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African Journal of Microbiology Research

September 2019
ISSN 1996-0808
DOI: 10.5897/AJMR
www.academicjournals.org

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Vol. 13(27), pp. 464-473, September, 2019
DOI: 10.5897/AJMR2019.9164
Article Number: 2CC499E61808
ISSN: 1996-0808
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African Journal of Microbiology Research

Full Length Research Paper

Antimicrobial and antioxidant activities of endophytic fungi extracts isolated from *Carissa carandas*

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Received 2 July, 2019; Accepted 22 August, 2019.

This is the first report of endophytic fungi derived from *Carissa carandas* producing bioactive compounds in Thailand. The aims of this research were to evaluate the antimicrobial and antioxidant activities of extracts from endophytic fungi, identify the potential fungal isolates by phylogenetic analysis and analyze the composition of the potential crude extract by gas chromatography-mass spectrometry (GC-MS). The endophytic fungus *Nigrospora guilinensis* TSU-EFHA009 produced the most active extracts. Broth ethyl acetate extract (BE) had the strongest activity against *Cryptococcus neoformans* at a minimum inhibitory concentration (MIC) of 4 µg/mL and a minimum fungicidal concentration of 8 µg/mL. Moreover, the antimicrobial activity was confirmed using scanning electron microscopy. The target cells were morphologically damaged. In addition, this active extract had the highest antioxidant activity with an inhibitory concentration (IC_{50}) value of 0.03 mg/mL. The total phenolic content of the target extract was detected by using the colorimetric method. This extract contained a total phenolic content of 41.20±0.40 mg GAE/g of the extract. The results indicated that the endophytic fungi from *C. carandas* are good sources of antimicrobial and antioxidant substances.

Key words: Antimicrobial activity, antioxidant activity, endophytic fungi, active metabolites.

INTRODUCTION

Nowadays, drug resistant microorganisms and free radical agents are gaining more attention. They lead to infectious diseases in humans and to various other diseases (cancer, asthma and cardiovascular disease). Infectious disease and free radicals are important issues found in all regions of the world (Racek et al., 2001; Hubalek, 2003; Lobo et al., 2010; Lindahl and Grace, 2015). Increasing attention has been paid to natural products, especially those from fungal endophytes. It is well documented that fungal endophytes are a good source of bioactive natural compounds that are effective, have low toxicity, and cause a minimum environmental impact (Jalgaonwala et al., 2011; Nisa et al., 2015). There are several definitions of endophytic fungi; one of the recent definitions is that it is fungus which colonizes host plant tissue without visible symptoms (Jia et al., 2016; Gouda et al., 2016). Recent studies have reported that the functional potential of fungal endophytes is derived from many terrestrial plant species (Joseph and Priya, 2011). In some cases, novel compounds from fungal

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endophytes showed strong antimicrobial activities against pathogenic microorganisms such as antibacterial, antifungal and anti-parasitic, and had strong antioxidant activities (Ascencio et al., 2014; Brissow et al., 2017; Raunsai et al., 2018). Active compounds (trichodermin and volatile compound) isolated from the fungal endophyte *Trichoderma* species had a strong activity against pathogenic bacteria and pathogenic fungi (Leylaie and Zafari, 2018). Fusaripeptide A isolated from the fungal endophyte *Fusarium* species which is isolated from roots of *Mentha longifolia* L. showed strong activity against *Plasmodium falciparum* (antimalarial) with an IC_{50} value of 0.34 µM, and against *Candida albicans, Candida glabrata, Candida krusei* and *Aspergillus fumigates* (antifungal) with IC_{50} values of 0.11, 0.24, 0.19 and 0.14 µM, respectively (Ibrahim et al., 2015). Furthermore, fungal endophytes can produce active antioxidant compounds (Huang et al., 2007; Khiralla et al., 2015). Bioactive natural products from fungal endophytes, in particular from *Carissa carandas*, have been rarely studied (Yadav et al., 2014; Tenguria and Firodiya, 2015, 2016). Previous studies on fungal endophytes which were isolated from *C. carandas* have been focused on biodiversity, distribution and cytotoxicity of endophytic extracts (Tenguria et al., 2012; Tenguria and Firodiya, 2015).

Carissa is a medicinal plant belonging to the Apocynaceae family comprised 20 to 30 species and is found in many parts of Asia, Africa and Australia. The common species of this genus are *C. carandas, Carissa macrocarpa, Carissa grandiflora, Carissa edulis, Carissa spinarum, Carissa lanceolata, Carissa opaca, Carissa congesta* and *Carissa bispinosa*. Many parts of this plant (stem, root, bark, fruit and seed) have been used in traditional medicine for thousands of years. Its fruit are used as a treatment for various ailments such as liver dysfunction, fever, digestion system problems and diarrheas (Arif et al., 2016). Recently, Toobpeng et al. (2017) reported the antibacterial activity of fruit extracts against *Pseudomonas aeruginosa, Klebsiella pneumonia, Escherichia coli, Staphyloccus aureus*, methicillin-resistant *S. aureus, Acinetobacter baumannii*, *Enterococcus faecalis,* and *Schleichera oleosa*. However, the biological activities, especially, antimicrobial and antioxidant activities of fungal endophytes from *C. carandas* have not been studied.

Thus, this study aimed to isolate endophytic fungi from healthy fresh leaves of *C. carandas* and to screen these microorganisms for their ability to produce antimicrobial metabolites against human pathogens and antioxidant substances that inhibit or delay the oxidation of biologically relevant molecules.

MATERIALS AND METHODS

Sample collection and fungal isolation

Healthy leaf samples of *C. carandas* were randomly collected during January to March 2018 from Trang, Thailand (7° 33' 22.79" N; 99° 36' 41.08" E). Samples were surface-sterilized with 10% ethanol (5 min), 3% sodium hypochlorite (15 s), and 10% ethanol (5 min), and rinsed with distilled water and dried on sterile tissue paper. Each of the leaf parts was cut into five segments (0.5 cm^2) and put onto Potato Dextrose Agar (PDA) provided with antibiotics (50 mg/L penicillin and streptomycin). Plates were incubated at room temperature for 4 weeks. Fungal isolates were subcultured in PDA without antibiotics until they were pure cultures. All fungal isolates were identified and selected for further study based on their morphological characteristics after the incubation period. Each pure fungal isolate on PDA was cut into small pieces and maintained in 20% glycerol at -80°C.

Fermentations and extractions

Fermentation and extraction methods were conducted according to Supaphon et al. (2010, 2013) with some modifications. Six agar plugs (1 cm^2) of fungal mycelium were inoculated in 500 mL Erlenmeyer flasks containing 250 mL potato dextrose broth (PDB) and were incubated for 3 weeks at room temperature for production of metabolites. The culture broth was filtered to separate the filtrate and mycelia. The filtrate was extracted with an equal volume of ethyl acetate (EtOAc) in a separating funnel two times. The combined EtOAc extracts were evaporated to dryness under reduced pressure at 45°C using a rotary vacuum evaporator to obtain the broth ethyl acetate extract (BE extract). The fungal mycelia were soaked in 500 mL of methanol (MeOH) for 3 days. The aqueous MeOH layer was concentrated using rotary evaporator to give the aqueous layer. The aqueous layer was extracted with an equal volume of hexane two times, followed by EtOAc twice. The combined EtOAc and hexane extracts were evaporated to dryness under reduced pressure at 45°C using a rotary vacuum evaporator to obtain the cell ethyl acetate and cell hexane extracts (CE and CH extracts). The stock extracts were kept in vials at 4°C. Working extracts were kept in vials at room temperature until used.

Antimicrobial assay

The dried extracts were dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions at 100 mg/mL. All the extracts at a final concentration of 200 µg/mL were screened for their antimicrobial activity against ten pathogenic microorganisms, including *S. aureus* ATCC25923, a clinical isolate of methicillin-resistant *S. aureus* (MRSA) SK1, *E. coli* ATCC25922, and *P. aeruginosa* ATCC27853, *C. albicans* ATCC90028, *C. albicans* NCPF3153, *Cryptococcus neoformans* ATCC90112, *C. neoformans* ATCC90113, a clinical isolate of *Microsporum gypseum* and *Talaromyces marneffei* by the colorimetric microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) with slight modifications (CLSI 2008, 2012). Microtiter plates were incubated at 35°C for 15 h, then 30 µL of resazurin solution (0.18%) was added to each well and incubated for 3 h under the same conditions (Supaphon et al., 2018). After incubation, the results were recorded as positive (blue color indicated growth inhibition) and negative (pink color indicated microbial growth). After that, the active extracts from the screening test were determined by the same method for the minimum inhibitory concentrations (MICs) at a concentration range of 0.25 to 128 µg/mL. The lowest concentration of extract that inhibited growth was recorded as MIC. The concentration of extract at MIC and more than MIC were determined by streaking onto nutrient agar (NA) plates for bacteria, Sabouraud's dextrose agar (SDA) plates for yeasts and PDA plates for filamentous fungi and incubated under appropriate conditions. The lowest concentration of extract that showed no growth was recorded as the minimum bactericidal

concentration (MBC) for yeast and the minimum fungicidal concentration (MFC) for filamentous fungi. Commercial antibiotics were used as standard agents for positive inhibitory controls (vancomycin for Gram-positive bacteria, gentamicin for Gram-negative bacteria, amphotericin for yeasts and *T. marneffei* and miconazole for *M. gypseum*).

