., 2006; Mgbeahuruike et al., 2018). Studies have shown that the most frequently isolated fungal genera in both private and commercial feed mills in Nigeria is *Aspergillus* spp which is about 40% of mould, *Penicillium* spp, which is 20% in private feed mills and 13% in commercial feed mills (Adeniran et al., 2013). Commercial feed companies in Nigeria depend on feed ingredients which are produced locally for livestock feed production. Such companies purchase large quantities of grains and other feed materials during the production seasons; and these feed stuffs are stored and used gradually for feed production throughout the year. Additionally, the long storage of cereals during post-harvest periods and improper storage conditions are known to favour fungal growth, resulting in aflatoxin production in feed. Most feed mills suffer huge losses due to diseases caused by poor processing of feeds and feed contamination with microbial metabolites (Chelkowski, 1991; Hussein and Basel, 2001). The poultry industry in Nigeria has an annual growth rate of 2.17% (Killebrew and Plotnick, 2010). It is an important agricultural subsector and it provides food, employment and other economic resources for the country (Killebrew and Plotnick, 2010). Some of the birds raised in Nigeria as poultry include chickens, turkeys, ducks, guinea fowls, pigeons and ostrich (Killebrew and Plotnick, 2010). In Nigeria, there is no regulatory framework guiding levels of concentration of aflatoxin in poultry feeds. As a result, aflatoxin contamination of most livestock feeds in Nigeria are on the increase from the delivery of grains from harvesting point, to storage for feed manufacturing and finally to poultry farms in the form of feed. Commercial poultry sector in Nigeria ranges from the small scale peri-urban or rural operations to very large farms with well-integrated facilities. About 90% of the meat from broilers is sold in frozen, fried or roasted forms to consumers through fast food companies, commercial distributors, supermarkets and hotels or other hospitality industry operators (Adene and Oguntade, 2006). Since the meat and eggs sourced from the birds are major protein sources for many Nigerians (USDA-FAS, 2010), there is a need for appropriate monitoring and legislation of all aspects of quality issues in this sector. However, in other countries, maximum concentration of aflatoxin, DON, ZEN and ochratoxin A (OTA) are regulated in poultry feeds at various levels (FAO, 2004). In Africa, poultry industry suffers annual losses to the tune of over USD 670 million due to diseases caused by poor processing of feeds and feed ingredients (Bankole and Adebanjo, 2003). Because of these prevailing challenges, there is need to understand the rate of occurrence, distribution and concentrations of regulatory aflatoxin in poultry feed from different sources in Nigeria. Presently, there is no baseline data on the distribution of aflatoxin in feed mills in different parts of Nigeria. Additionally, there is a limited information on the major fungal species causing aflatoxin contamination of poultry feeds in Nigerian feed mills. The present study was carried out to investigate the prevalence of fungi and aflatoxin in commercial poultry feed mills across 2 geopolitical zones in Nigeria. Hence, we can have a base line data on aflatoxin distribution and fungal contamination in poultry feeds across the different geopolitical regions in Nigeria.

MATERIALS AND METHODS

Trial feed

The poultry feeds used in this trial were purchased from commercial

feed dealers in Ogige Market Nsukka. All the feeds were freshly prepared feeds of 2-3 weeks old and they were purchased from 7 different commercial feed dealers. From our inquiry, 4 of the dealers purchased their feeds from 4 different companies A, B, C and D in Kaduna State, North-western part of Nigeria while the remaining 3 dealers purchased their feeds from 3 different feed mills E, F and G in Enugu State, the South-eastern part of Nigeria. After purchasing the feeds, they were placed in dark, water/air proof bags and kept in a storage room with a humidity of 80-100% and temperature of 25-30°C. The storage period for all the studied feed was two weeks.

Aflatoxin measurement

Aflatoxin analysis was done using high-performance liquid chromatography system consisting of model 600 pump, 717 autosampler, an in-line degasser and model 470 scanning fluorescence detector. The Aflatoxin extraction was carried out following the procedure described in (Mgbeahuruike et al., 2018). Briefly, 10 g of each feed sample was ground with mortar and pestle and transferred to extraction tubes containing water and acetonitrile (20:80%, v/v). The solution was vigorously mixed by shaking in a rotary shaker for 45 min before it was subjected to the extraction process. About 1 g of sodium chloride and 20 ml of n-hexane were added to each tube and mixed thoroughly. The resulting extract was filtered through folded filter paper (Schleicher and Shuell, 597½) and the filtrate was centrifuged for 10 min at 4000 g. The upper hexane phase was discarded while the lower methanol phase was used for immune affinity cleanup. An aliquot (1 ml) was diluted with 40 mL of de-ionized water, mixed thoroughly and the resulting solution was purified on immuno affinity columns (VicamAflaTest, Waters Corp.). The purified extract was further analyzed using reverse phase High Performance Liquid Chromatography (HPLC) (Shimadzu Corp.) with isocratic elution and fluorescence detection after post column derivatization with bromine by KOBRA CELL® (Rhone Diagnostics, Glasgow UK). The four known fractions of aflatoxin (Aflatoxin B1, B2, G1 and G2) were analyzed.

Fungal isolation

The feed samples from the different sources were finely ground for fungal isolation. The fungi were isolated by the dilution plate technique as reported by Ezekiel et al. (2014). One gram of each sample was suspended in 9 ml of sterile distilled water and vortexed for 2 min by hand inversion. A 0.1 ml aliquot of the suspension was spread plated in triplicates on Potato Dextrose Agar (PDA) and plates were incubated at 28°C for 48 h after which the colonies in each plate were counted and recorded as the fungal load per sample and the Colony Forming Unit (CFU/g). Each fungal colony from the mixed culture in each plate was carefully picked with a sterile toothpick and transferred again into sterile solid PDA plates for final purification prior to DNA extraction (Adebajo and Diyaolu, 2003). The fungal colonies were isolated based on their morphological characteristics such as color of isolate and physical appearance on agar (Pitt and Hocking, 2009).

Molecular characterization of fungal isolates and phylogenetic analysis

The extraction of the genomic DNA of the fungal isolates was done with the Zymo Research kit (Zymo-Research fungal/Bacterial Soil Microbe DNA, D6005, USA) supplied by Bio lab, South Africa, according to the manufacturer's instructions. Amplification of the Internal Transcribed Spacer Region (ITS rDNA) of the fungal isolates from the poultry feeds was carried out with PCR using universal primers ITS 1 and ITS 4 using established protocols (Mgbeahuruike et al., 2012). Cycling parameters included an initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72℃ for 30 s, with final extension at 72℃ for 8 min. PCR products were analyzed in 1% agarose gel and electrophoresed after staining with ethidiumbromide, then visualized under ultraviolet (UV) lights. Single PCR products were cleaned using Sephadex G-50 Fine DNA Grade (GE Healthcare, Sweden) followed by sequencing at Inqaba Biotechnical Industrial (Pty) Ltd, Pretoria, South Africa with PRISM™ Ready Reaction Dye Terminator Cycle Sequencing Kit, using the dideoxy chain termination method and electrophoresed with a model ABI PRISM® 3500XL DNA Sequencer (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions. Sequenced amplicons were used for BLAST search at the National Center for Biotechnology Information (NCBI). The obtained sequences were aligned by ClustalW following the method by Thompson et al. (1994) as implemented in MEGA 7 (Kumar et al., 2016). The aligned sequences were used to reconstruct a phylogenetic tree using Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993).

Statistical analysis

The data from the different aflatoxin fractions were analyzed using descriptive statistics and the average from different aflatoxin fractions were compared and represented in tables as means and standard deviations. Phylogenetic data was analysed using bootstrap method with 1000 replications per run. Branch support was placed at 100% and values that were less than 70% were excluded from the analysis.

RESULTS

Feed samples from North West

The feed samples from Kaduna State, North-western Nigeria had very low of aflatoxin concentration except the sample from feed mill A where the aflatoxin level was high (Table 1). The aflatoxin level in the samples from feed mill A ranged from 2.6 ± 0.83 µg/kg for aflatoxin B2 to 78 ± 26 µg/kg for aflatoxin G1. However, aflatoxin B1 was relatively high but not as high as AFG1, in this same feed mill. The total aflatoxin content (AFB1+ AFB2+AFG1+AFG2) of feed samples from Feed mill A was 120 ± 38 µg/kg. In feed mills B, C and D, the different aflatoxin fractions (AFB2, AFG1 and AFG2) were low except AFB1 that appeared to be relatively higher than the other fractions (Table 1). In all the sampled feed mills in this region, AFG2 was abysmally low.

Feed samples from south east

The feed samples from feed mills in Enugu State, the South-eastern part of Nigeria presented a similar trend in aflatoxin concentration. AFB1 was relatively higher in the three feed mills sampled compared to the other aflatoxin fractions (Table 2). The total aflatoxin in the three feed

Table 1.[1]Aflatoxin level in poultry feeds from Kaduna State North-western Nigeria.

Feed source	B1 (µg/kg)	B2 (µg/kg)	G1 (µg/kg)	G2 (µg/kg)	Aflatot (µg/kg)
Feed mill A	28 ± 9.1	2.6 ± 0.83	78 ± 26	7.0 ± 2.3	120 ± 38
Feed mill B	8.4 ± 2.7	0.72 ± 0.24	3.4 ± 1.1	<0,2	13 ± 4.1
Feed mill C	13 ± 4.2	1.1 ± 0.34	3.7 ± 1.2	<0,2	18 ± 5.7
Feed mill D	11 ± 3.5	0.91 ± 0.30	4.0 ± 1.3	<0,2	16 ± 5.1

[1]Aflatoxin level was measured using reverse phase HPLC and expressed as means±standard deviation of 3 technical replicates. Abbreviations: B1 = Aflatoxin B1, B2 = aflatoxin B2, G1 = aflatoxin G1 and G2 = aflatoxin G2, Aflatot = Total aflatoxin.

Table 2.[1]Aflatoxin levels in poultry feeds from Enugu State South-eastern Nigeria.

Feed source	B1 (µg/kg)	B2 (µg/kg)	G1 (µg/kg)	G2 (µg/kg)	Aflatot (µg/kg)
Feed mill E	23 ± 7.5	3.0 ± 0.98	7.4 ± 2.4	<0,2	34 ± 11
Feed mill F	19 ± 6.2	1.8 ± 0.57	12 ± 4.0	0.83 ± 0.27	34 ± 11
Feed mill G	22 ± 7.2	5.0 ± 1.6	2.5 ± 0.82	<0,2	30 ± 9.6

[1]Aflatoxin level was measured using reverse phase HPLC and expressed as means ± standard deviation of 3 technical replicates. B1 = Aflatoxin B1, B2 = aflatoxin B2, G1 = aflatoxin G1 and G2 = aflatoxin G2, Aflatot = Total aflatoxin.

mills from South-eastern Nigeria ranged from 30 ± 9.6 µg/kg in Feed mill G – 34 ± 11 µg/kg in Feed mill E. AFG2 was very low in all the feed mills sampled in this region (Table 2). In the three feed mills from this region, AFB1 was relatively higher than the other fractions aflatoxin analyzed. The feed samples from South-eastern Nigeria showed a slightly higher level of aflatoxin concentration compared to the samples from some feed mills in the North-western part of the country.

Molecular identification and evolutionary relationship of the isolated fungi

The initial fungal culture on PDA produced several colonies of fungi with mixed morphology. Purification of the mixed fungal morphologies on fresh PDA produced pure cultures of each fungal isolate. Sequencing of the fungal ITS regions and subsequent BLAST searches in NCBI database showed that the major fungal contaminants in the feed samples were *Penicillium crustosum, Alternaria alternate, Aspergillus amstelodami, Monascus purpureus, Aspergillus niger* (Table 5). The sequences of the identified fungal species were deposited in GenBank with accession numbers JF731272, MG831329, KF986418, KY828866, KR085975 respectively. The percentage similarity of the identified fungal isolates with the fungal sequences in the NCBI database ranged from 78-100%. The identified fungal species belong to four major fungal genera. *Aspergillus* species, *A. amstelodami* and *A. niger* were the most common fungal species found in the feed samples. From the phelogenetic analysis, one major clade was observed and this cluster was made up of the following fungi, *P. crustosum, A. niger, M. purpureus* and *A. amstelodami* while *A. alternate,* formed the root of the tree showing its distant relationship from the other fungal species. In the order of evolutionary relationship, *P. crustosum* and *A. niger* appeared to be more closely related in this study, while *M. purpureus and A. amstelodami* appeared to be closer sisters in the evolutionary tree (Figure 1).

DISCUSSION

The total aflatoxin concentrations in feeds from three (B, C and D) out of the four feed mills in Kaduna state were below the 20 µg/kg recommended by the United States Food and Drug Administration (USFDA) (Table 4). However, aflatoxin in these three feed mills including feed mill A were higher than the 10 µg/kg threshold recommended by the European Union (Mgbeahuruike et al., 2018). Feed mill A from the same state (Kaduna) had a reasonably high level of aflatoxin. The probable reason for this high level of aflatoxin could be due to poor method of preservation of feed ingredients. Due to high level of demands and supply, production of cheap low-quality feeds with the main aim of fast profit making becomes a common practice in some feed mills. In all the feed mills from the North-western state, AFB1 was considerably higher than the other aflatoxin fractions, except in feed mill A. Analysis of aflatoxin concentration in all feed types in Nigeria has shown that AFB1 contaminated all chick feed, while layers mash, grower mash, finisher feed and starter feed had 46.4, 53.6, 62.5 and 72.7% contamination respectively (Ezekiel et al., 2012). AFB1 is a very potent fraction of the known aflatoxins (Mgbeahuruike et al., 2018). AFB1 has been implicated in increased mortality of birds in poultry farms, decreased blood cell count, lower egg production, lower

Table 3. Aflatoxin level in poultry feeds from 5 locations in Ogun State, South-west Nigeria.

Location	Total aflatoxin concentration (µg/kg)	Absorbance at 450 nm
Obantoko	43.2	1.522
Idi-aba	13.5	2.391
Omida	24.0	2.168
Kuto (K)	29.7	1.981
Lafenwa (L)	95.1	0.840

Source: Kehinde et al. (2014).

Table 4. USFDA approved action levels for aflatoxins in different commodities.

Commodity	Concentration(µg/kg)
All products, except milk, designated for humans	20
Milk	0.5
Corn or peanut products for immature animals and dairy cattle	20
Corn or peanut products for breeding beef cattle, swine and mature poultry	100
Corn or peanut products for finishing swine	200
Corn or peanut products for finishing beef cattle	300
Cotton or peanut products seed meal (as a feed ingredient)	300
All feedstuff other than corn	20

Source: Centre for Applied Special Technology (CAST), 2003.

feed consumption rate, impaired resistance to infectious diseases, reduced vaccination efficiency and induced pathological damage to the liver and other organs (Mgbeahuruiek et al., 2018; Kamalavenkatesh et al., 2005). The high concentration of this aflatoxin fraction in this region could be attributed to the ubiquitous nature of the producing fungi. For the South eastern part of Nigeria, the AFB1 concentration in the feed samples was high in the three feed mills sampled when compared with the other fractions.

The total aflatoxin (AFB1+AFB2+AFG1+AFG2) in the three feed mills sampled in Enugu state, South-eastern Nigeria were relatively higher than the total aflatoxin level in three out of the four feed mills (B, C and D) sampled in Kaduna state, North western part of Nigeria. The reason for this could be due to differences in weather conditions between the two regions. While the weather in the North is hot, dry and sunny, the South-eastern part of the country has a hot and humid tropical weather condition which provides the toxigenic fungal species with more favorable conditions for growth. In Ogun State, the South-western part of Nigeria, an independent study by Kehinde et al. (2014) showed a similar trend in the total aflatoxin level in poultry feed samples obtained from 5 different locations in the state (Table 3). Although the authors did not report the concentration of the different aflatoxin fractions, the total aflatoxin concentration in the feed mills studied were comparable to the results obtained from this study in the other two geopolitical regions (South-eastern and North-western Nigeria). In the survey from the South-west region, the highest level of aflatoxin concentration, 95 µg/kg was found in a feed mill from Lafenwa town, this was followed by Obantoko town with aflatoxin concentration of 43.2 95 µg/kg (Table 3). Feed samples obtained from the other towns Idi-aba, Omida and Kuto had different concentrations of aflatoxin respectively (Kehinde et al., 2014). Molecular identification of the fungal contaminants in the feeds showed five different fungal species *P. crustosum, A. alternate, A. amstelodami, M. purpureus, A. niger* belonging to four major genera of fungi (Table 5). Although four fungal genera were observed in the feeds, only two (*Penicillium* and *Aspergillius*) are known for mycotoxin production and may have contributed to the different fractions of aflatoxin observed in the feeds. However, *Aspergillius* species are known as the major producers of aflatoxin in the tropics (Mgbeahurike et al., 2018). *Aspergillus* species were the most abundant fungal species isolated from the feed samples. *Aspergillus* species such as *A*. *tamarii*, *A. nominus, A. flavus, A. parasiticus* and *A. tubingensis* have been implicated in many studies as the primary source of aflatoxin contamination of poultry feeds (Mgbeahuruike et al., 2018; Peterson et al., 2001; Klich et al., 2000). However, the two major *Aspergillus* species *A. flavus* and *A. parasiticus* known for heavy aflatoxin production in poultry feeds (Donner et al., 2009) were not found in this study. Heavy contamination of poultry feeds by *A. niger* has also been reported in other studies

Table 5. Fungal isolates from the feed samples screened in this study.

Sequence ID	Fungal identity	Accession number	% similarity
Seq1	*Penicillium crustosum*	JF731272	78
Seq2	*Alternaria alternata*	MG831329	100
Seq3	*Aspergillus amstelodami*	KF986418	100
Seq4	*Monascuspurpureus*	KY828866	99
Seq5	*Aspergillus niger*	KR085975	99

Amplification and sequencing of the Internal Transcribed Spacer Region (ITS rDNA) of the fungal isolates from the poultry feeds was done using the dideoxy chain termination method and electrophoresed with a model ABI PRISM® 3500XL DNA Sequencer (Applied Biosystems).

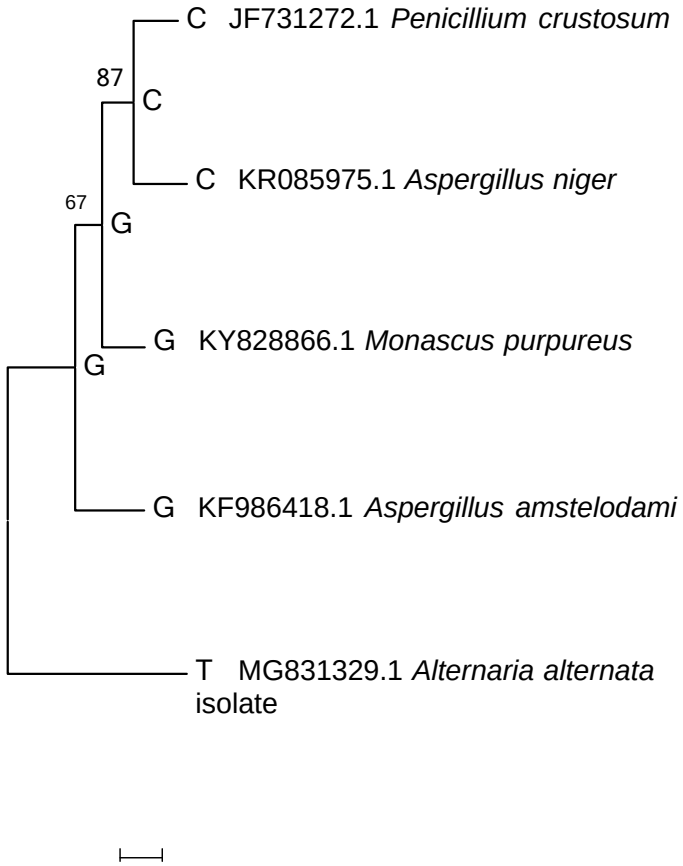

Figure 1. Molecular Phylogenetic analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model and the tree with the highest log likelihood (-1598.8496) was chosen. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 5 nucleotide sequences with 1000 replications for bootstrap support. Branch supports that were less than 70% were excluded from the tree. Letters C, G and T indicate different branches of the tree.

(Adeniran et al., 2013). *Penicillium* species are known to produce the mycotoxin, Trichothecenes, including DON (Rodrigues and Naehrer, 2012; Tiemann and Dänicke, 2007). However, in this study, we did not screen for the presence of Trichothecenes and other mycotoxins in the feed. *Alternaria* species and *M. purpureus* are not known as aflatoxin producing fungi, they could be in the feed as contaminants but not for aflatoxin production. Phylogenetic analysis of the identified fungal species confirmed an evolutionary relationship between the fungal isolates.

Conclusion

The study has been able to make a comparative analysis of aflatoxin distribution in major poultry feed mills across some geopolitical zones in Nigeria. The observed aflatoxin level in most of the feed mills was slightly above the recommended levels of aflatoxin in poultry feeds (20 µg/kg) by the United States Food and Drug Administration (USFDA). The data from this study will act as a baseline data for aflatoxin mapping in the study areas and it will also act as a guide for poultry feed producers in the country. From the study also, we have succeeded in isolating and identifying some of the major fungal contaminants of poultry feeds in some feed mills in Nigeria.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Molecular epidemiology of schistosomiasis in Central River Region of The Gambia

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Schistosomiasis is the second most prevalent tropical parasitic disease after malaria, and one of the leading cause of morbidity and mortality in developing countries especially in Africa. This study was carried out among students, farmers and fishermen/women from four villages in the Central River Region of The Gambia. The aim of this study was to show the prevalence value of schistosomiasis in Central River Region of The Gambia. Questionnaires were administered to acquire data such as age, gender, prior schistosomiasis infection and treatment. One hundred and ninety-five blood and 192 urine samples collected from 117 females and 78 males were examined. Microscopy, ELISA and Polymerase Chain Reaction (PCR) techniques were used to detect and characterize schistosome isolates from the biological samples. Prevalence of *Schistosoma haematobium* was 28.7 with 41.0% in males and 23.9% in females. The highest prevalence value among the villages was in Brikama Ba with 53.1% while the age group 6-15 years had the highest prevalence of 50.0%. *Schistosoma mansoni* was only detected in Jahally village (1.5%). Schistosomiasis detection was highest using ELISA (40%) and lowest using microscopy (24.5%). PCR gave a 28.7% prevalence value.

Key words: Prevalence, *Schistosoma*, diagnostic techniques, Central River Region.

INTRODUCTION

Schistosomiasis is a chronic disease also well known as bilharzia or snail fever, parasitic flukes of the genus *Schistosoma* cause it. It remains extremely prevalent in many low-income and middle-income countries (Steinauer et al., 2008; Colley et al., 2014; Adenowo et al., 2015: Oboh et al., 2018; Li et al., 2019). The estimated global prevalence showed that at least 229 million people required preventive treatment in 2018 and more than 97.2 million people were reported to have been treated (GBD, 2016; WHO, 2017, 2020). The name bilharzia was coined from the name of Theodor Bilharz, a German surgeon. He was the first to recognize the etiological agent *Schistosoma haematobium* in 1851 while working in Cairo, Egypt (Nour, 2010).

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Schistosomiasis is one of the neglected tropical diseases (NTDs) in Africa. NTDs are hidden epidemics of enormous health and economic significance for African countries (Adenowo et al., 2015; Li et al., 2019). They are hidden because many African countries are unable to establish and address the associated health issues due partly to inadequate resources for an appropriate understanding of the biological and social characteristic of these diseases and they have been mostly wiped out in the more developed parts of the World also (Adenowo et al., 2015). Schistosomiasis is the second most prevalent tropical parasitic disease after malaria, and is a leading cause of morbidity and mortality for developing countries especially in Africa (Adenowo et al., 2015). Adult schistosomes are white or greyish worms of 7-20 mm in length with a cylindrical body that features two terminal suckers, a complex tegument, a blind digestive tract, and reproductive organs (Gryseels et al., 2006; Adenowo et al., 2015). Infection is acquired by exposure to fresh water that contains cercariae (the parasitic form that is infective for final host) released by infected snails (the intermediate host) (Corachan, 2002; Ntounifor and Ajayi, 2007). The transmission cycle needs contamination of surface water by excreta, specific freshwater snails as intermediate hosts, and human water contact (Gryseels et al., 2006). Numerous species of *Schistosoma* are pathogenic parasites of humans: *S. haematobium* is responsible for urinary schistosomiasis. *Schistosoma mansoni, Schistosoma japonicum*, *Schistosoma mekongi* and *Schistosoma malayi* are responsible for gastrointestinal (GI) and hepatosplenic schistosomiasis. *Schistosoma intercalatum* affects the GI tract but are associated with a lower morbidity. *Schistosoma matheei* and *Schistosoma bovis* are occasional parasites of human and *Schistosoma incognitum* may also show infective strains to humans (Corachan, 2002; Oboh et al., 2018). The species differ in their final location in the human host, the species of the intermediate host(snail), the pathology they induce, and the number, size and shape of the eggs produced (IARC, 2012). *Schistosoma* transmission is extremely dependent on environmental conditions, mainly those affecting the snail host. Climate change modifies aquatic environments and subsequently the transmission and distribution of waterborne diseases (Mas-Coma et al., 2009). Schistosomiasis is typically found in poor rural communities, where fishing and agricultural activities are dominant. Domestic and recreational activities such as clothes washing, water fetching and swimming expose women and children to infection (WHO, 2017). The river Gambia is at the focus of activities for people living in the Central River Region (CRR). Folks living in these communities wash their clothes and take a bath in the river. This region has numerous fresh water bodies and the main irrigated rice fields of the country. The river is fresh water hence the high prevalence of *Schistosoma* in the region (Gambia NTD Mapping Report, 2015; Sanneh et al., 2017). The Gambian Government depends on the region to reach its goal of rice self-sufficient production among other agricultural development objectives. The high prevalence of schistosomiasis hamper the attainment of these goals. Most of the children in these regions help their parents in farming, when they are sick, their parents will have to stop farming and look after these children which lead to a double losses for them. Laboratories in The Gambia use stool to detect *S. mansoni*. This specie is sometimes challenging to detect as most people are unwilling to take their stool to the laboratories for testing causing late detection and subsequently leads to severe schistosomiasis. This can give rise to bladder cancer, damaged of organs and development issues in children (WHO, 2017). Earlier studies on the prevalence of schistosomiasis in humans were done using only microscopy in The Gambia (Gambia NTD Mapping Report, 2015). The prevalence of schistosomiasis in The Gambia is put at 4.3%, with a higher prevalence in the Central River Region (CRR) at 14.2% followed by Upper River Region at 9.4% (Gambia NTD Mapping Report, 2015). The River Gambia remains fresh throughout the year in these two regions. The two regions also have several fresh water bodies and are the major irrigated rice fields in the country. However, all other regions apart from North Bank East Region (NBER) are also endemic for schistosomiasis (Gambia NTD Mapping Report, 2015; Sanneh et al., 2017). The Government of The Gambia needs to embark on a nationwide sensitization work since, the awareness level about schistosomiasis continues to be very low, and this may imply that a great deal of the Government's pledge to schistosomiasis control programs is extremely needed (Barrow et al., 2020). Microscopy as a detection tool for Schistosoma is not only associated with sensitivity limitation but it is also highly unspecific as it cannot characterize the parasite into species and sub-species level. To the best of our knowledge, no investigation/research has been conducted using molecular techniques or immunological tests in The Gambia. Molecular tools which are the most sensitive and specific tools will be used to detect *S. mansoni* in blood and urine. However, effective and efficient surveillance systems need to be put in place to curb the spread of this vector borne diseases across The Gambia (Kargbo and Kuye, 2020). The study aims of this study were to show the prevalence of schistosomiasis in the Central River Region of The Gambia.

MATERIALS AND METHODS

Study area

This study was done in the Central River Region of The Gambia from March to April 2017 (Figures 1 and 2). This region is divided into two locations, north and south. It is separated by the river Gambia, four villages were selected at random from both locations of the region; Jahally (13°33` 40.09``N 14°58'19.53``W), Wali Kunda (13°34` 0``N 14°53` 0``W), Brikama Ba (13°32` 11.96``N

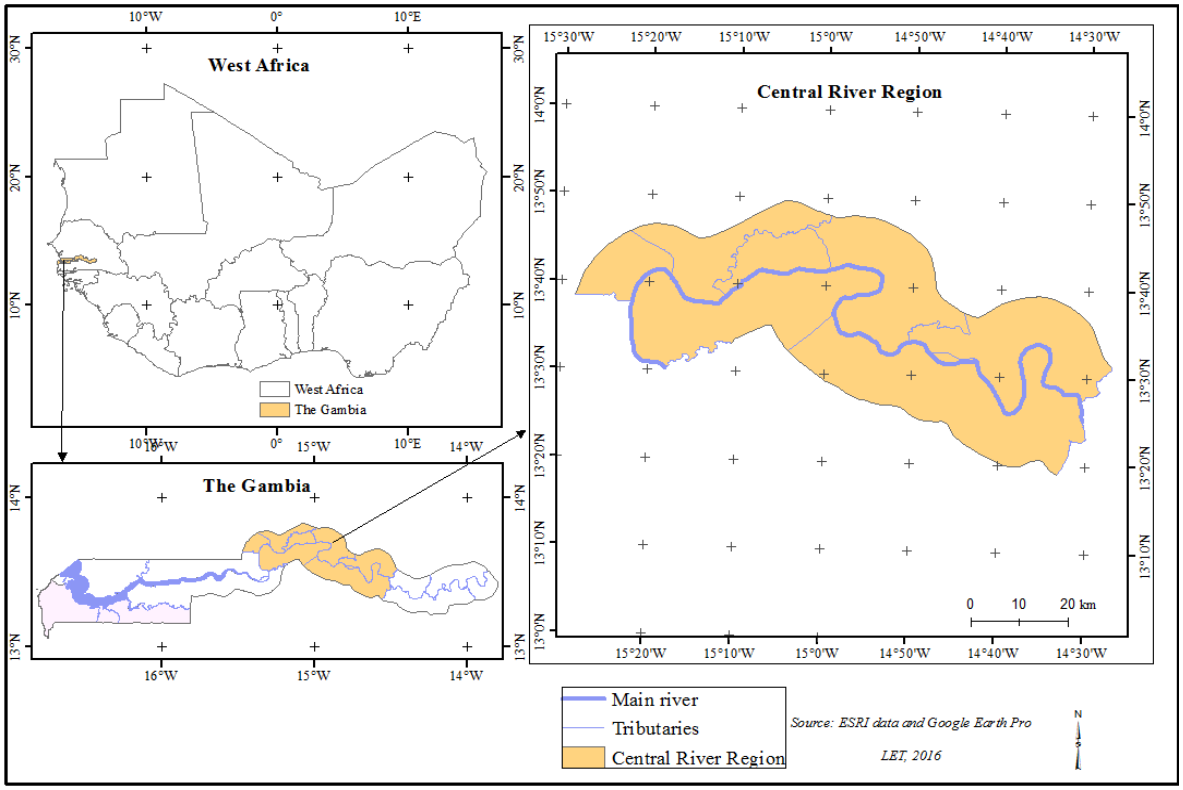

Figure 1. Location of The Gambia in West Africa and Central River Region in The Gambia.

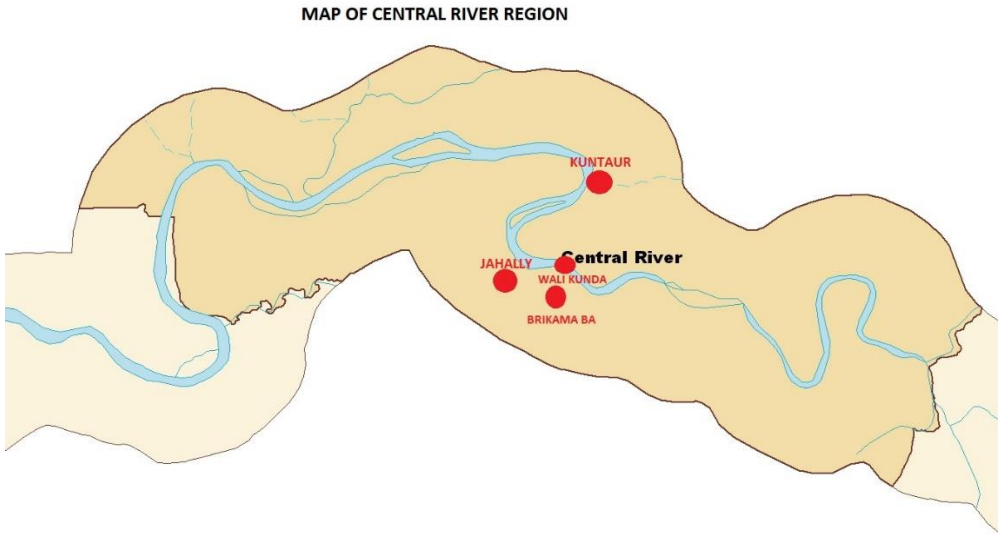

Figure 2. Location of Central River Region in The Gambia showing the Kuntaur, Jahally, Wali kunda and Brikama Ba.

14°55 53.78`W), Kuntaur (13°40` 14.74``N 14°53` 23.90``W). Children (both sex) from the ages of 6-15 were randomly selected. Children less than 6 years old were not included in the study. Farmers and Fishermen/women were also selected using simple random sampling.

Sampling

The sample size was calculated using the formula described by Thrusfield (2007). The prevalence of *Schistosoma* used in CRR was 14.2% (Gambia NTD Mapping Report, 2015). A total of 188

samples were collected.

Ethical clearance

Ethical clearance was attained from the Scientific Coordinating Committee of The Gambia government and Medical Research Council Joint Committee. Before sample collection, permission was obtained from the Ministry of Basic and Secondary Education as well. The study was described to each participant for their understanding and cooperation. Moreover, an informed written consent form was signed by each study participant, and for the children, consent was obtained from their parents/guardians.

Microscopy of urine samples

Urine samples were collected in 50 ml conical tubes between the hours of 10.00 and 14.00 when schistosome eggs excretion is known to be highest (Obeng et al., 2008; Orsot et al., 2018). All the urine samples were examined and recorded as either clear amber, clear and cloudy or bloody before centrifugation for microscopy. These samples were examined by centrifugation technique (Ukaga et al., 2002). Ten ml of each urine sample was centrifuged at 1,500 rpm) for five minutes. The supernatant was decanted and the sediment examined under the microscope. Pasture pipette was used to add two drops of the sediment to a frosted microscope slide and covered with a cover slip slightly without the formation of air bubbles. The slide was examined under the microscope at low magnification (x10 and x40 objective lenses) for the presence of eggs of *S. haematobium* and *S. mansoni.* For quality control, duplicate slides were prepared for all samples. An experienced microscopist read all the positive slides and 10% of the negative (Sousa et al., 2019).

ELISA test of blood samples

Blood samples were obtained by the finger prick method. The finger was cleaned with alcohol wipes and left for about three seconds to dry. Then using a pricker, the finger was pricked and gently squeezed to drop blood on the spots created on the filter paper. The spotted papers were left to dry away from direct sunlight packed in a sealable bag with desiccants and kept at ambient temperature for transportation to the Department of Biochemistry at Ahmadu Bello University Zaria in Nigeria. A regular paper puncher was used to cut 6 mm size paper discs from each filter paper and put in a well of the ELISA plate. To each sample in a well, 300 µl of phosphate buffer saline (PBS) containing 0.05% Tween 20 was added and incubated overnight in a refrigerator at 4°C (Gruner et al., 2015). Fifty microliters of the elute was used to test for IgG antibody response directed against schistosome egg antigens using *Schistosoma* IgG ELISA Kit. The ELISA plates are pre-coated with *Schistosoma* antigens. On adding of the elutes to the plate, antibodies in the samples were bonded to the antigens in the test well during the first incubation. After washing, enzyme conjugate was added and incubated at room temperature to allow the enzyme complex to bind to the antigen-antibody complex. After a few washings to remove unbound enzymes, a substrate was added that developed a blue coloration in the presence of the enzyme complex and peroxide. The plate was read on an ELISA plate reader at 450 nm with a reference filter at 620 nm (AccuDiag™ *Schistosoma* IgG ELISA Kit pamphlet).

DNA extraction from dried blood samples

A regular paper puncher was used to cut 6 mm discs from each blood soaked filter paper and put in a labelled 1.5 ml Eppendorf tube. Between the cuttings, the paper puncher was cleaned with 70% ethanol and allowed to dry to avoid contamination. 500 µl of phosphate buffer saline was added to the tubes and incubated (Lodh et al., 2013). The DNA was extracted using Quick-DNA Miniprep Plus Kit (ZYMO RESEARCH) according to the manufacturer's protocol. The DNA concentration was measured using the NanoDrop spectrophotometer. Extracted DNA samples were stored at −20°C until required.

Molecular polymerase chain reaction (PCR) identification

Nested schistosome-specific PCR was performed using the DNA extracted from each blood sample. Each of the samples was tested for both *S. mansoni* and *S. haematobium* using specifically designed primers to amplify variable regions 600 and 770 bp within cox1mitochondrial DNA (mtDNA) of *S. mansoni* and *S. haematobium* respectively. For the *S. haematobium*, the schistosome cox1 mitochondrial DNA (mtDNA) region was amplified using an outer primer for *S. haeamtobium* as follows Sh1 (5′-CGTATTTTAGGTTTATGG-3′) Sh2 (5′-CGAACTACACTTCCTAAGCA-3′) and inner primer Sh3 (5′-CGTGGTTTCATTAGATGTTTA-3′) with inner reverse primer Sh4 (5′- CGACAAATCAATCCATAATAC-3′). For *S. manson,* the PCR was carried out by using outer forward primer Sm1 (5`-CGTTGATTAAGAAGATTATGA-`3) with outer reverse primer Sm2 (5`-CGTGAAATTGACAGATCCA-`3), and inner forward primer Sm3 (5`-ATGTTACGATGTCTGTTCGGT-3`) with inner reverse primer Sm4 (5`- CGATAAAGGAGGATATAGAGTTC-3`). The cox1 mitochondria DNA gene was used as a DNA barcode to detect animal species because its mutation rate is often fast enough to distinguish between closely related species. It is highly abundant in the cell and has highly conserved regions and structures among con-specifics (Sady et al., 2015).

PCR amplification was performed in 25 µl reaction mixture and consisted of 14.75 µl of nuclease free water, 2.5 µl of PCR buffer containing 25 mM $MgCl_2$, 2.5 µl of dNTPs, and 0.5 µl of each of the primers, 0.25 µl of Taq polymerase and 4 µl of DNA. The outer primers were used for the first round of the nested PCR with the following PCR cycling conditions for the first reaction was: an initial denaturing step of 95°C for 5 min, followed by 30 cycles of 95°C for 60 s, the annealing temperature of 60°C for 30 s, elongation at 72°C for 45 s and final extension at 72°C for 4 min. The second round in which 4 µl of the products of the first round was used as DNA, with the inner primers and the same volume of the other components (nuclease free water, PCR buffer dNTPs and Taq polymerase with the following PCR conditions: an initial denaturing step of 95°C for 5 min followed by 30 cycles of 95°C for 60 s, the annealing temperature of 55°C for 30 s, elongation at 72°C for 45 s and final extension at 72°C for 4 min. The alteration in annealing temperature was due to the expected amplicon size; for the outer primers it is expected to be around 770 bp whiles for the inner, it is around 600 bp. Amplicons were visualized on a 1% agarose gel stained with Ethidium bromide using 100 bp ladder to estimate band sizes.

Statistical analysis

The prevalence of schistosomiasis in the area was calculated. The Chi-squared test was used to determine the association between factors and prevalence. Odds ratio (OR) was determined to show the association between the factor and infection rate/prevalence. Overall, age and sex specific prevalence of the disease was calculated and expressed as a percentage (%).

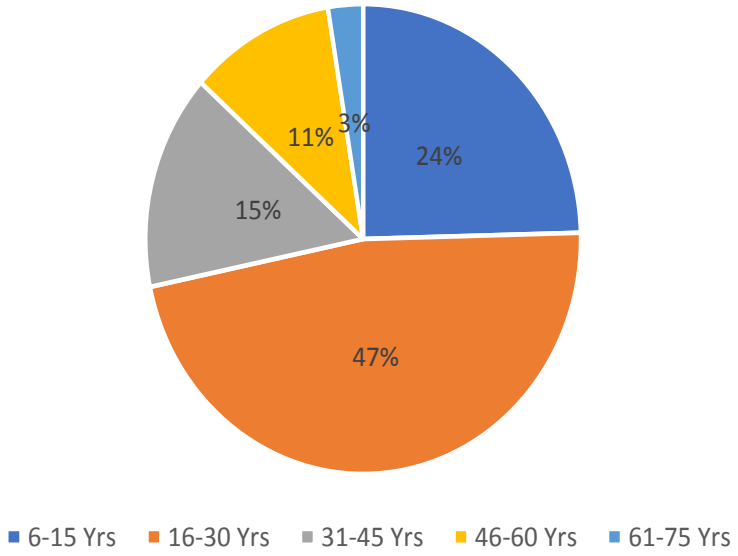

Figure 3. Age group distribution of participants in years.

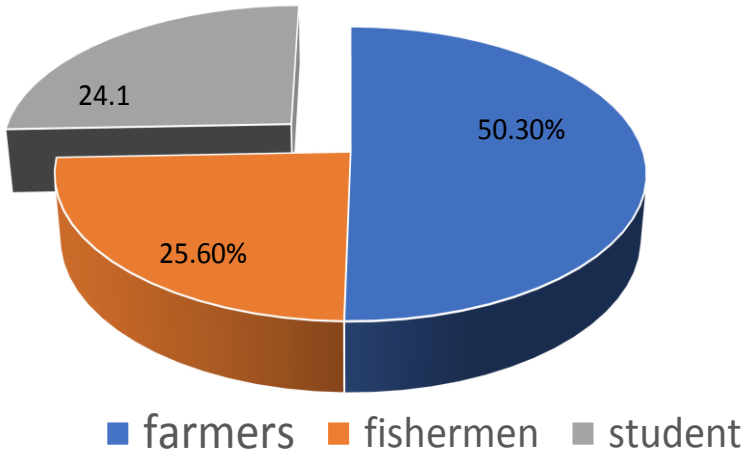

Figure 4. Occupational distribution of participants.

RESULTS

Analysis of demographic distribution of participants

Age distribution of the study participant (Figure 3) shows that adults of the age bracket 16-30 years constituted almost half of the participants from which blood and urine sample were obtained. Children of school age were about one-quarter while those above 30 years represented the remaining. Analysis of the occupational status of the participants also showed that farmers formed approximately half of the participants, with students and fishers each accounting for about one quarter (Figure 4). A large proportion of the population from Jahally village had not received any drug treatment for schistosomiasis.

Macroscopic results

Urine examination showed that Amber clear urine (44.8%) was more frequent than cloudy urine (39.9%) or bloody (10.9%) urine with clear urine (4.7%) being less frequent. Microscopy revealed an overall prevalence of 24.5% (47 of 192) for *S. haematobium* infection in the urine samples examined in this study (Figure 5). Figure 6 shows that, Brikama Ba recorded the highest prevalence among the four villages with 44.8% (13 of 29) whiles Kuntaur had the lowest prevalence with 8.2% (5/61). Jahally had a prevalence of 35.3% (24 of 68) and Wali Kunda 14.7% (5 of 34). The prevalence was higher in males (33.3%) than in females (18.8%). The infection distribution by age shows that school children from the

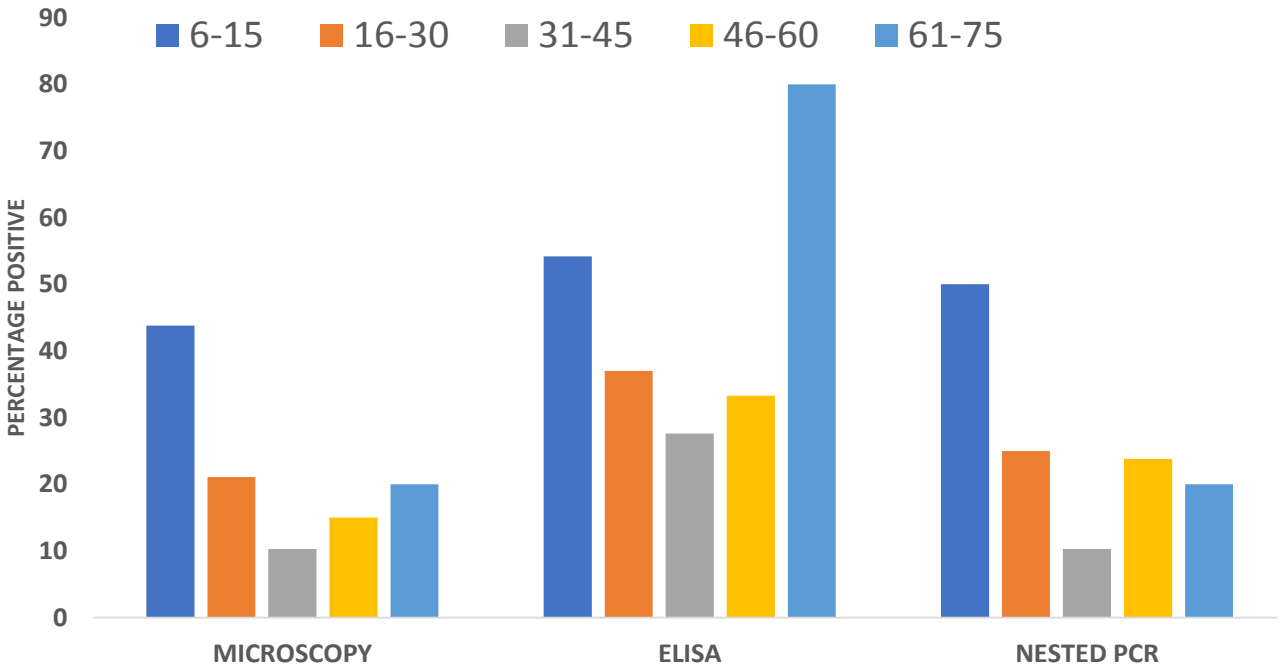

Figure 5. Percentage positive prevalence rates of the different analytical method for the different age groups.

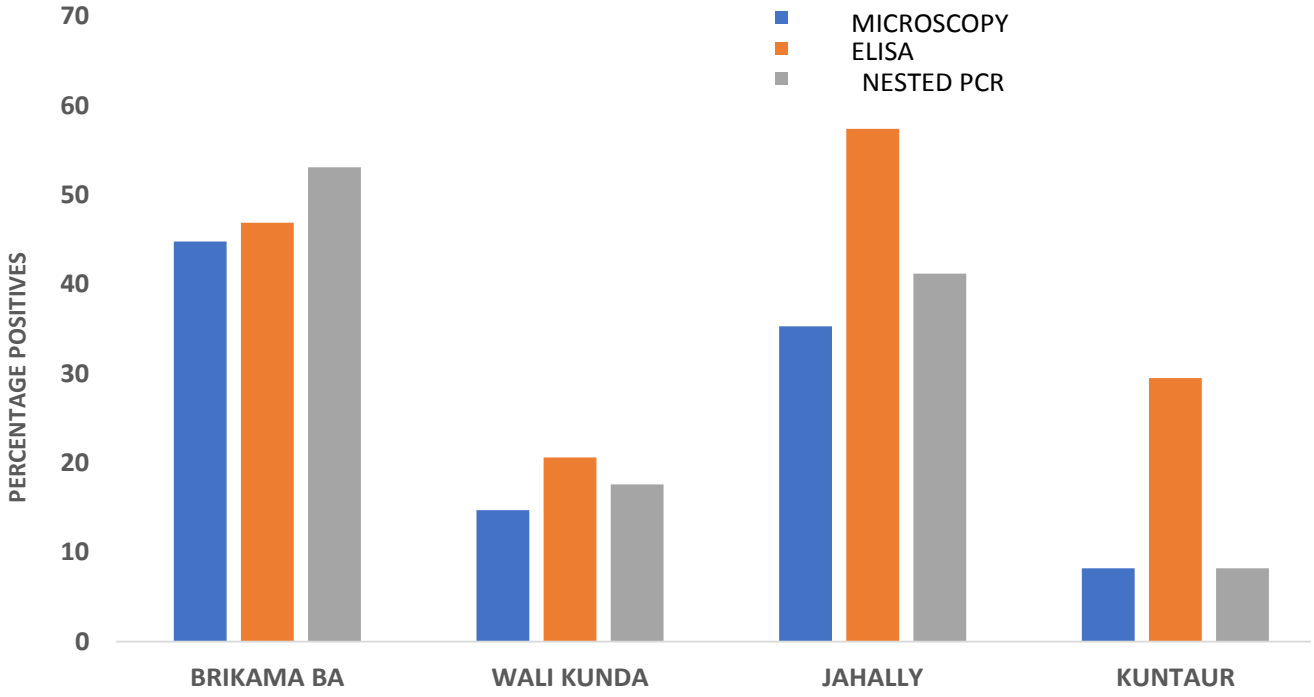

Figure 6. Percentage positive prevalence rates of the different tests for the different villages.

ages of 6 to 15 years have the highest prevalence as shown in Figure 7 with 43.8% (21 of 48) followed by age group 16-30 years with 21.1% (19 of 90). The oldest age groups 61-75 years has a prevalence of 20% (1 of 5) and 46-60 years recorded 15% (3 of 20). The lowest prevalence occurred in the age group 31-45 years with 10.3% (3 of 29) (Figure 3). In terms of occupation, fisherwoman have the lowest prevalence with 14.8% (4

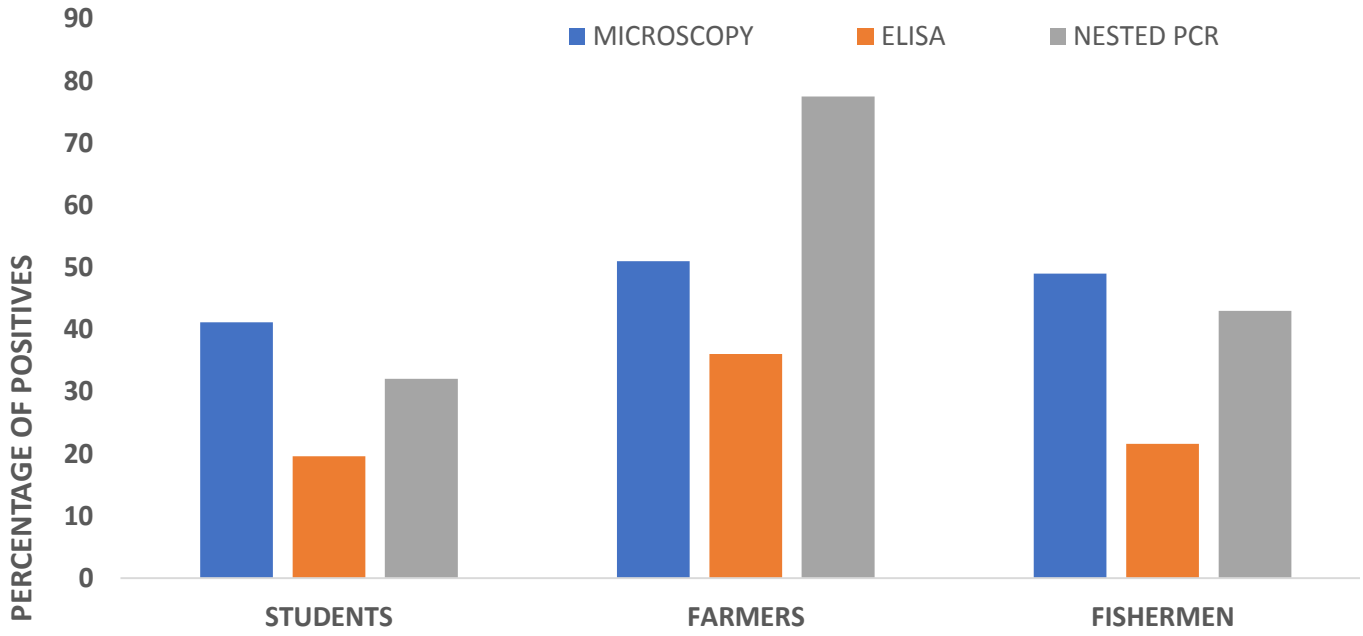

Figure 7. Percentage positive prevalence rates of the different tests for the different profession.

of 27) whiles fishermen recorded 23.5% (3 of 18). Students had the highest prevalence with 41.2% (21 of 51) followed by farmers with 19.6% (19 of 97) as indicated in figure 4. 28.8% (44 of 153) of those that were positive had never received drug (praziquantel) for treatment of schistosomiasis. In comparison 7.7% (3 of 49) had recently been treated with praziquantel. Statistical analysis (Table 2) showed that there was a statistically significant difference in prevalence between males and females and between all age groups (P<0.05).

Nested PCR

There was a prevalence of 28.7% (56 of 195) based on the PCR results for *S. haematobium.* Brikama Ba had the highest prevalence among the four villages with 53.1% (17 of 32) while Kuntaur had the lowest prevalence 8.2% (5 of 61). Jahally has a prevalence of 41.2% (28/68) whiles Wali Kunda had a prevalence of 17.6% (6 of 34). Plate 1 shows some of the bands at about 700 bp for *S. haematobium* for blood samples while Plate 2 shows the bands for *S. mansoni* in urine samples corresponding to band 600 bp. The infection distribution by age shows that school children from the ages of 6-15 had the highest prevalence of 50.0% (24 of 48) followed by age group 16-30 years with 25.0% (23 of 92). Age group 46-60 years had a prevalence of 23.8% (5 of 21). The oldest age group 61- 75 years had a prevalence of 20% (1 of 5) while the age group 31-45 years had the lowest prevalence with 10.3% (3 of 29) as shown in Table 1. In terms of occupation, fisherwomen have the lowest prevalence with 19.2% (5 of 26) whiles fisherman had a prevalence of 23.8% (5 of 21). Students had the highest prevalence with 49.0% (25 of 51) and farmers with a prevalence of 21.6 % (21 of 97). 34.0% (53 of 156) of those that are positive have never received any drug (praziquantel) for treatment of schistosomiasis whilst 7.7% (3 of 39) have recently received praziquantel. Generally, a higher infection was recorded among males 41.0% (32/78) than in females 23.9% (28 of 117). Statistical analysis showed that there was a statistically significant difference in incidence between males and females (P<0.05) as well as in all other risk factors (age, the drug received and occupation). Table 2 shows the distribution of the s*chistosoma* species detected in the urine and blood sample. While *S. mansoni* was detected only in urine samples from Jahally, *S. haematobium* was identified in the blood samples from four villages. The highest percentage of *S. haematobium* was in Brikama while the lowest was in Kuntaur.

ELISA

ELISA revealed an overall prevalence of 40.5% (79 of 195) for *Schistosoma* and this method was the most sensitivity method when compared to microscopic and molecular detection methods used in this study Figure 5. Jahally had the highest prevalence with 57.4% (39 of 68) and Wali Kunda with the lowest prevalence with 20.6% (7 of 34). Brikama Ba has an incidence of 46.9% (15 of 32) as against 29.5% for Kuntaur. Statistical analysis on Table 3 shows that there was a statistically significant

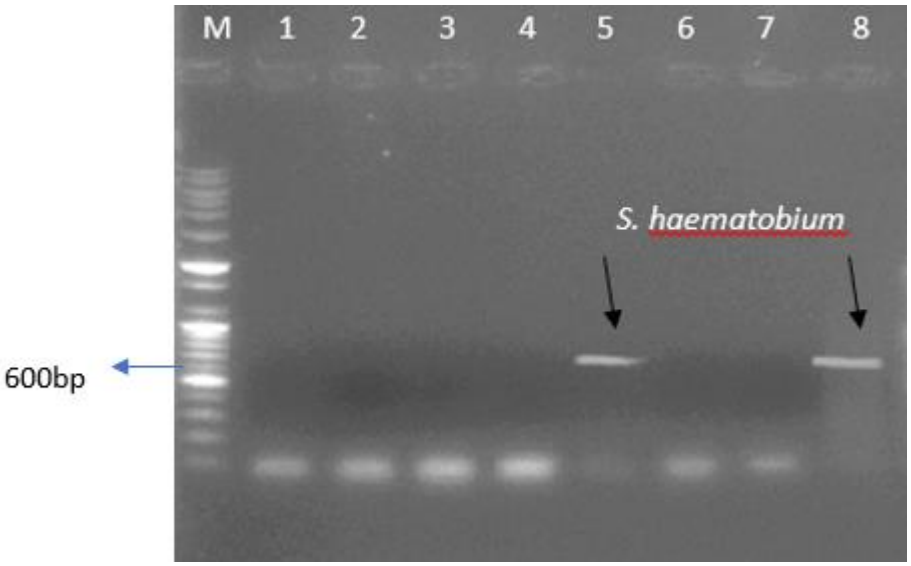

Plate 1. Molecular identification of *Schistosoma* spp from mitochondria DNA (mDNA) was prepared from the parasites and it was used for nested PCR with generic primers targeting COX-1 mDNA gene of the organisms in genus *Schistosoma*. The PCR product was then resolved on 1% agarose gel. The size of 770 bp the signal was calculated by comparing its mobility to that of the standards' bands in the marker lane as demonstrated.
Source: Sady et al. (2015).

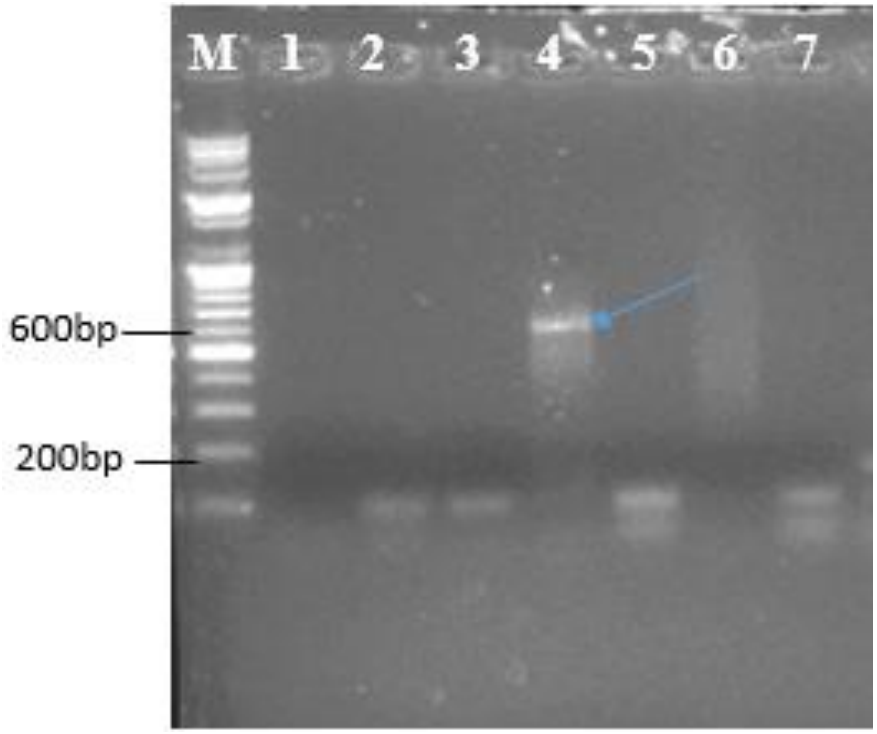

Plate 2. Molecular identification of *S. mansoni* from mitochondria DNA (mtDNA) was prepared from the parasites and it was used for nested PCR with generic primers targeting COX-1 mDNA gene of the organisms in genus *Schistosoma*. The PCR product was then resolved on 1% agarose gel. The size of 600 bp the signal was calculated by comparing its mobility to that of the standards' bands in the marker lane as demonstrated.
Source: Sady et al. (2015).

Table 1. Statistical analysis of PCR results showing the different risk factors and their prevalence.

Risk factor	Number analyzed	Number positive	Prevalence (%)	P value
Age				
6-15	48	24	50.0	
16-30	92	21	25.0	0.016*
31-45	29	5	17.2	
46-60	21	6	10.3	
61-75	5	1	20	
Total	195	56	28.7	
Drug treatment				
No	156	53	34.0	0.001*
Yes	39	3	7.7	
Occupation				
Farmer	97	21	21.6	
Fishermen	47	10	43	0.001*
Student	51	25	49.0	

Table 2. Distribution of different species of Schistosomes in the villages using nested PCR.

Sample area	Frequency (%)	
	S. haematobium	***S. mansoni***
Brikama Ba (n=32)	17(53.1)	0
Wali kunda (n=32)	6 (17.6)	0
Jahally (n=68)	26 (41.2)	3 (4.4)
Kuntaur (n=6)	5 (8.2)	0
Total	54 (28.7)	3 (1.5)

Table 3. Statistics analysis of elisa results.

Risk factor	Odd ratio	Confidence interval	P value
Age			
6-15	1		
16-30	0.73	0.26-1.91	
31-45	1.37	0.41-4.50	0.091
46-60	1.16	0.40-3.42	
61-75	0.86	0.29-2.44	
Drug treatment			
No	1	0.279 – 1.250	0.194
Yes	0.59		
Occupation			
Farmer	1		
Fishermen	1.77	0.66-4.96	0.336
Student	0.58	0.29-1.15	

prevalence difference between male and female (P<0.05). Chi-square test showed that there was no significant difference between the prevalence rates of the different age groups (P>0.05).

DISCUSSION

This study revealed that there is endemicity of *S. haematobium* in CRR of The Gambia with a prevalence of 28.7% among school children, farmers and fishermen/women. This is in agreement with findings from the previous study that revealed high transmission rate of 14.2% for *S. haematobium* infection in CRR (Gambia NTD Mapping Report, 2015). The higher prevalence obtained in this study could be due to the fact the PCR used in this study is more sensitive and can detect the *Schistosoma* in all stages of infection (Corcoran and da Silva, 2014) compared with microscopy that was employed in the diagnosis and characterization of the *Schistosoma* species in previous studies. The lowest prevalence of schistosomiasis recorded in Kuntaur may be attributed to mass administration of anti-schistosomal drug carried out in early 2017, few weeks before the start of sample collection in this village. Questionnaire survey attested to the fact that, 60.7% of people in this village benefited from the mass drug administration held in early 2017. The other villages were not included in the mass drug administration. The study also revealed a higher prevalence in males compared with their female counterpart. There was a significant association between the disease and sex. This result is similar to the NTD mapping study done in The Gambia which showed a higher prevalence in male than in female (Gambia NTD Mapping Report, 2015). A similar survey in Senegal also showed a higher prevalence in boys than in girls (Senghor et al., 2014). This could be because males (especially the students) have more frequent contacts with water than females because in traditional African settings, young females are more associated with indoor activities than their male counterpart. In the study population, 73.9% of the positive students (6-15) are boys. Boys are fond of going to the streams and ponds to swim, wash domestic animals, bath etc. Generally, prolonged and more frequent contact with water causes more exposure to the snail intermediate hosts in the water bodies. Since the villages have pipe-borne water, the girls usually stay at home and generally use tap water for housework, thus reducing their contacts with other water sources. It is for this difference in their exposure to water bodies where the snail intermediate vectors habit that may explain the differences in the infection rates rather than their gender differences. The prevalence of infection in age groups 6-15 and 16-30 shows that there is a significant association between the age and prevalence of the disease. The higher prevalence among the age group 6- 15 years is most likely because they spend more time in the water swimming, bathing, washing, fishing and other water activities. On the other hand, there was no association in the age group of 61-75 years. The association between praziquantel administration and prevalence of schistosomal infection is aptly demonstrated in this study. Communities which had received mass drug administration against schistosomal disease recorded much lower incidence as compared with those had did not. This shows that praziquantel protects against *Schistosoma* in the region although there is still a considerable level of infection persistence. This find was similar to that of Woldegerima et al. (2019), who showed that the used 40 mg/kg of Praziquantel against *S. mansoni* was highly efficient in the elimination and control of Schistosomiasis. The cure rate was not associated with age, occupation or gender. Students had the highest prevalence this correlates with the age group 6-15 years as all the students are within this age group. The more prolonged exposure of this age group to the water bodies has been adduced for this observation. This study is in an agreement with the findings of Sacolo-Gwebu et al. (2019) and; Exum et al. (2019), who also showed that, age group of students was highly correlated to infection rate. While the higher prevalence in fishermen/women (though not significant) compared to farmers may be attributed to longer contacts with waters, the higher prevalence recorded among fishermen compared with the figure in fisherwomen is unexplainable.

The higher detection rate with ELISA compared with PCR and Microscopy (Figure 5) might be since ELISA detects antibodies to *Schistosoma* and cannot distinguish between active and past infection, with parasite-specific antibodies remaining in the system long after the disease has been cured (Doenhoff et al., 2004; Sousa et al., 2019). ELISA cannot distinguish between the different species of *Schistosoma* prevalence recorded in this study is for both *S. mansoni* and *S. haematobium* and possible other species, even though there is no report of any other parasitic species in the region or within the country. The manual washing carried out during the ELISA test because of the lack of automated ELISA plate washer, may also have contribute to the observed higher prevalence. The low prevalence of *S. mansoni* reported for CRR in this study agrees with previously reported low prevalence for *S. mansoni* in the country NTD Mapping Report, 2015). The NTD mapping study reported a prevalence of 0.4% for *S. mansoni* in CRR. The detection of *S. mansoni* in blood and urine using nested PCR with appropriate primers is revealing as this organism (*S. mansoni*) is usually detected in stool samples only of infected patients. This may be attributed to the higher sensitivity of PCR compared to ELISA and microscopy.

Conclusion

The study showed that prevalence of schistosomiasis

infection varied among different age groups and occupation in the central River Region of the Gambia and was largely dictated by level of contact with water bodies and previous drug treatment with anti-schistosomal drug and previous drug of people living in the Central River Region of The Gambia. Schistosomiasis is still a serious public health problem in this region. The molecular technique showed that *S. haematobium* is the dominant causative nematode of Bilharzias in the Gambia. The study also indicated that *S. mansoni* can be detected in blood and urine. To reduce the prevalence of schistosomiasis in The Gambia, mass drug administration using praziquantel should be carried out in the villages that have not benefited from it yet. A community health eradication campaign and adequate health education should be promoted on the control of the disease. Since the infection of children can be evaded, the community should be educated on the mode of transmission of the disease and the pathology of the disease and therefore encourage them to adopt control measures. Since there is the availability of running water, children should be discouraged from swimming and washing in the river.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

AUTHOR'S CONTRIBUTION

YKE and TTB conceived and designed the experiments; AM, AK and MEE collected the data, performed the experiments, analysed the data and as well wrote the paper.

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Wolbachia infected anthropophilic mosquitoes in The Gambia are not infected with filarial nematode

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***Wuchereria bancrofti* has been reported to cause 90% of all cases of lymphatic filariasis in Africa. wolbachia endosymbiot infect a wide range of insects and nematodes. This study was conducted in 48 settlements (8 from each division). A total of 2003 mosquitoes was pooled into 120 clusters from the 48 settlements in The Gambia. Polymerase chain reaction (PCR) was used to detect the presence of Wolbachia and filarial nematode and further differentiate Wolbachia into super groups among the positive samples. The level of association between Wolbachia and the genera of anthropophilic mosquitoes in The Gambia was also determined. Microscopic results showed 64.9% Anopheles, 32.0% Culex, 3.0% Aedes. PCR showed that, 34.17% of Wolbachia in the mosquito obtained varied among the three mosquito genera, with highest being among *Anopheles*. Only Wolbachia super group B was identified in *Culex* and *Aedes*. The purpose of this study carried out in The Gambia was to describe the most common mosquito species and to identify, by means of PCR, their association with the Wolbachia bacteria and the presence of nematodes responsible for filiariasis, in order to understand the role that this bacterium plays in the chain of filarial transmission.**

Key words: *Wuchereria bancrofti*, Filariasis, *Anopheles gambiae, Culex quinquefasciatus*, elephantiasis.

INTRODUCTION

Lymphatic filariasis, typically known as elephantiasis and it is one of the neglected tropical disease caused by a microscopic parasite which can affect man (WHO, 2020). It can disfigure and damage the lymphatic system and can cause abnormal enlargement of body parts, thereby causing grave pain, severe disability and stigma. About 893 million people in 49 countries worldwide are at risk of contracting this disease (WHO, 2020). It is caused by a thread- like filarial worms (microfilariae): *Wuchereria bancrofti*, *Brugia malayi* or *Brugia timori* (Lambert, 2005). The adult worms which could measure up to 4 -12 cm long, house and mature as adults in the body's lymph nodes and lymph vessels for a period of 4 to 8 years (Bockarie et al., 2009; Pfarr et al., 2009). Three species that causes lymphatic filariasis, *W. bancrofti* is most widely spread and is responsible for more than 90% of

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infection (Rebollo et al., 2015a). Microfilariae of *W. bancrofti* exhibit nocturnal periodicity (Nwoke et al., 2010); during the day, they are found in the deep veins and during the night between 10 pm and 4 am, they migrate to the peripheral circulation. This behaviour gives them the chances of being picked up by the night biting vectors during their blood meals (Nwoke et al., 2010). The species of mosquitoes capable of transmitting these filarial nematodes are many but varies between geographical regions. In most urban and semi urban areas, *W. bancrofti* is vectored mainly by *Culex* mosquito while in the rural areas, transmission from one person to another is mainly by *Anopheles* mosquito (Singh et al., 2013). The main vectors in The Gambia are *Anopheles gambiae* in the rural areas while in the urban areas transmission is by *Culex quinquefasciatus*. Studies have revealed the presence of an endosymbiont, Wolbachia in arthropods and nematodes (Bouchery et al., 2012). Wolbachia is a Gram-negative intracellular α-proteobacterium belonging to the Order *Rickettsiales* (Bouchery et al., 2012). The specie *Wolbachia pipientis* was first identified in Culex mosquito (Hertig and Wolbach, 1924; Porennan et al., 2008). It is classified into sixteen sub groups (A-Q) (Guan et al., 2016). Super group G have been removed because its status is currently not clear (Baldo and Werren, 2007). Of the sixteen sub groups, C and D are commonly found in filarial nematodes (Werren et al., 2008), while A and B are found in mosquitoes (Ravikumar et al., 2011). According to Dyab et al., (2016), Wolbachia are maternally inherited and infect a wide range of insects and nematodes and play an important role in the development and pathogenesis of the filariae *Onchocerca volvulus*, *Brugia malayi* and *W. bancrofti*. Wolbachia as a parasite, relates with its host in a number of ways, some of which are parasitism while others are mutualism. The parasitic relationship includes feminization of genetic males, pathenogenetic induction resulting in the development of unfertilized eggs, the killing of male progeny from infected females and sperm-egg incompatibility (Werren et al., 2008). Wolbachia appear to contribute to the inflammation process which is a major pathological symptom of filarial infections (Dyab et al., 2016). The most common effect of Wolbachia infection in mosquitoes is cytoplasmic incompatibility. Mating of infected male with the uninfected female mosquitoes of the same species, does not result into fertilization (Sinkins, 2004). Wolbachia has the potential to be used as biocontrol in both pest and in biomedical applications (Zabalou et al., 2004; Engelstadter and Hurst, 2009). In 2013, the World Health Organization listed The Gambia as among 73 countries considered endemic for lymphatic filariasis (WHO, 2013) and according to Cano et al. (2014), Global Atlas of Helminths Infection, The Gambia still had a high probability of transmission. Lymphatic filariasis is mainly characterized by the occurrence of inflammatory pathogenesis, and Wolbachia bacterium which is an endosymbiont of filarial nematode appear to contribute to this inflammation (Barton et al., 2010; Bouchery et al., 2013). The endosymbiont Wolbachia could be genetically transformed to modify the disease transmitting abilities of mosquitoes (Yeap et al., 2011; Fraser et al., 2017). It is crucial to know which Wolbachia strains are present in population before releasing engineered infected individuals because pre-existing natural infections can interact with and alter the dynamics of introduced strains (Duron et al., 2010; Atyame et al., 2011). Understanding the role of Wolbachia in filarial transmission can provide a template for breaking the transmission chain of the disease. Effective and efficient surveillance systems need to be put in place for most arthropod-borne diseases across The Gambia (Kargbo and Kuye, 2020). The purpose of this study carried out in The Gambia was to describe the most common mosquito species and to identify, by means of polymerase chain reaction (PCR), their association with the Wolbachia bacteria and the presence of nematodes responsible for filiariasis, in order to understand the role that this bacterium plays in the chain of filarial transmission.

MATERIALS AND METHODS

Study area

The Gambia is a West African country that lay between latitude 13° and 14° and longitude 13° and 17°. The country is almost completely surrounded by Senegal except on the western side where it bordered with the Atlantic Ocean. The Gambia is divided into eight Local Government Areas namely: Banjul, Kanifing, Brikama, Mansa Konko, Kerewan, Kuntaur, Janjanbureh and Basse. For the purpose of this research, the former divisions as shown in Figure 1 was used; Banjul, Western, North Bank, Lower River, Central River and Upper River division.

Sample size

The sample size was determined by using the formula

$N = Z^2 pq/ d^2$

as described by Thrusfield (2007) based on 95% confidence interval and prevalence of 50%.
Where;
N is the sample size of pools of mosquito (to be calculated)
Z = 1.96 for 95% confidence level
p= 0.5 (Prevalence)
q= Complementary probability (1-0.5)
d= 0.05
N = 1.96×1.96x0.5×0.5/0.05x0.05
N = 0.9604/0.0025
N = 384 pools of mosquitoes

Sampling techniques

Multi stage sampling technique was employed. For each division, 8 settlements were selected through balloting, and for each settlement, 8 compounds were selected through balloting. The

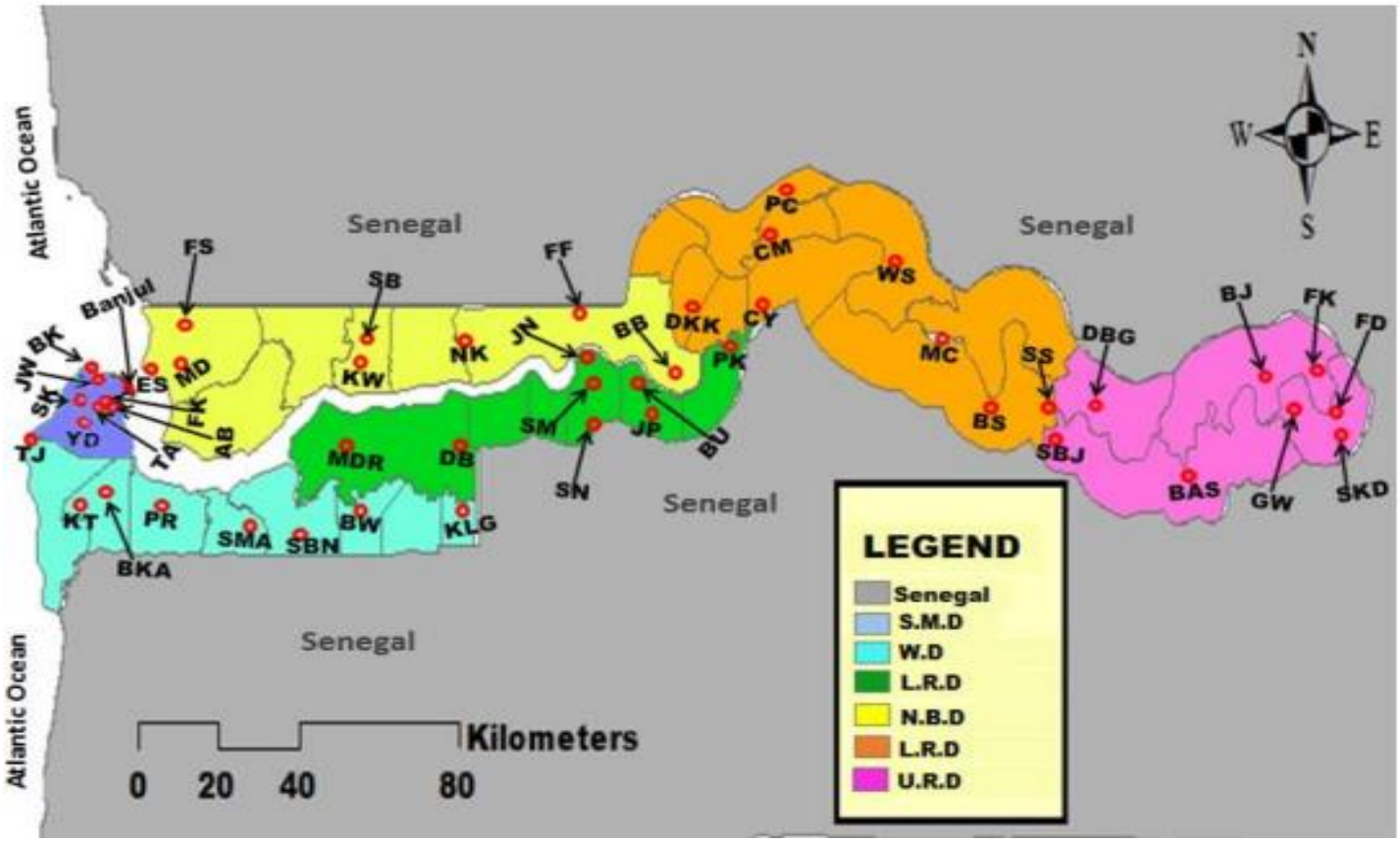

Figure 1. Map of The Gambia showing the sample site. SMD= Saint Mary's Division, WD= Western Division, NBD= North Bank Division LRD= Lower River Region, CRD= Central River Region; FK = Fagi Kunda, TJ = Tanji, ES = Essau, SM = Soma, MC= Macaty; TA = Tallinding, PR = Pirang, KW= Kerewan, MDR = Manduar, CY – Choya; SK = Sukuta, BW = Bwiam, NK= No Kunda, JN = Jinoi, DKK=Dankunku; BK = Bakau, SMA = Somita, FS = Fass, PK = Pakaliba, CM= Chamen; JW = Jeswang, SBN = Sibonor, SB = Saaba, SN = Senoba, PC= Panchang; AB = Abuko, KT = Kiti, FF = Farafenni, DB = Dumbuto, WS= Wassu; YD = Yundum, BKA = Brikama, BB = Bambali, BU = Buiba, SS = Sare Sofi; Banjul = Banjul, KLG = Kanglagi, MD = Madina, JP = Japineh, BS= Bansang; BAS= Basse, SKD = Song Kunda, FT= Fatoto, GW= Garawol, BJ= Baja, DBG = Diabugu, SBJ = Sare Bojo; FD = Foday Kunda.

compounds selected are the sampling points/pools. This equates to 64 sampling points/pools for each division giving a total of 384 sampling points/pools for the entire country (Table 1).