Scanning electron microscopy analysis

The effect of the most active extract on cell morphology was determined using scanning electron microscopy (SEM). The sample was prepared according to previous studies with slight modifications (Supaphon et al., 2018). Briefly, cell suspension (10^8 CFU/mL) was treated with four times MIC concentration of the active extract and incubated for 24 h. For the controls, antibiotics and DMSO were used as positive and negative controls, respectively. The treatments were fixed with 2.5% glutaraldehyde in a phosphate buffer solution for 1 h and washed three times with PBS, pH 7.2. Each treatment was serially dehydrated with 50, 70, 80, 90 and 100% ethanol. Then, cell samples were dried in a lyophilizer, smeared on a silver stub, mounted with gold and observed using SEM at the Scientific Equipment Center, Prince of Songkla University.

Gas chromatography-mass spectrometry analysis (GC-MS)

GC-MS was used to determine the active extracts. This experiment was performed according to the previous study with some modifications (Supaphon et al., 2018). The separation and identification of the compounds (extract from *Nigrospora guilinensis*) used the HP5MS capillary column (30 m × 0.25 mm ×0.5 µm). The temperature program was performed as follows: initial temperature of 50°C (2 min), raised to 160°C at the rate of 8°C/min (5 min), then raised to 270°C at the rate of 8°C/min (8 min), Helium was used as the carrier gas at the rate of 10 mL/min. The fragmentation of the MS range from 40 to 1000 m/z was conducted by electronic impact mode (ionization energy, 70 eV, 300°C) and scanned at the rate of 3.0 scans/s. GC-MS was analyzed for 50 min.

Determination of antioxidant activity (DPPH) assay

1, 1- diphenyl-2-picryl-hydrazyl (DPPH) scavenging activity of endophytic fungi extracts was evaluated according to the previous report of Yadav et al. (2014) with slight modifications. Briefly, the DPPH solution was prepared by dissolving 2.4 mg DPPH in 100 mL methanol, and the stock solution was kept at -20°C until used. The extract solution at a concentration of 10 mg/mL (50 µL) was added to 50 µL of 1 mM DPPH solution in 96-well microtiter plates. The mixture was shaken and stored at room temperature for 30 min in the dark, and then the absorbance was recorded at 515 nm using a spectrophotometer. For the control, the absorbance of ascorbic acid solution was used as a standard for making the calibration curve using 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL concentrations of the standard. All determinations were performed in triplicate. The percent scavenging activity was calculated using the formula: $[A_0-A_s/A_0] \times 100$, where A_0 and A_s represent the absorbance values of the control and extract, respectively. The active extracts that provided ≥50% scavenging activity were identified by interpolation from linear regression analysis.

Determination of total phenolics contents

The total phenolic content of the broth ethyl acetate extracts was measured as described by Yadav et al. (2014) with some modification. The extracts were prepared and diluted from stock solutions (1 mg/mL). 100 µL of fungi extract was diluted with 7 mL water and then was mixed with 500 µL of Folin-Ciocalte. The mixture solution was incubated at room temperature for 4 min. Then 1.5 mL of sodium carbonate (7.5%) was added and kept in the dark at room temperature for 2 h. The results were recorded by measuring the absorbance at 765 nm using a spectrophotometer (Thermo Scientific). Gallic acid was used as positive control, and a standard curve at the concentrations of 0.2, 0.4, 0.6, 0.8 and 1 mg/mL in 70% of methanol was constructed. The results were reported as mg gallic acid equivalent dry weight (GAE/g of extract). Each treatment was performed in triplicate.

Molecular traits

The most active isolate as determined by the antimicrobial and antioxidant activity assays was further identified by molecular methods based on ITS sequence analyses. Extraction of fungal mycelium used the modified CTAB method from O'Donnell et al. (1997). Afterwards, the qualities of genomic DNA were estimated with 1% agarose gel electrophoresis in 1% TAE buffer. Internal transcribed spacer (ITS) rDNA was amplified by PCR with the primer pairs ITS5/ITS4 (White et al., 1990). The PCR reactions used Taq DNA polymerase (Thermo Scientific) following the manufacturer's instructions and were performed in a T100TM Thermal cycler (BIO-RAD laboratories, Inc). Furthermore, the amplification conditions of ITS rDNA were performed as follows: initial denaturation at 94°C for 2 min, followed by 35 cycles at 94°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 2 min, with a final extension period of 72°C for 10 min. The PCR products were checked on 1% agarose electrophoresis gels and stained with RedSafe DNA stain (20,000X). After that, PCR products were purified by Macrogen Inc. in South Korea for direct DNA sequencing.

Phylogenetic analysis

Nucleotide sequences in this study were compared with the related sequences from GenBank (http://www.ncbi nlm.nih.gov) and following Wang et al. (2017). All sequences were assembled with BioEdit version 7.2.5 (Hall, 2005) and aligned with Muscle program version 3.8 (Edgar, 2004). While, the phylogenetic tree was constructed by maximum parsimony (MP) and maximum likelihood (ML) analyses. Maximum parsimony analyses were performed in PAUP*4.0b10 (Swofford, 2002). The most parsimonious trees (MPTs) results were evaluated, followed by heuristic searches: 100 replicates of random stepwise addition of sequence, branch-swapping algorithm: tree-bisection-reconnection (TBR) and equal weight characters. Maximum parsimony bootstrap supports of the clades were approximated by 1000 replicates (stepwise addition of sequence, 10 replicates of random addition of taxa, TBR branching-swapping algorithm). Additionally, the maximum likelihood and bootstrap analyses were generated on the CIPRES web portal (Miller et al., 2010) through RAxML 8.2.4 (Stamatakis, 2014) with the BFGS method to optimize GTR rate parameters. The phylograms were visualized using FigTree v1.4.3 (Rambaut, 2016). Moreover, the sequences analyzed in this study were deposited in the GenBank databases and are shown in Table 4. The alignment result was submitted to TreeBASE (submission number: 24397).

RESULTS AND DISCUSSION

Fungal isolation

One-hundred and nine fungal isolates from 900 segments

of *C. carandas* were identified based on their morphology as *Penicillium,* 20 isolates; *Cladosporium,* 13 isolates; *Fusarium*, 6 isolates; *Curvularia*, 5 isolates; and *Nigrospora* 3 isolates. The remaining fungal isolates (62 isolates) did not produce any reproductive structure and were classified as unidentified endophytic fungi. All endophytic fungal isolates were grouped into 36 morphotypes based on their morphology. Representative isolates were selected from each group for antimicrobial and antioxidant activity tests.

Antimicrobial assay

The preliminary screening of crude extracts (108 extracts) from selected endophytic fungi (36 isolates) revealed the presence of bioactive compounds. The antimicrobial activity was determined by using the colorimetric broth microdilution assay. The activities of crude extracts were evaluated at a concentration of 200 µg/mL. Fourteen extracts (BE = 6, CE = 5 and CH = 3) exhibited significant inhibition against at least one test microorganism. The MIC test showed potential antagonism against two strains of Gram-positive bacteria (*S. aureus* ATCC25923 and a clinical isolate of methicillin-resistant *S. aureus* (MRSA) SK1), four strains of yeasts (*C. albicans* ATCC90028, *C. albicans* NCPF3153, *C. neoformans* ATCC90112 and *C. neoformans* ATCC90113) and two strains of filamentous fungi (clinical isolate of *M. gypseum* and *T. marneffei*) at concentrations ranging from 4 to 128 µg/mL, but not against Gram-negative bacteria (*E. coli* ATCC25922 and *P. aeruginosa* ATCC27853). The BE from isolate TSU-EFHA009 inhibited the growth of *C. neoformans* ATCC90112 at the lowest concentration of 4 µg/mL and produced an MFC at the lowest concentration of 8 µg/mL; while this extract inhibited other test microorganisms at moderate to high concentrations ranging from 32 to 128 µg/mL shown in Table 1.