Collection of mosquito samples

G'rumba is a name of a container in Jola language made of clay. Jola is a tribe in the Senegambia region and some parts of Guinea Bissau deeply rooted in herbal medicine. The container is mostly used by herbalists in the preparation and storage of concoctions. It has been observed that while in storage, a scourge of mosquitoes seeks shelter and a source of water for drinking in such containers. The container could also be a breeding ground if kept in that position for weeks. It is from this concept; this study formulated a trap for collecting mosquitoes for purpose of this research. The materials used here are buckets and transparent piece of clothes sawed into sacs with a control valve made of rope. In the setup, water is filled into the buckets to one quarter full. Small branches with leaves of shrubs were then put inside to create a bushy environment that can be a temporary home for mosquitoes and other flies within that environment. The harvesting processes involved carefully capping the mouth of the bucket with the mouth of the designed sac such that when the bucket is shaken the mosquitoes will run into the sacs. The sac can then be removed carefully and the valve closes to prevent any trapped mosquito from escaping. The activities of mosquitoes are time bound. Some genera are active during the night while others are active in some parts of the day. At around midday, mosquitoes are generally not active. During such periods, they could be found hanging on walls in dark areas or any other surfaces nearby. In this method of collection, the traps were set to provide such resting platforms for the mosquitoes during such inactive periods of their time. The traps were positioned in baths rooms, verandas, sitting rooms and other immediate environments. The compound owners and the community were sensitized on the whole operation. The selected area for the traps was cleared of any other material that could provide a resting platform for the mosquitoes. The traps were laid from 8 am in the mornings to 2 pm the next day (a period of 30 h). Harvests were made at 2 pm as described above. The live mosquitoes were left in the sacs for 48 h to weaken and then transferred into tubes containing silica in sachets (for preservation) and labelled to indicate division, pool number and date of harvest. Once harvested, the small branches with leaves were discarded and the water poured to avoid the setup being used by mosquitoes for breeding (Sankung et al., unpublished method of trapping

Table 1. The sampling sites for each division in The Gambia.

Division	Sampling sites
SMD	Fagikunda , Banjul, Tallinding, Sukuta, Bakau, Jeswang, Abuko and Yundum
WD	Tanji, Pirang, Bwiam, Somita, Sibanor, Kitti, Brikama and Kalagi
NBD	Essau, Kerewan, No kunda, Fass , Saaba, Farafenni, Bambali and medina
LRD	Soma, Manduarr, Jinoi, Pakaliba, Senoba, Dumbuto, Buiba and Japineh
CRD	Macaty, Choya, Dankunku, Chamen, Panchang, Wassu, Sare- Sofi and Bansang
URD	Basse, Song-Kunda, Fatoto, Garawol, Baja, Diabuqu, Sarebojo and Foday kunda

SMD= St Mary's Division, WD= Western Division, NBD= North Bank Division, LRD= Lower River Division, CRD= Central River Division, URD Upper River Division.

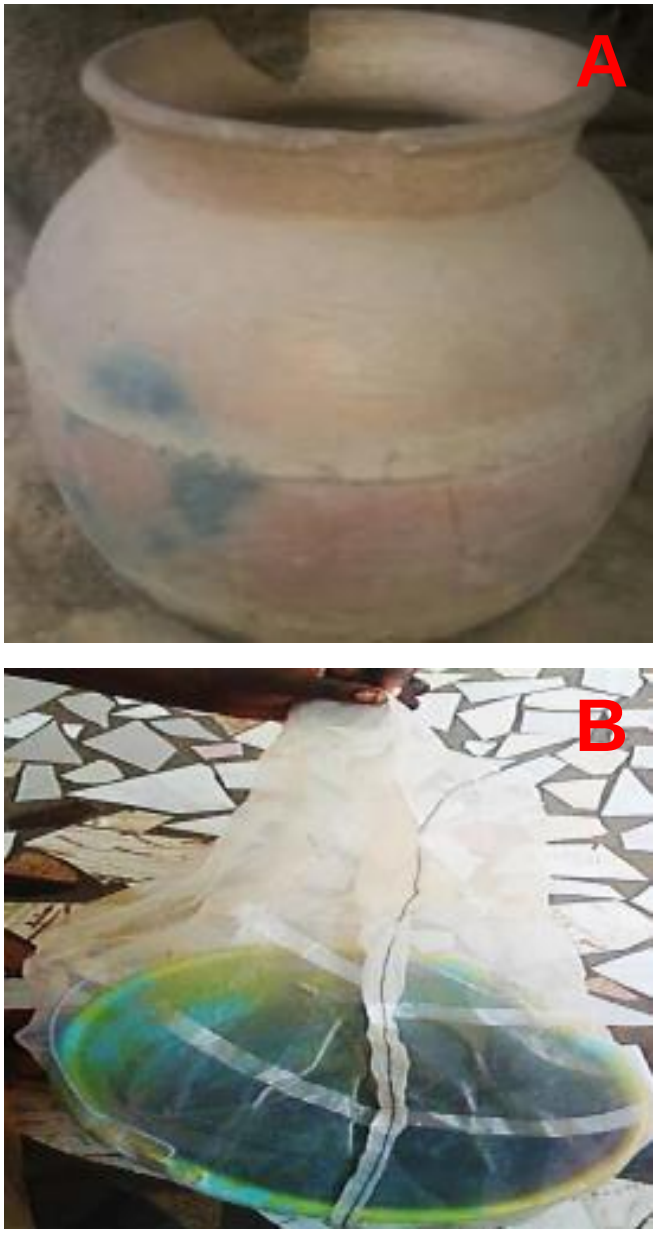

Figure 2. (a) G'ruumba (b) G'ruumba Trap (Modified mosquito trap).

mosquitoes). The mosquitoes were then transported to Department of Biochemistry Ahmadu Bello University, Zaria Kaduna State, Nigeria for laboratory analysis (Figure 2).

Morphological identification of the mosquitoes

The mosquitoes were identified per pool into genera level according to Stone et al. (1959) using microscopy at the Department of Veterinary Parasitology and Entomology Laboratory Ahmadu Bello University Zaria. The main taxonomic features used were; body colour, spotted and unspotted wings, length of palps in comparison with their proboscis and presence of black strips on body and legs. The number of each genera of mosquito for each pool were established and placed in tubes and labelled. The sex of the mosquitoes was also determined for every pool based on the presence of bushy feathers around the antenna. For improving the sensitivity and cost effectiveness in the DNA analysis by Polymerase Chain Reaction (PCR), each genera of mosquitoes in the sampling points/pools of each settlement/cluster were pooled together. This translates into 48 clusters of Anopheles genera mosquitoes, 48 clusters of Culex genera mosquitoes and 24 clusters of Aedes genera of mosquitoes. DNA was extracted from 120 clusters of mosquitoes.

Identification of Wolbachia and microfilariae

DNA extraction

Dry mosquito samples by cluster were homogenized by grinding into powder in eppendorf tubes (one for each cluster) using fresh micro pestle for each cluster. 100 µl of distilled water was added to each of the samples with less than 5 mosquitoes while 200 µl of distilled water was added to samples with more than 5 mosquitoes. The samples were briefly vortexed to distribute the particles evenly. Quick-DNA^{TM} miniprep plus kit (Zymo) was used with strict adherence to manufacturer instructions (Vanek et al., 2011).

DNA quantification

The DNA samples were removed from the freezer, thawed and briefly vortexed. The lower and the upper pedestals of the Nano drop Spectrophotometer (Denovix DS- 11+) were cleaned and blanked with 2 µl of deionized water. The samples were then run one after the other with intermittent cleaning of the lower and the upper pedestals with tissue paper after every sample run. The concentration of the DNA and the absorbance of 260/280 ratio (for purity) were recorded.

Identification of Wolbachia DNA using 16SrDNA and wsp gene

Detection using 16S rDNA

To determine the presence of Wolbachia DNA, PCR technique was employed using 16S Wolb F and 16S Wolb R (forward and reverse primers) specific primers: 16S rDNA (W-Specf 5'-CATACCTATTCGAAGGGATAG-3', W-Specr 5'-AGCTTCGAGTGAAACCAATTC-3') (Baldini et al., 2014). The amplification were carried out with a Gradient Thermo Cycler and the reaction mixture for each of the 120 cluster sample consisted of 4 µl of DNA template, 2.5 µl of 10X buffer, 2.5 µl of 2.5 mM (each) dNTPs, 0.35 µl of 20 µM each of forward and reverse primers (16SWolbF/16SWolbR), 0.25 µl of 5U/µl Taq DNA polymerase and a volume of 15.05 µl DNAse free water to make a final reaction volume of 25 µl. The amplification reaction protocol used was as follows initially of 2 min at 95°C then 40 cycles of 30 s at 95°C, annealing at 55°C for 30 s and extension at 72°C for 30 s and a final extension of 3 min at 72°C with an expected ban size of 438 bp (Baldini et al., 2014; Dyab et al., 2016).

Detection of Wolbachia super group a using wsp136F and wsp691R

All positive samples from 16SrDNA screening were subjected to wsp136F/wsp691R screening. The primer sequence are wsp136F 5'TGAAATTTTACCTCTTTTC 3 and wsp691R 5'AAAAATTAAACGCTACTCCA 3'. These primers are specific to Wolbachia super group A. PCR amplification were carried out for all the positive samples for 16SrDNA. A total reaction volume for a single reaction used was 25 µl. The mix contained 4 µl of DNA template, 2.5 µl of 10x reaction buffer, 2.5 µl of dNTPs mix, 0.35 µl each of forward (wsp136f) and reverse (wsp691r) primer, 0.127µl of 5U/µl Taq polymerase and 15.175 µl of DNAse free water. The PCR reaction conditions were as follows; 1 cycle of initial denaturing for 1 min at 94°C followed by 35 cycles of 15 s at 94°C of denaturing, 30 s at 55°C of annealing, extension at 72°C for 1 min and a final extension for 7 min at 72°C with an expected amplicon size of 556 bp (Nugapola et al., 2017).

Detection of Wolbachia super group B using wsp81F and wsp522R

PCR amplification was carried out for all the positive samples for 16srDNA using Forward primer wsp81F 5'TGGTCCAATAAGTGATGAAGAAC 3'and a reverse primer wsp522R 5' ACCAGCTTTTGCTTGATA 3'. These primers are specific to Wolbachia super group B. A total reaction volume for a single reaction used was 25µl. The mixture contained 4µl of DNA template, 2.5 µl of 10x reaction buffer, 2.5 µl of dNTPs mix, 0.35 µl each of forward (wsp136f) and reverse (wsp691r) primer, 0.127 µl of 5 U/µl Taq polymerase and 15.175 µl of DNAse free water. The PCR reaction conditions were as follows; initial denaturing of 1 cycle of 1 min at 94°C followed by 35 cycles of denaturing at 94°C for 15 s, 30 s at 55°C of annealing, extension at 72°C for 1 min and a final extension for 7 min at 72°C with an expected amplicon size of 442 bp (Nugapola et al., 2017).

Identification of microfilariae specie

Wuchereria bancrofti

The sspI gene is a repeat sequence which is a signature for *W. bancrofti*. PCR with specific primers for the SspI gene were used to detect the presence of *W. bancrofti* in the sample. The forward primer for the SspI gene of *W. bancrofti* was *sspI* F 5'-CGT GAT GGC ATC AAAGTA GGG-3', and the reverse primer for the SspI gene was *SspI*R 5'-CCC TCA CTT ACC ATA AGA CAAC-3'. The PCR amplification for 48 cluster samples were carried out in a gradient thermal cycler. A total reaction volume of a single reaction of 25 µl, contained 4 µl of DNA template, 2.5 µl of 10x reaction buffer, 2.5µl of dNTPs mix, 0.35 µl each of forward and reverse primer, 0.127 µl of 5 U/µl Taq polymerase and 15.175 µl of DNAse free water. The PCR reaction conditions were as follows: initial denaturing at 95°C for 5 min followed by 38 cycles of 1min at 94°C denaturing, 1 min at 56°C of annealing, 1 min at 72°C of extension and a final extension at 72°C for 10 min (Nugapola et al., 2017).

Brugia malayi

The *HhaI* gene in *Brugia malayi* is a tandem repeat sequence of about 320 bp. The *HhaI* gene is the target in the detection of *Brugia malayi*. The forward primer used was *HhaI* F 5'-GCG CAT AAA TTT ATC AGC-3'and the reverse primer was *HhaI* R 5'- GCG CAA AAC TTA ATTACA AAA GC-3'. The amplification of the gene was carried out using a Gradient Thermal cycler (Rebollo et al., 2015b). A total reaction volume of each of the 73 cluster samples used was 25 µl. This contained 2 µl of DNA sample, 2.5 µl of 10 x reaction buffers, 2.5 µl of dNTPs mix, 0.35 µl of each of forward and reverse primers, 0.125 µl of 5 U/µl of Taq polymerase and 15.175 µl of DNase free water. The reaction protocol was as follows; 95°C for 5 min, 38 cycles of 30 s at 95°C, 30 s at 56°C 30 s at 72°C, 5 min at 72°C, with an expected product size of 320 bp. The products from the nested PCR were subjected to gel electrophoresis (35 min at 100V) in 1% agarose gel stained with Ethidium and visualized using Chem-Doc Imaging System (BIO-RAD).

Statistical analysis

The association between Wolbachia and different genera of mosquitoes were analysed using bivariate fit of quadratic regression of Statistical Analysis System (SAS 9.4). Relationship between variables was determined using correlation analysis. Charts were fitted in Microsoft Excel. Significant association was set at $p < 0.05$.

RESULTS

Distribution of Anthropophilic Mosquito Genera in The Gambia. A total of 2003 anthropophilic mosquitoes from six divisions in The Gambia were classified into genera by microscopy method (magnification X10). Three genera of anthropophilic mosquitoes were identified as Anopheles, Culex and Aedes. Mansonia genera of mosquito was not detected in the total anthropophilic mosquitoes collected (Table 2). A total of 65.0% of the anthropophilic mosquitoes in The Gambia are Anopheles genera, 31.9% are Culex genera, while 3.1% belong to Aedes genera. None of the anthropophilic mosquitoes belongs to the Mansonia mosquito genera. The distribution pattern of the genera was similar across the six divisions. Figure 3 shows the pooled samples of anthropophilic mosquitoes in different divisions in The Gambia. There was significant differences ($p<0.05$) among the mosquito genera across the division. SMD and CRD had the highest population of mosquitoes which was highly significant ($p<0.05$) different from those caught in the other regions.

Table 2. Population of anthropophilic mosquitoes by genera in different divisions.

Division	Frequency (%)		
	Anopheles	Culex	Aedes
SMD	216 52.6	165 40.1	30 7.3
WD	265 74.7	84 23.9	5 1.4
NBD	207 70.4	81 27.6	6 2
LRD	176 69.3	68 26.8	10 3.9
CRD	255 65.5	129 33.1	6 1.5
URD	180 60	114 38	5 2

SMD = St Mary's Division; WD = Western Division; NBD = North Bank Divisio; LRD = Lower River division; CRD = Central River division; URD = Upper River Division.

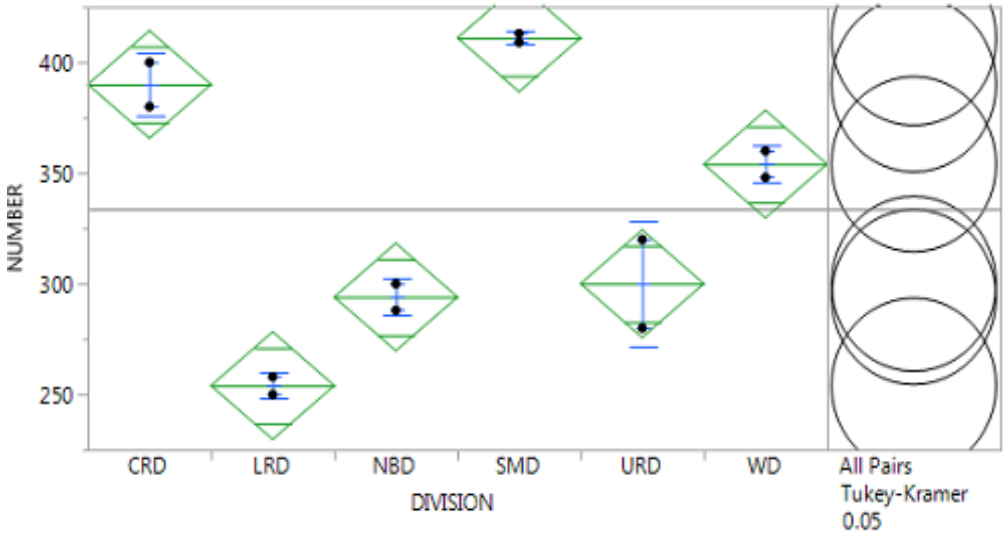

Figure 3. Pooled samples of anthropophilic mosquitoes in different divisions in The Gambia. CRD = Central River Division; LRD = Lower River Division; NBD = North Bank Division; SMD = St Marys Division; URD = Upper River Division.

Sexual dimorphism of anthropophilic mosquito genera

Sexual dimorphism of 2003 anthropophilic mosquito from the six divisions in The Gambia were determined by microscopic technique, of which 82.1% were female while 13.0% were males. (Table 3) There is a significant different ($p<0.05$) in sexual dimorphism among

Table 3. Sexual dimorphism of anthropophilic mosquitoes in different divisions in The Gambia

Division	Frequency %		
	Female	Male	Aedes
SMD	386 93.9	25 6.1	30 7.3
WD	299 84.4	55 15.5%	5 1.4
NBD	232 78.9	64 21.8	6 2
LRD	206 81.1	48 18.9	10 3.9
CRD	352 90.3	38 9.7	6 1.5
URD	269 89.7	31 10.3	5 2

SMD = St Marys Division, WD = Western Division; NBD = North Bank Division; LRD = Lower River Division; CRD = Central River Division; URD = Upper River Division.

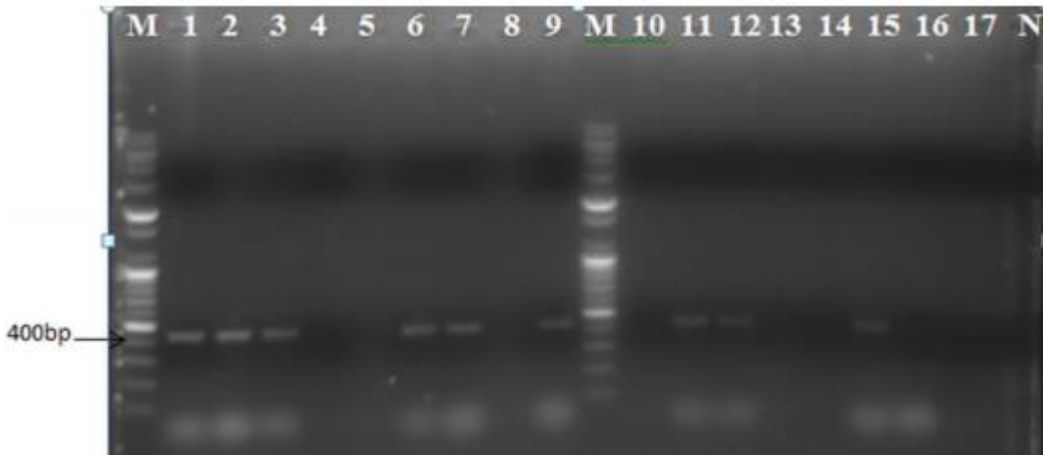

Figure 4. Molecular detection of Wolbachia in anthropophilic mosquitoes.

anthropophilic mosquitoes in The Gambia.

Detection of Wolbachia and classification into super group

Results of the DNA analysis from 120 clusters of mosquitoes for presence of Wolbachia and super group using 16SrDNA and wsp primers are presented in Figure 4 and 5. The results indicated a 34.17% incidence (Table 4) of Wolbachia in anthropophilic mosquitoes in The Gambia. All the three genera of mosquitoes were infected with Wolbachia (Figure 4). A higher proportion of the Anopheles harboured Wolbachia (50%), while Aedes had a significantly lower rate. The Wolbachia detected in Culex and Aedes belong to Super group B (Figure 5). Though Wolbachia was detected in Anopheles genera using 16SrDNA, the super group could not be determined

Table 4. Pooled samples of Wolbachia prevalence in anthropophilic mosquito genera.

	Frequency		
Mosquito genera	**Cluster total**	**No. positive**	**No. negative**
Anopheles	48	24 50%	24 50%
Culex	48	15 31.3%	33 68.8%
Aedes	24	2 8.3%	22 91.7%

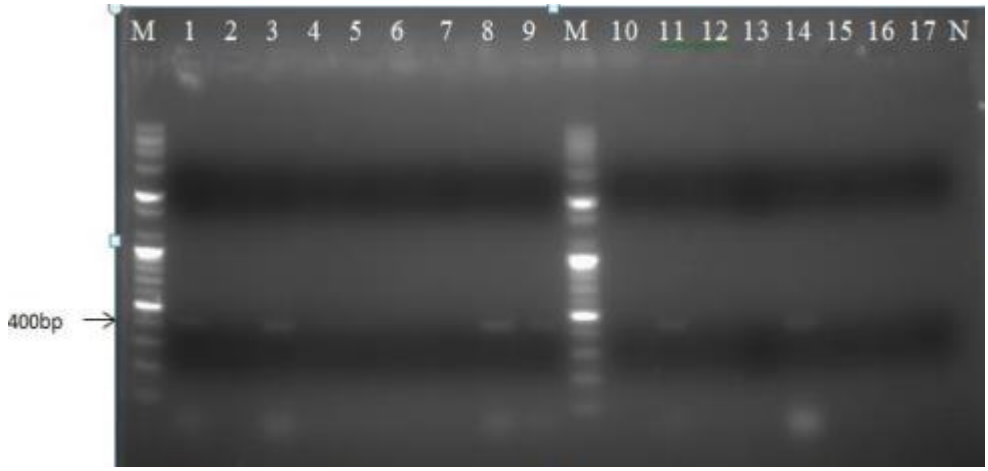

Figure 5. Molecular detection of Wolbachia super group B in Aedes samples.

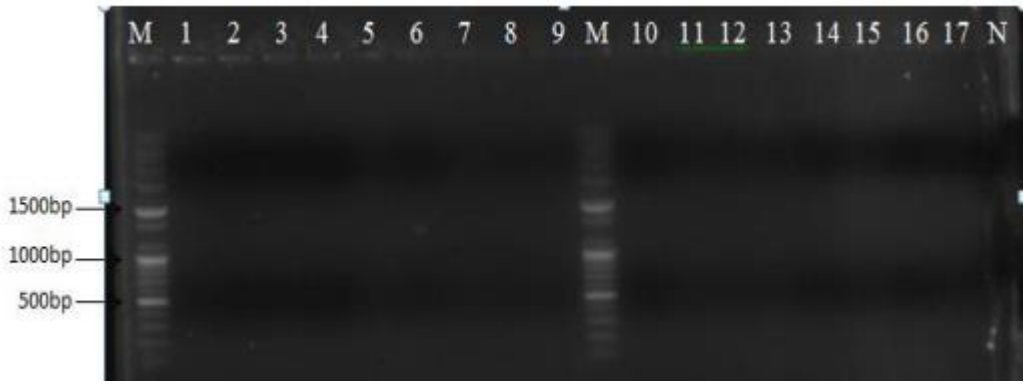

Figure 6. Molecular detection of *W. bancrofti* and *B. malayi* in anthropophilic mosquitoes.

using wsp primers. This was due to the insensitivity of wsp primers in detecting Wolbachia in Anopheles. However, no filiarial nematode was detected in mosquitoes as seen Figure 6.

Wolbachia in anthropophilic mosquitoes in The Gambia. Lane M is the DNA marker. Lane N was the negative controls, that is: PCR reaction without gDNA. Samples from mosquitoes are represented in the

Table 5. Distribution of Wolbachia super groups among anthropophilic mosquito genera in the Gambia

Mosquito			% Frequency	
Genera	**No. Cluster**	**No. positive**	**Wolbachia A**	**Wolbachia B**
Anopheles	48	0	0	0
Culex	48	15	0	31.3
Aedes	24	2	0	8.3
	120	**17**	**0.0**	**14.17**

Table 6. Bivariate fit of anthropophilic mosquito genera and Wolbachia

Genera	RMSE	R^2	r	P value
Culex	36.4	0.63	0.79	0.05*
Anopheles	84.35	0.36	0.60	0.29
Aedes	7.63	0.37	0.61	0.25

RMSE – Root mean square error; R^2- Coefficient of determination; r - Correlation
p- Probability of regression coefficient I

following lane. PCR amplification of the 16SrDNA (438 bp) of Wolbachia in anthropophilic mosquitoes Lanes 1, 2, 3, 6 and 7 were positive for Anopheles samples. Lanes 9, 11, 12 and 15 was positive for Culex samples (Baldini et al., 2014; Dyab et al., 2016) (Figure 5). Wolbachia in anthropophilic mosquitoes in The Gambia. Lane M is the DNA marker. Lane N was the negative controls, that is: PCR reaction without gDNA. Samples from mosquitoes are represented in the following lane. Lane M is the DNA marker. Lane N was the negative controls, that is: PCR reaction without gDNA. Samples from mosquitoes are represented in the following lane. Lanes 1, 3, 6, 7, 8, 9, 11, 12 was positive for Wolbachia super group B in Culex samples Lane 14 was positive for Wolbachia super group B in Aedes samples. PCR amplification of the wsp (442 bp) for Wolbachia Super group B in anthropophilic Mosquitoes. This size of the signal were calculated by comparing its mobility to that of the standards' bands in the marker lane as demonstrated by (Nugapola et al., 2017) (Table 5).

Wolbachia – mosquito association

The association of Wolbachia and anthropophilic mosquito's genera in The Gambia was analysed using bivariate fit of quadratic regression of statistical analysis system (SAS 9.4). The analysis in Table 6 shows that Wolbachia has high and significant association ($p<0.05$) with Culex. The association between Wolbachia and Culex was strong and positive ($r=0.79$) with coefficient of determination of 63%. Association of both Anopheles and Aedes with Wolbachia is not significant ($p>0.05$), though the correlation was high between Wolbachia and Anopheles ($r=0.6$), the correlation between Wolbachia and Aedes is low (0.61).

Screening for the presence of *W. Bancrofti* and *B. Malayi* DNA in anthropophilic mosquitoes

Results of the screening of the DNA of the mosquitoes for filarial nematode using ssp1 and *HhaI* gene primers (Figure 6) indicated that there were no nematode in the sampled mosquitoes Both *W. bancrofti* and *B. malayi* were absent in the mosquitoes in the six divisions of The Gambia.

Wolbachia in anthropophilic mosquitoes in The Gambia. Lane M is the DNA marker. Lane N was the negative controls, that is: PCR reaction without gDNA. Samples from mosquitoes are represented in the following lane. Lanes 1, 2, 3,4,5,6,7,8,9 were all negative for Anopheles samples, Lanes 10,11,12,13 and 14 were negative for Culex samples Lanes 15, 16 and 17 were all negative for Aedes samples. PCR amplification of the sspI (188 bp) and hhaI (320 bp) of *W. bancrofti* and *B. malayi* in anthropophilic mosquitoes Nugapola et al., (2017).

DISCUSSION

Various genera of mosquitoes in The Gambia are distributed across all the divisions of the country, with the exception of Mansonia genera which was not detected among the anthropophilic family of mosquitoes sampled. Availability of fresh, clean or turbid water plays an important role in the distribution of mosquitoes. Fresh water body from rivers or ponds constitutes the highest environments for mosquito breeding sites. Although SMD and WD lacked with fresh river waters, urbanization has played a major role in providing mosquito breeding grounds through numerous sewages and waste water bodies produced daily by human activities (M'koumfida et

al., 2018). In CRD and URD, the high population could be attributed to the existence of fresh water bodies from the river which could provide a good breeding grounds for the mosquitoes. NBD and LRD registered the lowest in anthropophilic mosquito population compare to the other divisions. These two divisions characterized by sparsely distributed populations produces less sewages and waste water bodies couple with salty river waters reduces the chances of mosquito breeding compared to other divisions. The mosquito population in these two divisions are thus hampered by lack of adequate fresh water bodies all year round. The high proportion of Anopheles genera among mosquitoes in The Gambia is not unexpected. Anopheles is not restricted to clearly defined habitats for breeding. The genera have the potentials to breed in all open water bodies (Laporta et al., 2011). The lower proportion of Anopheles in SM when compared to other divisions, could be attributed to the fact that SMD is the most highly dense in human population with the highest industrial activities. SMD has also been designated as the most polluted divisions. Anopheles mosquitoes generally prefer unpolluted water bodies for breeding (Geissbühler et al., 2007; Laporta et al., 2011). It has been reported that pollution as a result of urbanization has eliminated certain species of Anopheles from urban centres (Geissbühler et al., 2007; Laporta et al., 2011). The least populated among the three existing genera in anthropophilic mosquitoes is the Aedes genera. Aedes genera generally breeds in artificial containers such as pots, tyres, open barrels containing clean water mainly of rain or natural containers of rain water such as holes in trees (Paupy et al., 2009; Nazri et al.,2013). The increased awareness in the fight against mosquito borne diseases which have resulted in the cleaning of the environment, by collecting discarded pots, tyres or any container which could hold water for mosquito breeding and the decline in amount of rain water received in recent times might have contributed to the low occurrence of Aedes mosquito genera in The Gambia. Difference in sexual dimorphism among anthropophilic mosquitoes in The Gambia is significantly higher in SMD, WD, CRD and URD where Wolbachia infection was evident in this study. The relationship between these two factors (mosquito sex and Wolbachia) has been explained by several researchers. Wolbachia induces cytoplasmic incompatibility in arthropods and distort the sex ratio (Bordenstein et al., 2001; Charlat et al., 2006; Telschow et al., 2007). Through cytoplasmic incompatibility Wolbachia modifies the spermatozoa such that male mosquitoes dies early in embryogenesis and also Wolbachia was also reported to have the potentials to feminize male mosquitoes during early embryogenesis. The significant differences in sexual dimorphism observed in this study may be attributed to the presence of Wolbachia in anthropophilic mosquito populations. The three genera of mosquitoes; Anopheles, Culex and Aedes among anthropophilic mosquitoes in The Gambia are infected with Wolbachia. The incidence of infection is greater in Anopheles than Culex and Aedes. Although the mosquito-Wolbachia association is significant only in Culex, only the super group B of Wolbachia was identified in Culex and Aedes anthropophilic mosquitoes in The Gambia. The super group in Anopheles could not be detected by wsp primers in this study as also reported by (Kittayapong et al., 2000; Wiwatanaratanaburt, 2013; Nugapola-nalaka et al., 2017). The insensitivity of wsp primers in detecting Wolbachia in Anopheles mosquitoes could be attributed to some form of mutation in the surface protein gene in Wolbachia. The inability of wsp primers in detecting Wolbachia has previously led to the conclusion that Anopheles genera are not naturally infected with Wolbachia (Nugapola et al., 2017). This conclusion spurred scientists in the World to attempt introducing Wolbachia artificially into natural population of Anopheles. However, screening with 16srDNA has now revealed that Anopheles mosquito can also harbour Wolbachia. The super group of the Wolbachia in Anopheles will be better determined by sequencing. None of the 120 clusters of anthropophilic mosquitoes (comprising 2003 mosquitoes) was positive for *W. bancrofti* and *B. malayi*, suggesting absence transmission of lymphatic filariasis in The Gambia at the sampling time. This finding is in agreement with the conclusion of Rebollo et al. (2015a) who stated that there is no longer active filarial transmission in the country as at the time of their study. It is probable that the high incidence of Wolbachia infection in anthropophilic mosquitoes could have contributed to this. This assumption is further strengthened by the very high dimorphic difference among the mosquito sexes.

Conclusion

Anopheles mosquitoes constitute the predominant anthropophilic mosquito genera in The Gambia. A high proportion of anthropophilic mosquitoes in the country are infected with Wolbachia, super group B, particularly in Culex and Aedes. *W. bancrofti* and *B. malayi* DNA were not detected in anthropophilic mosquitoes in The Gambia and hence no active transmission of lymphatic filariasis in the Gambia exist at the time of this study in The Gambia.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Genetic characterization of indigenous Tswana pig population using microsatellite markers

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The indigenous Tswana pig is currently listed as an endangered animal genetic resource and if not conserved, might go extinct. The objective of this study was to assess the genetic diversity (genetic characterization) of the indigenous Tswana pig population. Blood samples were collected from 30 randomly selected Tswana pigs in Kgatleng and South-East districts of Botswana for the assessment of genetic diversity using a panel of 12 FAO-recommended microsatellite markers. All the microsatellite markers screened in indigenous Tswana pigs were polymorphic and the number of observed alleles per marker varied between 3 (SW2406) and 9 (SW225) with mean number of alleles per marker of 6.33. The observed heterozygosity ranged from 0.16 (SW2405) to 0.875 (SW2465) with average observed heterozygosity across all 12 loci of 0.647. The expected heterozygosity was lower than the observed heterozygosity and ranged between 0.143 (SW2405) and 0.776 (SO385) with mean expected heterozygosity across all loci of 0.603. The allelic diversity and levels of heterozygosity indicate high levels of genetic diversity in Tswana pig population. The within-locus inbreeding coefficient (F_{is}) ranged between -0.321(S0120) and 0.234 (SW35) with inbreeding coefficient of the entire population of -0.012 indicating that the Tswana pig population is relatively outbred.

Key words: Genetic diversity, microsatellite markers, heterozygosity.

INTRODUCTION

Indigenous pigs are kept by the rural populace under the low-output free range production system. Indigenous Tswana pigs are mostly owned by women, usually survive in harsh, low input environments and strive under high disease, parasite prevalence and nutrients shortages (Chabo et al., 2000). During the 1980's, indigenous Tswana pigs were found in South East, Kgatleng and Kweneng districts of Botswana while nowadays they are fairly well distributed in the south east district of the country in and around Ramotswa village (Nsoso et al*.,* 2006). The farmers who keep indigenous pigs in Botswana have a tendency to keep low numbers to match herd size with available feed resources. Notable attributes of indigenous Tswana pigs include disease resistance, high fertility, parasite and heat tolerance, low protein requirements, ability to utilize course fibrous rations and strong feet which make them suitable for free range low-intensity management systems affordable to

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Figure 1. Indigenous Tswana pigs at Department of Agricultural Research, Sebele.

the rural poor (Gandin and Oldenbroek, 1999; Lekule and Kyvsgaard, 2003).

The indigenous Tswana pigs are usually black or black with white stripes (Figure 1) and have a body of medium stature (Nsoso et al., 2006). Indigenous Tswana pigs are however shunned away from commercial production systems due to their inferior growth and reproductive performance and carcass traits relative to exotic breeds (Moreki and Montsho, 2012). Resource-poor farmers in rural areas also view genetic improvement of indigenous Tswana pigs as synonymous to crossbreeding, grading up and possible breed replacement with exotic breeds (Nsoso et al., 2004).

Extensive system coupled with undeveloped markets for indigenous Tswana pigs and lack of a clear policy on the conservation of indigenous animal genetic resources in the country is leading to the disappearance of indigenous Tswana pigs. This poses a risk of worsening poverty levels for most of the rural women populace who own most of the indigenous Tswana pigs since the fast-growing exotic pigs require high levels of inputs and management unaffordable to the resource-poor and highly marginalized farmers. The population of indigenous Tswana pigs has declined drastically in the last three decades and the indigenous Tswana pig is currently listed as an endangered animal genetic resource (Podisi, 2001). Rege and Lipner (1992) argued that some indigenous animal genetic resources of Africa are endangered and may even be lost before they are described and documented, and the indigenous Tswana pig is one classic example. Research to evaluate the indigenous Tswana pig has been sporadic and inadequate; consequently, the indigenous Tswana pig has not been sufficiently characterized. Information on phenotypic characteristics and production performance of Tswana pigs is still very scarce and there has been no attempt till date aimed at their genetic characterization. Genetic characterization of Tswana pigs by microsatellite markers is important to assess the degree of genetic diversity in the remaining population, the extent of inbreeding and will inform future conservation and management practices. The objective of this study was therefore to assess the genetic diversity of the indigenous Tswana pig population using microsatellite markers.

MATERIALS AND METHODS

Population sampling

Blood samples were collected from 30 unrelated Tswana pigs in the Southern half of the country in Kgatleng and South-East districts following the guidelines of Measurement of Domestic Animal Diversity FAO (2011) programme. Blood samples were collected from the ear vein of the animals in vacutainer tubes containing

Table 1. Locations where indigenous Tswana pigs were sampled and number of samples per location.

Sampling location	District	No. of samples per location
Ditshweneng lands	Kgatleng	3
Lesetlhane lands	South East	3
Metsimaswaane lands	South East	3
Mmopane	Kweneng	3
Mogobane	South East	4
Ramotswa	South East	6
Sebele (DAR)	Gaborone	2
Segakwaneng lands	Kgatleng	3
Taung	South East	3
Total		**30**

EDTA as the anticoagulant. Blood samples were then transported to the laboratory at 0-4°C (under ice cubes) and stored overnight at -20°C prior to DNA extraction. Information on sampling locations and number of samples per sampling location is given in Table 1.

DNA extraction

Genomic DNA was isolated from whole blood using Zymo Quick-gDNA miniPrep kit following the manufacturer's protocol. Briefly, 400 µl of Genomic Lysis Buffer was added to 100 µl of whole blood and completely mixed by vortexing for 4-6 s. The mixture was allowed to stand for 5-10 min at room temperature, transferred to a Zymo-Spin™ Column in Collection Tube and centrifuged at 10,000 ×g for a minute. The collection tube with the flow through was discarded and the Zymo-Spin™ Column transferred into a new collection tube. 200 µl of DNA Pre-Wash Buffer was added to the spin column and centrifuged at 10,000 ×g for a minute. 500 µl of g-DNA Wash Buffer was added to the spin column and centrifuged at 10,000 ×g for a minute. The spin column was then transferred to clean micro centrifuge tube and 60 µl of DNA Elution Buffer was added and incubated 2-5 min at room temperature. The spin column was then centrifuged at top speed for 30 s to elude the gDNA. The concentration of eluded gDNA was measured using a spectrophotometer (Nanodrop, 2000) and the purity of the gDNA was verified by the 260/280 absorbance ratio (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Microsatellite markers amplification and analysis

A panel of 12 microsatellites recommended by Food Agriculture Organisation (FAO)/ISAG-FAO Advisory Group on Animal Genetic Diversity FAO (1995) were used for genetic characterization of Tswana pigs. The markers used in the study (with chromosome position) were: SW2456 (X/Y), S0165 (3), SW225 (13), SW2008 (11), SW35 (4), SW2406 (6), S0385 (11), S0120 (18), S0073 (4), SW2443 (2), SW949 (X/Y), and SW2410 (17). Selective amplification of different microsatellites was achieved by polymerase chain reaction using the thermocycler GeneAmp PCR system 9700 (Applied Bio systems, Forster City CA, USA) and PCR reagents synthesized by Fermentas Life Sciences Opelstrasse, Germany. Each 25 µl PCR reaction comprised approximately 100 ng gDNA, primers (60 ng each), dNTPs (40 mm each), 10X ammonia-based PCR buffer (2.5 µL), 1.5 mm $MgCl_2$, 1 unit of Taq DNA polymerase and PCR grade deionized water. The PCR reaction was accomplished by initial denaturation for 5 min at 94°C, followed by 33 cycles of denaturation at 94°C for 30 s, primer annealing for 45 s at the desired temperature and DNA replication at 72°C for 1 min. The final extension step was run at 72°C for 10 min. The resulting PCR products were denatured at 98ºC for 3 min and rapidly cooled by placing on ice. The PCR products were separated by capillary electrophoresis on ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster city, CA, USA) according to the manufacturers recommendations and allele sizing was achieved by using the internal size standard of Genescan-500 LIZ (Applied Biosystems, Foster city, CA, USA). Data on allele sizes was done using Genescan Analysis software v.3.1.2 and the identification of different alleles for each marker was performed by Genotyper 2.5 software.

Statistical analysis

The within breed genetic diversity parameters for Tswana pigs which included observed heterozygosity (H_o), expected heterozygosity (H_e), polymorphism information content (PIC) and mean number of alleles (MNA) were calculated using Microsatellite Toolkit software (Kim et al., 2005). The inbreeding coefficient (F_{is}) for each locus was computed using the program FSTAT (Goudet, 2001). The probability test approach (Gou and Thompson, 1992) implemented in the GENEPOP software (Gou and Thompson, 1992) was used to test each locus for Hardy-Weinberg equilibrium.

RESULTS AND DISCUSSION

All the microsatellite markers screened in indigenous Tswana pigs were polymorphic (Table 2). A total of 76 alleles were detected in 12 microsatellite markers screened and the allele size range varied from 83-107 bp at locus S0073 to 220-234 bp at marker locus SW2406. The number of observed alleles per marker varied between 3 (SW2406) and 9 (SW225) with mean number of alleles per marker of 6.33 (Table 2). The range of observed number of alleles per marker and mean number of alleles per marker observed in this study are comparable to 3.38-8.71 and 6.25, respectively, found in local Criollo pig breeds from the Americas (Revidatti et al., 2014) but lower than the range of 5-12 alleles per marker and mean number of alleles per marker of 7.04

Table 2. Observed and effective number of alleles found in Tswana Pigs.

Marker	Allele	Na	Ne
SW2456	205, 207, 191, 209, 211, 189,	6	3.56
S0165	140, 142, 156,159, 137,136, 134,135,	8	5.01
SW225	106, 108, 94, 96, 118, 112, 116, 110, 114,	9	4.35
SW2008	94, 98,96,90,88, 92,	6	3.13
SW35	133, 127, 132, 135, 100,	5	3.58
SW2406	220, 234, 222,	3	1.11
S0385	173, 179, 273, 171, 177, 175, 151,	7	4.53
S0120	169, 171, 151, 153, 163, 165,	6	2.18
S0073	91, 107, 113, 85, 83, 105, 90, 101,	8	3.99
SW2443	209, 211, 213, 203, 207, 201,	6	3.93
SW949	182, 184, 202, 204, 172, 188,	6	2.50
SW2410	107, 121, 118, 105, 117, 123,	6	1.88
	Means	6.33±1.56	3.31±1.18

Na=Observed number of alleles; Ne=effective number of alleles.

reported in indigenous Andaman Desi pig of India (De et al., 2013) and mean number of alleles per marker of 8.45 found in indigenous pigs of Mozambique (Swart et al., 2010). The range of observed number of alleles per marker found in this study is however, higher than the range of 3.98- 5.54 reported by Swart et al. (2010) in commercial pig breeds of South Africa (Landrace, large white and Duroc).

The mean number of alleles per marker of 6.33 found in this study is comparable to 6.18 found in indigenous South African Kolbroek breed (Swart et al., 2010) but higher than 5.72 found in Uruguayan Pampa Rocha pigs (Montenegro et al., 2015), 3.93 and 5.97 in Namibia and Kune-kune breeds (Swart et al., 2010). Effective number of alleles in Tswana pigs ranged between 1.11 (SW2406) and 5.01 (S0165) with mean effective number of alleles per marker of 3.31±1.18. Revidatti et al. (2014) reported a lower mean effective number of alleles per marker of 3.33±1.56 in Criolli pig breeds of the Americas which is comparable with the present study. The mean effective number of alleles per marker in Tswana pigs is however lower than the mean effective number of alleles per marker of 5.09±0.20 found in Andaman Desi pigs of India. According to Pandey et al. (2006), FAO specified a minimum of four alleles per marker for effective screening of genetic differences between breeds and all the markers used in this study with the exception of SW2406 exhibited sufficient polymorphism for evaluation of genetic variation within breed and genetic differences between breeds.

Apart from the number of alleles per locus and mean number of alleles for all loci, other measures of genetic diversity include observed heterozygosity, expected heterozygosity and polymorphic information content (PIC) and those are depicted in Table 3.

The observed heterozygosity for individual markers ranged from 0.16 (SW2405) to 0.875 (SW2465) with average observed heterozygosity across all 12 loci of 0.647. The expected heterozygosity was lower than the observed heterozygosity and ranged from 0.143 (SW2405) to 0.776 (S0385) with mean expected heterozygosity across all loci of 0.603. For markers to be useful in measuring genetic variation they should have average heterozygosity between 0.3 and 0.8 (Takezaki and Nei, 1996) and therefore all the markers used in this study with the exception of SW2405 were appropriate for measuring genetic variation in Tswana pigs. According to Nei and Kumar (2000), observed heterozygosity and expected heterozygosity are highly correlated but expected heterozygosity also known as Hardy-Weinberg heterozygosity is considered a better estimator of the genetic variability present in a population.

More heterozygous loci than expected in Tswana pigs is consistent with Setyawan et al. (2015) who observed a similar pattern in most Indonesian Native cattle breeds. Unlike in Tswana pigs, most pig genetic characterization studies report heterozygote deficiencies than heterozygote excesses (De et al., 2013) due to inbreeding resulting from limited population sizes and selective breeding in pig improvement programs. The average expected heterozygosity of 0.603 and observed heterozygosity of 0.647 indicate high level of genetic variability or genetic diversity in Tswana pigs since it is interpreted as such when the heterozygosity values exceed 0.5 (Melendez et al., 2014). High degrees of genetic diversity have also been reported in indigenous pigs of Cerete-Colombia, Andaman Desi pig, Criollo pig breeds from the Americas, Uruguayan Pampa Rocha pigs and Chinese village pigs with expected heterozygosity values of 0.527, 0.77, 0.622, 0.603 and 0.826, respectively (Melendez et al., 2014; De et al., 2013; Revidatti et al., 2014; Montenegro et al., 2015;

Table 3. Measures of genetic diversity in Tswana pigs.

Locus	Ho	He	PIC	HWE p-value	F_{is}
SW2465	0.875	0.679	0.47	0.648	-0.242
S0165	0.586	0.637	0.422	0.591	0.240
SW225	0.819	0.693	0.499	0.791	-0.075
SW2008	0.71	0.724	0.524	0.729	-0.029
SW35	0.556	0.578	0.424	0.730	0.234
SW2405	0.16	0.143	0.094	1.00	-0.044
S0385	0.736	0.776	0.569	0.548	-0.008
S0120	0.694	0.542	0.39	0.914	-0.321
S0073	0.622	0.704	0.517	0.698	0.218
SW2443	0.823	0.723	0.525	0.776	-0.102
SW949	0.743	0.551	0.378	1.00	-0.251
SW2410	0.403	0.485	0.321	0.889	0.238
Mean	0.647	0.603	0.428	0.776	-0.012

Fang et al., 2009). The expected heterozygosity value of 0.603 found in Tswana pigs is comparable to those found in other Southern African pig breeds such as Mozambique indigenous pig, South African Kolbroek and South African Kune-Kune with Hardy-Weinberg heterozygosity values of 0.692, 0.634 and 0.675, respectively (Swart et al., 2010). Compared to commercial pig breeds, the average expected heterozygosity of the indigenous Tswana (0.603) is similar to 0.60 of the large white (Oh et al., 2014) but slightly higher than 0.580 and 0.531 of the South African Landrace and Duroc breeds, respectively (Swart et al., 2010). The high level of genetic variation or diversity in Tswana pigs might be attributed to lack of selective breeding or improvement programs targeted towards the breed and possible existence of population substructure (Genetic uniqueness in terms of alleles of Tswana pigs coming from different villages).

The polymorphic information content (PIC) values of the 12 markers employed in the characterization of Tswana pigs ranged from 0.094 for SW2405 to 0.569 for S0385 with average PIC value of all the markers of 0.428 (Table 3). According to Montenegro et al. (2015), markers with PIC values greater than 0.5 are highly informative, those with PIC values between 0.25 and 0.5 are moderately informative and those with PIC values less than 0.25 are uninformative. Following the same classification criterion, four markers (SW2008, S0385, S0073 and SW2443) were highly informative, seven (SW2465, S0165, SW225, SW35, S0120, SW949 and SW2410) were moderately informative and one (SW2405) was uninformative in Tswana pigs. Moderately informative and highly informative markers are more variable and therefore more suitable for genetic diversity studies in indigenous Tswana pigs.

All the 12 microsatellite markers used in the current study were in Hardy-Weinberg Equilibrium clearly indicating the high genetic stability of indigenous Tswana pigs kept by farmers under extensive management system. The high genetic stability of indigenous Tswana pigs confirm that Tswana pigs are mostly random mating under free running management system practised by majority of farmers, is not undergoing any artificial selection (no improvement program for indigenous Tswana pig), the effects of random genetic drift common in small populations like that of Tswana pigs are negligible and Tswana pigs are not subjected to other evolutionary forces such as mutation and migration capable of altering gene, genotype frequencies and causing significant departures form Hardy-Weinberg equilibrium.

The within-locus inbreeding coefficient (F_{is}) ranged between -0.321(S0120) and 0.234 (SW35) with multilocus inbreeding coefficient of the entire population of -0.012. The negative inbreeding coefficient of Tswana pigs might be due to avoidance of mating among closely related animals (Hui-Fang et al., 2010) which resulted in significant excess of heterozygotes in the population. All the markers with the exception of SW35 and S0073 contributed to the negative inbreeding coefficient of the Tswana pigs. Markers SW35 and S0073 exhibited significant deficit of heterozygotes probably due to genetic drift or linkage disequilibrium of the marker with loci under either natural or artificial selection (Ibeagha and Erhardt, 2005).

Conclusions

Moderate levels of genetic diversity and no inbreeding exist within the Tswana pig population in Southern Botswana. This genetic diversity in the Tswana pigs showed that there is random mating and the animals are not undergoing any form of artificial selection. If deliberate efforts towards conservation are not put in place, this valuable genetic resource with its hardiness,

disease resistance and heat tolerance genes might become extinct within the next decades, even before it has been fully characterized. The conservation of indigenous Tswana pigs should be given high priority because it contains valuable genes (disease resistance and heat tolerance genes) for future breed developments and genetic engineering applications to counter the effects of global warming or climate change on pig production and productivity.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Natural occurrence of products of cryptic pathway metabolites of pharmacological importance using liquid chromatographic-mass spectrometric techniques in date palm fruit, garlic bulb and groundnuts from North-West Nigeria markets

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Investigation of secondary metabolites from the cryptic metabolic pathways in date palm fruit, garlic bulb and groundnuts from North-West Nigeria was conducted using liquid chromatography-mass spectrometry and mass spectrometry (LC-MS/MS). Results indicated the presence of several compounds which include Valinomycin, asperglaucide, asperphenamate, cyclo (L-Pro-L-Tyr). Cyclo (L-Pro-L-Val), emodin, physcion, integracin A and B, Monocerin, and fallacinol were detected at different concentrations in the different sampled materials. Asperglaucide, asperphenamate, cyclo (L-Pro-L-Tyr), cyclo (L-Pro-L-Val), and emodin were found in all the tested samples at different concentrations. Garlic bulb contains valinomycin, emodin and physcion which are compound that may have a potential inhibitory effect on severe acute respiratory syndrome coronavirus (SARS-CoV) with their maximum concentration of 55.15, 20.25 and 419.45 µg/kg respectively in the tested materials. Integracin A and B which are compound with potential inhibitory effect on the HIV-1 integrase enzyme found in Human immune deficiency virus (HIV) were detected in garlic bulb samples at the maximum concentration of (3.98 and 18.68 µg/kg) respectively. This study has identified the presence of a compound of immense pharmacological importance, and it may provide a veritable lead for natural product discovery.

Key words: Asperphenamate; cryptic pathway; date fruits; garlic bulb; groundnut seed; metabolites.

INTRODUCTION

Secondary metabolites are compounds of natural origin that are often produced specifically in the cells of some animals, plant, fungi and bacteria. Secondary metabolites are involved in the homeostasis, modulation of organism health system such as excretion and overall health status of the body system (Baral et al., 2018). Production of

these metabolites by some organism is regarded as an adaptive capacity of coping with stress caused by challenges of changing growth environment. This lead to overproduction of complex chemical types and it involves interaction in their structural and functional stabilization through cell signaling processes and pathway (Tanaka et al., 2013).

The biosynthetic capabilities of fungal and bacteria cryptic pathways in producing secondary metabolites which can be used as natural product is much more than it's currently appreciated. Cryptic biosynthetic pathway involves manipulation and activation of biosynthetic genes. It has been previously enhanced using several methods which include, epigenetic modifier and genetic engineering (Jordan et al., 2016; Tanaka et al., 2013). Mixed fermentation using microbial cultivation of two or more microorganism has been reported to induce expression of cryptic pathway, leading to the production of different microbial natural products (Becerril and Susana, 2018). It can be deduced that competition between microbes encourages production of secondary metabolites via signaling molecules (auto-regulator/quorum sensing molecules and siderophore) in their environment (Jordan et al., 2016).

Alternatively, epigenetic modification in the producers strain may be related to the activation of metabolites precursors by producer active enzymes (Rutledge and Challis, 2015). However, it is worth noting that in many cases, direct contact is necessary between bacteria and fungi to elicit this effect. Recent studies have demonstrated that co-cultivation or co-contamination may be a remarkable successful approach for discovering new natural bioactive products (Becerril and Susana, 2018; Lal and Lal, 2011). Their biosynthesis can be greatly influenced by manipulating the type of host plant parts and concentration of the nutrient in the culture media (Jordan et al., 2016).

Some bacteria and fungi have cryptic gene clusters that produce secondary metabolites which is a leading source of drug discovery (Shen, 2015). Many of these microbial secondary metabolites are leading drug candidates as fungal bio-control agent of plants and animal pathogens, anti-metastatic agent, anti-inflammatory, antiviral and antibacterial agents (Abia et al., 2017). Cryptic products have been identified in tomato, maize meal, cassava fermented products, cashew nuts and animal feeds in different countries (Abia et al., 2017; Adetunji et al., 2019).

There is need to determine the differences in where these microbial organisms produce these cryptic pathway metabolites among the crop samples which are susceptible to fungal and bacteria co-contamination.

Date palm (*Phoenix dactylifera* L.) is grown in many tropical countries and is the most popular fruit eaten in Northern Nigeria as high calorie appetizer (Arias et al., 2016). Date fruits have sweet flavour and high nutrition profile providing important essential nutrients like protein, fibre, carbohydrates, fat and minerals (Chandrasekaran and Bahkali, 2013). Groundnut (*Arachis hypogea* L seed has abundant protein, fat, vitamins, minerals and fibre. It is eaten locally by diabetic patient because it low glycaemic index (Nautiyal, 2002). Groundnut is very popular for its seeds which can be eaten raw, boiled, roasted or dried. Also, the seed oil is used for cooking food and has industrial application (Goswami et al., 2014). Garlic (*Allium sativum* L) bulb is commonly consumed for its medicinal and culinary use worldwide (Sharma et al., 2013). In Nigeria like other parts of the world, it is used in seasoning meat and meat products. Its medicinal properties include lowering of blood pressure, treat viral and bacterial diseases (Rastogi et al., 2016).

Several reports of fungal and bacterial contamination or co-contamination of date fruit, groundnut seeds and garlic bulb have been reported (Al-Meamar et al., 2017; Kachapulula et al., 2017; Moharam et al., 2013). The interaction between fungal and bacteria flora in these substrates may lead to the expression of the cryptic metabolic pathway and subsequent production of some useful metabolites. This study is focused on identifying products from the cryptic pathway which may be of pharmacological importance and relating it with substrate where they are found using high throughput screening with liquid chromatography-mass spectrometry/mass spectrometry.

MATERIALS AND METHODS

Sampling and sample collection

Samples of garlic bulbs (16), groundnut seeds (30) and date palm fruits (45) were collected from three retail market points in each of the six zones of Zaria (Samaru, Gaskiya, Sabongari, Tudunwada, Kongilla and Tudunwada) located between Lat. 11° 06' 40''N and Long. 7°43'21''E in Kaduna State, North West Nigeria. Thirty bulk samples (1 kg each) of randomly measured sample materials were obtained and kept in clean zip-lock bags. They were transported to the laboratory of Crop Protection Department, Ahmadu Bello University, Samaru-Zaria, Nigeria. A total of 91 samples (each representing a pack of the bulbs, fruits and the seeds, weighing approximately 500 g) were randomly collected. Each sample was quartered and about 100 g was taken and pulverized using a commercial Blender (Waring Commercial Blender 8010BU, Model HGBTWT, Connecticut, USA). The representative samples were stored at -4ºC to prevent further metabolite liberation by fungi and bacteria within samples prior to multi-mycotoxin analysis. Samples were kept in polypropylene bags and transported to the Center for Analytical Chemistry Laboratory in the Department of Agro-biotechnology, University of Natural Resources and Life Sciences, Vienna, Austria for analysis.

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Analysis of mycotoxin

Reagents

Liquid chromatographic grade methanol (CH_3OH) and acetonitrile were purchased from Merck (Germany) and VWR (Belgium) respectively. The Mass Spectrometry grade ammonium acetate and standards for fungi metabolite were brought from Sigma-Aldrich (Austria). Decontamination of water was carried out consecutively through reverse osmotic pressure and ultra-analytic system purchased from Veolia water (UK). A total of 34 working solutions were made and kept at -20°C in the fridge but were brought to 25°C before use. Fresh final working solution was mixed accordingly for the spiking experiment.

Garlic bulb, groundnut seeds and date fruits extraction

Each garlic bulb, groundnut seeds and date fruit were milled using a cyclone pulverizer which has one millimetre square sieve (Cyclotech, Sweden) before being homogenized. Five grams each were measured into the centrifuge tube (0.05 L polypropylene). Twenty millilitres of the separation solvent (acetic acid/water/acetonitrile 1:20:79, v/v/v) were added before being vortexed using a vortexed using laboratory rotary shaker (Model GFL 3017, Germany). Ratio of the dilution of the sample with the solvent was 1:1 and 5 ml of the dilution obtained from the extract were injected into the LC-MS/MS.

LC-MS/MS parameters

Detection and quantification of extracts was achieved through a described procedure (Malachova et al., 2014). Briefly, a QTrap 5500 multimycotoxin LC-MS/MS system (Applied Biosystem, California, United State of America) furnished with TurboV spray ESI source and Ultra High Performance Liquid Chromatography system (UHPLC) (Agilent, Germany). The Chromatographic separation of extracts was performed at 25°C on a 150 × 4.6 mm, 5-µm Gemini C18-column equipped with a C18 security guard cartridge, 4 × 3 mm i.d. (Phenomenex). Elution was carried out in binary gradient mode. Both mobile phases contained 5 mM ammonium acetate and were composed of methanol/water/acetic acid 10:89:1 (v/v/v; eluent A) and 97:2:1 (v/v/v; eluent B), respectively. After an initial time of 2 min at 100% A, the proportion of B was increased linearly to 50% within 3 min. Further linear increase of B to 100% within 9 min was followed by a hold-time of 4 min at 100% B and 2.5 min column re-equilibration at 100% A. The flow rate was 1000 µL/min. ESI–MS–MS was performed in the scheduled multiple reaction monitoring (sMRM) mode both in positive and negative polarities in two separate chromatographic runs. The sMRM detection window of each analyte was set to the respective retention time ±27 s and ±42 s in positive and in negative mode, respectively. The target scan time was set to 1 s. The settings of the ESI source were as follows: source temperature: 550 °C; curtain gas: 30 psi; ion source gas 1 (sheath gas): 80 psi; ion source gas 2 (drying gas): 80 psi; ion-spray voltage: −4500 V and +5500 V, respectively; collision gas: (nitrogen) medium. Confirmatory identification was obtained through the acquisition of two sMRMs per analyte, which yields 4.0 identification points according to commission decision 2002/657/EC.

The method precision was tested through proficiency testing organized by Bureau Interprofessionel des Etudes Analytique (BIPEA) (Gennevilliers, France) in accordance with ISO 13525:2015. All the results of the extracts were between -2< x < 2 which was a satisfactory range. The percentage of contaminated samples, maximum and median concentration ($\mu g kg^{-1}$) of toxins and metabolites were determined from the data collected for each of the samples analysed.

Metabolites were quantified by external calibration (1/*x* weighted) using a multi-component standard prepared from authentic standards.

RESULTS AND DISCUSSION

Secondary metabolites produced through the expression of the cryptic pathway may be of great pharmacological importance. The tested samples are: defective date fruit (DDF) (21), healthy date fruits (HDF) (24), garlic bulb (16) and groundnut seed (30) samples and result presented in Table 1.

Valinomycin was detected only in 9/16 (56%) of the garlic sample with a median and maximum values of (44.99 and 55.15) µg/ kg (Table 1 and Figure 1a). Valinomycin is a product of bacterial contamination. It is found in garlic from different markets in the sampled area. It is a cyclic peptide which readily dissolves in the membrane lipid bilayer and thus enhanced its bioavailability in target cells. It is an antibiotic which greatly affects the metabolic pathways of several bacteria (Shen, 2015).

Asperglaucide was found naturally occurring in all the samples tested. The lowest value found in the food samples tested was 2.42 µg/ kg in groundnut seeds while the maximum concentration of 2886.40 µg/ kg was found in garlic bulb. Also, the maximum values of 13.52 µg/ kg of asperglaucide was obtained in defective date fruits.

Asperphenamate was also detected in all tested crop samples. Its median values are (2.41, 2.56, 267.24, and 3.41) µg/ kg in DDF, HDF, garlic bulbs and groundnut seeds (Table 1 and Figure 1b). Presence of Asperglaucide in all tested samples with its highest concentration of 2886.40 µg/kg in garlic showed that the metabolites can be produced in different substrates. It was also observed that it was detected in 100% of all samples which indicated that the growth environment for this microbial contaminant is conducive for the biosynthesis of Asperglaucide. Natural occurrence of Asperglaucide has been previously reported at different concentration in different substrates in several countries (Abass et al., 2017; Andersen and Frisvad, 2004; Humer et al., 2016).

Cyclic dipeptide identified in the samples was cyclo (L-Pro-L-Tyr) and cyclo (L-Pro-L-Val). Garlic bulb samples were positive for these two cyclo dipeptides. The maximum values recorded were (1458.00 and 1481.00) µg/ kg for cyclo (L-Pro-L-Tyr) and cyclo (L-Pro-L-Val) respectively (Table 1 and Figure 1c). A total of 18/21 (86%) of DDF and 9/24 (38%) HDF were positive for cyclo (L-Pro-L-Val). Their respective median values were 8.42 and 3.71 µg/kg. The result of this study showed that the two cyclo dipeptides may be a natural contaminant of garlic bulb. These cyclo-peptides have been isolated from *Streptomyces* species, they possess antibacterial activities and has inhibitory effect on some plant pathogens (Nishanth et al., 2012).

Table 1. Products of cryptic pathway metabolites detected in the liquid chromatography-mass spectrometry (LC-MS)/mass spectrometry (MS) analysed samples, specifying the proportion of positive samples (*p* pos), the percentage of positive samples (% pos), as well as the median, maximum concentration (max. concentration) and range in µg/kg.

Metabolites	Source	% Positive (proportion)	Median (µg/kg)	Maximum (µg/kg)	Range (µg/kg)
Valinomycin	Defective date fruit	-*	-	-	-
	Healthy date fruit	-	-	-	-
	Garlic bulb	56.25 (9/16)	44.99	55.15	30.76
	Groundnut seeds	-	-	-	-
Asperglaucide	Defective date fruit	100 (21/21)	4.6	13.52	12.15
	Healthy date fruit	100 (24/24)	3.64	5.56	4.53
	Garlic bulb	100 (16/16)	697.31	2886.40	2835.83
	Groundnut seeds	75 (24/30)	1.22	2.42	1.64
Asperphenamate	Defective date fruit	100 (21/21)	2.41	4.39	3.94
	Healthy date fruit	100 (24/24)	2.56	5.56	5.21
	Garlic bulb	100 (16/16)	267.24	492.15	437.63
	Groundnut seeds	96 (29/30)	3.41	4.10	3.96
Cyclo (L-Pro-L-Tyr)	Defective date fruit	86.71 (18/21)	7.52	17.93	15.87
	Healthy date fruit	37.5 (9/24)	3.86	10.88	9.9
	Garlic bulb	100 (16/16)	1107.67	1458.00	1034.21
	Groundnut seeds	86.66 (26/30)	8.52	11.85	7.87
cyclo (L-Pro-L-Val)	Defective date fruit	71.42 (15/21)	8.42	13.18	12.32
	Healthy date fruit	37.5 (9/24)	3.71	8.40	7.69
	Garlic bulb	100 (16/16)	561.53	1481.60	965.66
	Groundnut seeds	93.33 (28/30)	7.14	175.21	171.64
Emodin	Defective date fruit	33.33 (7/21)	1.27	99.46	99.19]
	Healthy date fruit	4.16 (1/24)	2.38	2.38	2.38
	Garlic bulb	100 (16/16)	10.01	20.25	12.4
	Groundnut seeds	26.66 (8/30)	11.28	131.38	129.31
Physcion	Defective date fruit	4.76 (1/21)	698.24	698.27	698.27
	Healthy date fruit	4.16 (1/24)	20.96	20.96	20.96
	Garlic bulb	50 (8/16)	258.15	419.45	298.34
	Groundnut seed	-	-	-	-
Integracin A	Defective date fruit	-	-	-	-
	Healthy date fruit	-	-	-	-
	Garlic bulb	62.5 (10/16)	3.11	3.98	3.98
	Groundnut powder	-	-	-	-
Integracin B	Defective date fruit	-	-	-	-
	Healthy date fruit	-	-	-	-
	Garlic bulb	68.75 (11/16)	5.62	18.68	15.57
	Groundnut powder	-	-	-	-
Monocerin	Defective date fruit	-	-	-	-
	Healthy date fruit	-	-	-	-
	Garlic bulb	81.25 (13/16)	1.66	7.97	3.45
	Groundnut seeds	6.67 (2/30)	18.12	35.57	32.12
Fallacinol	Defective date fruit	4.76 (1/21)	0.41	0.41	-
	Healthy date fruit	-	-	-	-
	Garlic bulb	50 (8/16)	18.12	35.57	29.91
	Groundnut seeds	-	-	-	-

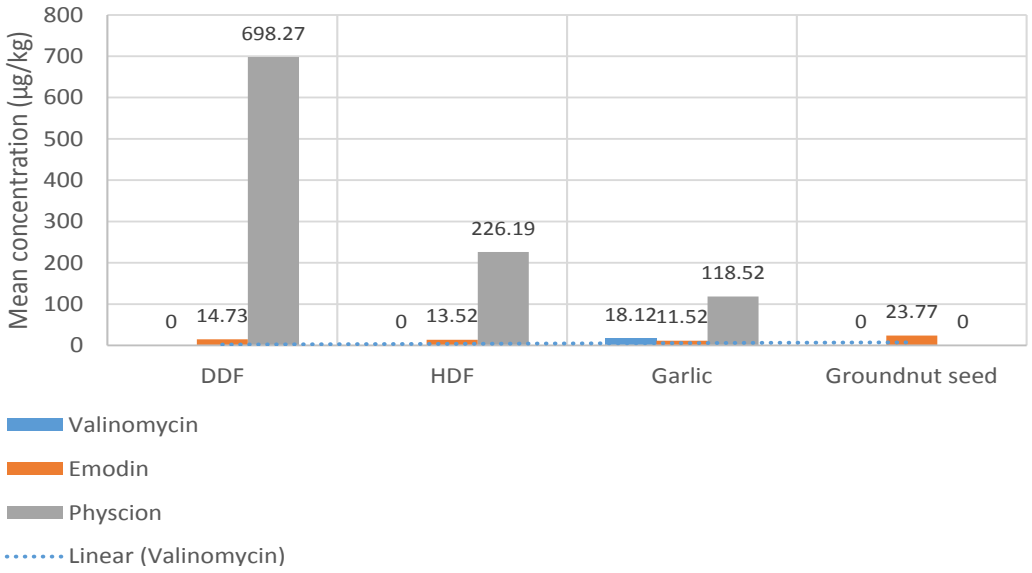

Figure 1a. Mean concentration (µg/kg) of compounds with activities against severe acute respiratory coronavirus (SARS-CoV) naturally occurring in DDF, HDF and garlic bulb samples.

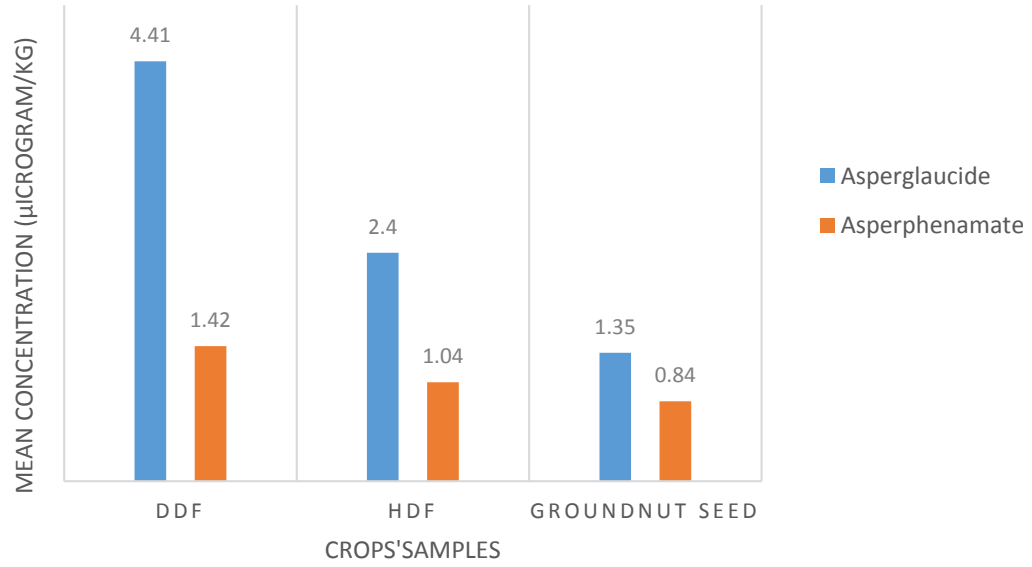

Figure 1b. Mean concentration (µg/kg) of compounds with activities against cancer and inflammatory diseases naturally occurring in DDF, HDF and groundnut seed samples.

Emodin was found in 100% of garlic samples, 7/21 (33.33%) samples of DDF, 1/24 (4.1%) in HDF and 8/30 (27%) in groundnut seeds. Their respective maximum values were 99.46, 2.38, 20.25, 131.38 and 1.48 µg/ kg (Table 1 and Figure 1a). Natural occurrence of emodin in unripe fruits, vegetables and different herbs has been previously reported (Dong et al., 2016); to the best of our knowledge this is the first report of emodin in garlic bulbs, groundnut seeds and date fruits in Nigeria. Unlike emodin, physcion is found in 50% of the garlic bulb samples and only one sample each of DDF and HDF (Table 1 and Figure 1a). The presence of physcion in half of the garlic samples tested indicated its appreciable presence in the study area. These metabolites can be exploited for its documented pharmacologically activities. Emodin and physcion have been documented to possess anti-SARS-CoV activities (Ho et al., 2007). They have also been used in Traditional Chinese Medicine in the treatment of microbial infections, liver diseases and are good anti-inflammatory agent (Ahirwar and Jain, 2015).

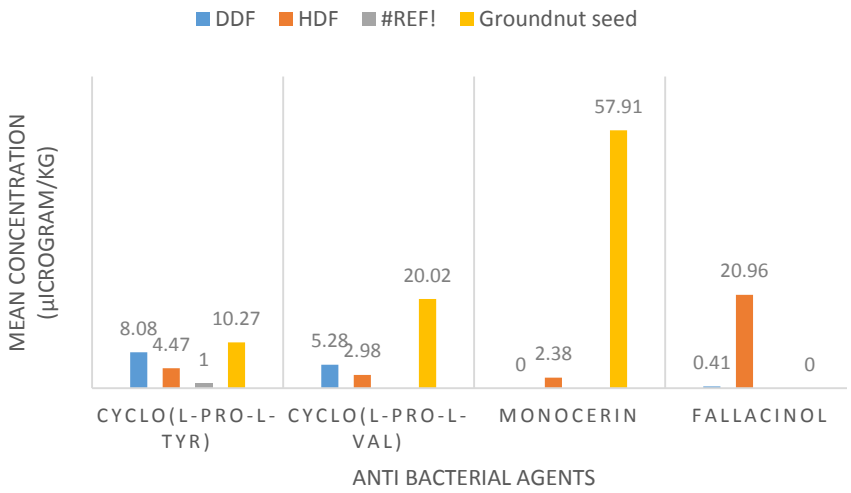

Figure 1c. Mean concentration (µg/kg) of compounds with antifungal and antibacterial activities naturally occurring in DDF, HDF and groundnut seed samples.

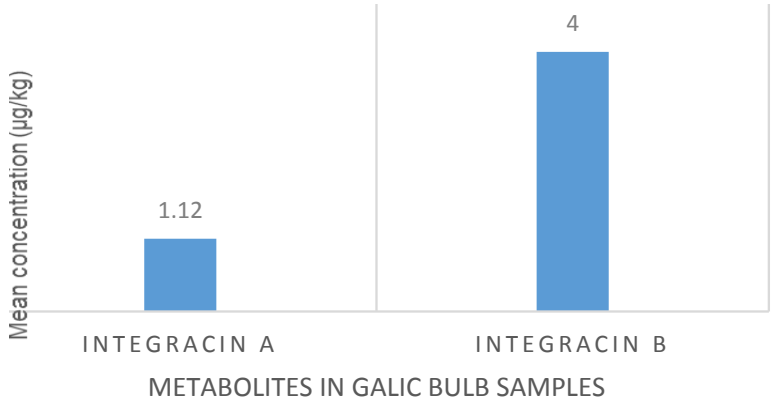

Figure 1d. Mean concentration (µg/kg) of compounds with anti-HIV activities in garlic bulb samples.

Integracin A and integracin B are found only in garlic samples at maximum values of (3.98 and 18.68) µg/ kg respectively (Table 1 and Figure 1d) while Monocerin was detected in garlic 11/16 (69%) and groundnut seeds 2/30 (7%) (Table 1 and Figure 1e). Their respective median values are 1.66 and 55.41 µg/ kg respectively. These fungi metabolites are products of the cryptic metabolic pathway with beneficial medicinal properties. They have been reported to have activities against HIV-1 integrase enzymes (Sivro et al., 2019). In addition, Monocerin has shown appreciable pharmacological activities against plant pathogens like powdery mildew of wheat (Robeson and Strobel, 1982).

Fallacinol (Table 1 and Figure 1e) is found in two samples of garlic bulb and DDF with their respective maximum values of 0.41 and 35.54 µg/kg. Fallacinol is an antimicrobial metabolites found in some medicinal plants (Bitchagno et al., 2015).

The microbial secondary metabolites identified in this study are of significant pharmacological importance. This study will serve as a template for further work on isolation and subsequent development of natural biologically active product that can be effective in managing several diseases of plants and animals.

Conclusion

Several products of cryptic metabolic pathways of pharmacological importance were identified using LC-MS/MS chromatographic techniques. Valinomycin, integrin A and C were found naturally occurring in only

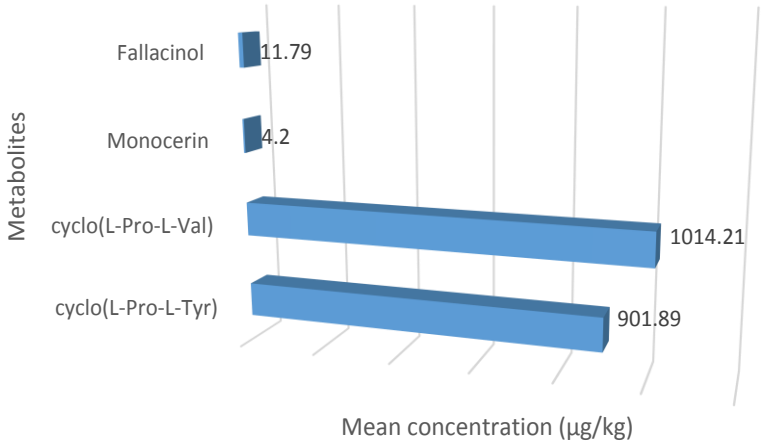

Figure 1e. Mean concentration (µg/kg) of compounds with antifungal and antibacterial activities found in the analysed samples.

garlic bulb samples while Asperglaucide, Asperphenamate, the Cyclo dipeptides and Emodin are found as natural contaminants of all the sample tested. This study detected Asperglaucide and Asperphenamate in 100% of DDF, HDF and groundnut seed tested. These secondary metabolites detected in this study may be an important lead in medicinal plant drug discovery.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Comparative study of single nucleotide polymorphisms (SNPs) of a candidate growth gene (IGF-I) in *Oreochromis niloticus* and *Sarotherodon melanotheron*

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In teleost fishes, the regulation of growth performance by the GH and IGF system also seems to be highly conserved. Adult *Oreochromis niloticus (Nile* tilapia) could reach up to 60 cm maximum length while *Sarotherodon melanotheron* (black chin Tilapia) has a maximum length of 28 cm. This study describes the analysis of Single Nucleotide Polymorphism in isolated, amplified, and sequenced DNA from two common Tilapia species (*O. niloticus* and *S. melanotheron)* with the aim of identifying genetic variation and single nucleotide polymorphisms (SNPs) in one of the main genes, Insulin like growth Factor-1 (IGF-I), related to growth in the Tilapia species. The extracted DNA from the clipped caudal fins of the Tilapia species samples using Sambrook and Russell's modified chlolorophenol/isoamyl alcohol DNA extraction method were further amplified in a thermal cycler with designed IGF-I forward and reverse primers of 447 bp which were subsequently sequenced with an automated analyzer. The PCR product was separated on 1.5% ethidium stained agarose gel electrophoresis and the bands revealed on the gel were all of the same length (447 bp). The Sequence alignment revealed a total of five single nucleotide polymorphisms which were detected in the forward reaction at the positions 181, 199, 328, 362 and 369 of the sequences and in the reverse reaction at positions 18, 20, 54, 183 and 201. A total of 138 amino acid sequence was translated from the DNA sequence with variations sequence at positions 1, 3, 4, 61, 67, 121 and 123. These results showed variations among these two fish species which could explain differential growth performance between them.

Key words: Single nucleotide polymorphism (SNP), *Oreochromis niloticus* (ON), *Sarotherodon melanotheron* (SM), Tilapia and insulin-like growth factor (IGF).