The SEM analysis

The cell surface morphology of *C. neoformans* ATCC90112 was observed after treatment with the most active extract (BE extract from TSU-EFHA009) using SEM. Cells after treatment with the extract at a concentration of 4X MIC (16 µg/mL) appeared to be shrunken, with broken and wrinkled cell surfaces, similar to cells treated with amphotericin B at a concentration of 1 µg/mL (positive control). Whereas, the morphology of cells treated with 1% DMSO (negative control) exhibited a normal cell surface without any damage (Figure 1). These SEM analyses indicated that the BE extract had effects on cell wall of *C. neoformans.*

Antioxidant activity and total phenolic content

Crude extracts of 36 fungal isolates were screened for their antioxidant properties (DPPH assay) in comparison with an antioxidant agent (ascorbic acid). From the DPPH radical scavenging activity results, three out of the 108 extracts showed strong antioxidant activity with 90% inhibition, while ascorbic acid gave a 95% inhibition. Among the active extracts (three extracts) had an excellent scavenging effect, especially the BE from TSU-EFHA009. The inhibitory concentration value (IC_{50}) of this extract was 0.03 mg/mL (Table 2).

Phylogenetic relationship of active fungal isolates

ITS rDNA sequences analyses were used to classify the selected endophytic fungi. The phylogenetic trees were performed by MP and ML analyses. The sequence similarity of sequences retrieved from GenBank databases was determined. Subsequently, BLAST search results of ITS rDNA sequences indicated that the isolates belonged to the class Sordariomycetes, order Xylariales, and family Apiosporaceae. The generated phylogenetic alignment consisted of 48 taxa (Table 4), with *Amphisphaeria sorbi* (MFLUCC 13-0721), *Phlogicylindrium eucalyptorum* (CBS111689) and *Phlogicylindrium uniforme* (CBS131312) as outgroup.

The dataset constituted 607 total characters; 385 characters were constant; 191 characters were parsimony informative and 31 variable characters were parsimony uninformative. The best tree inferred a length of 443 steps [consistency index (CI) = 0.693, retention index (RI) = 0.873, relative consistency index (RC) = 0.605, homoplasy index (HI) = 0.307]. One of the ten MPTs is as shown in Figure 2; the best topology was determined by the K-H test (Kishino and Hasegawa, 1989). The maximum likelihood tree illustrated a similar topology to the MP tree (data not shown).

The phylogenetic results demonstrated that our strain (TSU-EFHA009) was assigned to genus *Nigrospora* with strong statistic support as shown in Figure 2. It is grouped together with the *N. guilinensis* clade (LC3481 and LC7301), with strong support (65% BSMP and 64% BSML) and their numbers of nucleotide substitutions exhibited 507/515 = 98.5% similarity with eight substitutions. Hence, this strain should be classified taxonomically as *N. guilinensis*.

GC-MS analysis

The CE extract from isolate TSU-EFHA 009 was analyzed by GC-MS as shown in Table 3. In this study, retention indices were also compared to the published values. An agreement above 90%, of the spectra, was considered for identification of constituents. The CE could be divided into 10 components, the majority of which are 4-(cyclopentyloxy) cyclohex-2-en-1-yl acetate (21.89%), 5,7a-dimethyloctahydro-1-inden-3a-yl)(phenyl)methanone (12.37%) and 2-methylcyclohexanone (10.02%). Three

Table 1. The minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC) of the active extracts from fungal endophytes at a final concentration range of 0.25 to 128 µg/mL.

Extract	Fungal code	Test microorganisms									
		MIC/MBC or MFC values (µg/mL)									
		Bacteria				Yeasts				Filamentous fungi	
		SA	MRSA	EC	PA	CA1	CA2	CN1	CN2	MG	TM
BE	TSU-EFHA 001	64/128	-	-	-	128/128	128/128	128/128	-	-	-
	TSU-EFHA 003	128/128	-	-	-	128/128	-	-	128/128	-	-
	TSU-EFHA 005	64/128	64/128	-	-	-	-	-	-	-	-
	TSU-EFHA 006	-	-	-	-	64/128	32/64	-	-	-	-
	TSU-EFHA 008	8/16	128/200	-	-	-	64/128	128/128	-	-	128/128
	TSU-EFHA 009	32/64	64/128	-	-	64/128	32/64	4/8	64/128	128/128	-
CE	TSU-EFHA 003	128/128	-	-	-	-	-	-	128/128	-	-
	TSU-EFHA 006	128/128	-	-	-	-	-	-	-	-	-
	TSU-EFHA 008	32/64	32/64	-	-	-	-	-	-	-	-
	TSU-EFHA 003	-	64/128	-	-	-	-	-	-	-	-
	TSU-EFHA 012	-	128/128	-	-	-	-	-	128/128	-	-
CH	TSU-EFHA 003	128/128	-	-	-	-	-	-	-	-	-
	TSU-EFHA 006	128/128	-	-	-	-	-	-	-	-	-
	TSU-EFHA 008	128/128	-	-	-	-	-	-	-	-	-
Control	Vancomycin	0.25/0.5	0.5/1.0	ND	ND	ND	ND	ND	ND	ND	ND
	Gentamicin	ND	ND	0.5/2	0.5/2	ND	ND	ND	ND	ND	ND
	Amphotericin B	ND	ND	ND	ND	0.125/0.5	0.125/0.5	0.25/1.0	0.25/1.0	ND	ND
	Crotimazole	ND	ND	ND	ND	ND	ND	ND	ND	2/4	1/2

CH, Hexane extract from fungal mycelium; BE, ethyl acetate extract from culture broth; CE, ethyl acetate extract from fungal mycelium. MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; MFC, minimum fungicidal concentration. SA, *Staphylococcus aureus* ATCC25923; MRSA, methicillin-resistant *Staphylococcus aureus* SK1; EC, *Escherichia coli* ATCC25922; PA, *Pseudomonas aeruginosa* ATCC27853; CA1, *Candida albicans* ATCC90028; CA2, *Candida albicans* NCPF 3153; CN1, *Cryptococcus neoformans* ATCC90112 (flucytosine-sensitive); CN2, *Cryptococcus neoformans* ATCC90113 (flucytosine-resistant); MG, *Microsporum gypseum* clinical isolate; TM, *Talaromyces marneffei* clinical isolate.

main components were identified in the BE fraction and represented over 10% of the peak area. The remaining components were present at <10% of the peak area.

Many secondary metabolites are produced by the fungal endophytes. Thus, this study aimed to evaluate the antimicrobial and antioxidant activities of such fungal isolates. They may be a renewable source of novel bactericidal, fungicidal and antioxidant activities. The effects of the endophytic extracts in this study against tested pathogenic microorganisms were significant, except against Gram-negative bacteria. This might be because of the structure of these bacteria. There is an outer membrane that prevents a sufficient active agent

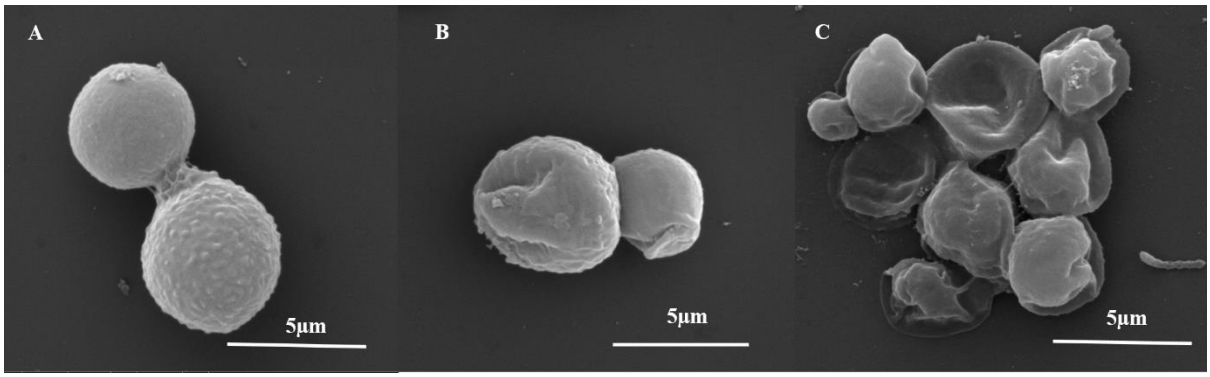

Figure 1. Scanning Electron Micrograph of *Cryptococcus neoformans* ATCC 90112. (A) Cell surface of *C. neoformans* after treatment with 1% DMSO. (B) Cell surface of *C. neoformans* after treatment with amphotericin B (1 µg/mL). (C) *C. neoformans* after treatment with active extract (Broth ethyl acetate extract from isolate TSU- EFHA 009) at 4X MIC value (16 µg/mL).