INTRODUCTION

Single nucleotide polymorphism (SNP) markers are the method of choice for genetic analyses including diversity and quantitative trait loci studies (Thu et al., 2017). Determination of the genetic variability was described by Lupchinski Jr et al. (2011) as an essential step for the implementation of genetic improvement programs that

are focused on the selection of faster growing fish with lower feed conversion rates and resistant to diseases. Studies of genetic diversity at DNA level represents in expansion field in aquaculture aimed at finding out those DNA variations associated with productive phenotypes, so as to use them as tools for assisting the offspring selection at early stage and possibly predict their performance (Na-Nakorn and Moeikum, 2009). This strategy is known as gene-assisted selection (GAS) (De-Santis and Jerry, 2007). Growth performance is often used as an indicator of the status of individuals and populations in culture and the wild, and therefore, major effort has been applied towards garnering a more comprehensive understanding of how multiple components of the GH (growth hormone) and IGF (insulin-like growth factor) system interact to control growth and metabolism (Picha et al., 2008; Beckman, 2011). To improve growth and growth efficiency in aquaculture, an advanced understanding of the physiological mechanisms that regulate growth in fishes are needed. The growth hormone/insulin-like growth factor (GH/IGF) in the endocrine axis regulates growth in all vertebrates, including fishes as described by Davis et al. (2008).

Tilapia is the common name for nearly a hundred species of cichlids from the tilapinne cichlid tribe. Tilapia are mainly freshwater fish inhabiting shallow streams, ponds, rivers and lakes and less commonly found living in brackish water. The survey carried out by Oguntade et al. (2014) shows that some fish species including Tilapia are fast disappearing in Nigerian water bodies such as Brass and Nun River of Niger Delta.

In 2017, according to FAO statistics, Nile tilapia (*Oreochromis niloticus)* culture alone was ranked fourth among the most cultured in the world, in terms of both production and value with a total aquaculture production of 4.1 million tonnes (FAO, 2019). The other top four species were silver carp, grass carp, common carp and other Cyprinids (FAO, 2019). Nile tilapia represents approximately 86% of total global tilapia production (FAO, 2019). In 2017, it is anticipated that global Nile Tilapia production will reach nearly 4 million tonnes (FAO, 2017). Adult *Oreochromis niloticus (Nile* tilapia) reach up to 60 cm maximum length while *Sarotherodon melanotheron* (black chin Tilapia) has a maximum length of 28 cm (Olaosebikan and Raji, 1998) when subjected to the same environmental condition. This justifies the higher value and demand for Nile tilapia, hence higher production of other Tilapia species is needed to meet the demand for Tilapia. Black chin Tilapia on the other hand thrive well in high salinity region but are constrained by their growth and as such do not meet market value. Since IGF-I also regulate growth in fishes; the need to study its variation in the two Tilapia species arises with the aim of identifying a possible growth factor that will promote a higher production of *S. melanotheron* from saline environment to complement the production of *O. niloticus* from fresh water bodies to meet global tilapia demand. This would be achieved by detecting the genetic variations and single nucleotide polymorphisms (SNPs) in one of the main growth genes, Insulin like growth Factor-1 (IGF-I) in two Tilapia species.

MATERIALS AND METHODS

Fish sample collection and identification

Ten (10) live female tilapia fishes comprising five *Oreochromis niloticus* (ON1, ON2, ON3, ON4 and ON5) and five *Sarotherodon melanotheron* (SM1, SM2, SM3, SM4 and SM5) were obtained from Nigerian Institute for Oceanography and Marine Research (NIOMR), Badore outstation (latitude 6°25'60"N and longitude 3°51'0"E), and Oluwo market sourced from Epe river, Epe (latitude 6°35'2"N and longitude 3°59'0"E), Lagos state, Nigeria respectively. The samples of caudal fins were clipped and transferred into ten different plain 10 mL sterile tubes each containing 4 mL absolute ethanol.

Fish samples were identified from description checklist and identification keys (FAO, 1996; Froese and Pauly, 2003; Uyeno and Fujii, 1984).

DNA extraction and amplification

DNA was isolated from the caudal fin tissue of the ten sampled fish using modified chlorophenol/ isoamyl/alcohol protocol according to Sambrook and Russell (2001) on bench at biotechnology laboratory of NIOMR, Badore outstation, Nigeria. The integrity of the DNA was checked on 1% ethidium bromide stained agarose gel electrophoresis and the isolate was stored at -20°C prior PCR amplification.

The PCR amplification was run with the specific primers (IGF-I forward- 5'-CTTGGACGAGTAGGAGGCAAATG-3' and IGF-I reverse- 3'-GAAATACAAGCAAGCGATAAGAA-5') of 447 bp designed to amplify coded regions (exons) of the IGF-I gene sequences (IGF-I, GenBank accession AF033797) which was re-sequenced and used. The DNA amplification was carried out by polymerase chain reaction (PCR) in a with 20 ng of genomic DNA using Thu et al. (2017) protocol, 20 ul reactions containing 0.2 uM of each primer, 200 uM of dNTPs, 50 mM KCL, 10 mM Tris HCL (pH 8.3), 1.5 mM $MgCl_2$ and 0.5 units of Taq DNA polymerase with Eppendorf thermocycler with an amplification profile of initial denaturation at 95°C for 10 min, followed by 35 cycles with 95°C for 30s, annealing temperature at 60°C for 45s, extension at 72°C for 45s and final extension at 72°C for 5 min. The product was checked on 1.5% agarose gel electrophoresis at 70v for 1.5 h in 1x TBE buffer and the gel was stained with ethidium bromide for visualization through Fisher Scientific UV transilluminator.

DNA sequencing

Purified PCR products from the amplification of the ten Tilapia

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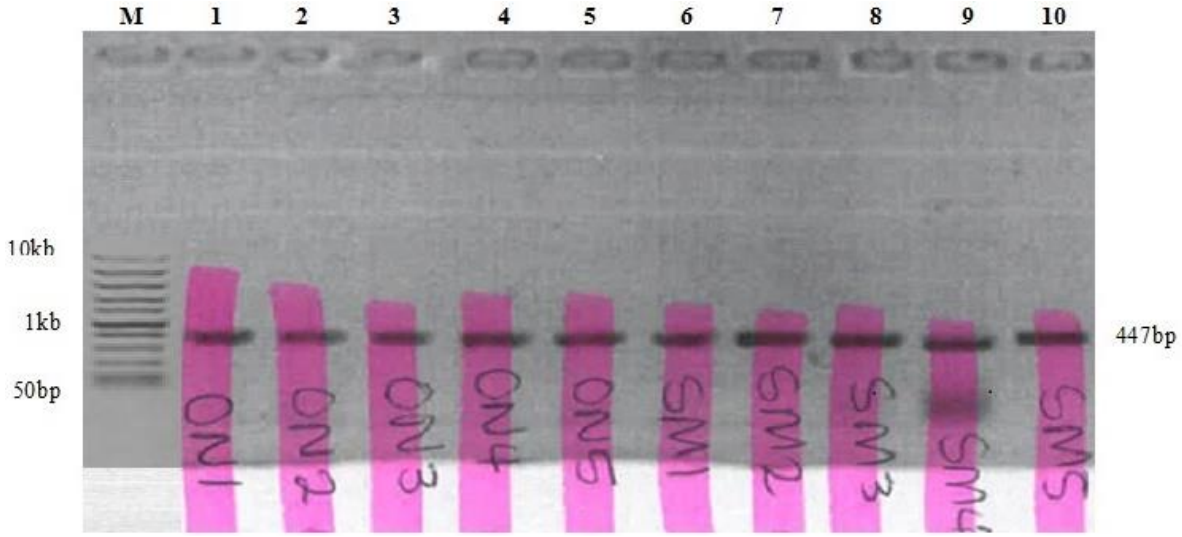

Plate 1. Picture of 1.5 % ethidium bromide stained agarose gel electrophoresis picture of the amplified DNA isolate from the ten Tilapia samples of *O. niloticus* (ON1-ON5) and *S. melanotheron (SM1-SM5).*

fishes, *O. niloticus* (5) and *S. melanotheron* (5) were bidirectional sequenced in an automatic sequencer (ABI 3500XL Genetic Analyzer).

Nucleotide sequences obtained were edited and aligned using clustal O (version1.2.4) multiple sequence alignment software, the Single nucleotide Polymorphisms (SNPs) were discovered by visual analysis and dendogram was also created while translation of the DNA sequence of each species was done using biolign alignment software (version 4.0.6.2).

RESULTS AND DISCUSSION

DNA extraction and amplification

The IGF-I amplified PCR product of the extracted DNA run on 1.5% ethidium bromide stained agarose gel demonstrated that IGF-I genes had the same bands which demonstrated equal fragment length of the ten Tilapia fishes, *O. niloticus* (5) and *S. melanotheron* (5) as shown on Plate 1, where M is the known 50 bp- 10 kb DNA ladder.

DNA sequencing and analyses

The sequence alignment generated for the forward and reverse primers of IGF-1 of the ten tilapia fish (5 *Oreochromis niloticus* and 5 *Sarotherodon melanotheron)* were shown in Figures 1 and 2 respectively while Tables 1 and 2 show the five single nucleotide polymorphisms (SNPs) detected at the positions 181(T/C), 199(T/C), 328(C/G), 362(C/A), and 369(A/C) of the forward reaction sequence and those detected in the reverse reaction sequence at positions 18(T/A), 20(T/G), 54(C/G), 183(G/A), and 201(G/A) respectively.

The dendogram revealed lower similarities between SM1-SM5 and ON1-ON5 and higher similarities among SM1-SM5 and among ON1-ON5 (Figure 3). The lower similarity between SM and ON might imply a high genetic variation and could be due to the fact that they are different species and do not have common ancestry. This finding is on the contrary with the report of Usman et al. (2013) who obtained a high similarity coefficient of 78% between *T. guineensis* and *S. melanotheron* from the wild.

A total of 138 amino acid sequence was translated from the DNA sequence of *O. niloticus* and *S. melanotheron* as shown in Figures 4 and 5. The alignment of these sequences revealed seven (7) Variations at positions 1, 3, 4, 61, 67, 121 and 123 as shown in Figure 6.

The variations observed are as follows; position 1 R (arginine) in SM to V (valine) in ON, position 61 Q (glutamine) in SM to *(stop codon) in ON (Table 3).

This study described the use of Single Nucleotide Polymorphism (SNP) markers to validate genetic variation in a candidate growth gene (IGF-I) in *O. niloticus* and *S. melanotheron.* The DNA sequences gotten from primer used in this study was about 447 bp in agreement with the result obtained by Cuevas-Rodríguez et al. (2016).

Among the growth genes, IGF-I is said to contribute in a variety of physiological processes, such as growth, metabolism, reproduction and osmoregulation (Reinecke et al., 2005) in teleosts. Thus variation of IGF-I might be a good reason for the growth difference between *O. niloticus* and *S. melanotheron.*

It was summarized that there are indeed variations in the candidate growth gene, IGF-I of the two Tilapia species and this might be a reason for the significant difference in their growth rate. Thus improved varieties of

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CLUSTAL O(1.2.4) multiple sequence alignment

SM1_IGF-1F      ----------------AGTCTGTGTATGTAGATAAATGTGAGGGATTTTCTCTCTAAATC  44
SM3_IGF-1F      -------CTTGCAAATGTCTGTGTAATGTAGATAAATGTGAGGGATTTTCTCTCTAAATC  53
SM4_IGF-1F      --CTTTTCTGTTGAATGTCTGTGTAATGTAGATAAATGTGAGGGATTTTCTCTCTAAATC  58
SM5_IGF-1F      -CGCTTTCTGTTGAATGTCTGTGTAATGTAGATAAATGTGAGGGATTTTCTCTCTAAATC  59
SM2_IGF-1F      CGTTTTCTTGTTGAATGTCTGTGTAATGTAGATAAATGTGAGGGATTTTCTCTCTAAATC  60
ON1_IGF-1F      GTTTTTTCTTTTGAATGTCTGTGTAATGTAGATAAATGTGAGGGATTTTCTCTCTAAATC  60
ON4_IGF-1F      --CGCCACTGATGAATGTCTGTGTAATGTAGATAAATGTGAGGGATTTTCTCTCTAAATC  58
ON2_IGF-1F      -------------ATGGTCTGTGTAATGTAGATAAATGTGAGGGATTTTCTCTCTAAATC  47
ON3_IGF-1F      -CTTTTCTGGTTGAATGTCTGTGTAATGTAGATAAATGTGAGGGATTTTCTCTCTAAATC  59
ON5_IGF-1F      -GTTTTCTTGTTGAATGTCTGTGTAATGTAGATAAATGTGAGGGATTTTCTCTCTAAATC  59
                                        **********************************

SM1_IGF-1F      CGTCTCCTGTTCGCTAAATCTCACTTCTCCAAAACGAGCCTGCGCAATGGAACAAAGTCG  104
SM3_IGF-1F      CGTCTCCTGTTCGCTAAATCTCACTTCTCCAAAACGAGCCTGCGCAATGGAACAAAGTCG  113
SM4_IGF-1F      CGTCTCCTGTTCGCTAAATCTCACTTCTCCAAAACGAGCCTGCGCAATGGAACAAAGTCG  118
SM5_IGF-1F      CGTCTCCTGTTCGCTAAATCTCACTTCTCCAAAACGAGCCTGCGCAATGGAACAAAGTCG  119
SM2_IGF-1F      CGTCTCCTGTTCGCTAAATCTCACTTCTCCAAAACGAGCCTGCGCAATGGAACAAAGTCG  120
ON1_IGF-1F      CGTCTCCTGTTCGCTAAATCTCACTTCTCCAAAACGAGCCTGCGCAATGGAACAAAGTCG  120
ON4_IGF-1F      CGTCTCCTGTTCGCTAAATCTCACTTCTCCAAAACGAGCCTGCGCAATGGAACAAAGTCG  118
ON2_IGF-1F      CGTCTCCTGTTCGCTAAATCTCACTTCTCCAAAACGAGCCTGCGCAATGGAACAAAGTCG  107
ON3_IGF-1F      CGTCTCCTGTTCGCTAAATCTCACTTCTCCAAAACGAGCCTGCGCAATGGAACAAAGTCG  119
ON5_IGF-1F      CGTCTCCTGTTCGCTAAATCTCACTTCTCCAAAACGAGCCTGCGCAATGGAACAAAGTCG  119
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SM1_IGF-1F      GAATATTGAGATGTGACATTGCCCGCATCTCATCCTCTTTCTCCCTGTTTTTAATGACTT  164
SM3_IGF-1F      GAATATTGAGATGTGACATTGCCCGCATCTCATCCTCTTTCTCCCTGTTTTTAATGACTT  173
SM4_IGF-1F      GAATATTGAGATGTGACATTGCCCGCATCTCATCCTCTTTCTCCCTGTTTTTAATGACTT  178
SM5_IGF-1F      GAATATTGAGATGTGACATTGCCCGCATCTCATCCTCTTTCTCCCTGTTTTTAATGACTT  179
SM2_IGF-1F      GAATATTGAGATGTGACATTGCCCGCATCTCATCCTCTTTCTCCCTGTTTTTAATGACTT  180
ON1_IGF-1F      GAATATTGAGATGTGACATTGCCCGCATCTCATCCTCTTTCTCCCTGTTTTTAATGACTT  180
ON4_IGF-1F      GAATATTGAGATGTGACATTGCCCGCATCTCATCCTCTTTCTCCCTGTTTTTAATGACTT  178
ON2_IGF-1F      GAATATTGAGATGTGACATTGCCCGCATCTCATCCTCTTTCTCCCTGTTTTTAATGACTT  167
ON3_IGF-1F      GAATATTGAGATGTGACATTGCCCGCATCTCATCCTCTTTCTCCCTGTTTTTAATGACTT  179
ON5_IGF-1F      GAATATTGAGATGTGACATTGCCCGCATCTCATCCTCTTTCTCCCTGTTTTTAATGACTT  179
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SM1_IGF-1F      CAAACAAGTTCATTTTCGCCGGGCTTTGTCTTGTGGAGACCCGTGGGGATGTCTAGCGCT  224
SM3_IGF-1F      CAAACAAGTTCATTTTCGCCGGGCTTTGTCTTGTGGAGACCCGTGGGGATGTCTAGCGCT  233
SM4_IGF-1F      CAAACAAGTTCATTTTCGCCGGGCTTTGTCTTGTGGAGACCCGTGGGGATGTCTAGCGCT  238
SM5_IGF-1F      CAAACAAGTTCATTTTCGCCGGGCTTTGTCTTGTGGAGACCCGTGGGGATGTCTAGCGCT  239
SM2_IGF-1F      CAAACAAGTTCATTTTCGCCGGGCTTTGTCTTGTGGAGACCCGTGGGGATGTCTAGCGCT  240
ON1_IGF-1F      TAAACAAGTTCATTTTCGTCGGGCTTTGTCTTGTGGAGACCCGTGGGGATGTCTAGCGCT  240
ON4_IGF-1F      TAAACAAGTTCATTTTCGTCGGGCTTTGTCTTGTGGAGACCCGTGGGGATGTCTAGCGCT  238
ON2_IGF-1F      TAAACAAGTTCATTTTCGTCGGGCTTTGTCTTGTGGAGACCCGTGGGGATGTCTAGCGCT  227
ON3_IGF-1F      TAAACAAGTTCATTTTCGTCGGGCTTTGTCTTGTGGAGACCCGTGGGGATGTCTAGCGCT  239
ON5_IGF-1F      TAAACAAGTTCATTTTCGTCGGGCTTTGTCTTGTGGAGACCCGTGGGGATGTCTAGCGCT  239
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SM1_IGF-1F      TTTTCCTTTCAGTGGCATTTATGTGATGTCTTCAAGGTAACTTACCTGATTTCCTTTGAC  284
SM3_IGF-1F      TTTTCCTTTCAGTGGCATTTATGTGATGTCTTCAAGGTAACTTACCTGATTTCCTTTGAC  293
SM4_IGF-1F      TTTTCCTTTCAGTGGCATTTATGTGATGTCTTCAAGGTAACTTACCTGATTTCCTTTGAC  298
SM5_IGF-1F      TTTTCCTTTCAGTGGCATTTATGTGATGTCTTCAAGGTAACTTACCTGATTTCCTTTGAC  299
SM2_IGF-1F      TTTTCCTTTCAGTGGCATTTATGTGATGTCTTCAAGGTAACTTACCTGATTTCCTTTGAC  300
ON1_IGF-1F      TTTTCCTTTCAGTGGCATTTATGTGATGTCTTCAAGGTAACTTACCTGATTTCCTTTGAC  300
ON4_IGF-1F      TTTTCCTTTCAGTGGCATTTATGTGATGTCTTCAAGGTAACTTACCTGATTTCCTTTGAC  298
ON2_IGF-1F      TTTTCCTTTCAGTGGCATTTATGTGATGTCTTCAAGGTAACTTACCTGATTTCCTTTGAC  287
ON3_IGF-1F      TTTTCCTTTCAGTGGCATTTATGTGATGTCTTCAAGGTAACTTACCTGATTTCCTTTGAC  299
ON5_IGF-1F      TTTTCCTTTCAGTGGCATTTATGTGATGTCTTCAAGGTAACTTACCTGATTTCCTTTGAC  299
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SM1_IGF-1F      ACTATACATTATCACCTTGATTCTTCAGTTGCTCACTATTTGCACAGAGCATCCTCGCCT 344
SM3_IGF-1F      ACTATACATTATCACCTTGATTCTTCAGTTGCTCACTATTTGCACAGAGCATCCTCGCCT 353
SM4_IGF-1F      ACTATACATTATCACCTTGATTCTTCAGTTGCTCACTATTTGCACAGAGCATCCTCGCCT 358
SM5_IGF-1F      ACTATACATTATCACCTTGATTCTTCAGTTGCTCACTATTTGCACAGAGCATCCTCGCCT 359
SM2_IGF-1F      ACTATACATTATCACCTTGATTCTTCAGTTGCTCACTATTTGCACAGAGCATCCTCGCCT 360
ON1_IGF-1F      ACTATACATTATCACCTTGATTCTTCACTTGCTCACTATTTGCACAGAGCATCCTCGCCT 360
ON4_IGF-1F      ACTATACATTATCACCTTGATTCTTCACTTGCTCACTATTTGCACAGAGCATCCTCGCCT 358
ON2_IGF-1F      ACTATACATTATCACCTTGATTCTTCACTTGCTCACTATTTGCACAGAGCATCCTCGCCT 347
ON3_IGF-1F      ACTATACATTATCACCTTGATTCTTCACTTGCTCACTATTTGCACAGAGCATCCTCGCCT 359
ON5_IGF-1F      ACTATACATTATCACCTTGATTCTTCACTTGCTCACTATTTGCACAGAGCATCCTCGCCT 359
                ************************** ******************************

SM1_IGF-1F      AATTTAAACAGAAACAATAAAAGGGGATTCTTATCGCTTGTTTTGTATTTCA-------- 396
SM3_IGF-1F      AATTTAAACAGAAACAATAAAAGGGGATTCTTATCGCTTGTTTTGTATTTCAACACC--- 410
SM4_IGF-1F      AATTTAAACAGAAACAATAAAAGGGGATTCTTATCGCTTGTTTGTATTTCA--------- 409
SM5_IGF-1F      AATTTAAACAGAAACAATAAAAGGGGATTCTTATCGCTTGTTTGTATTTCA--------- 410
SM2_IGF-1F      AATTTAAACAGAAACAATAAAAGGGGATTCTTATCGCTTGCTTTGTATTTCAGCA----- 415
ON1_IGF-1F      ACTTTAAAAAGAAACAATAAAAGGGGATTCTTATCGCTTGCTTTGTATTTCA-------- 412
ON4_IGF-1F      ACTTTAAAAAGAAACAATAAAAGGGGATTCTTATCGCTTGTTTTGTATTTCACCGCTTTT 418
ON2_IGF-1F      ACTTTAAAAAGAAACAATAAAAGGGGATTCTTATCGCTTGTTTTGTATTTCA-------- 399
ON3_IGF-1F      ACTTTAAAAAGAAACAATAAAAGGGGATTCTTATCGCTTGTTTTGTATTTCA-------- 411
ON5_IGF-1F      ACTTTAAAAAGAAACAATAAAAGGGGATTCTTATCGCTTGTTTTGTATTTCA-------- 411
                * ****** ******************************** **    **

SM1_IGF-1F      ------------------------------------------------------------ 396
SM3_IGF-1F      ------------------------------------------------------------ 410
SM4_IGF-1F      ------------------------------------------------------------ 409
SM5_IGF-1F      ------------------------------------------------------------ 410
SM2_IGF-1F      ------------------------------------------------------------ 415
ON1_IGF-1F      ------------------------------------------------------------ 412
ON4_IGF-1F      TTTCGACTGCATTCATGTGGATTTATTTATATTTGCCTCTCTACTCATGCAAGCAAGA   476
ON2_IGF-1F      ------------------------------------------------------------ 399
ON3_IGF-1F      ------------------------------------------------------------ 411
ON5_IGF-1F      ------------------------------------------------------------ 411
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Figure 1. Alignment of the forward reaction of the IGF-1 of the ten tilapia fish (5 *O. niloticus* and 5 *S. melanotheron)* . *Mean similarities among the sequence bases of the ten fishes.

Table 1. SNPs detected between the forward nucleotide sequence reaction of the ten Tilapia fishes ***(****Oreochromis niloticus* and *Sarotherodon melanotheron).*

SNP position	ON	SM
181	T	C
199	T	C
328	C	G
362	C	A
369	A	C

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CLUSTAL O(1.2.4) multiple sequence alignment

SM2-IGF-1R      -----TATTGTTTTGTTTATTAGGCGAGGATGCTCTGTGCAAATAGTGAGCAACTGAAGA 55
SM1-IGF-1R      --TTTTTGTTCCTGTTTTATTAGGCGAGGATGCTCTGTGCAAATAGTGAGCAACTGAAGA 58
SM5-IGF-1R      ----TATTGTTTTGTTTTATTAGGCGAGGATGCTCTGTGCAAATAGTGAGCAACTGAAGA 56
SM3-IGF-1R      ---TTATTCTTTCTGTTTATTAGGCGAGGATGCTCTGTGCAAATAGTGAGCAACTGAAGA 57
SM4-IGF-1R      ---TTTTGGTTTCTGTTTATTAGGCGAGGATGCTCTGTGCAAATAGTGAGCAACTGAAGA 57
ON4-IGF-1R      -TTATTGTTTCTTTTTTAAGTAGGCGAGGATGCTCTGTGCAAATAGTGAGCAAGTGAAGA 59
ON3-IGF-1R      TTTTGGTTTTCTTTTTTAAGTAGGCGAGGATGCTCTGTGCAAATAGTGAGCAAGTGAAGA 60
ON1-IGF-1R      ---TTTTTCTTCTTTTTAAGTAGGCGAGGATGCTCTGTGCAAATAGTGAGCAAGTGAAGA 57
ON2-IGF-1R      --TATTGGTTTCTTTTTAAGTAGGCGAGGATGCTCTGTGCAAATAGTGAGCAAGTGAAGA 58
ON5-IGF-1R      -TTATTGGTTTCTTTTTAAGTAGGCGAGGATGCTCTGTGCAAATAGTGAGCAAGTGAAGA 59
                         ** * ********************************** ******

SM2-IGF-1R      ATCAAGGTGATAATGTATAGTGTCAAAGGAAATCAGGTAAGTTACCTTGAAGACATCACA 115
SM1-IGF-1R      ATCAAGGTGATAATGTATAGTGTCAAAGGAAATCAGGTAAGTTACCTTGAAGACATCACA 118
SM5-IGF-1R      ATCAAGGTGATAATGTATAGTGTCAAAGGAAATCAGGTAAGTTACCTTGAAGACATCACA 116
SM3-IGF-1R      ATCAAGGTGATAATGTATAGTGTCAAAGGAAATCAGGTAAGTTACCTTGAAGACATCACA 117
SM4-IGF-1R      ATCAAGGTGATAATGTATAGTGTCAAAGGAAATCAGGTAAGTTACCTTGAAGACATCACA 117
ON4-IGF-1R      ATCAAGGTGATAATGTATAGTGTCAAAGGAAATCAGGTAAGTTACCTTGAAGACATCACA 119
ON3-IGF-1R      ATCAAGGTGATAATGTATAGTGTCAAAGGAAATCAGGTAAGTTACCTTGAAGACATCACA 120
ON1-IGF-1R      ATCAAGGTGATAATGTATAGTGTCAAAGGAAATCAGGTAAGTTACCTTGAAGACATCACA 117
ON2-IGF-1R      ATCAAGGTGATAATGTATAGTGTCAAAGGAAATCAGGTAAGTTACCTTGAAGACATCACA 118
ON5-IGF-1R      ATCAAGGTGATAATGTATAGTGTCAAAGGAAATCAGGTAAGTTACCTTGAAGACATCACA 119
                ************************************************************

SM2-IGF-1R      TAAATGCCACTGAAAGGAAAAAGCGCTAGACATCCCCACGGGTCTCCACAAGACAAAGCC 175
SM1-IGF-1R      TAAATGCCACTGAAAGGAAAAAGCGCTAGACATCCCCACGGGTCTCCACAAGACAAAGCC 178
SM5-IGF-1R      TAAATGCCACTGAAAGGAAAAAGCGCTAGACATCCCCACGGGTCTCCACAAGACAAAGCC 176
SM3-IGF-1R      TAAATGCCACTGAAAGGAAAAAGCGCTAGACATCCCCACGGGTCTCCACAAGACAAAGCC 177
SM4-IGF-1R      TAAATGCCACTGAAAGGAAAAAGCGCTAGACATCCCCACGGGTCTCCACAAGACAAAGCC 177
ON4-IGF-1R      TAAATGCCACTGAAAGGAAAAAGCGCTAGACATCCCCACGGGTCTCCACAAGACAAAGCC 179
ON3-IGF-1R      TAAATGCCACTGAAAGGAAAAAGCGCTAGACATCCCCACGGGTCTCCACAAGACAAAGCC 180
ON1-IGF-1R      TAAATGCCACTGAAAGGAAAAAGCGCTAGACATCCCCACGGGTCTCCACAAGACAAAGCC 177
ON2-IGF-1R      TAAATGCCACTGAAAGGAAAAAGCGCTAGACATCCCCACGGGTCTCCACAAGACAAAGCC 178
ON5-IGF-1R      TAAATGCCACTGAAAGGAAAAAGCGCTAGACATCCCCACGGGTCTCCACAAGACAAAGCC 179
                ************************************************************

SM2-IGF-1R      CGGCGAAAATGAACTTGTTTGAAGTCATTAAAAACAGGGAGAAAGAGGATGAGATGCGGG 235
SM1-IGF-1R      CGGCGAAAATGAACTTGTTTGAAGTCATTAAAAACAGGGAGAAAGAGGATGAGATGCGGG 238
SM5-IGF-1R      CGGCGAAAATGAACTTGTTTGAAGTCATTAAAAACAGGGAGAAAGAGGATGAGATGCGGG 236
SM3-IGF-1R      CGGCGAAAATGAACTTGTTTGAAGTCATTAAAAACAGGGAGAAAGAGGATGAGATGCGGG 237
SM4-IGF-1R      CGGCGAAAATGAACTTGTTTGAAGTCATTAAAAACAGGGAGAAAGAGGATGAGATGCGGG 237
ON4-IGF-1R      CGACGAAAATGAACTTGTTTAAAGTCATTAAAAACAGGGAGAAAGAGGATGAGATGCGGG 239
ON3-IGF-1R      CGACGAAAATGAACTTGTTTAAAGTCATTAAAAACAGGGAGAAAGAGGATGAGATGCGGG 240
ON1-IGF-1R      CGACGAAAATGAACTTGTTTAAAGTCATTAAAAACAGGGAGAAAGAGGATGAGATGCGGG 237
ON2-IGF-1R      CGACGAAAATGAACTTGTTTAAAGTCATTAAAAACAGGGAGAAAGAGGATGAGATGCGGG 238
ON5-IGF-1R      CGACGAAAATGAACTTGTTTAAAGTCATTAAAAACAGGGAGAAAGAGGATGAGATGCGGG 239
                ** ***************** ************************************

SM2-IGF-1R      CAATGTCACATCTCAATATTCCGACTTTGTTCCATTGCGCAGGCTCGTTTTGGAGAAGTG 295
SM1-IGF-1R      CAATGTCACATCTCAATATTCCGACTTTGTTCCATTGCGCAGGCTCGTTTTGGAGAAGTG 298
SM5-IGF-1R      CAATGTCACATCTCAATATTCCGACTTTGTTCCATTGCGCAGGCTCGTTTTGGAGAAGTG 296
SM3-IGF-1R      CAATGTCACATCTCAATATTCCGACTTTGTTCCATTGCGCAGGCTCGTTTTGGAGAAGTG 297
SM4-IGF-1R      CAATGTCACATCTCAATATTCCGACTTTGTTCCATTGCGCAGGCTCGTTTTGGAGAAGTG 297
ON4-IGF-1R      CAATGTCACATCTCAATATTCCGACTTTGTTCCATTGCGCAGGCTCGTTTTGGAGAAGTG 299
ON3-IGF-1R      CAATGTCACATCTCAATATTCCGACTTTGTTCCATTGCGCAGGCTCGTTTTGGAGAAGTG 300
ON1-IGF-1R      CAATGTCACATCTCAATATTCCGACTTTGTTCCATTGCGCAGGCTCGTTTTGGAGAAGTG 297
ON2-IGF-1R      CAATGTCACATCTCAATATTCCGACTTTGTTCCATTGCGCAGGCTCGTTTTGGAGAAGTG 298
ON5-IGF-1R      CAATGTCACATCTCAATATTCCGACTTTGTTCCATTGCGCAGGCTCGTTTTGGAGAAGTG 299
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SM2-IGF-1R      AGATTTAGCGAACAGGAGACGGATTTAGAGAGAAAATCCCTCACATTTATCTACATTACA 355
SM1-IGF-1R      AGATTTAGCGAACAGGAGACGGATTTAGAGAGAAAATCCCTCACATTTATCTACATTACA 358
SM5-IGF-1R      AGATTTAGCGAACAGGAGACGGATTTAGAGAGAAAATCCCTCACATTTATCTACATTACA 356
SM3-IGF-1R      AGATTTAGCGAACAGGAGACGGATTTAGAGAGAAAATCCCTCACATTTATCTACATTACA 357
SM4-IGF-1R      AGATTTAGCGAACAGGAGACGGATTTAGAGAGAAAATCCCTCACATTTATCTACATTACA 357
ON4-IGF-1R      AGATTTAGCGAACAGGAGACGGATTTAGAGAGAAAATCCCTCACATTTATCTACATTACA 359
ON3-IGF-1R      AGATTTAGCGAACAGGAGACGGATTTAGAGAGAAAATCCCTCACATTTATCTACATTACA 360
ON1-IGF-1R      AGATTTAGCGAACAGGAGACGGATTTAGAGAGAAAATCCCTCACATTTATCTACATTACA 357
ON2-IGF-1R      AGATTTAGCGAACAGGAGACGGATTTAGAGAGAAAATCCCTCACATTTATCTACATTACA 358
ON5-IGF-1R      AGATTTAGCGAACAGGAGACGGATTTAGAGAGAAAATCCCTCACATTTATCTACATTACA 359
                ************************************************************

SM2-IGF-1R      CAGACATTTTCAACAGGAAACAGCTGGGGCAGCATTTGCCTCCTCTTTCGTCCAAGA    412
SM1-IGF-1R      CAGACATTTTCAACAGGAAACAGCTGGGGCAGCATTTGCCTCCTCTTCGTCCAAGA-    414
SM5-IGF-1R      CAGACATTTTCAACAGGAAACAGCTGGGGCAGCATTTGCCTCCTATTCGTCCAAGA-    412
SM3-IGF-1R      CAGACATTTTCAACAGGAAACAGCTGGGGCAGCATTTGCCTCCTCTTCGTCCAAGA-    413
SM4-IGF-1R      CAGACATTTTCAACAGGAAACAGCTGGGGCAGCATTTGCCTCCTCTTCGTCCAAGA-    413
ON4-IGF-1R      CAGACATTTTCAACAGGAAACAGCTGGGGCAGCATTTGCCTCCTATTTCGTCCAAGA    416
ON3-IGF-1R      CAGACATTTTCAACAGGAAACAGCTGGGGCAGCATTTGCCTCCTCTTCGTCCAAGA-    416
ON1-IGF-1R      CAGACATTTTCAACAGGAAACAGCTGGGGCAGCATTTGCCTCCTCTTCGTCCAAGA-    413
ON2-IGF-1R      CAGACATTTTCAACAGGAAACAGCTGGGGCAGCATTTGCCTCCTATTCGTCCAAGA-    414
ON5-IGF-1R      CAGACATTTTCAACAGGAAACAGCTGGGGCAGCATTTGCCTCCTCTTCGTCCAAGA-    415
                ****************************************** **    * *
```

Figure 2. Alignment of the Reverse Reaction of the IGF-I of the ten tilapia fish (5 *Oreochromis niloticus* and 5 *Sarotherodon melanotheron).* *Mean similarities among the sequence bases of the ten fishes.

Table 2. SNPs detected between the reverse nucleotide sequence reaction of the two Tilapia species *(O. niloticus and S. melanotheron).*

SNP position	SM	ON
81	T	A
20	T	G
54	C	G
183	G	A
201	G	A

Table 3. Variations detected between the amino acid sequence of the two Tilapia species *(O. niloticus and S. melanotheron).*

Sequence position	SM	ON
1	R	V
3	L	S
4	V	F
61	Q	*
67	P	S
121	N	T
123	N	K

*Mean stop codon.

S. melanotheron with bigger sizes might be achievable. Also, regions of higher salinity where *S. melanotheron* strive well can be encouraged to grow the improved varieties and the demand for *S. melanotheron* increases.

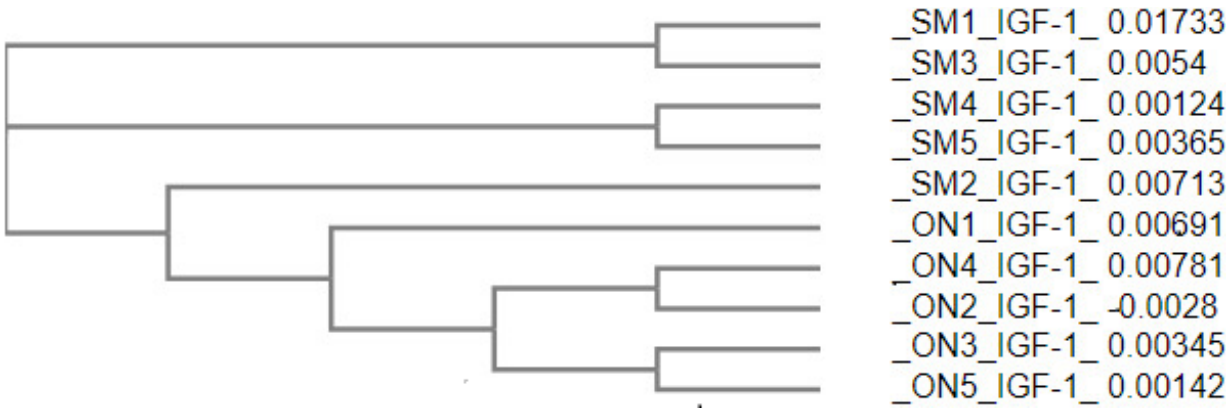

Figure 3. Dendogram (phylogenetic tree) of the DNA sequence from IGF-I for the ten Tilapia (5 *O. niloticus* and 5 *S. melanotheron*).

>(ON1_IGF-1)

```
1     GTT TTT TCT TTT GAA TGT CTG TGT AAT GTA GAT AAA TGT GAG GGA   45
1      V   F   S   F   E   C   L   C   N   V   D   K   C   E   G   15

46    TTT TCT CTC TAA ATC CGT CTC CTG TTC GCT AAA TCT CAC TTC TCC   90
16     F   S   L   *   I   R   L   L   F   A   K   S   H   F   S   30

91    AAA ACG AGC CTG CGC AAT GGA ACA AAG TCG GAA TAT TGA GAT GTG   135
31     K   T   S   L   R   N   G   T   K   S   E   Y   *   D   V   45

136   ACA TTG CCC GCA TCT CAT CCT CTT TCT CCC TGT TTT TAA TGA CTT   180
46     T   L   P   A   S   H   P   L   S   P   C   F   *   *   L   60

181   TAA ACA AGT TCA TTT TCG TCG GGC TTT GTC TTG TGG AGA CCC GTG   225
61     *   T   S   S   F   S   S   G   F   V   L   W   R   P   V   75

226   GGG ATG TCT AGC GCT TTT TCC TTT CAG TGG CAT TTA TGT GAT GTC   270
76     G   M   S   S   A   F   S   F   Q   W   H   L   C   D   V   90

271   TTC AAG GTA ACT TAC CTG ATT TCC TTT GAC ACT ATA CAT TAT CAC   315
91     F   K   V   T   Y   L   I   S   F   D   T   I   H   Y   H   105

316   CTT GAT TCT TCA CTT GCT CAC TAT TTG CAC AGA GCA TCC TCG CCT   360
106    L   D   S   S   L   A   H   Y   L   H   R   A   S   S   P   120

361   ACT TTA AAA AGA AAC AAT AAA AGG GGA TTC TTA TCG CTT GCT TTG   405
121    T   L   K   R   N   N   K   R   G   F   L   S   L   A   L   135

406   TAT TTC   411
136    Y   F
```

Figure 4. Translation of DNA sequence of IGF-1 of *O. niloticus* (ON) to amino acid. *Mean stop codon.

```
>(SM2_IGF-1)

1     CGT TTT CTT GTT GAA TGT CTG TGT AAT GTA GAT AAA TGT GAG GGA   45
1      R   F   L   V   E   C   L   C   N   V   D   K   C   E   G    15

46    TTT TCT CTC TAA ATC CGT CTC CTG TTC GCT AAA TCT CAC TTC TCC   90
16     F   S   L   *   I   R   L   L   F   A   K   S   H   F   S    30

91    AAA ACG AGC CTG CGC AAT GGA ACA AAG TCG GAA TAT TGA GAT GTG   135
31     K   T   S   L   R   N   G   T   K   S   E   Y   *   D   V    45

136   ACA TTG CCC GCA TCT CAT CCT CTT TCT CCC TGT TTT TAA TGA CTT   180
46     T   L   P   A   S   H   P   L   S   P   C   F   *   *   L    60

181   CAA ACA AGT TCA TTT TCG CCG GGC TTT GTC TTG TGG AGA CCC GTG   225
61     Q   T   S   S   F   S   P   G   F   V   L   W   R   P   V    75

226   GGG ATG TCT AGC GCT TTT TCC TTT CAG TGG CAT TTA TGT GAT GTC   270
76     G   M   S   S   A   F   S   F   Q   W   H   L   C   D   V    90

271   TTC AAG GTA ACT TAC CTG ATT TCC TTT GAC ACT ATA CAT TAT CAC   315
91     F   K   V   T   Y   L   I   S   F   D   T   I   H   Y   H   105

316   CTT GAT TCT TCA GTT GCT CAC TAT TTG CAC AGA GCA TCC TCG CCT   360
106    L   D   S   S   V   A   H   Y   L   H   R   A   S   S   P   120

361   AAT TTA AAC AGA AAC AAT AAA AGG GGA TTC TTA TCG CTT GCT TTG   405
121    N   L   N   R   N   N   K   R   G   F   L   S   L   A   L   135

406   TAT TTC AGC   414
136    Y   F   S
```

Figure 5. Translation of DNA sequence of IGF-1 of *S. melanotheron* (SM) to amino acid. *Mean stop codon.

```
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    5         15         25         35         45         55
(SM2_IGF-1      RFLVECLCNV DKCEGFSL*I RLLFAKSHFS KTSLRNGTKS EY*DVTLPAS HPLSPCF**L
(ON1_IGF-1      VFSFECLCNV DKCEGFSL*I RLLFAKSHFS KTSLRNGTKS EY*DVTLPAS HPLSPCF**L

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    65         75         85         95        105        115
(SM2_IGF-1      QTSSFSPGFV LWRPVGMSSA FSFQWHLCDV FKVTYLISFD TIHYHLDSSV AHYLHRASSP
(ON1_IGF-1      *TSSFSSGFV LWRPVGMSSA FSFQWHLCDV FKVTYLISFD TIHYHLDSSL AHYLHRASSP