Table 2. DPPH scavenging activity and total phenolic content of the potential crude extracts.

Crude extract	DPPH scavenging activity (%)	IC_{50} values (mg/mL)	Total phenolic content (mg of GAE/g of extract)
TSU-EFHA 005	92	0.10	19.83±0.11
TSU-EFHA 009	95	0.03	41.20±0.40
TSU-EFHA 010	90	0.25	4.30±0.25
Ascorbic acid	98	0.001	-

effect (Beveridge, 1999). This is the first report that evaluated the antimicrobial and antioxidant properties of endophytic fungi from *C. carandas*, while other reports focused on the biological activities and compound identification from *C. carandas*.

Pawle and Singh (2014) isolated fungal endophytes (*Nigrospora* species) from the living fossil *Ginkgo biloba*. The ethyl acetate extracts from culture broth showed antimicrobial activity with MIC values of 2.5 mg/mL against *E. coli, Klebsiella* species, *S. aureus, C. albicans* and *Geotrichum* species, while the ethyl acetate extract from *N. guilinensis* TSU-EFHA009 in this study yielded high activity against *C. neoformans* with an MIC value of 4 µg/mL and MFC of 8 µg/mL. These results give credence that extracts from fungal endophytes show great antimicrobial activity. Molecular identification showed that the active fungi could be classified as Sordariomycetes and identified as *N. guilinensis*; which was also reported to be the major endophytic group from various plant species (Zhang and Yao, 2015). *Nigrospora* spp. was commonly found as an endophyte in several species of plants (Sharma and Rangari, 2015; Tenguria and Firodiya, 2015; Kucerova-Chlupacova et al., 2016; Saad et al., 2019). Furthermore, there are many reports about metabolites from *Nigrospora* spp. (Arumugam et al., 2014; Rathod et al., 2014; Ibrahim et al., 2018) that displayed good activity against pathogenic micro-organisms. Some secondary metabolites (griseofulvin, spirobenzofuran and pyrazine) from *Nigrospora* spp. have been reported as antifungal and antibacterial substances (Kratky et al., 2012; Roymahapatra et al., 2012; Sharma and Rangari, 2015; Kucerova-Chlupacova et al., 2016). In addition, the ethyl acetate extract of *N. guilinensis* (TSU-EFHA009) contained three main compounds which are 4-(cyclopentyloxy)cyclohex-2-en-1-yl acetate, 5,7a-dimethyloctahydro-1-inden-3a-yl)(phenyl) methanone and 2-methylcyclohexanone. It is possible that they might have an important role in antimicrobial and antioxidant activities. However, these compounds have not been previously reported from *Nigrospora* spp. and there are no reports about their biological activities.

Phenolic compounds are secondary metabolites that stabilize lipid oxidation. The amount of phenolic content in crude extracts seems to have an important role in antioxidant activity. The total phenolic content of fungal extracts has been previously determined (Bharwaj et al., 2015; Madhuchhanda et al., 2017). Total phenolic content in this study ranged from 4.30±0.25 to 41.20±0.40 gallic acid equivalents (GAE mg/g of extract) of dry weight of extracts (Table 2). Total phenolic content was lower than the extracts of endophytic fungi from *Eugenia jambolana.* The endophytic extracts having high phenolic contents showed a high antioxidant activity which ranged from 58 to 60 GAE mg/g of extract and produced a 50 to 80% inhibition (Yadav et al., 2014). The antioxidant content range in this study was different from previous studies

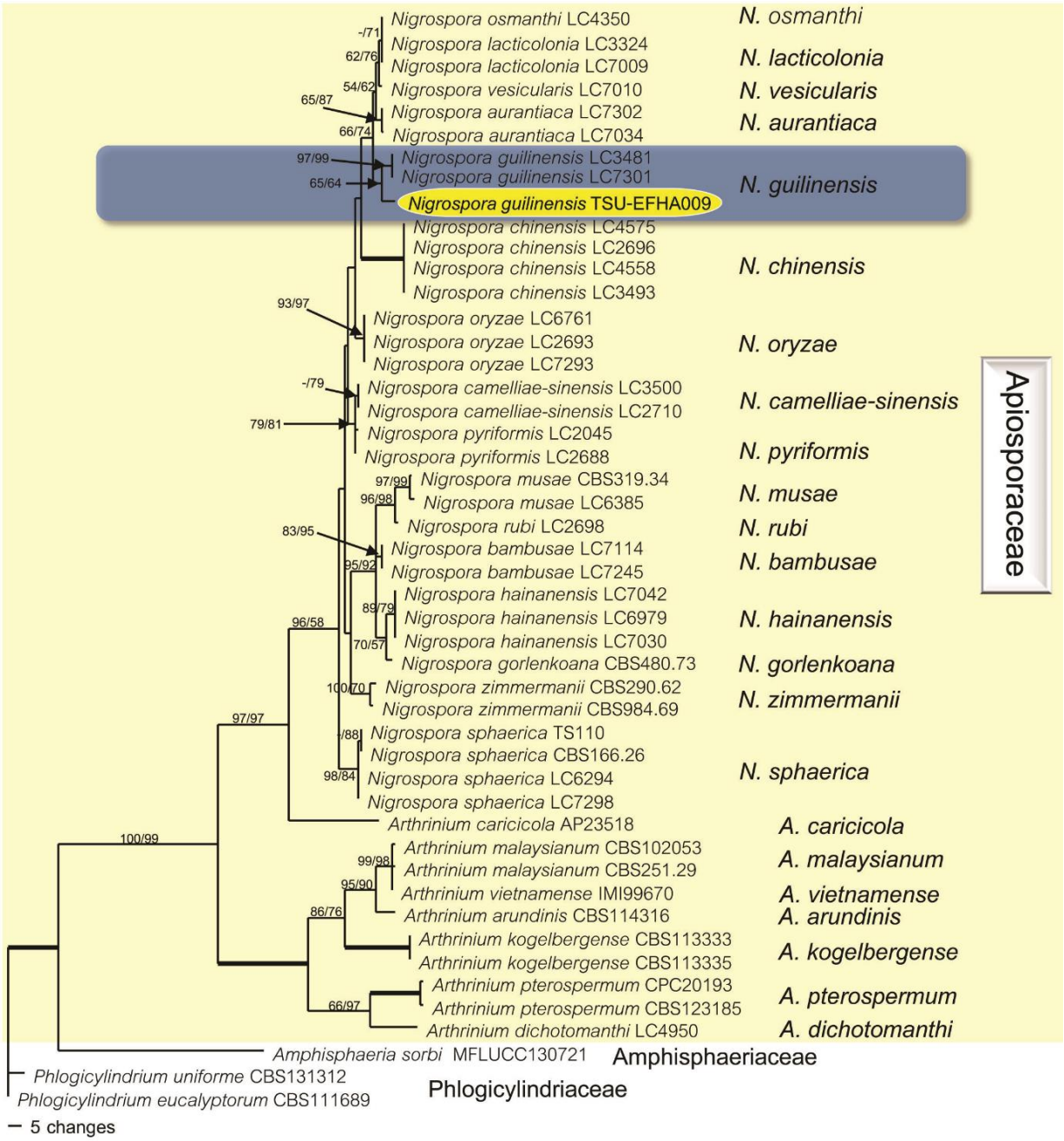

Figure 2. The most parsimonious trees using the ITS rDNA gene with *Amphisphaeria sorbi* (MFLUCC 13-0721), *Phlogicylindrium eucalyptorum* (CBS111689) and *P. uniforme* (CBS131312) as outgroup. The best phylogeny performed by maximum parsimony analyses. Maximum parsimony bootstrap values (BSMP, left) and maximum likelihood (BSML, right) bootstrap values equal or greater than 50% are shown above each branch. The bold line shows strong supported by all bootstrap values (100%).

Table 3. Major constituents of broth ethyl acetate (BE) fraction of *N. guilinensis*.

No.	Compound	% Peak area
1	4-(cyclopentyloxy)cyclohex-2-en-1-yl acetate	21.89
2	5,7a-dimethyloctahydro-1-inden-3a-yl)(phenyl)methanone	12.37
3	2-methylcyclohexanone	10.02

Table 4. GenBank accession number of generated ITS rDNA sequences.