                ....|....| ....|...
                   125        135
(SM2_IGF-1      NLNRNNKRGF LSLALYFS
(ON1_IGF-1      TLKRNNKRGF LSLALYF.
```

Figure 6. Amino acid sequence alignment from the translation of the DNA sequence of IGF-1 of *Oreochromis niloticus* (ON) and *Sarotherodon melanotheron* (SM). * Mean stop codon.

This study serves as baseline information in selective breeding whereby the amino acids present in the IGF-I of *O. niloticus* may be fed orally to *S. melanotheron* by adding them to their feed while growing.

CONFLICTS OF INTERESTS

The authors have not declared any conflict of interests.

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Molecular identification of indigenous bacteria isolated from pesticides heavily contaminated soils

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This study was carried out to isolate and identify indigenous bacteria associated with pesticides heavily contaminated soil in Sudan. The samples were collected from pesticides contaminated soil in different locations. The predominant bacterial isolates were then purified by subculturing on selective media. Seven isolates were selected based on their capability to degrade pesticides and three of them having the highest performance in biodegradation of frequently reported pesticide contaminants were subject to identification by molecular tools using 16S rDNA gene sequence analysis. Amplification of 16S rDNA gene was done from genomic DNA and sequenced. Construction of phylogenetic tree was done after aligning of multiple sequences. The results reveal that the three bacterial isolates are *Bacillus cereus* ATCC 14579, *Bacillus subtilis* subsp. *inaquosorum* KCTC 13429 and *Bacillus safensis* FO-36b. This is the first record of pesticides biodegradation by *B. safensis* FO-36b which can be considered as a novel contribution in this field. Since these strains were found effective and are living naturally in an environment heavily contaminated with pesticides, therefore, they might have potential in removing pesticides from contaminated soils, especially soils of pesticide stores and dumping sites in developing countries.

Key words: Bacillus, gene sequencing, pesticides-polluted soil, Sudan.

INTRODUCTION

Pesticide residues in soil and plants are one of the major environmental issues, which affect directly or indirectly humans and animal's health and act as a potential threat to the environment. Microorganisms with high efficient biodegradation rates could be considered as potential bioremediation agents and could be used for biotechnological applications. Biodegradation by naturally occurring microorganism to remove or detoxify pesticides (Huang et al., 2018), insecticides (Ishag et al., 2016, 2017), herbicides (Kanissery and Sims, 2011) and oil (Abbas et al., 2018) residues from contaminated soils is a useful and eco-friendly approach.

Many genera of different types of bacteria such as *Alcaligenes*, *Bacillus*, *Flavobacterium* and *Pseudomonas* had been reported to effectively degrade different types of pesticides (Ishag et al., 2016; Huang et al., 2018). Moreover, Trama et al. (2014) concluded that microorganisms that originated from contaminated environments usually are strong and powerful in bioremediation. The success of the pesticides bioremediation process is affected by the type of microorganism which affects the rate of bioremediation, environmental factors and nature (Lovecka et al., 2015; Dzionek et al., 2016; Abdelbagi et al., 2018).

The potential of local existing soil microorganisms in remediation of heavily contaminated soils in Sudan was reported by Abdelbagi et al. (2000, 2003). Further investigation was done by Ali (2005), who studied the biodegradation of endosulfan and *γ*-HCH and confirmed the efficiency of the local existing soil microorganisms in degradation process of tested pesticides. Subsequent works on endosulfan biodegradation were carried out by Elsaid et al. (2009, 2010a, b) who provided further evidence for the potential of local microorganisms in biodegradation of tested pesticides and they found that the degradation rate could be accelerated by activators such as farm manure and synthetic fertilizers (Elsaid et al., 2009). The biodegradation of pendimethalin, endosulfan, chlorpyrifos, malathion, and dimethoate utilizing local bacteria were investigated by Ishag et al. (2016, 2017) and Sharef et al. (2013). The latter reported that the pendimethalin could best be mineralized by *Pseudomonas aeruginosa.* All the previous works did not use molecular biotechnological tools method such as 16s rDNA gene (Aakra et al., 1999; Bosshard et al., 2006) and rather depends on biochemical identification, except Ishag et al. (2016; 2017) which utilized both biochemical and molecular biotechnological tools, however, these papers displayed the degradation pathways and the kinetics of the reaction. The molecular identification findings were considered in details in the current paper. Therefore, this study present the molecular identification (using 16s rDNA gene) data of some of the indigenous bacteria found in pesticides highly polluted soil. The significance of the method 16s rRNA gene includes the followings; it could identify bacteria at the species level and assist in differentiating between closely related bacterial species. Moreover, the 16s rRNA is part of the translation process, in all types of bacteria and therefore it represent a perfect universal target. Besides that it has a multi-copy gene, which increases the detection sensitivity. Furthermore, it consists of conserved and highly variable regions, which increases its detection specificity and also allows for the use of universal primer In addition it evolves at relatively constant rates, which allows inferring phylogenetic relationships (Patel, 2001, Clarridge 2004, Woo et al., 2008).

MATERIALS AND METHODS

Soil samples collection method

Various pesticides contaminated storage soils (Alrahad, Barakat, and Hassahisa) were randomly sampled, using a soil auger of 10 cm length and 5 cm diameter. Soil samples were collected aseptically from upper layers (0 -30 cm) of the pesticide store where the maximum population of microorganisms is expected to exist. Five augers of soil samples were taken from each of the three stores and mingled carefully to produce composite sample (1 kg). Taken samples were wrapped in tapestry cloth sacks and labeled and transferred at once to pesticides center, University of Khartoum for isolation and identification.

Sample preparation

The samples were spread on the bench overnight to dry under room temperature. Samples were blended carefully and the aggregated particles and clods were broken manually under aseptic conditions to a reasonable and homogeneous size. Crushed samples were then wrapped in tapestry cloth sacks, labelled and sent directly to Plant Pathology Laboratory, University of Khartoum for Isolation and Identification.

Media preparation method

Nutrient agar (NA)

The medium was prepared by adding 5.0 g of peptone, 5.0g of NaCl, 1.5 g of beef extract, 1.5 g of yeast extract and 15.0 g of agar to a liter of distilled water, then the final pH was adjusted to 7.4 ± 0.2 (at 25°C) (Tepper et al., 1993).

Nitrate agar (NA)

The NA was prepared following the method described by Society of American Bacteriologist (SAB) (1944) by adding 5.0 g of peptone, 3.0 g of beef extract, 1.0 g of potassium nitrate and 12.0 g of agar to a liter of distilled water and the final pH was adjusted to 6.8 ± 0.2 (at 25°C).

Preparation of soil suspension

The soil suspension was prepared by dissolving 1 g of soil in 10 ml of sterile distilled water followed by gentle shaking. One gram of soil was dissolved in 10 ml of sterile distilled water to make soil suspension and shake gently. Serial dilution was carried out to reduce microbial density and to get a single colony isolate. One millilitre of soil suspension from each of the six soil samples was taken using micropipette and added to sterilized test tube containing nine ml of distilled water and shaken well to give six serial dilutions.

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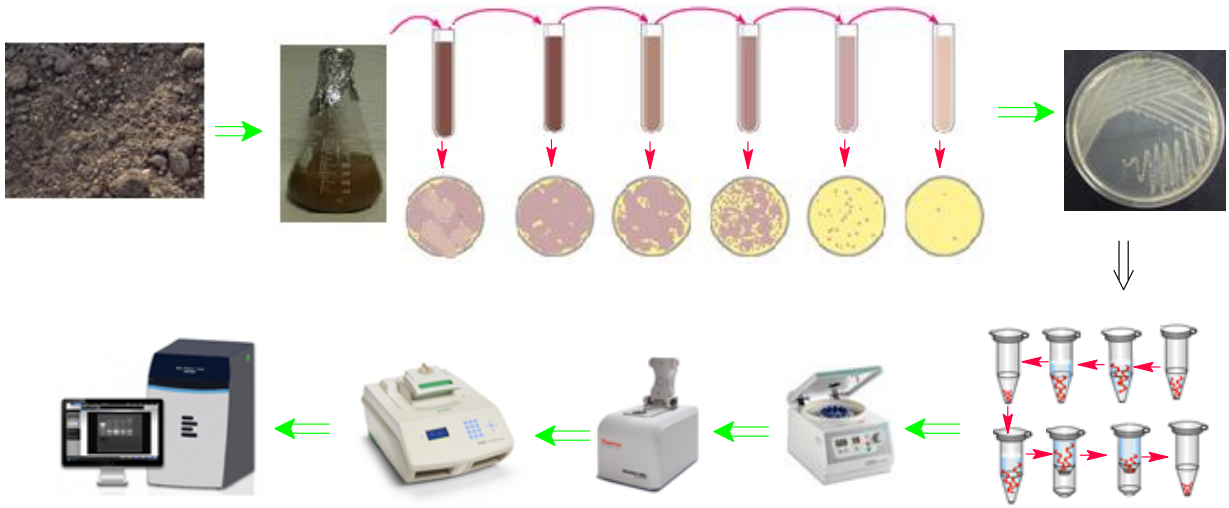

Figure 1. A simplified diagram for the method used.

Isolation of microorganisms from pesticide-polluted soil

One hundred microliters from each serial dilution were added to the surface of the nutrient agar (NA) medium on the center of each Petri dish and distributed carefully to the whole plate. The cultures were incubated at 30°C for 24 h. Hundred microliters from each serial dilution was added to the nutrient agar (NA) surfaces in the center of each plate and distributed carefully to the whole plate. The inoculums were spread on the NA surfaces using flamed glass spreader and incubated for 24 h at 30°C (Figure 1).

Purification and identification of the bacterial isolates

The dominant morphologically different bacterial colony types were chosen from the plate count agar. Predominant bacteria from morphologically different colony types were selected from plate count agar. The purification of different isolates was carried out by repeated subculturing on the selective media of nitrate agar. This was followed by streaking the isolates into sterile nutrient agar and left to propagate at 37°C for 24 h. The propagated bacterial colonies were sub-cultured on slope nutrient agar medium and preserved in a refrigerator at 4°C for further tests.

Culture and morphological characteristics

Barrow and Felthman (2003) procedure was followed for identification and purification of the isolates. Morphological tests performed include; Grams staining, shape, endospore staining, motility, and growth in the presence of air. The set of bio-chemical tests used in the identification of microorganisms are catalyzed test, oxidize test, glucose (acid) and O\F test.

Molecular identification

Genomic DNA extraction

Purified isolated bacterial cells were picked from pure culture and re-suspended in 10 µl of nuclease-free water. Genomic DNAs were extracted from isolates at Macrogen Ltd., Korea, using the following protocol; Cells were boiled in water bath at 95ºC for 3 min followed by cooling down on ice for 1 min. The suspensions were then centrifuged at 1500 rpm for 5 min. The extracted genomic DNAs were used as a template for PCR analysis.

PCR amplification of 16S rDNA gene

The 16S rDNA gene was amplified from genomic DNA using Macrogen universal primer sets 27F 5'-AGAGTTTGATCMTGGCTCAG -3' and 1492R 5'-TACGGYTACCTTGTTACGACT-3'. Amplification was performed according to the standard protocol using a EF-Taq (SolGent, Korea) as follows: initial denaturation at 95ºC for 2 min, 35 cycles of 95ºC for 1 min, 55ºC for 1 min, 72ºC for 1 min and a final extension step of 10 min at 72ºC. PCR products were separated by gel electrophoresis on 1.2% agarose gels, stained with ethidium bromide and visualized with a UV transilluminator.

Purification of PCR products

PCR products were cut from the gel, purified with a multiscreen filter plate (Millipore Corp., Bedford, MA, USA) and quantified with Nano Drop spectrophotometer before sequencing.

Sequencing of 16S rDNA gene

Purified PCR products were sequenced using PRISM Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). The PCR reaction was performed with 20 ng genomic DNA as a template and both forward primer (27F) and reverse primer (1492R) in a 30 µl reaction mixture in separate reactions. The PCR reactions were performed under the same conditions described above. The DNA samples containing the extension products were added to Hi-Di formamide (Applied Biosystems, Foster City, CA). The mixture was incubated at 95ºC for 5 minutes, followed by 5 minutes on ice and then analyzed by ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA). The homology of the 16S rDNA gene

sequences was checked with the 16S rDNA sequences of other organisms that have been submitted to a database of NCBI (https://www.ncbi.nlm.nih.gov). The determined sequences of 16S rDNA gene were analyzed in the EZBIOCLOUD database (http://www.ezbiocloud.net/eztaxon).

Phylogenetic analysis

Multiple sequence alignment was performed using CLUSTALW program (http://www.genome.jp/tools-bin/clustalw) and edited using BioEdit 7.2.5 software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The sequences used in this study were as follows: AM: 747812 (*Brevibacterium halotolerans* DSM 8802T), JH: 600280 (*Bacillus mojavensis* RO-H-1T), AMXN: 0100002 1(*Bacillus subtilis* subsp. *inaquosorum* KCTC 13429T),CP: 002905)(*Bacillus subtilis subsp. spizizenii* NRRL B-23049T), AYTO: 01000043 (*Bacillus tequilensis* KCTC 13622T), JH: 600273 (*Bacillus vallismortis* DV1-F-3T), ABQL: 01000001 (*Bacillus subtilis subsp. subtilis* NCBI 3610T), JTKJ: 01000077 (*Bacillus methylotrophicus* KACC 13105T), FN: 597644 (*Bacillus amyloliquefaciens subsp. amyloliquefaciens* DSM 7T), AJVF: 01000043 (*Bacillus siamensis* KCTC 13613T), CP: 000560 (*Bacillus amyloliquefaciens subsp. plantarum* FZB42T), AB: 021181 (*Bacillus atrophaeus* JCM 9070T), AYTN: 01000016 (*Bacillus sonorensis* NBRC 101234T), AE: 071333 (*Bacillus licheniformis* ATCC 14580T), AJ: 831843 (*Bacillus aerius* 24KT), ASJD: 01000027 (*Bacillus safensis* FO-36b^T), ABRX: 01000007 (*Bacillus pumilis* ATCC 7061^T), Aj: 831844 (*Bacillus aerophilus* 28K^T), ASJC:01000029 (*Bacillus altitudinis* 41KF2b^T), AJ: 831841 (*Bacillus stratosphericus* 41KF2a^T), AMSH: 01000114 (*Bacillus xiamenensis* HYC-10^T), CP: 000764 (*Bacillus cytotoxicus* NVH 391-98^T), ACMX: 01000133 (*Bacillus pseudomycoides* DSM 12442^T), ACNF: 01000156 (*Bacillus thuringiensis* ATCC 10792^T), CP:006863 (*Bacillus toyonensis* BCT-7112^T), AE: 016879(*Bacillus anthracis* Ames), AE:016877 (*Bacillus cereus* ATCC 14579^T), ACMU: 01000002 (*Bacillus mycoides* DSM 2048^T), BAUY: 01000093 (*Bacillus weihenstephanensis* NBRC 101238^T), and K: I271266 (*Lactobacillus brevis* ATCC 14869^T). The phylogenetic tree was constructed using a Neighbor-joining method utilizing a Mega software version 6.0 (Tamura et al*.,* 2013).

RESULTS AND DISCUSSION

Previous work from this group (Elsaid et al*.,* 2009; Elsaid et al*.,* 2010a, b; Sharef et al*.,* 2013; Abdurruhman et al*.,* 2015; Ishag et al*.,* 2016, 2017) evaluated the degradation efficiency of microorganisms especially bacterial strains isolated from pesticide-polluted storage soil in Sudan. The isolated strains have shown promising capability in degrading many of the frequently detected pesticide contaminants in storage soils and dump sites in the Sudan including; chlorpyrifos, malathion, dimethoate, pendimethalin, endosulfan alpha and endosulfan beta.

In this study, seven isolates were subject to preliminary biodegradation tests against the most frequently reported contaminants of hot spots in the Sudan. Three of them have shown high capability in the removal of pesticide contaminants from contaminated soils and therefore were selected for further genetic identification.

The previously described works did not use molecular biotechnological tools and rather depend on biochemical identification, except Ishag et al. (2016, 2017) who utilized both biochemical and molecular biotechnological tools, however, their published work (Ishag et al*.,* 2016, 2017) displayed the degradation pathways and the kinetics of the reaction only. Their molecular identification findings were considered in details in the current paper. This paper describes the molecular identification and characterization of these strains using 16S rDNA gene. The use of 16S rDNA gene for identifications of genus and species of bacteria has been reported by Barghouthi (2011), Kinuthia et al. (2010), Karisham and Hari (2014) and Maryam et al. (2014). This method (16s rRNA gene) can identify bacteria at the species level as well as assist in differentiating between closely related bacterial species. Moreover, the 16s rRNA is part of the translation process, in all types of bacteria and therefore it represent a perfect universal target. Besides it has a multi-copy gene, which increases the detection sensitivity. Furthermore, it consists of conserved and highly variable regions, which increases its detection specificity and also allows for the use of universal primer. In addition it evolves at relatively constant rates, which allows inferring phylogenetic relationships (Patel, 2001; Clarridge, 2004; Woo et al., 2008). The genomic DNA was extracted and analyzed on an agarose gel which indicated good quality of band for PCR analysis (Figure 2). Extracted DNA from the isolates was subject to PCR amplification of 16S rDNA gene using universal primers. These primers amplify a specific fragment of 16S rDNA gene in all isolates at the length of 1500 bp. Similar results of band amplification of 16S rDNA gene were obtained by Orengo et al. (2010) using those primers.

The amplified PCR products were purified and sequenced using the Sanger-dideoxy method. The results of sequencing indicated that the 16S rDNA gene consists of 1487, 1480 and 1479 nucleotides in isolates 1, 2 and 3 respectively. In order to identify genus and species, analysis of partial 16S rDNA sequences (Figures 3 to 5) was used for BLAST search against GeneBank database of Bacillus species in NCBI. The sequences were aligned by multiple sequence alignment using CLUSTALW program.

The Neighbor-joining method was used to construct the phylogenetic tree using Mega 6.0 software. The phylogenetic analysis found that the three isolates belong to the genus Bacillus. The 16S rDNA gene of isolate 1, isolate 2 and isolate 3 showed 100 , 99.9 and 99% similarity with 16S rDNA gene of the *B. cereus* ATCC 14579, *B. subtilis* subsp. *inaquosorum* KCTC 13429 and *B. safensis* FO-36b, respectively (Table 1 and Figure 6).

This result indicated that the three isolates belong to species *cereus, subtilis* and *safensis*. The efficiency of the identified isolates in degrading frequently detected

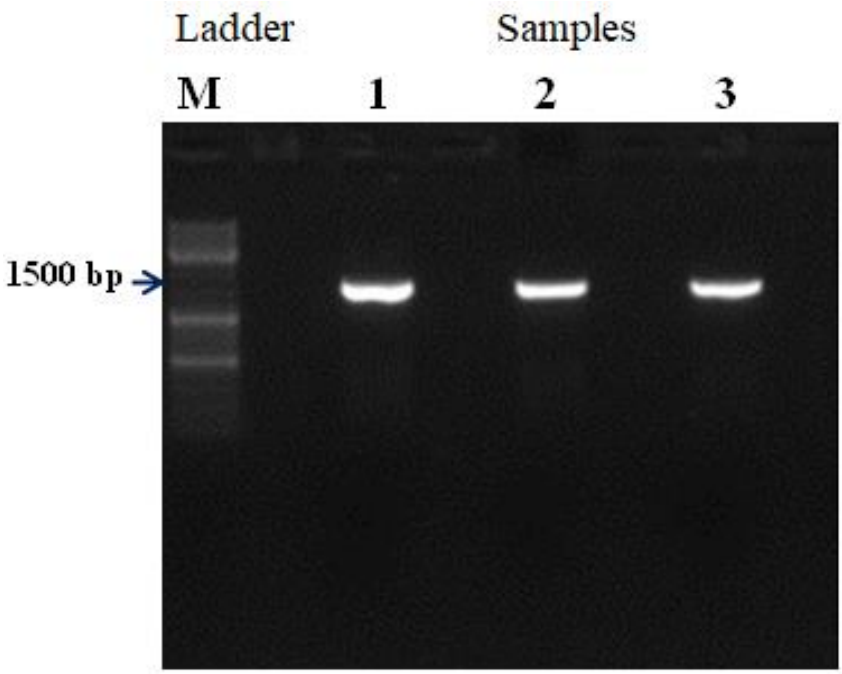

Figure 2. Genomic PCR analysis of Bacillus strains PCR amplification of 16S rDNA gene using universal primers. M, Marker; 1, Bacillus strain 1, 2; Bacillus strain 2; 3, Bacillus strain 3.

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   1 GCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAG
  76 TTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAA
 151 TACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCG
 226 TCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCA
 301 CACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTG
 376 ACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGT
 451 TGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACG
 526 TAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCC
 601 CACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTA
 676 GCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGC
 751 GCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGA
 826 GGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACT
 901 CAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCA
 976 GGTCTTGACATCCTCTGAAAACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTG
1051 TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTA
1126 AGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTT
1201 ATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTCATA
1276 AAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGC
1351 ATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTC
1426 GGTGGGGTAACCTTTTTGGAGCCAGCCGCCTAAGGTGGGACAGATGATTGGGGTGAAGTCGT
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Figure 3. The 16S rDNA gene sequences of Bacillus strain 1 (1487 bp).

pesticide contaminants (chlorpyrifos, malathion, dimethoate, pendimethalin, endosulfan alpha, endosulfan beta, temphos and fenthion) was described elsewhere (Ishag et al., 2016, 2017; Abdelbagi et al., 2018). These findings agree with those of Kinuthia et al. (2010), Karisham and Hari (2014) and Maryam et al. (2014) who utilized 16S rDNA gene sequences to identify different species of Bacillus from pesticides polluted soil. The

Figure 4. The 16S rDNA gene sequences of Bacillus strain 2 (1480 bp).

Figure 5. The 16S rDNA gene sequences of Bacillus strain 3 (1479 bp).

Table 1. Phylogenetic neighbors of bacteria on the basis of similarity to the partial 16S rDNA sequence.

Accession No.	Species (16S rRNA gene analysis)	Pairwise Similarity (%)	Diff/total nt	Identity (%)
AE016877	*Bacillus cereus* ATCC 14579T	100	0/1487	100
AMXN01000021	*Bacillus subtilis subsp. inaquosorum* KCTC 13429T	100	0/1479	99.90
ASJD01000027	*Bacillus safensis* FO-36bT	100	0/1479	99

Diff ≡ different; nt ≡ nucleotide.

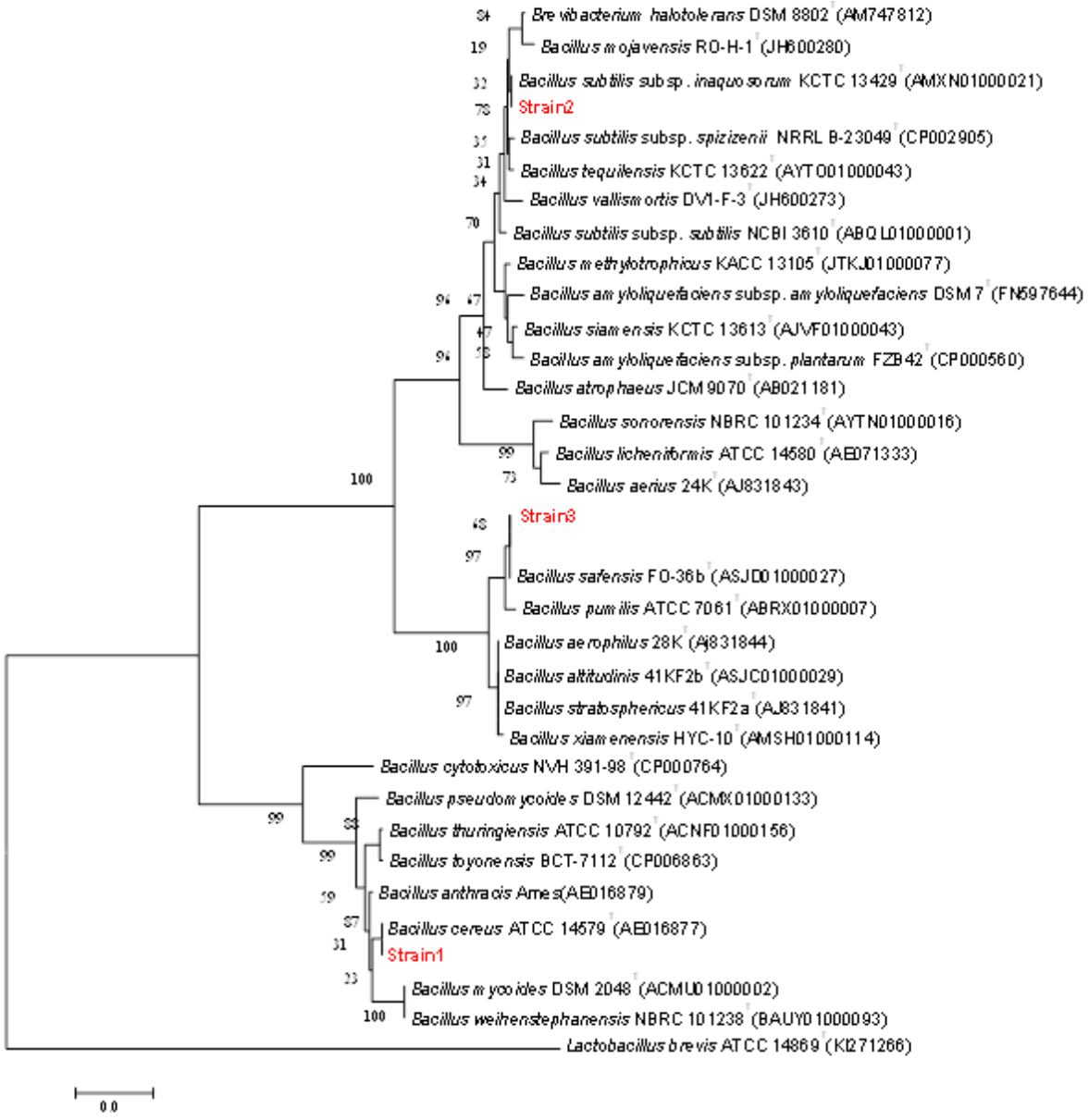

Figure 6. Phylogenetic relationships between the different strains of Bacillus isolated*. Numbers above and below branches indicate bootstrap values from Neighbor-Joining analysis. *Based on the analysis of partial 16S rDNA sequences.

identified isolates in these studies were also found capable of degrading pesticide contaminants. Further research should be done to clone genes responsible for degradation of pesticides to engineer indigenous microorganisms for bioremediation of pesticide in polluted soils and dumping sites.

Conclusion

Three indigenous isolates of *Bacillus* were isolated from pesticide heavily contaminated soils in Sudan and were identified by16S rDNA gene as *B. cereus*, *B. subtilis* and *B. safensis*. The identified isolates have shown promising potential in degrading many pesticide contaminants and can be further developed for future remediation of pesticides heavily polluted soils.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Comparative studies between growth regulators and nanoparticles on growth and mitotic index of pea plants under salinity

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To our knowledge, no research study has been carried out on the effects of ascorbic acid (ASA), 5-Aminolevulinic acid (ALA) and Nano selenium (N-Se) on the cytological parameters of pea seedlings under salinity stress. Salinity treatment (60 and 120 mM NaCl) was applied. Two concentrations of ASA (50 and 100 ppm), ALA (25 and 50 ppm), and N-Se (10 and 20 ppm), respectively were used individually and in combination with NaCl (60 and 120 mM). Modifications in shoot length, number of leaves, leaf area, chromosomal aberrations and mitotic index were determined. Salinity treatment (120 mM) caused the highest reduction in shoot length, leaf area and mitotic index. A significant increase of chromosomal abnormalities percentage (%) was detected in salinity treatments compared with control. ASA (100 ppm), ALA (50 ppm) and N-Se (10 ppm) treatments significantly reduced the damaging effect of salinity stress on growth attributes, mitotic index and chromosomal abnormalities percentage (%) and improved seedlings' performance. These treatments can be recommended for the improvement of pea plants' productivity under salt stress.

Key words: Ascorbic acid, 5-aminolevulinic acid, nano selenium, salt stress, mitosis, chromosomal aberrations, *Pissum sativum* L.

INTRODUCTION

Salt stress adversely affects the morphological, physiological and biochemical responses of plant species (Nazar et al., 2011). Several researchers found that the chlorophyllian pigments were reduced with an increase in salinity level. This may be due to the disruption of the fine structure of chloroplasts and pigment-protein complex or chlorophyll stability, which can result in chlorophyll oxidation (Saha et al., 2010; Helaly et al., 2016; Elsheery et al., 2020c) and disturb plant growth and development (Sairam and Tyagi, 2004). Tang et al. (2017b) established that salinity inhibits plant growth, reduces yield in many crop plants and affects their commercial value (Helaly et al., 2016; Elsheery et al., 2020c). So, salinity stress inhibits growth of basil plants by

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decreasing a significant number of leaves/plant and plant height (Khan et al., 2009; Nassar et al., 2019). Also, the retardant effects of salinity stress on growth, physiological aspects and productivity were also recorded on other different plants species; for instance, Reda (2007) on *Senna occidentalis*, Dawood et al. (2014) on Faba bean, Bargaz et al. (2016) on *Phaseolus vulgaris*, Nassar et al. (2016) on Leucaena and Elsheery et al. (2020b) on mango. There are many ways to improve salinity tolerance in plants such as using of biofertilizer and amino acids (Helaly et al., 2016) and grafting in vegetable crops (Elsheery et al., 2020a; Helaly et al., 2016; Al-Mayahi, 2016). This study was carried out to investigate the effects of ascorbic acid (ASA) under salinity stress on growth of pea plant. Some biochemical constituents that can promote growth and increase productivity of many species of plants grown under normal or abiotic stress conditions are highly recommended (Sharma et al., 2019). Ascorbic acid (ASA) is a small water soluble antioxidant molecule which acts as an essential substrate in the cyclic pathway of enzymatic detoxification of hydrogen peroxide. Ascorbic acid (ASA) is a naturalist product that acts as an antioxidant and enzyme and also improves cofactor. It engages in a variety of procedures. It correlates with chloroplasts in the oxidative stress of photosynthesis (Latif et al., 2016). Furthermore, ASA has a number of roles in protein modification and cell division in plant cells (Hussein et al., 2019). Nowadays, it plays an essential role in a series of physiological processes such as cofactor of key enzyme, plant defense against oxidization, growth, development, cell division, cell extension, senescence and counteracts the deleterious effects of biotic and abiotic stresses (Zhang and Sonnewald, 2017). Therefore, it is chosen to be one of the substances of the subject of our present study.

5-Aminolevulinic acid (ALA) is a type of non-protein amino acid that supports plant stress tolerance. However, the underlying physiological and biochemical mechanisms are not entirely understood (Anwar et al., 2020). ALA is found in all plants and animals. 5-aminolevulinic acid (ALA) is and a key precursor for the biosynthesis of porphyrins such as chlorophyll, heme and plant hormones. In addition, it has newly been reported that ALA regulates the expression level of fructose-1, 6-bisphosphatase (FBP), triose-3-phosphate isomerase (TPI), and ribulose-1, 5-bisphosphate carboxylase/ oxygenase small subunit (RBCS), which activate the Calvin cycle of photosynthesis under drought stress (Liu et al., 2016). It was found that, ALA is one of plant growth regulators (PGRs) and mitigates salinity stress effect in germinating seeds and ameliorates seedling growth. Foliar application of 5-aminolevulinic acid at low concentrations has been shown to promote salt tolerance in a lot of plants (Tang et al., 2017a). On the other hand, ALA is involved in the chlorophyll biosynthesis pathway under salt stress conditions (Wu et al., 2011) and motivates antioxidant enzyme efficiency and accumulation of endogenous hormone under many stress factors such as low-temperature in cucumber seedlings (Anwar et al., 2018). Under drought stress, spray application of ALA up-regulated the chlorophyll fluorescence indexes in oilseed rape (*Brassica napus* L.) (Liu et al., 2014) and gas exchange indexes, such as net photosynthetic average (Pn), stomatal behavior (gs), intercellular CO_2 concentration (Ci) and the rate of transpiration (Tr), which were adversely influenced by abiotic stress (Wu et al., 2018). It is also reported that foliar application of ALA may confer plant tolerance to diverse abiotic stresses, such as chilling, high temperature, salinity, drought, weak light, and heavy metals (Wu et al., 2019a). Previous studies demonstrated that ALA encourages abiotic stress tolerance by activation of numerous types of transcription factors, signal transduction, and chlorophyll and carbohydrate biosynthesis (Nishihara et al., 2003; Anwar et al., 2020). These results submit that ALA can broadly minimize the harmful effects of environmental stress. Increasing attention has been paid to the beneficial impacts of many nanoparticles (NPs) used in low doses on diverse crops (Jampílek and Kráľová, 2017; Rastogi et al., 2019; Kumar et al., 2020; Elsheery et al., 2020c). A lot of researchers like Sonkaria et al. (2012) and Prasad et al. (2014) established that, using of NPs can promote plant growth, warrant food goodness and decrease waste. Nano-Selenium (N-Se) as Nano fertilizer has been recently used in the field (Shang et al., 2019; Elsheery et al., 2020a; Elsheery et al., 2020b). There is less documented information on the biological effects of N-Se and its application (Chau et al., 2007; Cushen et al., 2012). Bhattacharjee et al. (2014) and Kamle et al. (2020) suggest that N-Se plays a role as a reactive oxygen species (ROS) scavenger in plants under stress conditions. So, the purpose of our study was: To evaluate the (ASA, ALA and N-Se) morphological and cytological effect of application of our treatments (Soaked and foliar) on pea plants under salinity stress using hydroponic methods.

MATERIALS AND METHODS

Plant material

The pea variety used in this study was obtained from El Korma Company, Egypt for seeds import. The experiment was carried out at the greenhouse of the Agriculture Botany Department, Faculty of Agriculture, Tanta University, Egypt, during winter of the two growing seasons of 2018 and 2019. In both growing seasons, the average of the daily temperature ranged between 11 and 26°C and relative humidity between 60 and 65%.

Hydroponic experiment for evaluating responses of tested cultivar of pea to salt stress

The seeds were washed and soaked for 6 h before they were

planted in the treatment solutions [ASA (50, 100 ppm), ALA (25, 50 ppm) and N-se (10, 20) ppm]. Then they germinated in polyethylene bags (8 seeds per bag) filled with washed sand on a half-strength Hoagland,s nutrient solution used as macronutrient sources (Hoagland and Arnon, 1950). Then, the bags were placed in dishes containing 1 L of Hoagland,s solution (pH 5.8) in greenhouse at a temperature range of 20 to 26° during the day and 11 to 16° during the night. Nutrient solutions were added every day and renewed every 3 days. Four replicates (each with 10 polyethylene bags containing 8 plants) were planted. After the emergence of the first real leaves (15 days after germination), the number of plants per bag was adjusted to five, by thinning out weak and less vigorous ones. Seedlings were exposed to two levels of salinized water (salt mixture of 60 and 120 mM) of a mixture salts. When the 4th true leaf emerged, foliar spraying was done twice (after 30 and 33 days from sowing) at the same concentrations [ASA (50,100 ppm), ALA (25, 50 ppm), N-Se (10, 20 ppm)]. Foliar treatments were not applied on nine pots: The first 3 pots were treated with saline; the second pot was treated with a salt mixture of 60 mM and the third was treated with a salt mixture of 120 mM.

The plants were collected after seven days of foliar application and their morphological parameters were recorded.

Growth parameters

The following parameters were recorded: Shoot length (cm), number of leaves and leaf area per plant (cm^2) using the formula:

Leaf area /plant with weight method (cm^2/plant) = B=L*S/ Z

B = Green leafy area on one plant; S = Circle space for tablets; L = Total weight of the leaves on the plant; Z = Weight of tablets.

For shoot length from each treatment, shoots of 10 pea plants were separated from roots; they were washed using distilled water and dried carefully with wish tissue paper. The number of leaves per plant was counted.

Cytological parameters

For mitotic screening physiologically, uniform and healthy seeds of *Pisum sativum* L. were used to study the effect of ASA, ALA and N-Se on the growth of pea plants under salinity stress conditions (Darlington, 1976). The dividing cells were observed and recorded. Cells were examined under a light microscope for mitotic index, numbers and types of abnormalities. At least 3000 cells were examined per treatment (1000 cell/replicate). Mitotic index (MI) and percentage of abnormal cells were calculated using the following formulas:

$$\text{Percentage of abnormal cells} = \frac{\text{Total abnormal cells}}{\text{Total dividing cells}} \times 100$$

$$\text{Mitotic index (MI)} = \frac{\text{Total dividing cells}}{\text{Total dividing and non dividing cells}} \times 100$$

RESULTS AND DISCUSSION

Growth parameters

Plant height (shoot length), fresh and dry biomass of shoot and root per pea plant, number of leaves and leaf area per plant were affected by salt stress levels. ASA, ALA, and N-Se soaking and foliar application improved the plant's tolerance to salt stress (Table 1).

The data presented show that increased salt levels induced significant (mean value for two seasons) stress which resulted in a significant reduction of all growth parameters compared to control. ALA, ASA and N-Se treatments reduced the harmful effect of salinity on plants treated compared with non-treated plants.

Salinity is an essential environmental factor which curbs crop plants from attaining their full genetic potential; therefore, salt stress in pea plant induces a lot of growth limitation (Gama et al., 2007). Pea plant subjected to higher salt will experience delay in growth which could be attributed to the inhibition of cell elongation (Taïbi et al., 2013). These results are consistent with those obtained on rice by Yu et al. (2019), on flax by Wu et al. (2019b) or other species.

The inhibitory effects of salt stress on pea plant and the reduction in dry mass might be due to the toxic effect of salt stress as a result of high osmotic pressure. Salinity stress causes a significant increase of growth inhibitors and decrease of growth promoters. Disturbance of water in plants grown under salinity restricts absorption or plants are not able to uptake the water and nutrients required by them (Memon et al., 2010) (Colla et al., 2006a, b).

A similar tendency was observed by Shi et al. (2013). The suppression effects of salinity lead to disturbance in ionic homeostasis, stomatal closure, reduction in photosynthesis, accumulation of toxic ions (Na^+, Cl^-) which restricted the absorption of water (Shahzad et al., 2020; Kamran et al., 2020) and inhibited growth and productivity. The same trend was observed by Nassar et al. (2019) and Bargaz et al. (2016).

The results presented in Table 1 show that all growth characteristics of "leaf area, shoot height and number of leaves" decreased significantly with increased salt stress level.

Leaf area

Data indicate that the leaf area has been significantly affected by different salinity concentrations (Table 1). Soaking and foliar applications significantly increased the leaf area under salinized and non-salinized conditions ($P < 0.05$).

Under salinity level of 120 mM, the highest increase in leaf area was with ASA (100 ppm) (99.96 cm^2) followed by ALA (50 ppm) (96.71 cm). In contrast, the lowest amounts of leaf area recorded using N-Se (20 ppm) (62.50 cm^2) were 99.96, 96.71 and 62.50 cm^2, respectively, compared with control plants.

Foliar application of ASA and ALA increased all previous parameters, improved plants' tolerance to NaCl toxicity and minimized reduction in growth caused by

Table 1. Effect of ASA, ALA and N-Se soaking and foliar application on growth parameters of field pea plant grown under different salt stress levels.

Salinity level (mM NaCl)	Treatments	Concentration (ppm)	Leaf area (cm^2)	Shoot length (cm^2)	No. of leaves
Control 0 mM NaCl	Control		131.07	28.96	6.00
	ASA	50	135.33	29.64	7.00
		100	168.26	38.81	9.00
	ALA	25	132.77	30.61	7.00
		50	140.04	35.66	8.00
	N-Se	10	137.50	32.70	7.00
		20	137.18	29.24	6.33
60 mM NaCl	Control		97.08	22.73	6.00
	ASA	50	133.52	29.03	7.00
		100	167.14	35.82	8.00
	ALA	25	99.98	27.95	7.00
		50	138.57	34.68	7.00
	N-Se	10	137.01	30.84	7.00
		20	122.33	26.45	6.00
120 mM NaCl	Control		49.84	17.75	5.00
	ASA	50	68.11	25.34	6.00
		100	99.96	32.69	7.00
	ALA	25	58.64	24.79	6.00
		50	96.71	28.00	6.00
	N-Se	10	85.39	27.24	6.00
		20	62.50	22.39	5.00
L.S.D. (0.05)					
Salinity			1.491**	0.403**	0.540**
Treatment			2.277**	0.616**	0.825**
Salinity x Treatment			3.944**	1.067**	0.040**

NaCl. Meanwhile, it is evident that ASA and ALA play a vital role in the regulation of a number of metabolic processes in plants exposed to salinity. It has been concluded that, the typical effect of salt stress on plants is growth retardation due to the inhibition of cell elongation (Hanafy et al., 2013).

ALA is one of the existing PGRs used for the improvement of plants' stress tolerance (Hotta et al., 1997; Naeem et al., 2012) and an essential precursor for the biosynthesis of tetrapyrroles such as heme and chlorophyll. It was found that ALA is formed in all animals and plants. Recently, it was found that low concentrations of ALA had a promotive effect on growth and yield of several crops and vegetables (Watanabe et al., 2000).

Ascorbic acid is an essential main metabolite in plants that is utilized as an enzyme cofactor, an antioxidant and a cell signaling modulator in crucial physiological processes, in biosynthesis of the cell wall, secondary metabolites and phytohormones, stress tolerance, photoprotection, cell division and growth (Elkelish et al., 2020).

Selenium spraying treatment had a considerable positive effect on all studied characteristics such as plant height, number of leaves, fresh weight of shoots and roots, dry weight of leaves and shoots and chlorophyll content. It could be proved that foliar application of nano selenium at 10 and 20 ppm increased vegetative growth, yield and quality as well as mineral contents in leaves of pea plants. Furthermore, the best selenium used as a foliar spray is the nano type because it is safer and more environmental friendly compared to the chemical form. These results agree with Shedeed et al. (2018).

Shoot length

Shoot length was significantly affected by different salinity concentrations. ASA (100 ppm) showed higher shoot length followed by ALA (50 ppm) (32.69 and 28.00 cm, respectively). The lowest shoot length was obtained with

N-Se (20 ppm) treatment (22.39 cm) compared to untreated plants exclusively under high level of salt mixture (120 mM). Previous studies show that the application of some natural bio-stimulants used as a foliar spray and/or seed soaking improved growth and yield constituents of pea grown under salinity stress (Desoky et al., 2017).

Number of leaves

The data in Table 1 show that in both growing seasons, salinity caused a significant reduction in the number of leaves by different salinity concentrations. In contrast, in the treatments of ASA (100 ppm) and ALA (50 ppm), the number of leaves was higher (7 leaves and 6 leaves respectively). The lowest number of leaves was obtained with N-Se (20 ppm) treatment (5 leaves) in comparison to untreated plants, especially under high level of salinity (120 mM).

The decrease in growth and productivity could be attributed to the osmotic effect of salinity stress which caused increase of growth inhibitors and decrease of growth promoters, disturbance of water in plants grown under salt stress. It indicates that these inhibitory effects of salinity lead to stomatal closure, reduced photosynthesis, unrest in ionic homeostasis, accumulation of toxic Na^+, Cl^- and finally inhibit growth and productivity. Moreover, the decrease in the shoot length of stressed plants is actually due to senescence which is accompanied by loss and withering of plant organs as well as the transport of elaborated materials to the reproductive organs.

Pea seed and plant treated with ASA, ALA and N-Se used as foliar spray and seed soaking significantly promoted plant growth and productivity under the adverse effect of soil salinity. One of the compounds which has antioxidative characteristic is ASA (Zhang, 2013). This compound can reduce the harmful effects in plants under environmental stress. The ASA treatments influenced the passive effect of salinity on growth and productivity. This could be referred to as the biochemical functions of ASA which can be divided into categories, that is, antioxidant, that changes the lipophilic antioxidants such as tocopherol, vitamin E, and enzyme cofactor for hydroxylase enzymes involved in the synthesis of rich-hydroxyproline glycolproteins, and cell wall structural proteins (Desoky et al., 2017). It was observed that, foliar treatment with ALA stimulated growth and also partially enhanced the effects of toxic caused by high levels of salinity in root and shoot. Also, salinity damage can also be attributed to the physiological drought generated by salt stress (Hopkins, 1995; Sajid et al., 2020), due to the reduction in osmotic potential and relative water content. ALA application encouraged an increase in osmotic potential and relative water content of the stressed seedlings (Naeem et al., 2011). Many studies have shown that ALA can stimulate crop resistance, yield and quality and can be used as a new type of plant growth regulator (Rafaqat et al., 2015; Tang et al., (2017b). A concentration of 50 mg/L of ALA could significantly mitigate seeds' deterioration and seedlings of *Perilla frutescens* under NaCl stress and encourage salt resistance (Zhang et al., 2011). It was indicated that a high level of salinity damages cellular electron transport, leading to the generation of reactive oxygen species (ROS). This activates lipid peroxidation and cell membrane damage (Shalata et al., 2001).

Cytological parameters

The cytological effects on pea plants treated with ASA, ALA and N-Se under saline conditions are shown in Table 2.

Our results showed that, the mitotic index in root tip meristems of *P. sativum* treated with salt mixture (60 and120 mM) significantly decreased compared to seeds (in control treatment). Table 2 proves the effect of ASA, ALA and N-Se on mitotic index (%) in *P. sativum* root tips. The total number of proliferating cells and the numbers of cells at various mitotic stages of *P. sativum* meristemic cells were scored in root tips. Cytological analysis showed that, under harmful stress conditions (120mM NaCl), the highest value of mitotic index (%) was observed in pea treated with ascorbic acid at a concentration of 100 ppm (13.53%) followed by ALA 50 ppm (13.19%); nano selenium at a concentration of 20 ppm gave the lowest value (10.12%), compared to control (10.02%).

The mitotic index of root tips of seeds treated with ASA, ALA and N-Se remarkably increased in salinity samples. At the same time, ASA, ALA and N-Se + NaCl application indicated serious performance in improving the passive effects of salt stress on the mitotic activity. Çavuşoğlu et al. (2007, 2013) established that, growth regulator (exogenous application) may have a positive or negative effect on seed germination and seedling growth under non-stress conditions.

Thus, the study aims to test the effects of ASA, ALA and N-Se application on seedling growth in non-stress and stressed conditions. Our results indicated that the shoot length, number of leaves and leaf area of treated plants were generally amplified in comparison to the control. It was stated previously that saline conditions harmfully affect growth and development actions in general, even in halophytes (Çavuşoğlu et al., 2017; Ghoulam and Fares, 2001). The seedling growth and germination of *P.sativum* seeds, as anticipated, were prevented under saline conditions (Table 1).

Ali (2000) demonstrated that salinity could fulfill/replace its harmful effect in numerous ways. It may intervene with seed germination by converting the water status of the seed so that water reuptake is inhibited. Our outcomes

Table 2. Mitotic index and mitotic phase (%) of tip root cells of *pissum sativum* under salinity stress conditions and different applied treatments (ascorbic acid - ASA, 5-aminolevulinic acid - ALA and Nano selenium - N-Se).

Salinity level (mM)	Treatments		No. of screened cells	Mitotic phase (%)				Total no. of normal dividing cells	Mitotic index (%)
				Prophase	Metaphase	Anaphase	Telophase		
Control (0)	Control		1072.67	38.333	27.67	22.67	18.00	106.67	12.11
	ASA	50 ppm	1135.00	61.667	45.33	31.00	20.00	158.00	14.03
		100 ppm	1061.00	83.000	71.33	57.00	38.33	241.67	22.79
	ALA	25 ppm	1056.67	51.333	44.67	30.67	20.00	146.67	13.97
		50 ppm	1052.67	72.667	59.33	49.00	32.33	221.33	21.06
	N-Se	10 ppm	1007.00	67.667	50.00	38.33	24.67	180.67	17.95
		20 ppm	1021.67	47.667	33.33	23.33	15.33	119.67	12.11
60 mM	Control		1054.00	21.000	24.33	20.00	20.00	85.33	10.31
	ASA	50 ppm	1092.67	45.000	26.00	17.00	9.33	97.33	11.72
		100 ppm	1028.00	71.667	62.33	38.33	26.33	198.67	20.24
	ALA	25 ppm	1034.00	40.000	21.67	17.67	10.33	89.67	10.92
		50 ppm	1164.33	61.333	54.33	31.33	20.33	167.33	15.63
	N-Se	10 ppm	1058.33	54.333	41.00	24.67	15.33	135.33	14.17
		20 ppm	1131.33	37.333	27.33	15.67	9.00	89.33	10.74
120 mM	Control		1103.67	17.000	16.67	17.33	12.67	63.67	10.02
	ASA	50 ppm	1177.00	21.000	19.33	18.00	12.67	71.00	10.62
		100 ppm	1048.00	37.000	38.00	15.33	8.67	99.00	13.53
	ALA	25 ppm	1261.00	19.333	19.67	16.00	13.33	72.00	10.23
		50 ppm	1029.00	35.667	34.67	12.33	6.00	88.67	13.19
	N-Se	10 ppm	1077.33	30.667	27.67	14.67	7.33	80.33	12.12
		20 ppm	1066.67	21.667	18.67	16.33	15.33	68.33	10.12
L.S.D. (0.05)									
Salinity			62.618	1.624**	0.997**	1.065	1.058**	2.435**	0.429**
Treatment			95.650	2.481**	1.523**	1.626	1.616**	3.719**	0.636**
Salinity x Treatment			165.670	4.298**	2.638**	2.817	2.798**	6.442**	0.964**

showed the decrease in stem length, number of leaves and leaf area under saline conditions. Other studies showed that the inhibitive effect of salt on root might result from decreasing cell division (McCue and Hanson, 1990), protein synthesis and nucleic acid (Prakash et al., 1988). This may be demonstrated by the failure of the roots to receive enough water due to high osmotic pressure of the medium (Al-Karaki, 2001).

On the other hand, ASA, ALA and N-S treatments noticeably removed the inhibitor effect of salinity stress on growth parameters, so our