Species	Voucher/Culture	GenBank accession numbers
		ITS rDNA
Arthrinium arundinis	CBS114316	KF144884
Arthrinium caricicola	AP23518	MK014871
Arthrinium dichotomanthi	LC4950	KY494697
Arthrinium kogelbergense	CBS113333	KF144892
Arthrinium kogelbergense	CBS113335	KF144893
Arthrinium malaysianum	CBS102053	KF144896
Arthrinium malaysianum	CBS251.29	KF144897
Arthrinium pterospermum	CPC20193	KF144913
Arthrinium pterospermum	CBS123185	KF144912
Arthrinium vietnamense	IMI99670	KX986096
Nigrospora aurantiaca	LC7034	KX986093
Nigrospora aurantiaca	LC7302	KX986064
Nigrospora bambusae	LC7114	KY385307
Nigrospora bambusae	LC7245	KY385305
Nigrospora camelliae-sinensis	LC2710	KX985957
Nigrospora camelliae-sinensis	LC3500	KX985986
Nigrospora chinensis	LC3493	KX985984
Nigrospora chinensis	LC4558	KX986020
Nigrospora chinensis	LC4575	KX986023
Nigrospora chinensis	LC2696	KX985947
Nigrospora gorlenkoana	CBS480.73	KX986048
Nigrospora guilinensis	LC3481	KX985983
Nigrospora guilinensis	LC7301	KX986063
Nigrospora guilinensis	TSU- EFHA 009	MK033475
Nigrospora hainanensis	LC6979	KX986079
Nigrospora hainanensis	LC7042	KX986094
Nigrospora hainanensis	LC7030	KX986091
Nigrospora lacticolonia	LC3324	KX985978
Nigrospora lacticolonia	LC7009	KX986087
Nigrospora musae	CBS319.34	KX986076
Nigrospora musae	LC6385	KX986042
Nigrospora osmanthi	LC4350	KX986010
Nigrospora oryzae	LC2693	KX985944
Nigrospora oryzae	LC7293	KX985931
Nigrospora oryzae	LC6761	KX986056
Nigrosora pyriformis	LC2688	KX985941
Nigrospora pyriformis	LC2045	KX985940
Nigrospora rubi	LC2698	KX985948
Nigrospora sphaerica	CBS166.26	MH854878
Nigrospora sphaerica	LC6294	KX986044
Nigrospora sphaerica	LC7298	KX985937
Nigrospora sphaerica	TS-110	MG832530
Nigrospora vesicularis	LC7010	KX986088
Nigrospora zimmermanii	CBS290.62	KY385309
Nigrospora zimmermanii	CBS984.69	KY385310
Outgroup		
Amphisphaeria sorbi	MFLUCC 13-0721	KR092797
Phlogicylindrium eucalyptorum	CBS111689	KF251205
Phlogicylindrium uniforme	CBS131312	JQ044426

Bold letters: Strain in this study; AP: culture collection of Rene Jarling, Germany; CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; IMI: Culture Collection of CABI Europe UK Centre, Egham, UK; KFRD: culture from kiwifruit, Chenzhou, China; LC: culture collection of Lei Cai, China; MFLUCC: Mae Fah Luang University Culture Collection, Chiangrai, Thailand; TS: culture from kiwifruit, Taishun, China.

and this may be due to the fungal strains and extraction method (Srinivansan et al., 2010; Chowdhury et al., 2018). However, the results in this study confirmed that the endophytic fungus (TSU-EFHA009) has a high phenolic content and showed excellent activity against DPPH radicals.

Conclusions

Broth ethyl acetate extract of endophytic fungus *N. guilinensis* TSU-EFHA009 significantly showed strong antimicrobial and antioxidant activities. This finding confirms that endophytic fungi isolated from *C. carandas* were sources of the potential substances. Thus, this plant appears to be an interesting plant which harbors active fungal isolates for development as pharmaceutical agents in the future.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Vol. 13(27), pp. 474-482, September, 2019
DOI: 10.5897/AJMR2017.8722
Article Number: 2953AF961810
ISSN: 1996-0808
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Full Length Research Paper

Predisposing factors and cost Implications of pulmonary tuberculosis in patients attending federal medical centre Makurdi, Nigeria

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Received 3 October, 2017; Accepted 5 March, 2018

The predisposing factors and the cost implications of pulmonary tuberculosis (TB) in patients attending Federal Medical Centre, Benue State, Makurdi, was investigated. Information on socio-economic and demographic characteristics of the patients was obtained using a structured questionnaire. The female had a higher (56.5%) number of TB patients than the male (43.5%). Most of the TB patients were married (52%). A larger percentage had tertiary education (34%). Majority of the TB patients were civil servants (41%) and 78.5% earned less than ₦ 100,000 monthly. Those living in flats and bungalows made up a greater percentage of TB patients, and majority (55%) of them had more than five persons in their households (reflecting family size). It was also observed that many of the patients (54.5%) ate together or shared cutleries and about one-half (49.5%) of the patients reported that they were sleeping together on same bed with family members. In terms of house location most of the TB patients lived in North Bank (25.5%) and Wadata (22.5%). Mostly children (31.5%) and wives (30.5%) were affected by the TB disease; the proportion of affected husbands and relatives were lower. Many (71.5%) reported that they did not have health insurance and had stopped work because of TB ailment. Majority of the TB patients made substantial out-of-pocket expenses: up to 57.5% spent more than ₦500 on transport per visit, 52% spent more than ₦2000 on complementary test while 75.5% spent above ₦2000 on non-TB tests. From the findings of this study, it may be necessary to provide an enabling environment that facilitates treatment completion such as isolation of patients and adequate ventilation. A shorter treatment regimen eliminating visits to the healthcare should be encouraged.

Key words: Predisposing factors, cost, tuberculosis (TB), Makurdi.

INTRODUCTION

Tuberculosis (TB) is an infectious disease usually caused by the bacterium *Mycobacterium tuberculosis* (MTB) (WHO, 2005). Tuberculosis is spread through the air when people who have active TB cough, spit, speak, or

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sneeze (CDC, 2012). According to the World Health Organization (WHO), the global incidence of TB was 9 million in 2005, 13.7 million in 2010 (WHO, 2009). Approximately one-third of the world's population has been infected with *M. tuberculosis*, with new infections occurring in about 1% of the population each year (WHO, 2009). TB is a serious public health problem in Nigeria. Nigeria has an estimated prevalence of nearly 900,000 TB cases, and the second highest TB disease burden in Africa, ranking 5th among the 22 high TB burden countries in the world (WHO, 2010). The Nigerian Medical Association (2013) reports that Benue State is one of the States with the highest TB infection rate in the country. The disease mostly affects those between 25 to 34 years (36.6%) with Lagos, Kano, Oyo and Benue states being the states with the highest level of infections (NMA, 2013). The World Health Organization (WHO) estimated that over 13 million people have TB (WHO, 2010).

Many people in the developing world contract tuberculosis because of compromised immunity due to HIV infection and other factors such as malnutrition, overcrowding, lack of control measures, poor treatment and the high cost of therapy (WHO, 2010). Many studies have shown a strong association between poverty and TB and have demonstrated that the poor have a higher risk of TB infection; they have a higher prevalence of the disease, have worse outcomes and display worse TB care-seeking behaviors (Lonnroth et al., 2010; Fabricant et al., 1999; Elender et al., 1998). Many studies have assessed the association between poverty and TB, but only very few have assessed the direct financial burden TB treatment and care can place on households (Dim and Dim, 2013). Although financial difficulties is the reason for implementing a free-of-charge strategy on TB diagnosis and treatment but TB patients still faces huge out-of-pocket expenses (Ukwaja et al., 2012; Sanou et al., 2004). Financial constraint is known to lead to non-adherence to TB medication, delays or slower recovery and could result in drug resistance (Chang et al., 2004). Studies have evaluated the direct cost incurred by TB patients to be from fees, transport and food during TB treatment (Umar et al., 2012). It was also observed that TB patients also experience indirect cost such as job losses or opportunities forgone as a result of TB ailment (Umar et al., 2013; Ukwaja et al., 2012).

The WHO calculates that the average TB patient loses three to four months of work time and up to 30% of yearly household earnings (WHO, 2013a, b). Kemp et al. (2007) observed that patients spend about US$13 on treatment which is equivalent to about 18 days wages. In another study by Umar et al. (2012) he estimated time value for the hours spent seeking treatment for hospitalized and non-hospitalized patients was US$333.30 and US$79.13, respectively. These suggests that many households would be unable to cope with TB treatment without certain coping strategies like selling or leasing their assets as well as borrowing of loans (Laokri et al., 2013; Ukwaja et al., 2013; Collins et al., 2013).

The costs typically measured, referred to as direct costs, include transport costs to and from the health facility and any costs for medication or consultation incurred by individuals while seeking care (Ataguba, 2011). Indirect costs are costs associated with the time lost while being unable to work due to seeking care, or being too ill to work. These are used to capture the productivity and economic costs an individual or household incurs as a result of being ill or spending time seeking treatment (Barter et al., 2012; Drummond et al., 2007; Russell, 2004).

The purpose of this study is therefore to describe and estimate the predisposing factors and the cost implications of TB infection.

MATERIALS AND METHODS

Study area

Study area was Makurdi in Benue State. Makurdi Metropolis is located in North Central Nigeria along the River Benue. It lies at Latitude: 7° 43' 32" N and Longitude: 8° 33' 51" E. Makurdi is the capital of Benue State and covers an area of 34,059 km^2 and an estimated population of 500,797. Federal Medical Centre Makurdi, is the only hospital rendering the direct observation therapy (DOT) in the State, people from all over the State and beyond go there to get treatment.

Study design

This study was a three month hospital based cross-sectional survey which was carried out at the direct observation therapy (DOT) centre of Federal Medical Centre (FMC), Makurdi, Benue State, Nigeria.

Data collection techniques

Pre-structured questionnaires were administered to 200 patients confirmed to have pulmonary tuberculosis and attending the DOT centre for treatment.

The questionnaire comprised of three sections: section A, B and C. Method used was based on filling a questionnaire from three parts; demographic and socio-economic data of the patients, direct and indirect costs of TB infection.

Data preparation

Section A was designed to obtain demographic and socio-economic data of the patients such as age, sex, marital status, occupation, educational status, monthly income and the habits and living conditions of the patient. Section B had to do with the indirect costs of the patients such as the distance travelled by the patient to the health centre, time taken to reach the health centre, health insurance and the effect of TB ailment on the patient or household. while Section C dealt with the direct costs or the out-of-pocket expenditures of the patients and which included the money spent on transportation, non TB tests and drugs such as cough syrup,

Table 1. Socio-economic characteristics of tuberculosis patients.

Studied parameter	Frequency	Percentage
Sex		
Female	113	56.5
Male	87	43.5
Marital status		
Married	104	52.0
Single	96	48.0
Educational qualification		
Polytech/COE	68	34.0
University Degree	58	29.0
Secondary	57	28.5
Primary	17	8.5
Occupation		
Civil servants	82	41.0
Businessmen	51	25.5
Unemployed	35	17.5
Others	32	16.0
Monthly income (₦)		
50,000 to 10,000	65	32.5
10,000 to 50,000	63	31.5
>100,000	43	21.5
<10,000	29	14.5
House type		
Flats	47	23.5
Bungalow	45	22.5
Self-contained	40	20.0
Multi-tenants	37	18.5
Thatch house	26	13.0
Storey building	5	2.5

vitamins and antibiotics.

Data analysis

Data were entered using the Statistical Package (SPSS) to describe relevant variables. Descriptive statistics such as frequencies and percentages were used for categorical variables while Means and Standard deviations were used for continuous variables. All financial calculations were in the Nigerian currency (₦).

Ethical clearance

Ethical clearance was sought and given at FMC, Makurdi for the study. Informed consent was obtained from the participating patients. Patients names were not required and were not recorded.

RESULTS

The socio-economic characteristics of TB patients

The socio-economic characteristics of TB patients are shown in Table 1. Socio-economic information on two hundred tuberculosis patients were obtained. Most of the TB patients interviewed were female (56%); and only 43.5% were male. Married patients (52%) were more in number than the singles (48%). Most of the TB patients (34%) had OND/HND as their highest academic qualification, followed by University graduates (29%) and the infection was least in those with Primary School certificate (8.5%). Civil servants accounted for the highest proportion (41%) of TB patients studied, followed by the businessmen (25.5%). Those who were unemployed and

Table 2. Predisposing factors among tuberculosis patients.

Social habits	Frequency	Percentage
No of persons in the household		
1-5	75	37.5
6-10	110	55
11-14	15	7.5
Sleep together on same bed		
No	103	51.5
Yes	97	48.5
Eat together or use the same cup		
Yes	109	54.5
No	91	45.5
Household location		
High level	59	29.5
North bank	51	25.5
Wadata	45	22.5
Judges quarters	27	13.5
Others	18	9.0
Household position		
Child	63	31.5
Wife	61	30.5
Relative	39	19.5
Husband	37	18.5

other occupational groups made up the least proportion.

A high percentage of patients earned between ₦10,000 to ₦100,000 as monthly income. Those living in flats and bungalows made up a greater percentage of TB patients (20.0 to 23.5%), followed by those who had thatch houses or lived as tenants (13.0 to 18.5%).

Predisposing factors of TB patients

Table 2 depicts the predisposing factors of TB patients. Majority (55%) of them had more than five persons in their household and more number of the patients (51.5%) were not sleeping. It was also observed that a higher percentage of the patients (54.5%) ate together or shared cutleries. Mostly children (31.5%) and wives (30.5%) were found among the TB patients studied. Proportions of husbands and relatives affected by the disease were smaller.

Indirect cost associated with TB treatment

The indirect cost associated with TB treatment is summarized in Table 3. A higher percentage lived near the health care unit (51%), spent less than 30 min (44.5%) to reach the health care centre while 17% spent above one hour to get there. Majority of the patients had no health insurance (71.5%). Those who stopped work because of the ailment were 62.5%. Adherence to treatment was also high among the patients as a higher number of the patients adhered to treatment (64%) while only a few admit that they did not adhere to treatment (36%) due to one reason or the other.

Direct cost of TB patients

The out-of-pocket expenditure of TB patients was also determined (Table 4). More number of the TB patients spent above ₦500 on transportation to the health care. Those who spent above ₦2000 on complimentary were also more than those who spent below ₦2000 on complementary test.

Expenditure on non-TB drugs was high as a higher number of TB patients (75.5%) spent above ₦2000 on non-TB drugs, lower percentage (24.5%) spent below ₦2000. Money spent on daily feeding was also high. (47.5%) spent above ₦100 on daily feeding. A smaller number of the patients (23.5%), spent above ₦3000 on

Table 3. Indirect costs incurred by tuberculosis patients.

Cost Implication	Frequency	Percentage
Distance from healthcare		
Near	102	51.0
Far	98	49.0
Time to reach healthcare		
0 to 30 mins	89	44.5
31 mins - 60 mins	77	38.5
above 1hour	34	17.0
Health insurance		
No	143	71.5
Yes	57	28.5
Hospitalization		
No	139	69.5
Yes	61	30.5
No of days on hospitalization		
0	139	69.5
1-5	38	19.0
6-10	19	9.5
11-17	4	2.0
No of visits to clinic		
Twice a month	106	53.0
Once a month	51	25.5
Once a week	43	21.5
Effect on work		
Stopped work	125	62.5
Did not stop work	75	37.5
Adherence to treatment		
Yes	128	64.0
No	72	36.0

hospitalization and only 8% spent below ₦3000 on hospitalization. As high as 60% of the TB patient complained of spending so much on treatment while only 40% did not complain.

Total out-of-pocket expenditure incurred by TB patients

Total out-of-pocket expenditure incurred by TB patients or cost burden of TB patients attending F.M.C Makurdi is summarized in Table 5. The out-of-pocket expenditure of TB patients per visit to F.M.C, Makurdi, is as follows: Transportation ranged from ₦150 to ₦550, non TB drugs was between ₦1,000 to ₦3,000, complementary exams ₦1,000 to ₦3,000, feeding ₦300 to ₦1,500 and money spent on hospitalization was around ₦4,000 per day.

Patient's monthly income ranged between ₦5,000 and ₦200,000 while total expenditure was between ₦2,450 and ₦10,900 per visit. Total expenditure was increased if the number of visits was increased. For patients at the intensive phase of treatment which was twice a month, total expenditure could be ₦2,450 X 2 = ₦4,900. For those who spent as high as ₦10,900 on treatment, total expenditure could be ₦10,900 X 2 = ₦21,800. Mean monthly income was ₦77,550.00 while mean total expenditure was ₦4971.50. Standard error of monthly income was ₦4,860.48 and standard error of total

Table 4. Out-of-pocket expenditure among tuberculosis patients.

Cost implication	Frequency	Percentage
Money spent on transport		
Above ₦500	115	57.5
200 to ₦ 500	67	33.5
Below ₦ 200	18	9.0
Money spent on treatment		
High	120	60.0
Low	80	40.0
Money spent on complementary test		
Above ₦2000	104	52.0
Below ₦ 2000	96	48.0
Money spent on non-TB drugs		
Above ₦ 2000	151	75.5
Below ₦ 2000	49	24.5
Money spent on daily feeding		
Above ₦ 1000	95	47.5
₦500 to ₦ 1000	84	42.0
₦200 to ₦ 500	21	10.5
Money spent on hospitalization		
₦0	137	68.5
Above ₦ 3000	47	23.5
Below ₦3000	16	8.0

Table 5. Total out-of-pocket expenditure incurred by TB patients.