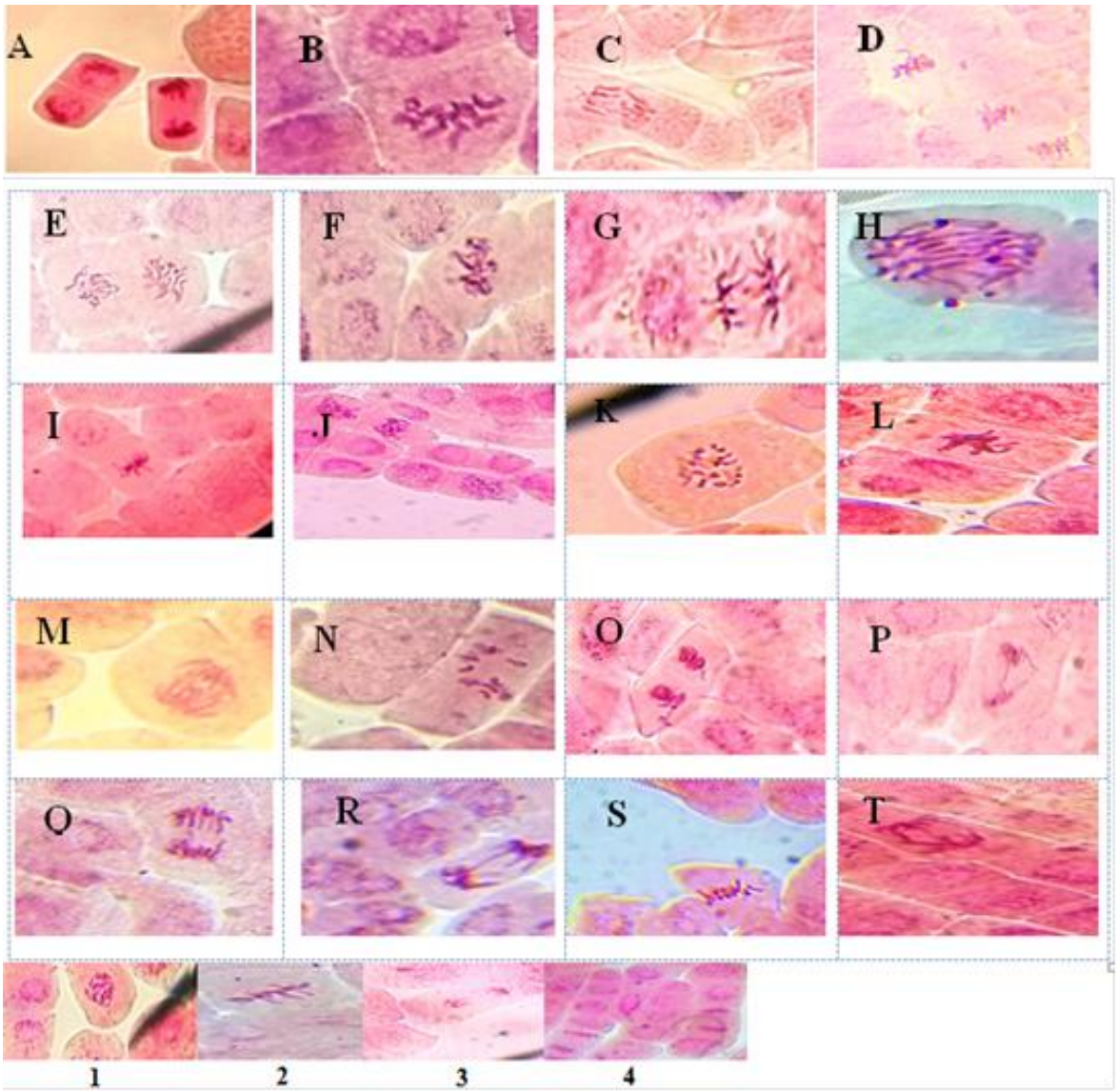

Figure 1. A: Sticky Anaphase, B: C- Metaphase stage, C: Vagrant chromosome in Anaphase, Chromosomal Laggard in late Anaphase, D: Overlapping chromosomes in Metaphase stage, E: Irregular prophase, F: Sticky Metaphase, G: Irregular Metaphase, H: Alignment anaphase, I: Vagrant chromosome in Metaphase, J- Irregular prophase. K: Granulation, L: Clumped metaphase, M: Multiple bridges in anaphase, N: Disrupted equatorial plate, O: Telophase with a fragment, P: Anaphase with laggard chromosome, Q: Anaphase with chromosome breakage, R: Anaphase with a single bridge and sticky chromosome, S: Metaphase with a fragment, T: Anaphase with a single bridge. 1: normal prophase, 2: normal metaphase, 3: normal anaphase, 4: normal telophase.

treatments alleviate salt stress on roots due to the reduction in the salts osmotic effects. ASA might have been effective in decreasing the inhibitive impact of salinity stress on the seed germination and seedling growth by rising nucleic acid and protein synthesis, providing steadiness of cell membranes or by raising the activity of antioxidant enzyme (Al-Kaisy et al., 2018). ASA has also received special attention because it is a highly efficient antioxidant and has free radical scavenging capacity (Acosta-Motos et al., 2020).

There are no present studies on the effects of ALA application under salinity conditions on cytogenetical parameters as studied here.

In Figure 1, It was concluded that aberrations in chromosomes induced by salinity at different stages of mitosis such as prophase, metaphase, anaphase, and telophase in root meristem cells are variable. In addition, a gradual decline in mitotic index and an increase in the abnormality index were observed as the concentration of salt mixture application duration was raised. Aberrations in chromosomal behavior such as sticky and disturbed chromosomes in metaphase and anaphase, c-metaphase,

bridges, laggard, and disturbed telophase were also observed. Bhattacharjee et al. (2014) confirmed that a significant reduction by N-Se in chromosomal aberration in bone marrow, and DNA damage in lymphocytes and bone marrow in mice treated with cyclophosphamide - induced hepatotoxicity and genotoxicity.

Types of chromosome aberrations in *P. sativum* root tips treated with ASA, ALA and N-Se under saline condition

In Table 3, various chromosomal aberrations like chromosomal bridges, stickiness, vagrant, broken, and lag chromosomes were recorded. In our study, the lowest value of abnormalities (%) under severe stress conditions (120 mM) was observed when the pea was treated with ascorbic acid at a concentration of 100 ppm (13.63%) followed by ALA 50ppm (14.01%); nano selenium at a concentration of 20 ppm gave the highest value. It was 17.45% compared with control which recorded 23.42%. Cytological effects and the data obtained from the analysis of root tips treated with ALA, ASA and N-Se are summarized in Tables 1 and 2 and Figure 1.

Previously, it was reported that salt stress conditions adversely affect growth and development events. It is recognized that salinity inhibits seedling growth (Abdul Qados, 2011; Ghezal et al., 2016).

Salinity that inhibits shoot length and leaf number may result from decreasing cell division (McCue and Hanson, 1990). The kinds of mitotic abnormalities detected in the present study were disturbed chromosomes, sticky, vagrant, laggard, bridges and C- shaped Metaphase.

On the other hand, ASA, ALA and N-Se application that markedly removed the inhibitor effect of salinity on growth parameters (Tables 1 and 2) is due to the decrease in the salts osmotic effects compared to the control. In addition to all these, ASA, ALA and N-Se might have been efficacious in decreasing the inhibitive effect of salt stress on the plant growth by increasing nucleic acid and protein synthesis, stabilizing cell membranes or raising the activities of antioxidant enzyme (Liu et al., 2014; Ekanayake et al., 2015; Germ et al., 2007).

According to some researchers, the harmful effects of salinity stress on mitotic activity have been known for a long time. Furthermore, the negative effects of salinity stress on chromosomal abnormalities have been planned in the last decade (Radic et al., 2009; Tabur and Demir, (2009, 2010a, b). It was demonstrated that a high concentration of salt entirely suppressed the activity of mitotic division and facilitated chromosomal abnormalities in root-tip meristem cells (Radić et al., 2009).

It is worth mentioning that salinity adversely affected the mitotic activity and chromosome behaviors in root meristem cells of *P. sativum*. Briefly, the results proved that under salinity, ASA, ALA and N-Se might act as a stimulator, triggering the protein synthesis necessary for the normal division of cells and acceleration of the mitotic cycle. Previous researches also proved that nanomaterials with low concentrations are better than high concentration alone or in a group with stress (Zedan and Omar, 2019). It was indicated that, the protective effect of selenium as nanoparticles versus numerous material induced cytotoxicity and genotoxicity effects (Bhattacharjee et al., 2014). A significant reduction by N-Se in chromosomal aberration was found in bone marrow, and DNA damage in lymphocytes and bone marrow in mice treated with cyclophosphamide -induced hepatotoxicity and genotoxicity.

Barakat (2003) established that, treating the roots with vitamin B6 or ascorbic acid presented a considerable increase in the mitotic index and reduced the inhibition effect of NaCl in wheat cultivars.

The previous results showed that ASA (100 ppm) in combination with NaCl does not only antagonize the inhibitory action of salinity but also activates the cells to inter mitosis and encourages a high mitotic activity. These results are in harmony with those obtained by Autifi et al. (2018) who proved that vitamin C reduces the influence of lead acetate on the mitotic activities. No perversion from the normal was seen in roots treated with vitamin C or vitamin B6; parallel results were observed by Bronzetti et al. (2001).

Harmful effects of salinity or any other stress conditions were attributed to lower endogenous levels of cytokinins and endogenous hormonal imbalance by some researchers (El-Mashed and Kamel, 2001). Plant growth inhibition refers to disturbances in natural growth regulators and mitotic chromosomal irregularities as additional factor (Hoda et al., 1991). So, the application with ascorbic acid can reform the genotoxic effect of the salinity which delays the cell in entering mitosis.

Conclusions

Considering the data of the present study it appears that when applied at high concentrations of salinity shows cytotoxic activity. In this study some growth regulators such as ASA, ALA and N-Se were used. It was concluded that salinity treatments stimulated the genotoxic effect to throw ROS generation inside the tissues which ultimately cause oxidative disturbance. This leads to redox homeostasis imbalance and genotoxic in addition to mito-depressive effects. Using these treatments (ASA, 100 ppm; ALA, 50 ppm and N-Se, 10 ppm) causes induction of mitotic index and reduces the chromosomal aberrations.

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Table 3. Effect of salt mixture (60 and 120 mM) ascorbic acid (ASA 50 and 100 ppm), 5-aminolevulinic acid (ALA 25 and 50 ppm) and Nano selenium (N-Se 10 and 20 ppm) and the combination between salinity and ASA, ALA and N-Se on frequency of chromosomal aberrations in *Pissum sativum* root tip meristems.

Salinity level mM	Treatments		No. of examined cells	No. of dividing cells	No. of abnormal cells	Mitotic aberration (%)									Abnormalities %
						Granulation %	C-metaphase%	Laggard %	Break%	Stickiness %	Vagrant %	Irregular %	Alignment %	Bridge %	
Control (0)	Control		1072.67	106.67	3.00	0.00	0.00	0.67	0.67	0.67	0.33	0.33	0.00	0.33	3.31
	ASA	50 ppm	1135.00	158.00	1.00	0.00	0.00	0.33	0.00	0.00	0.33	0.33	0.00	0.00	0.64
		100 ppm	1061.00	241.67	0.33	0.00	0.00	0.33	0.00	0.00	0.00	0.00	0.00	0.00	0.13
	ALA	25 ppm	1056.67	146.67	1.00	0.00	0.00	0.33	0.00	0.33	0.00	0.00	0.00	0.33	0.68
		50 ppm	1052.67	221.33	0.33	0.00	0.00	0.00	0.00	0.00	0.33	0.00	0.00	0.00	0.15
	N-Se	10 ppm	1007.00	180.67	0.67	0.00	0.00	0.33	0.00	0.00	0.33	0.00	0.33	0.00	0.55
		20 ppm	1021.67	119.67	1.33	0.00	0.00	0.33	0.00	0.00	0.33	0.00	0.00	0.33	1.11
60 mM	Control		1054.00	85.33	20.00	2.33	2.67	4.00	3.33	3.33	3.00	1.50	0.00	1.33	18.99
	ASA	50 ppm	1092.67	97.33	16.67	1.50	2.00	1.67	1.67	3.00	3.33	3.50	0.00	1.67	14.36
		100 ppm	1028.00	198.67	10.00	0.00	0.67	1.33	1.50	1.67	2.00	3.33	0.00	1.67	4.84
	ALA	25 ppm	1034.00	89.67	17.67	1.33	1.33	2.67	1.67	1.33	2.00	3.33	0.00	4.00	15.68
		50 ppm	1164.33	167.33	12.33	1.33	1.67	1.67	1.67	0.67	1.50	2.33	0.00	2.00	6.87
	N-Se	10 ppm	1058.33	135.33	11.00	1.67	1.00	1.00	1.67	2.00	0.67	1.33	1.33	1.67	7.36
		20 ppm	1131.33	89.33	18.00	1.00	2.00	1.67	2.00	2.00	2.67	3.00	0.33	3.67	16.56
120 mM	Control		1103.67	17.000	31.33	5.33	7.33	3.00	5.67	4.00	1.33	2.67	2.67	2.00	23.42
	ASA	50 ppm	1177.00	71.00	20.67	2.67	4.67	1.00	2.00	5.00	2.33	1.67	4.33	1.33	16.52
		100 ppm	1048.00	99.00	19.33	3.00	3.00	1.33	3.00	1.00	2.33	2.67	1.33	3.00	13.63
	ALA	25 ppm	1261.00	72.00	21.33	3.00	4.33	1.00	1.33	4.33	2.33	2.67	4.33	2.33	17.02
		50 ppm	1029.00	88.67	19.00	2.67	2.67	1.33	3.00	1.67	2.33	2.33	3.00	3.00	14.01
	N-Se	10 ppm	1077.33	80.33	20.33	3.67	4.00	0.33	1.33	2.67	2.67	2.67	3.67	3.00	15.61
		20 ppm	1066.67	68.33	22.33	2.67	3.67	2.00	1.67	4.67	2.67	2.00	4.33	3.00	17.45
L.S.D. (0.05)															
Salinity			62.618	2.435**	1.083**	0.551**	0.542**	0.582**	0.498**	0.710**	0.651**	0.628**	0.411**	0.487**	0.791**
Treat			95.650	3.719**	1.655**	0.842*	0.827**	0.889**	0.761**	1.084**	0.994	0.960	0.628**	0.743**	1.208**
Salinity x Treat			165.670	6.442**	2.866**	1.459	1.433**	1.540	1.319**	1.877	1.722	1.662	1.088**	1.288*	2.092**

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Study of the antibody stimulation potentials of *lactobacillus* spp isolated from palm wine

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In this study lactic acid bacteria were investigated for their presence in fresh palm wine and their effects on humoral immunity examined. The bacteria were isolated from fresh palm wine in a tenfold serial dilution. The isolates were purified by repeated subculture on De Man Rogosa Sharpe (MRS) agar and characterized phenotypically and genotypically. The identified *Lactobacillus* spp. were investigated for their effects on antibody (humoral immunity) - IgG, IgG1 and IgG2a secretion using sheep red blood cell as antigen (SRBC). *Lactobacillus* spp. were identified as *Lactobacillus brevis*, *Lactobacillus paracasei* subsp. *tolerans*, *Lactobacillus paracasei* and *Lactobacillus yonginensis*. The isolates produced no significant effect on IgG antibody after 4 days' post-secondary challenge. However, they had significant percentage stimulation at 9 days post-secondary challenge of 100.2, 122.9, 106.4 and 118.3% for *L. brevis*, *L. paracasei* sub. *tolerans*, *L. paracasei*, and *L. yonginensis*, respectively. The isolates had marginal and somewhat suppressive effects on IgG1 and IgG2a at both 4 and 9 days' post-secondary challenge. The results show that fresh palm wine contains *Lactobacillus* spp. capable of stimulating antibodies production.

Key words: Humoral immunity, antibody, palm wine, *Lactobacillus* spp.

INTRODUCTION

Lactobacilus spp. are important class of bacteria that have been extensively studied by scientists in recent times. They are generally regarded as safe (Gras) bacteria hence exploited in several ways to the benefit of man. *Lactobacillus* have been linked with the production of antimicrobial substances, antibiotic resistance patterns, improving digestive ability and antibody mediated response with demonstrable efficacy and safety (Hou et al., 2015; Wang et al., 2012). Studies over the years have proved that *Lactobacillus* spp are potent

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modulators of the immune system.

They are able to modulate both the adaptive and innate components of the immune system through the regulation of the functions of dendritic cells, macrophages, T and B lymphocytes. The success of this class of bacteria as an immunomodulator is linked to its ability to bind pattern recognition receptors (PRR) expressed on immune cells. These PRR in turn recognize conserved molecular structure known as microbe associated molecular patterns (MAMPS) and signal to induce the production of cytokines, chemokines and other innate effectors (Wells et al., 2010; Abreu, 2010; Kawai and Akira, 2010). Recent studies have shown that probiotics (e.g lactic acid bacteria) are becoming popular as an option in the treatment of inflammatory disorders (Evrad et al., 2011; Kwon et al., 2010). They also influence positively though indirectly, the regulatory T cells (Treg) by providing a favourable environment (Petersen et al., 2012). *Lactobacillus* spp administration/studies *in vivo* and *ex vivo* produced significant increase in all functions of peritoneal macrophages viz, increased microbiocidal and phagocytic activities and increased production of cytokines induced by macrophages (Marranzino et al., 2012). *Lactobacillus lactis* strains commonly found in food products directly stimulate plasmacytoid dendritic cells (pDC) resident in the intestinal draining mesenteric lymph nodes to produce not only type 1 IFN but also IFN-λ and augment the capacity of pDC to induce CD4+CD25+Treg generation (Jounai et al., 2012). *Lactobacillus acidophilus* increases the cytotoxic activity of natural killer cells (Corthesy et al., 2007). These beneficial and safe bacteria stimulate innate immune system in immune deficient individuals (Delcenserie et al., 2008) and also in immune deficient elderly persons where they stimulate both phagocytic activity of macrophages and natural killer cell function (El-Gaaly et al., 2016).

It is an established fact that *Lactobacillus* spp belong to a group of bacteria that have been extensively studied because of their enormous health benefits. The lyophilized forms of these bacteria are now used as therapeutics in the management of diarrhea. This study derived it uniqueness from the fact that these bacteria was isolated from a natural alcoholic beverage. *Elaeis guineensis* (Palm tree) sap (Palm wine) was studied for the presence of these important bacteria and their effect on humoral immunity. Palm wine is a whitish liquid produced as a result of natural fermentation by acetic acid bacteria, lactic acid bacteria and yeast (Santiago-Urbina and Ruiz-Teran, 2014). These organisms usually cause the breakdown of sugar in the sap into alcohol and other products (Obire, 2005). These organisms are responsible for the sour taste experienced on this beverage over time as the tapping process progresses. The chemistry of what happens is that at the point of tapping the beverage is rich in sugar hence very sweet. The activities of these beneficial bacteria tend to convert the sweet beverage into alcohol and ethanoic acid (sour taste experienced after about 24 h of fermentation) in a time dependent process (Eze et al., 2019). In West African sub-regions, this sweet and fresh alcoholic beverage is linked with some health benefits such as increased sperm in men and breast milk production in women, and high content of B1, B2, B3, and B6 vitamins (Mbuagbaw and Noorduyn, 2012). The beverages also possess high content of amino acid, potassium, zinc and iron (Carousel, 2015).

In this study, we investigated the presence of *Lactobacillus* as they are suspected to be involved in the fermentation of palm wine and the effect of the isolates on humoral immunity was determined.

MATERIALS AND METHODS

Sources of *Lactobacillus*

Fresh palm wine from oil palm tree *Elaeis guineensis*, Linex capsule (lyophilized lactobacillus capsule) Sandoz Pharmaceutical Slovenia.

Sources of antibodies and antigens used

Ovalbumin (Sigma-Aldrich, USA), Goat anti-mouse IgGFab HRP (Southern biotech, USA), Goat anti-mouse IgG1Fab HRP (Southern biotech, USA), Goat anti-mouse IgG2aFab HRP (Southern biotech, USA), albino mice (28-30 g), Sheep red blood cell (SRBC).

Collection of palm wine

The fresh palm wine samples were collected at about 6:30 am by Mr Anthony Idoko a local Palm wine tapper from Onicha Enugu Ezike, Igbo- Eze North Local government area of Enugu State, Nigeria. The samples were kept under cold conditions using ice pack to reduce the rate of fermentation while being transported to the laboratory.

Preparation of media and isolation of *Lactobacillus* spp

The media were prepared following the manufacturer`s specifications. The test organisms were isolated from Palm wine using quadrant streak plate method. A wire loop was used to collect a loopfull of the homogenized ten- fold serial dilution of the fresh Palm wine samples and streaked on the surface of sterile modified MRS media under aseptic conditions. The inoculated media were incubated using anaerobiosis generator at 37°C for 24-48 h. After the incubation, distinct colonies were subcultured on MRS agar to obtain pure cultures. The purified isolates were streaked on MRS agar slants and stored at 4°C for further use.

Phenotypic characterization of the culture

The isolates were presumptively identified based on cultural, morphological and some biochemical characteristics. The parameters investigated included colony morphology, Gram reactions, endospore formation, catalase production, motility, and sugar fermentation. The results were compared to Holt et al. (1994)

Bergey's Manual of Determinative Bacteriology.

Genotypic characterization of the Isolates

DNA extraction and polymerase chain reaction (PCR) amplification

DNA extraction was carried out on test organisms' isolated from palm wine using the Jena Bioscience Bacteria DNA Preparation Kit (http://www.jenabioscience.com). Polymerase chain reaction was carried out to identify the suspected lactic acid bacteria isolated from palm wine using the primer pair BSF8 (AGAGTTTGATCCTGGCTCAG) and BSR534 (ATTACCGCGGCTGCTGC). The primer pair are lactic acid specific. The PCR reaction was carried out using the Solis Biodyne 5X HOT FIREPol Blend Master mix. PCR was performed in 25 µl of a reaction mixture, and the reaction concentration was brought down from 5x concentration to 1X concentration containing 1X Blend Master mix buffer Buffer (Solis Biodyne), 1.5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphates (dNTP)(Solis Biodyne), 25 pMol of each primer (BIOMERS, Germany), 2 unit of Hot FIREPol DNA polymerase (Solis Biodyne), Proofreading Enzyme, 5 µl of the extracted DNA, and sterile distilled water was used to make up the reaction mixture. Thermal cycling was conducted in a Peltier thermal cycler (PTC100) (MJ Research Series) for an initial denaturation of 95°C for 15 min, followed by 35 amplification cycles of 30 s at 95°C; 1 min. at 58°C and 1 min 30 s at 72°C. This was followed by a final extension step of 10 min at 72°C. The amplification product was separated on a 1.5% agarose gel and electrophoresis was carried out at 80V for 1 h 30 min. After electrophoresis, DNA bands were visualized using ethidium bromide staining. 100 bp DNA ladder was used as DNA molecular weight standard.

Identification of the isolates

All the isolates were identified using 16S rRNA. All PCR products were purified and sent to Epoch Life science (USA) for Sanger sequencing. The corresponding sequences were identified using the online blast search at http://blast.ncbi.nlm.nih.gov/Blast.cgi.

Immunological studies

Grouping and dosing of the animals

The animals were grouped into 6 groups of 5 animals (mice) per group. The first 4 groups received 0.2 ml of 3×10^7 cfu/ml of each of the isolates determined by 0.5 McFarland standard. The 5th and the 6th group represent positive and negative control groups respectively. The positive control received linex capsule (0.2 ml) a brand of lyophilized *Lactobacillus* spp. at a dose of 1.2×10^5 cfu/ml.

Studies on humoral antibody HA response

This is a slight modification of the method by Sharma et al. (1996). Mice were immunized by an intraperitoneal (ip) injection of 0.2 ml of 1×109 SRBC ml-1 on (day 0) and challenged by similar i.p injection of the same amount on day 5. The animals were bled and the serum analysed for the antibody titre by ELISA on days 10 and 15. Briefly 1 mg/ml of ova in bicarbonate buffer (pH 9.6) was prepared and used to coat the 96 well ELIZA plates and the plates incubated at 4°C for about 14 h. The plates were washed with PBS-Tween (0.05% Tween-20 in 0.1 M PBS). The plates were blocked with 20 µl/well of 5% v/v solution of fat free milk in PBS-Tween and incubated at room temperature for 1 h. The plates were washed 3 times with PBS-Tween. The sera from the animals were diluted 1:20 with 2% fat free milk and added 100 µl/well in duplicates. The plates were washed with PBS-Tween. 100 µl of 1:1000 dilution of HRP-antigoat Immunoglobulin were added to the wells and incubated for 1 h at room temperature. The plates were washed four times with PBS-Tween. 100 µl of the quenching agent TMT were added per well and allowed for at least 15-20 min and the plates read in ELIZA machine at 405 nm. The LAB isolates were administered 3 days prior to immunization and continued on a daily dose of 3×107 cfu/ml until the 14th day. The immunoglobulin studied included IgG, IgG1 and IgG2a.

Statistical analysis

The statistical analysis was done using Graph Pad prism version 5.0. One-way ANOVA followed by Post-hoc Dunnet were used to compare mean ± SEM, and values were considered significant at $p < 0.05$.

RESULTS

The morphological and biochemical characteristics of the isolated *Lactobacillus*

The morphological and biochemical characteristics of the isolated *Lactobacillus* are as shown in Table 1.

Genotypic and blasted sequence results of the isolates

The result of the blasted sequenced showed the presence of four species of *Lactobacillus* namely: *L. brevis*, *L. paracasei subsp. Tolerans*, *L. paracasei* and *L. yonginensi*. Primer pairs - BSF8 (AGAGTTTGATCCTGGCTCAG) and BSR534 (ATTACCGCGGCTGCTGC) was used to identify the suspected lactic acid bacteria isolated from the palm wine.

The effect of *Lactobacillus* spp. on antibody secretion

Figures 1 and 2 shows the effect of *Lactobacillus* spp on IgG antibody secretion 4 days and 9 days post-secondary challenge in mice. The isolates produced insignificant effects on IgG antibody secretion 4 days post-secondary challenge; however, there was a significant increase of 100.2, 122.9, 106.4 and 118.3% for *L. brevis*, *L. paracasei subsp. Tolerans*, *L. paracasei* and *L. yonginensi* respectively when compared with the negative control. The effects of *Lactobacillus* spp on IgG1 and IgG2a secretion 4 and 9 days post-secondary challenge is as shown in Figures 3 to 6.

L. paracasei subsp.Tolerans, L. paracasei and *L. yonginensis* produced a significant percentage increase of 30.1, 48.6 and 31.7% on the albino mice 4 days post-secondary challenge while only *L. yonginensis* produced a significant percentage increase of 39% 9 days

Table 1. Morphological and biochemical characteristics of the isolates.

Suspected Organism	Gram stain	Endospore Test	Catalase	Motility	Glucose Fermentation
I	+	-	-	-	+/G –ve
II	+	-	-	-	+/G –ve
III	+	-	-	-	+/G –ve
IV	+	-	-	-	+/G –ve

Positive; (-) negative; +/G +ve gas production = heterofermentation; +/G –ve, without gas production = homofermentation.

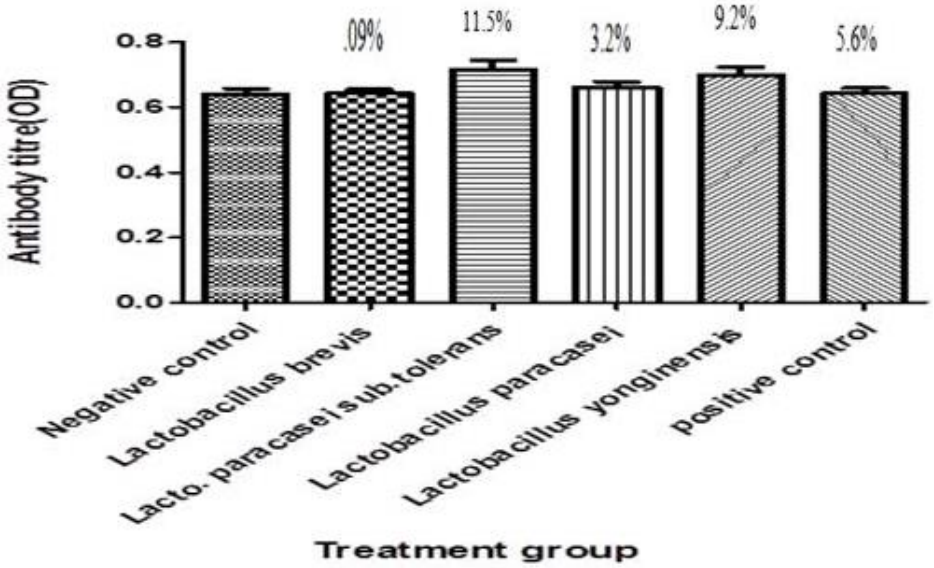

Figure 1. The effects of *Lactobacillus* spp on antibody titre (IgG) 4 days post-secondary challenge in mice.

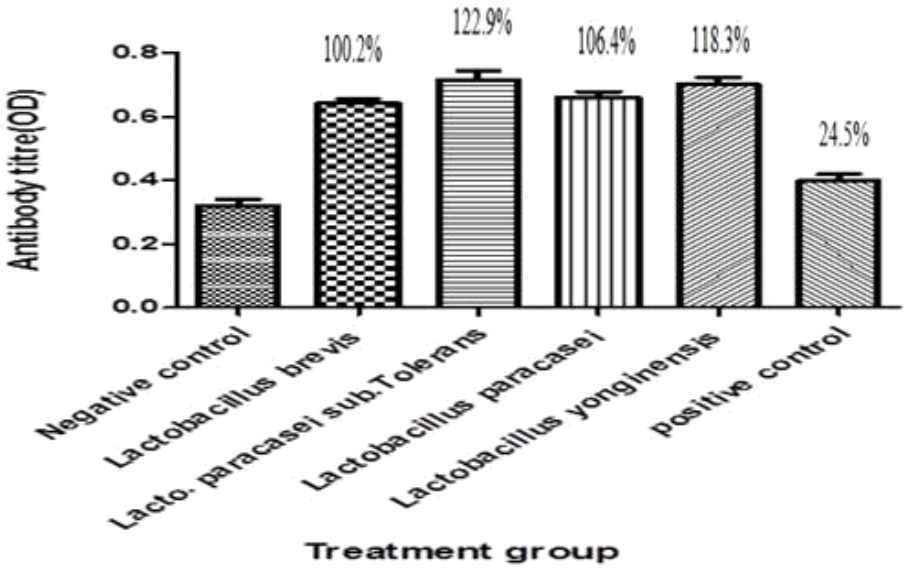

Figure 2. The effect of *Lactobacillus* spp on antibody titre (IgG) in albino mice 9 days post-secondary challenge.

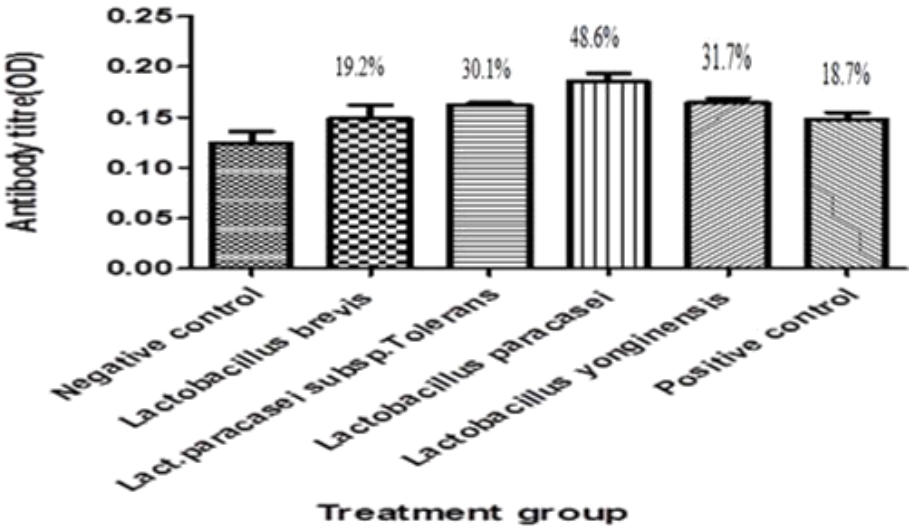

Figure 3. The effect of *Lactobacillus* spp on antibody titre (IgG1) on albino mice 4 days post –secondary challenge.

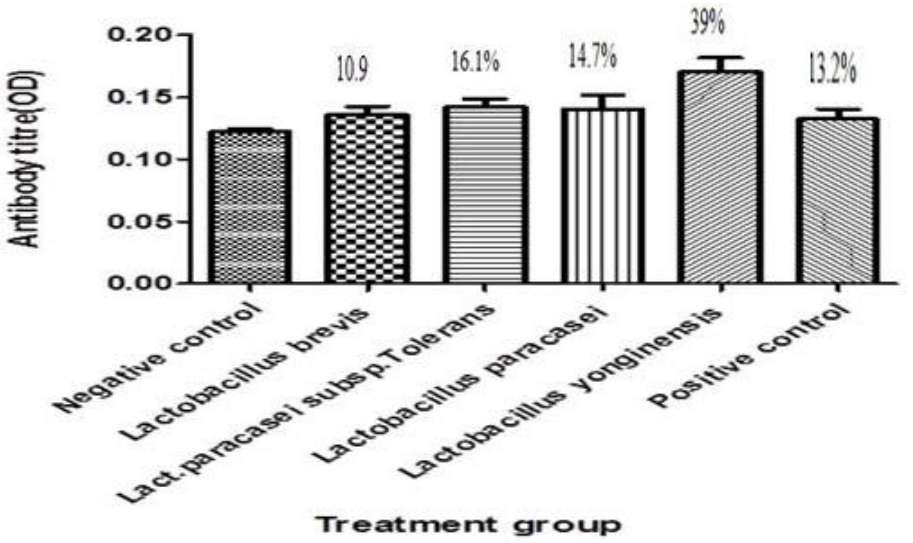

Figure 4. The effects of *Lactobacillus* spp. on antibody titre (IgG1) in albino mice 9 days post-secondary challenge.

post-secondary challenge when compared with the negative control. In the case of IgG2a there was no significant percentage stimulation at 4 and only *L. paracasei* produced significant percentage stimulation at 9 days post challenge.

DISCUSSION

Humoral immunity refers to antibody-mediated immune responses. Antibodies are produced by plasma cells and protect the host from infection in three main ways: by binding to pathogens to inhibit their toxic effects or infectivity (neutralization), by coating pathogens and facilitating their uptake and killing by phagocytes (opsonization) and by activating the complement cascade. Immunoglobulin G (Ig) is basically a major component of the circulating immunoglobulin. In mice it is sub classified into IgG1, IgG2a, IgG2b and IgG3 (Pelsue, 2019). The genotypic characterization and blasted sequence result confirmed the presence of *Lb. brevis, Lb. paracasei sub. Tolerans, Lb. paracasei* and *Lb. yonginensis.* The effects of the different *Lactobacillus* isolates on IgG, IgG1 and IgG2a secretion were

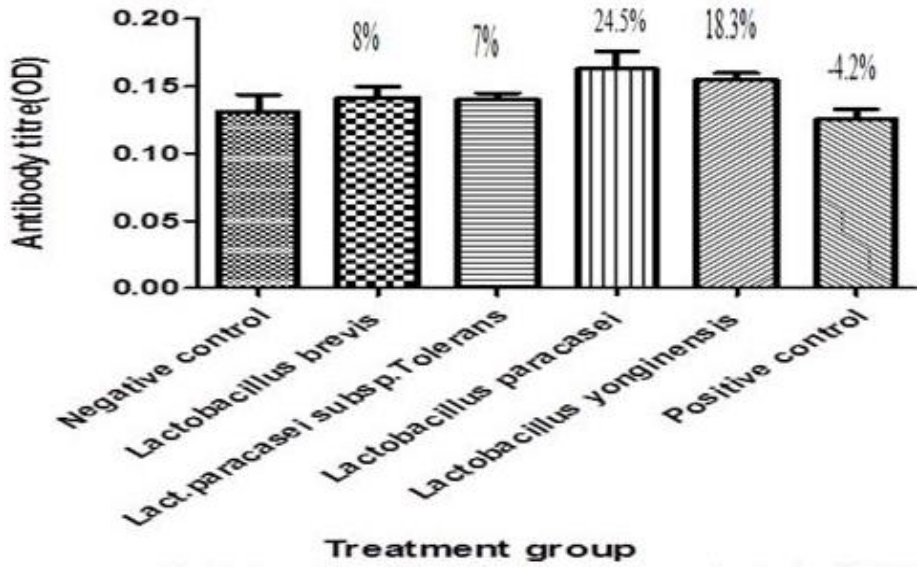

Figure 5. The effects of *Lactobacillus* spp on antibody titre (IgG2a) in albino mice 4 days post-secondary challenge.

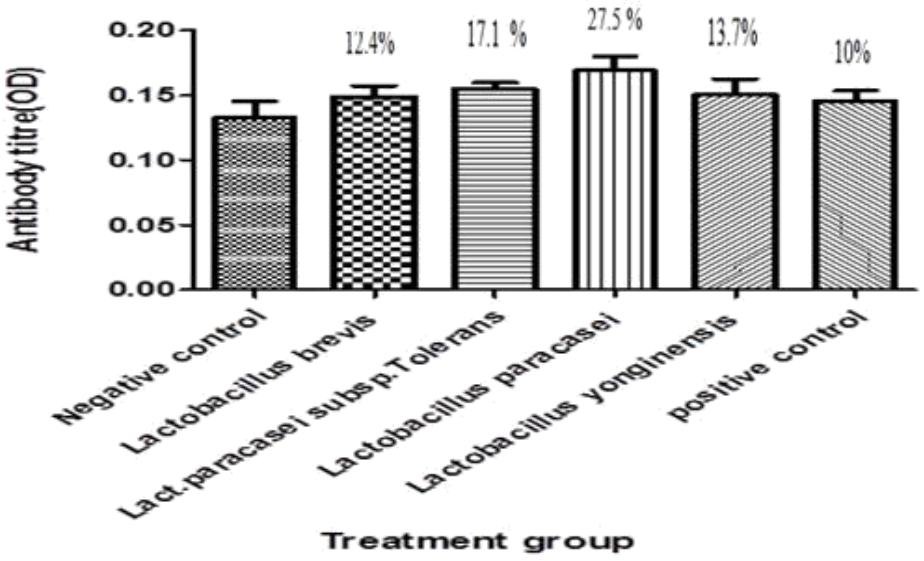

Figure 6. The effects of *Lactobacillus* spp on antibody titre (IgG2a) in albino mice 9 days post-secondary challenge.

investigated at 4 and 9 days post- secondary challenge on albino mice. The serum was analyzed for the antibody titre post-secondary challenge because IgM is the predominant immunoglobulin after initial exposure or post primary challenge. The result of the ELISA showed that there was an insignificant increase in the antibody titre for the different *Lactobacillus* species 4 days post-secondary antigenic challenge for IgG (Figure 1). However at 9 days post-secondary antigenic challenge for IgG, there was marked increase of 100.2, 122.9, 106.4 and 118.3% for *Lb. brevis, Lb. paracasei sub. Tolerans, Lb. paracasei,* and *Lb. yonginensis,* respectively (Figure 2). The non-significant effect experienced 4 days post challenge could be explained from the fact that the antibody secretion triggered by *Lactobacillus* may be T cell dependent. Antibody production that is T cell dependent involves a cascade of events thus: activated B and T cells remain complexed for 3 days and an exchange of signals, such as CD40/CD40L, leads to initiation of one of two fates for the B cell, some naïve B cells move into the extra

follicular region of the lymph node, where they differentiate into short-lived Plasma cells that produce only IgM as an initial response to infection while others migrate into the follicles where they form germinal centers (GC) (Paus et al., 2006). The GC is the site where B cells undergo affinity maturation, clonal expansion, and ultimately differentiate into high affinity, long lived plasma cells or memory B cells (Jackson and Elsawa, 2015). The sera of the experimental mice analyzed at 4 days post-secondary challenge showed that *Lb. brevis* produced an insignificant effect on IgG1 secretion while *Lb. paracasei sub. Tolerans, Lb. paracasei,* and *Lb. yonginensis* produced significant percentage increase as shown in Figure 3. The effect of the isolates on IgG1 9 day's post-secondary challenge showed that all the three isolates had insignificant effects on antibody production except *Lb. yonginensis* (Figure 4). Figures 5 and 6 showed that all the isolates produced no significant effect on IgG2a 4 days post- secondary challenge, however only *L. paracasei* produced significant antibody titre 9 days post challenge while the other three produced no significant titre.

The result of this experiment is in agreement with previous studies on the ability of *Lactobacillus* spp to stimulate antibody production (Davras et al., 2018; Easo et al., 2002). A comparison of the effects of the different isolates on the studied immunoglobulins, showed that the isolates produced better percentage stimulation of IgG and IgG1 when compared with the overall effect on IgG2a. This could be because in mice, IgG1 and IgE have been widely used as the surrogate markers of humoral antibody (T-helper [Th2] activation) responses as IL-4 secreted by Th2 cells induces Ig class switching into IgG1 and IgE subclasses (Wang et al., 2007). IL-4 plays an important role in antibody production by inducing the proliferation and differentiation of B cells into plasma cells. On the other hand, IgG2a and IgG3 is the surrogate markers of cellular immune response (Th1 activation) as IFN-γ produced by Th1 cells induces Ig class switching into IgG2a or IgG3 subclasses (Stevens et al., 1988).

Palm wine has shown that it is an alcoholic beverage with a difference since it is a natural habitat for immune enhancing, beneficial and generally regarded as safe (GRAS) bacteria, *Lactobacillus.* The four *Lactobacillus* spp were isolated from the fresh and sweet (high sugar content) beverage immediately (0-4 h) after fermentation. Therefore, since fresh palm wine has in abundance these beneficial bacteria, the locals who indulge in excessive consumption of fermented and soured palm wine should be educated on the health benefits of the fresh drink. This will enable them avoid the deleterious effects of alcohol while benefiting maximally from the enormous health benefit of this natural beverage.

Conclusion

Fresh *E. guinneesis* sap (palm wine) is home to *Lactobacillus* spp that has the capacity to positively stimulate the production of antibodies in mice.

RECOMMENDATION

The authors are proposing an increased research on this natural beverage especially as it concerns delay/control of fermentation by these beneficial bacteria. This will automatically transform this beverage to a healthier drink than alcoholic.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Characterization of crude invertase extracted from *Aspergillus* species CSA 35

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The enzyme invertase is very useful, especially in food industries. Invertase hydrolyzes the disaccharide sucrose into two monosaccharides, glucose and fructose. Invertase was extracted from *Aspergillus* sp. CSA35, and studied using spectrophotometric methods for enzyme kinetics and determination of other factors affecting enzyme activities such as salt tolerance, pH, temperature and substrate specificity. The invertase secreted by *Aspergillus* sp. CSA35 has a substrate concentration (km) value of 1.25 mg/ml and maximum velocity (Vmax) of 15.15 U/mg protein. It is tolerant to salt concentrations of 3 M NaCl for 24 h and shows a broad range of substrate specificity. The enzyme activity was optimal at pH 6.0 and at a temperature of 50°C. The Vmax of the enzyme was high compared to other invertases from fungal sources, previously reported in literature. This invertase could be useful in commercial processes where high activity invertase with salt-tolerant and broad specific properties are required.

Key words: *Aspergillus* sp CSA35, invertase, *Manihot esculenta.*

INTRODUCTION

Enzymes are biomolecules synthesized by the cells of an organism to allow biochemical reactions to occur under mild conditions and are known to catalyse many biochemical reactions (Li et al., 2018). They are generally considered to be very important in industrial processes (Roy and Prasad, 2017) due to the various advantages they have over inorganic catalysts. Some of these advantages include; high specificity, activity at mild pH and temperature conditions and reproducibility under laboratory conditions (Chapman et al., 2018).

A very useful enzyme used in industrial processes such as food processing is invertase (beta-fructofuranosidase, EC 3.2.1.26) (Ire et al., 2018). It is also known as saccharase, glucosucrase and beta-h-fructosidase (Ire et al., 2018). In food industries, invertase is usually preferred to glucose since they are sweeter and do not easily crystallize (Flores-Gallegos et al., 2012). Another enzyme related to these invertases are sucrases. Invertases and sucrases are known to hydrolyze sucrose, resulting in the same mixture of glucose and fructose which is commonly referred to as invert sugar (Bhalla et al., 2017). Invertases thus cleave the O-C (fructose)

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bond, whereas the sucrases cleave the O-C (glucose) bond (Schiweck et al., 2000). There are many industrial applications of invertase, amongst which includes its use in confectionary, beverage, bakery and pharmaceutical formulations (Kulshrestha et al., 2013; Nadeem et al., 2015).

For Industrial uses, enzymes are usually produced from microbial, animal or plant sources (Nascimento et al., 2019). In the majority of related research in literature, invertase was produced from microbial sources. Of these microbial sources, different species of Aspergillus have been utilized in synthesizing enzymes. For optimized conditions, Uma et al., (2010) observed high levels of invertase from *Aspergillus flavus* in submerged cultures. Also, high yields of thermostable extracellular invertase were obtained when *Aspergillus ochraceus* was cultured in Khanna medium (Khanna et al., 1995; Guimarães et al., 2007). Other species includes *Aspergillus niger* which was used by Dinarvand et al., (2012) to synthesize both Inulinase and Invertase.

Production of enzymes is labour-intensive and requires tedious experiments in the laboratory in order to determine optimum conditions. Production generally involves the use of high value added substrates which makes enzyme production not only a tedious but an expensive process (Nascimento et al., 2019). In order to reduce the high cost of production, most researchers have turned to agro industrial residues as substrate for enzyme production. Agro industrial products such as cassava flour and wheat bran was used by Giraldo et al., (2012) to produce high yields of invertase using *Paecilomyces variotii.* All of these prompted Oyedeji et al., (2017) to suggest that residues from agro industrial by-products are good candidates for use as substrates for production of enzymes and as well as other value added metabolites.

This study was aimed at producing invertase enzyme from *Aspergillus* CSA35 isolate and to evaluate parameters such as salt tolerance, kinetic properties, pH, temperature and substrate specificity in order to determine optimum conditions in which this crude invertase extracted from *Aspergillus* sp.CSA35 would maximally express its catalytic activity.

MATERIALS AND METHODS

Fungal isolate

The fungus, *Aspergillus* sp. CSA35 used in this study was provided by African Research Laboratories, Otorho-Agbon Delta State, Nigeria. It has previously been identified using microbiology and molecular biology techniques by Avwioroko et al., (2015), and studied for α amylase formation.

Preparation of invertase production medium

For invertase production, the fungus was grown in a medium made using the procedure of Uma et al. (2010) with little modification to contain 0.03 g of Ammonium ferrous sulphate, 0.14 g of potassium dihydrogen phosphate, 1 g of sodium nitrite, 0.03 g of magnesium chloride, 0.05 g of potassium chloride and 10 g of sucrose. All these were added into a 250 ml conical flask and dissolved with distilled water up to the 100 ml mark. Sterilization was done by autoclaving at 121°C for 15 min and cooled in a cold water bath.

Fungal growth and invertase production

The *Aspergillus* species CSA35 isolate was incubated for between 5 and 7 days. Fungal growth was filtered from the liquid medium using Whatman No. 1 filter paper, conical flask and funnel and used as the crude extract for enzyme assay.

Invertase activity assay

For invertase activity assay, the procedure of Bacon (1955) was used with slight modifications. 1 ml of crude invertase extract was collected and measured into a test tube and 1 ml sucrose (1% W/V) solution dissolved in acetate buffer pH 5.6 was added. The mixture was incubated for 15 min at 40°C and 2 ml of DNS reagent was added. The mixture was shaken and boiled for 5 min at a temperature of 95 to 100°C. Thereafter, 300 µl of Rochelle salt (40% sodium potassium tartrate) was added to stabilize the colour. Absorbance was read at 540 nm.

Determination of invertase salt tolerance

The effect of varying salt concentrations on crude invertase activity was determined. The crude invertase was mixed with different concentrations of NaCl (1, 2 and 3 M) and incubated at 4°C for 24 h; the substrate was added and invertase activity was determined.

Determination of kinetic parameters for crude invertase

The kinetic parameters [Michaelis-Menten constant (Km) and maximum velocity (Vmax)] of invertase crude activity from *Aspergillus* sp. CSA 35 was determined individually from line weaver Burk plot at a temperature of 40°C, pH 5.7 for sucrose concentration ranging from 1 to 5 mg/ml. Km and Vmax were investigated experimentally by measuring the rate of catalysis (V) for substrates at various concentrations (S). A straight line graph was obtained by plotting 1/V vs 1/S. The evaluation of this graph yielded the kinetic parameters for the crude invertase.

Procedure for substrate specificity

The specificity of the invertase enzyme obtained from the fungi was investigated by exposing the enzyme to other disaccharides or carbohydrate-containing substrates like table sugar, starch and sucralose. 1% W/V solution of the above substrates dissolved in acetate buffer was prepared and used as the substrate and the enzyme assay was carried out.

Effect of pH on invertase activity

To determine the effect of pH on invertase activity as well as the optimum pH for invertase activity, the effect of pH was studied under different pH ranging from 3.0 to 10.0 and the absorbance was read at 540 nm.

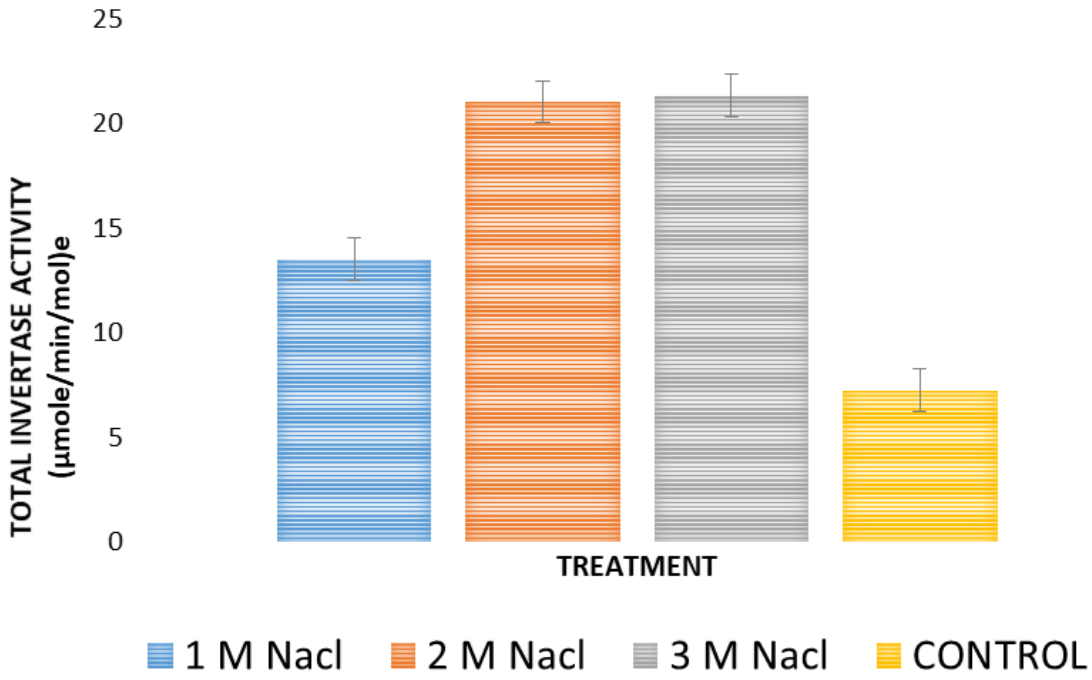

Figure 1. *Aspergillus* sp. CSA 35 invertase salt tolerance for 24 h.

Effect of temperature invertase activity

To determine the effect of temperature on invertase activity, invertase activity was measured at different temperatures ranging from 30 to 70°C using the same procedure described previously.

Statistical analysis

The results obtained were expressed in mean ± standard deviation and comparison of means for significant differences was done using One-way analysis of variance (ANOVA). Mean differences were said to be statistically significant at $P<0.05$.

RESULTS

Effect of salinity on invertase activity

The ability of invertase from *Aspergillus* species CSA 35 to withstand various concentrations of sodium chloride (1, 2 and 3 M) for 24 h was tested. The result (Figure 1) showed that activities of invertase increased with increasing salt concentration for 24 h.

Kinetic constants

Michaelis-Menten type kinetics was shown by the invertase isolated from *Aspergillus* species CSA 35. As calculated from the Lineweaver–Burk plots (Figure 2), the Vmax of invertase of *Aspergillus* sp. was 15.15 U/mg protein, while the affinity for substrate shown by invertase was 1.25 mg/ml.

Invertase substrate specificity

The specificity of enzyme activity was investigated by exposing the enzyme to other disaccharide containing substrates such as starch (containing amylose and amylopectin), table sugar (sucrose) and sucralose (chemically modified sucrose). It should be noted that all substrates were selected such that they contain mainly glucose (in the case of starch) or glucose and fructose (for sucrose and sacrulose) as their major components. Results obtained shows that crude invertase extracted from this species of *Aspergillus* shows a broad range of specificity as no significant difference was found in its ability to degrade other disaccharide containing substrates (Figure 3).

Effect of pH on invertase activity

The effect of pH on invertase was determined (Figure 4). There was a sharp rise in the activity of invertase from pH 3 to 4. Upon an increase in pH from 4 to 5, a slight increase in activity was again observed. This trend continues up till pH 6 when the maximum invertase activity was achieved and thereafter a rapid decline in enzyme activity with the lowest activity for the pH assayed observed at pH 9 (Figure 4).

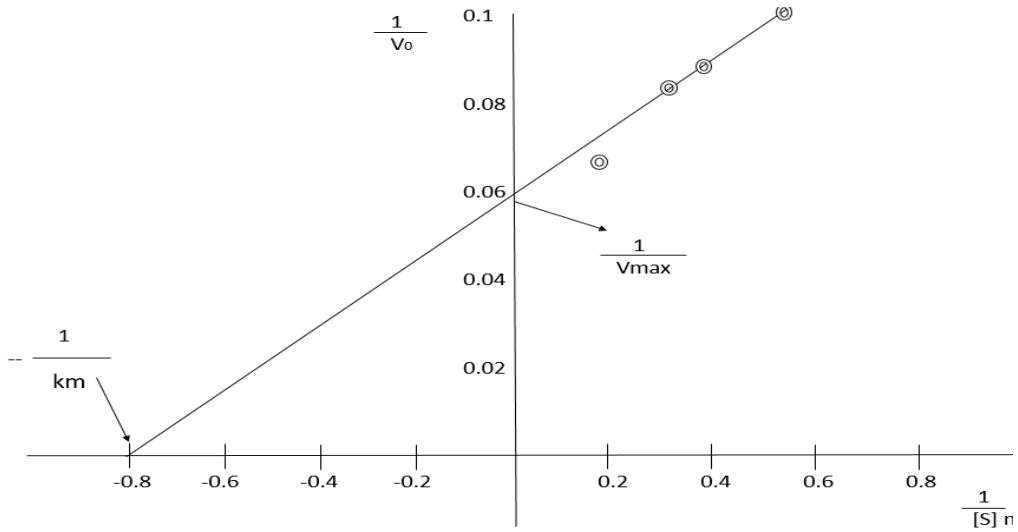

Figure 2. Lineweaver- Burk plot showing kinetic constants.

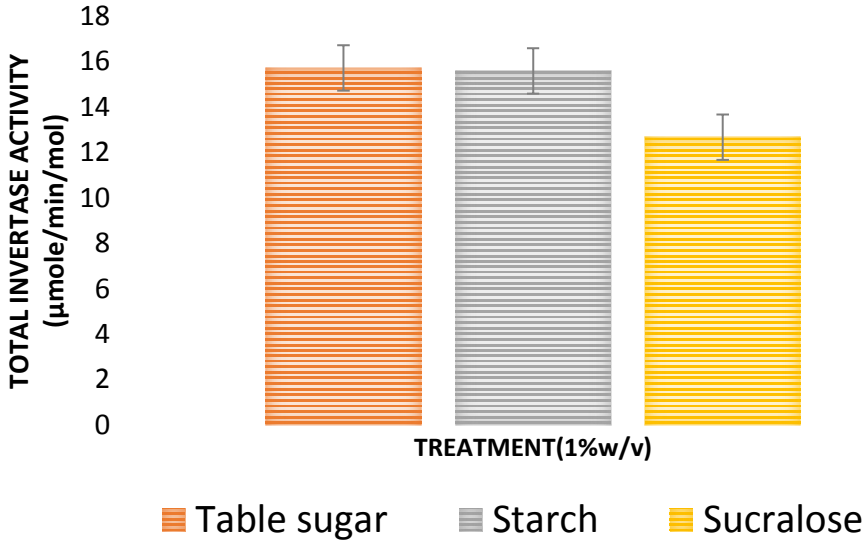

Figure 3. Aspergillus species CSA35 crude invertase substrate specificity.

Effect of temperature invertase activity

The effect of temperature on this enzyme is shown in Figure 5. Temperature ranging from 30 to 70ºC was used and it was observed that invertase activity was at its maximum at 50°C and the lowest activity was observed at 30°C (Figure 5).

DISCUSSION

In the present study, the ability of invertase from *Aspergillus* species CSA35 to tolerate different sodium chloride (NaCl) concentration was observed. Concentration ranging from 1 to 3 M was used and, invertase activity increased by increasing salt concentration from 1 to 2 M. However, on increasing NaCl concentration from 2 to 3 M, there was no significant difference in invertase activity. This could possibly suggest that beyond 2 M concentration, NaCl has no positive impact on invertase activity. Therefore, since invertase from *Aspergillus* species CSA35 can tolerate high salt concentration of 3 M for up to 24 h, it could be useful in industrial applications where such high salt treatments are required. Also, kinetic parameters (Km and Vmax) were determined at 37°C and pH 5.6 for

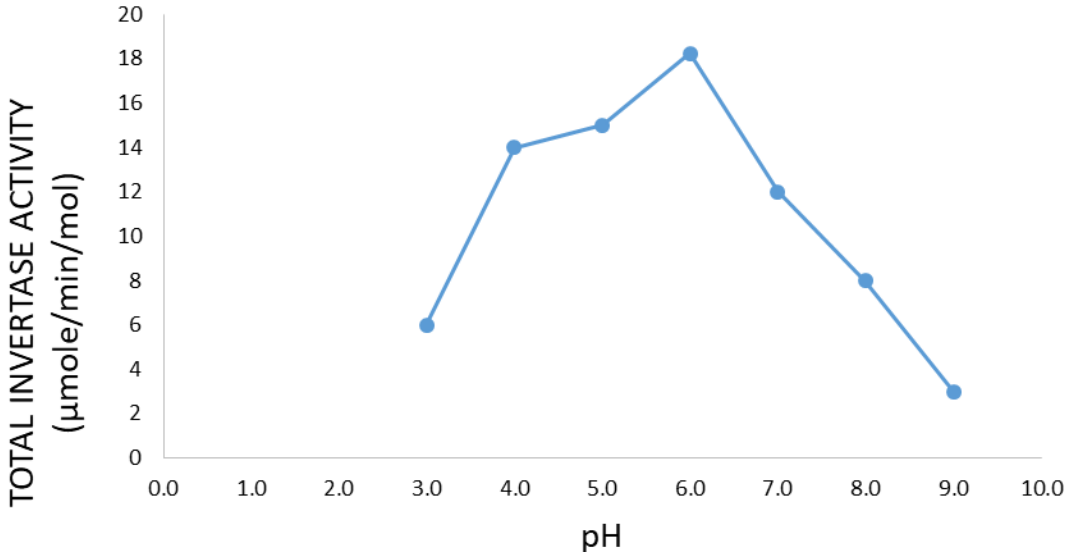

Figure 4. Effect of pH on crude invertase activity.

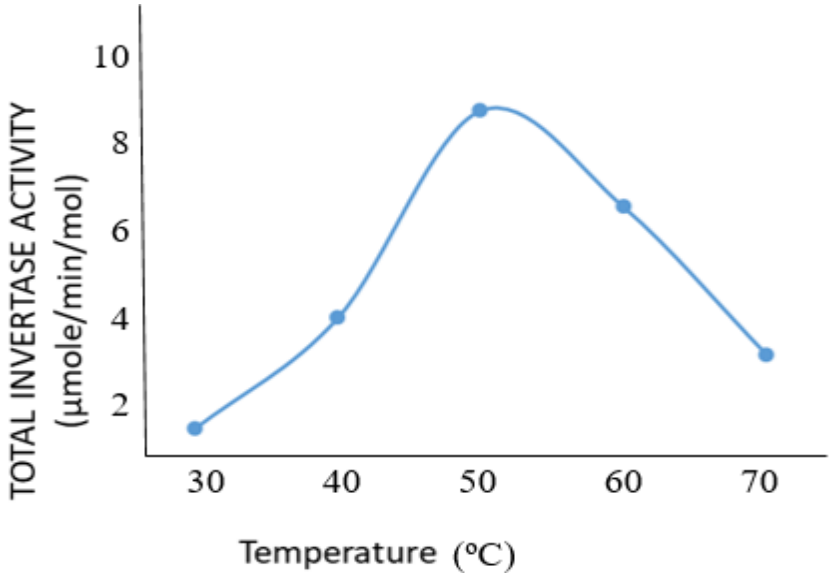

Figure 5. Effect of temperature on crude invertase activity.

Aspergillus CSA 35 for various concentrations ranging from 1 to 5 mg, using sucrose as substrate. The Km and Vmax values were 1.25 mg/ml and 15.15 U/mg protein, respectively. In a related research carried out by Uma et al., (2010), Km value of 0.23 mg/ml and Vmax of 15.8 U/mg were observed. Though the Vmax obtained from this study was similar to previous findings, the difference in Km value could likely be attributed to other factors such as different experimental conditions or even a difference in the source of invertase. These kinetic constants are at variance with those reported in a similar work by L'Hocine et al., (2000), whose Km and Vmax values were 0.6894 mg/ml and 0.3201 U/mg protein using *Saccharomyces cerevisae* MTCC 170. In another study, Hernalsteens and Maugeri (2008) reported a Km of 13.4 g/l and Vmax of 21 U/mg protein for sucrose by invertase in Candida species. It can thus be inferred that these km and Vmax values for invertase varies from one microorganism to another.

The hydrolytic activity of invertase from *Aspergillus* species CSA35 shows no significant difference in its ability to degrade sucrose or any disaccharide containing substrate. From Figure 3, table sugar (sucrose) shows a higher invertase activity when compared to sacrulose

which is a modified form of sucrose. The biochemical rationale behind this could be accounted for. It could probably be due to the fact that the refined sacrulose having undergone refinement processes and other forms of chemical modifications resulted in a lower rate of hydrolysis when compared to the normal sucrose, hence a lower invertase activity was observed for sacrulose. Hydrolysis of α-glucans such as starch also indicated a good invertase activity. It could therefore be said that *Aspergillus* sp.CSA35 invertase showed a broad range of substrate specificity. Also, other factors such as substrates molecular size and structure, type of bond in chain may also affect enzyme activity (Jackson et al., 2010).

On having a first glance of pH effect on invertase activity, it can be observed that maximum invertase activity was obtained at pH 6. This result agrees with Uma et al., (2010) and Patil et al., (2012), even though the microorganism used in their respective studies were different (*Cladosporium cladosporioides* for the former *and Aspergillus* sp M1 *for* the latter). Different results have also been reported from other related research. Shaker (2015) revealed that invertase extracted from *Aspergillus terreus* expresses it maximum activity at pH 2. Having optimum activity at such a low pH is not surprising, judging from the fact that this organism is being used extensively in producing useful organic acids. Similarly, Qureshi et al., (2012) recorded optimum activity for invertase extracted from *Mucorgeophillus at* pH 5 while Talekar et al., (2010) reported optimum activity at pH of 4.2 by *Saccharomyces cerevisiae* invertase*.* Generally, it could therefore suggest that differences observed could be attributed to the different sources of invertase.

The effect of temperature on invertase activity seems to vary from organism to organism because optimum temperature observed in this study was 50°C and this differs from related studies in literature where different microorganisms were used. Shaker (2015), worked on *A. terreus* and reported an optimum pH of 60°C while Talekar et al., (2010), observed peak invertase activity at a temperature of 30°C by *S. cerevisiae* MTCC 170.

Conclusion

Invertase from *Aspergillus* sp.CSA35 showed a broad range of substrate specificity and was salt tolerant to 3 M NaCl for 24 h even though there was no increase as was noticed when concentration was increased from 1 to 2 M. The Vmax of the enzyme was similar to many others from fungal sources previously reported in literature. Invertase activity was optimal at pH 6.0 and 50ºC. The findings in this study could be useful in industrial and commercial processes such as the production of confectionary, beverage, bakery products and pharmaceutical formulations in which high invertase activity with these properties are desired.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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