Expenditure	Range	Mean	Std. Error
Monthly income (₦)	5000.00 - 200000.00	77550.00	4860.49
Transport (₦)	150.00 – 550.00	234.00	8.36
Non TB drugs (₦)	1000.00 – 3000.00	1490.00	60.98
Complementary exams (₦)	1000.00 – 3000.00	1960.00	70.83
Feeding (₦)	300.00 – 1500.00	615.00	26.24
Money spent on hospitalization (₦)	0.00 – 4000	672.50	82.56
Total expenditure (₦)	2450.00 – 10900.00	4971.50	139.11

expenditure was ₦139.11.

DISCUSSION

The main objective of this study was to estimate and describe the predisposing factors and cost implications of TB infection in Makurdi Metropolis. It was discovered in this study that majority of the TB patient falls among those with lower levels of education (polytechnic 34%) and this agrees with the study of Rundi (2010), that lack of education and ignorance are major factors facilitating the spread of the disease. Among occupation of the patients, civil servants (41%) and businessmen (25.5%) had higher number of TB patients than the unemployed (17.5%) and other occupational groups (16%). It is believed that the civil servants and businessmen are always exposed to a larger number of people and also have the tendency to travel to various places thereby placing them at a higher risk of contracting TB. This is in

conformity with the reports of WHO (2010); Kumar et al. (2007); Peter et al., (2009). They reported that overcrowding and travelling are risk factors in the spread of the disease.

Poverty had also been linked to a greater risk of infection, poorer patients outcomes as well as affecting health seeking behavior (Harris et al., 2011; Oxlade and Murray, 2012; Wingfield et al., 2014). In this study, it was discovered that patients who earned between ₦10,000 to ₦100,000 had higher prevalence of TB infections than those who earned above ₦100, 000. It was also observed that a higher number of TB cases were found among those living in high level (29.5%) and Wadata (22.5%). Majority of the TB patients were also found among those living in flats (23.5%) and bungalows (22.5%). The above are characteristics of people who belong to the low socio-economic class of people and agrees with studies of Zumla et al. (2013), Fabricant et al. (1999) and Elender et al. (1998) which shows strong association between poverty and TB. These studies reported that the poor has a higher prevalence of the disease, this is because they are most vulnerable to acquiring TB. According to Ukwaja et al. (2012), malnutrition, overcrowding, poor air circulation and sanitation associated with poverty increase the risk of acquiring TB and developing the active disease. This is also in conformity with the report of Tony et al. (2015), which states that living conditions, unhygienic practices, overcrowding in homes, living in poorly designed, unplanned houses and environment may increase the persistence of TB in a locality. Ahlburg (2000), contradicts this report by saying that although TB is "tagged" as a disease of the poor, it is not absolutely true because a good number of those infected were literate, had good education, and earned good incomes.

High prevalence of TB was also found among those who had more than five persons in their household (55%) than among those who had less than five persons (37.5%). A larger percentage of the patients slept together on same beds (48.5%) and ate together or shared cutleries (54.5%). Rundi, (2010) reported that habits like sleeping together on the same bed and eating together played a major role in the spread of the disease. A larger proportion of TB patients were children (31.5%) and wives (30.5%). Proportions of husbands (18.5%) and relatives (19.5%) affected by the disease were smaller. Studies of TB in developing countries found that females in their reproductive years had a higher rate of progression to disease, a higher case-fatality rate, and higher mortality rates. When a woman suffers from TB, additional losses may result. The household loses the activities that the woman routinely performs in the household: cooking, cleaning, childcare, and managing the activities of the household. She can also easily pass TB to other family members.

Indirect costs of the patients were also analyzed in this study, forty-eight percent lived very far from the health centre, majority (71.5%) was found among those who had no health insurance. In this study, it was observed that a good number (62.5%) of the patients stopped work. According to other literatures, the largest indirect cost was income lost by being too sick to work. Other studies suggested that on average 3 to 4 months of work time were lost, resulting in about 20 to 30% of annual household income lost to TB infection (Umar et al., 2012). This agrees with reports from the following studies WHO (2005), Hansel et al., (2004), Paton and Ng (2006), Sagbakken et al. (2008) and Peter et al. (2009). They reported that TB can contribute to poverty by reducing patient's physical strength and ability to work and that most of the TB patients stopped work as a result of the ailment. It was also observed that a good number of TB patients had substantial out-of-pocket expenditures as 57.5% spent more than ₦500 on transportation, 52% spent above ₦2000 on complementary tests, 75.5% spent above ₦2000 on non-TB drugs and 47.5% spent above ₦1000 on daily feeding. 60% complained of spending so much on treatment despite the free-of-charge strategy. Reports from the following studies also showed high direct costs (Sanou et al., 2004; Chang et al., 2004; Ukwaja et al., 2012). Studies from South Africa also recorded high direct and indirect costs despite the free-of-charge treatment (Foster et al., 2015).

It was observed that patient's monthly income ranges between ₦5,000 and ₦200,000 while total expenditure was between ₦2,450 and ₦10,900 per visit. Total expenditure was increased if the number of visits to the health care centre was increased. For patients at the intensive phase of treatment which was twice a month, total expenditure could be ₦2,450 × 2= ₦4,900. For those who spent as high as ₦10,900 on treatment, total expenditure could be ₦10,900 × 2 = ₦21,800. If ₦4,900 was spent on treatment from a monthly income of ₦5,000, the patient will have little or no money left. According to Ukwaja et al. (2012), mean patient pre-diagnostic costs varied between US$36 and US$196 corresponding to respectively 10.4% and 35% of their annual income. 18 to 61% of patients received financial assistance from outside their household to cope with the cost of TB care (Ukwaja et al., 2012). Laokri et al., 2013 also reported that the poor would be unable to finance TB treatment without certain coping strategies.

Conclusions

It was observed in this study that most of the TB patients had lower levels of education, earned low monthly income, lived in overcrowded conditions, belonged to occupations that predispose them to high risk of TB infection and exhibited habits like sleeping together and eating together. The above characteristics could predispose individuals in the community to TB infection and this could result in TB re-occurring. It was also observed that TB patients made substantial expenses,

this further increase the impacts on the poor and the risks to others in society.

LIMITATIONS

Only descriptive statistics was used, this is because the study addresses problems facing TB patients alone. It describes the socio-economic and demographic characteristics of TB patients.

CONFLICTS OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors wish to appreciate the staff of Federal Medical Centre Makurdi where the research was conducted and the patients who completed the surveys. In particular, their thanks go to the Ethical Committee, Laboratory Technologists and Scientists of F. M. C, Makurdi, who contributed in one way or the other for the success of this work.

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Vol. 13(27), pp. 483-487, September, 2019
DOI: 10.5897/AJMR2017.8759
Article Number: CB0F0A161922
ISSN: 1996-0808
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Full Length Research Paper

Prevalence of intestinal schistosomiasis infections among school children in Danane, Western Côte D'Ivoire

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Received 10 November, 2017; Accepted 2 September, 2019

Intestinal schistosomiasis is very common in children and cause significant morbidity. Danané is located in the west of the Côte d'Ivoire which is an area where intestinal schistosomiasis transmission is established. The Ministry of Health of Côte d'Ivoire which have acceded to the global goal of eliminating these diseases as a public health problem, has adopted a five year strategic plan in 2011. The mass drug administration campaigns are one of the key elements of this strategic plan. The present study therefore examined the prevalence of *Schistosoma mansoni* among school children in Danané, Côte d'Ivoire. A cross-sectional study was undertaken in schools between November 2016 and February 2017. In total, 510 children, aged between 4 and 15, were included. Single stool samples from each child were collected and processed using the Kato-Katz method to diagnose schistosomiasis. Of the sample, 52 (10.2%; IC 95%: 7.9-13.1) were infected with *S. mansoni*, with intestinal schistosomiasis prevalence ranging from 3.3 to 26.7%, depending on the school. Possible associated factors were also examined, revealing that age over 9 years (p=0.015), living in urban areas (p=0.001), and the tendency to defecate outside at school (p<0.001) were factors that associated with intestinal schistosomiasis prevalence. In summary, this study has revealed that intestinal schistosomiasis is still moderately prevalent among school children in Danané, despite the national control program. Therefore, school-based interventions in urban areas that focus on prevention through education are recommended in this region.

Key words: Intestinal schistosomiasis, *Schistosoma mansoni*, children, schools, Côte d'Ivoire.

INTRODUCTION

Schistosomiasis is a particular problem due to its high prevalence and morbidity among children (WHO, 2013). Approximately, 207 million people are estimated to be infected by schistosomiasis (Steinmann et al., 2006), with a further 700 million at risk across 76 countries.

Schistosomiasis is also the second most commonly fatal parasitic disease (behind malaria) and is responsible for 280,000 deaths across Africa per year (van der Werf et al., 2003). Africa is by the far the most severally affected continent, possessing more than 90% of reported cases

(CDC, 2011). Among schistosomiasis, intestinal schistosomiasis is reported to be widespread and important in sub-Saharan Africa (WHO, 2013). Intestinal schistosomiasis, also known as intestinal bilharzia, is an infectious disease caused by *Schistosoma mansoni*, a parasitic worm in fresh water. The transmission to humans is led from snails infected (CDC, 2011). Praziquantel remains the drug of choice for treatment of schistosomiasis.

S. mansoni is endemic to 81 of the 83 sanitary districts in Côte d'Ivoire and has been a focus of intensive control efforts (Assaré et al., 2015; Chammartin et al., 2014). The department of Danané (Figure 1) is in the transmission zone of human schistosomiasis, which occurs in the western parts of the country. The city is located in the western part of the country, 681 km north of the coast. The climate of this mountainous region is typically hot and humid, with a rainy season lasting 7 to 9 months. During the rainy season, rainfall can reach 1676 mm, leading to particularly rich vegetation growth. The region is also irrigated by the Cavally and Ban rivers, in addition to various seasonal waterways. The primary deworming campaign began in 2012 and targeted school children aged from 4 to 15. Since then, there is a lack of assessment of the impact of this campaign due to lack of updated data about the prevalence or associated factors for schistosomiasis among this target population. To address this, the present study assessed the prevalence of *S. mansoni* infections in school children in the city of Danané, Côte d'Ivoire. In addition, detailed patient notes were collected via a questionnaire to determine various biological, behavioral, and socioeconomic characteristics. This combined approach revealed the current *S. mansoni* prevalence in this region of Côte d'Ivoire and also identified several associating factors that can be used to better target preventive strategies and may have implications for other soil-transmitted helminths.

Ethical consideration

The study was reviewed and approved by the relevant primary education inspectors in Côte d'Ivoire. The objectives and procedures were explained to participants in the presence of the teachers. Children that tested positive for infection were treated with 40 mg/kg praziquantel.

MATERIALS AND METHODS

Study design, area, and population

Between November 2016 and February 2017, a cross-sectional study was performed among primary school-aged children in the educational department of Danané, Côte d'Ivoire.

The study examined primary school children aged from 5 to 15 years old. All school children present during the investigation period and who lived in the department for at least 3 months prior to the start of the study, regardless of gender, were included. However, school children who had been dewormed within 3 weeks of the start of the study were excluded.

Sample size determination and sampling technique

The educational department of Danané included 306 primary schools, with 41,497 school children registered in the 2014-2015 school year [Department of strategies, planning and statistics (DSPS), 2014-2015]. To calculate the minimum number of school children to be included, sample size and power calculations were performed using Epi Info version 7 (CDC, Atlanta, GA, USA) to identify a theoretical prevalence of 50% with 5% precision and an α (type 1) rate less than 5%. This calculation resulted to a sample size of 384.16 school children. For the study, the size has been increased to 510.

The total number of classrooms to be assessed was set at 60. Each primary school typically has six classrooms, with each classroom corresponding to a level of study (forms 1 to 6). Each of the classroom levels in each school to ensure all age groups were represented was sampled.

The number of school children selected per classroom was obtained by dividing the previous number determined using sample size calculation by 60. School children were selected randomly per classroom. Finally, 10 schools were randomly selected for sampling from the list of the schools submitted by the Department of Strategies, Planning and Statistics (DSPS), 2014-2015.

Data collection and processing

Survey Questionnaire

Data were collected using a standardized questionnaire form. This contained questions concerning the age, gender, classroom, deworming history, origin of the pupil (either rural or urban), certain behaviors (for example defecating habits, visits to rivers), and the socio-economic status of the mother. The survey also included functional signs related to various stages of schistosomiasis, such as itching, headaches, stomach pain, or diarrhea.

Sample collection and laboratory procedures

Feces samples were collected from school children directly in plastic pots and analyzed using the Kato-Katz method. A single stool sample was collected for each child. This technique was used to identify *S. mansoni* eggs and the presence of other helminths, including roundworms, whipworms, hookworms, and *Taenia* sp.). Briefly, this technique involves cleaning helminth eggs from stool under wet cellophane that has previously been soaked with a reactive compound consisting of malachite green and 50% glycerin. The cleaning of eggs is obtained through the action of glycerin. Each sample was assessed at 10× then at 40x magnification using light microscopy. Observations were made 1 h and 24 h after

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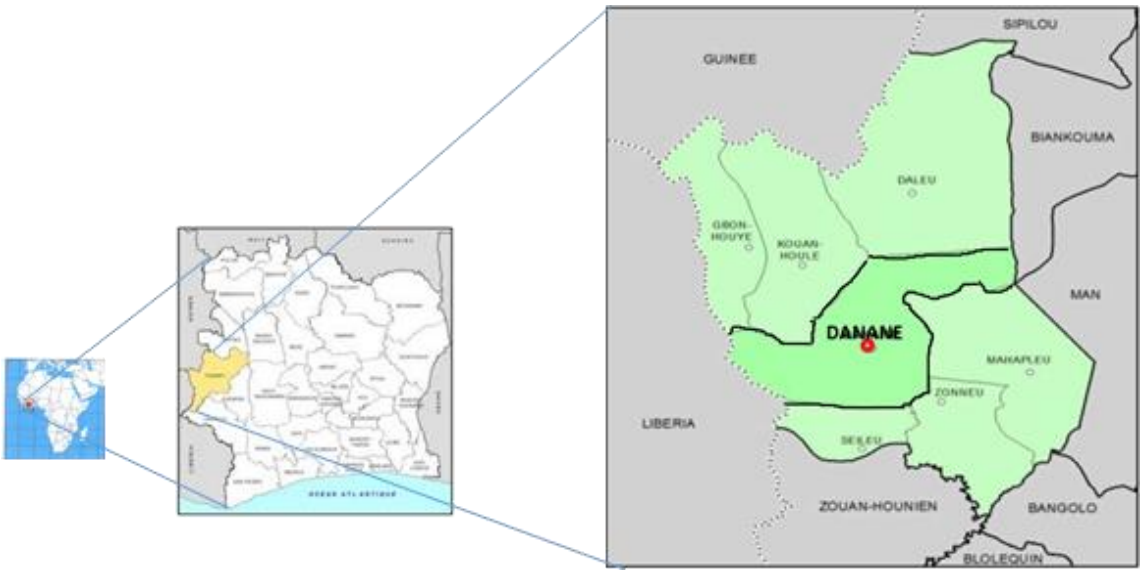

Figure 1. Map of Danané Department , 7° 16′ 00″ north, 8° 10′ 00″ western (RGPH, 2014).

staining to allow for better discrimination between hookworm and *S. mansoni* eggs.

Data management and analysis

Data were analyzed using Epi Data 3.1 (CDC) and SPSS 22 (IBM, Armonk, NY, USA). The proportions of the different modalities were estimated with 95% confidence intervals to estimate prevalence. Any associations between questionnaire answers and the occurrence of intestinal *S. mansoni* were evaluated using χ^2 tests. The statistical threshold of significance was set at $p<0.05$.

RESULTS

A total of 510 schoolchildren, aged between 5 to 15 years old, were included in the study. Most of the pupils (57.5%) were male with an average age of 8.9 years (±2.08 SD). Participants from 5 to 9 years were predominant (65.1%). More than two third of the schoolchildren (70.1%) lived in rural areas and the majority of them (92.3%) stated that they had been dewormed less than 6 months before the start of the study. Approximately, 63.9 and 71.8% of the schoolchildren' mothers were illiterate and homemakers, respectively. Finally, 73.9% of the households obtained drinking water from wells (Table 1).

Of the children examined, 52 (10.2%) were infected with *S. mansoni*, although the prevalence varied depending on the school and ranged from 3.3 to 26.7%. In addition, there was one school in which *S. mansoni* was not detected (Table 2). Most infections were found in urban areas, with significant difference at the threshold of $p<0.05$ ($p=0.001$). Children with age over 9 years ($p=0.0157$) and school children living in urban areas ($p=0.001$), were each significantly more likely to be infected by *S. mansoni*. Behaviorally, defecation outdoors when at school ($p<0.001$) also significantly associated with the incidence of parasites. However, *S. mansoni* infection prevalence did not significantly associate with gender ($p=0.97$), deworming history ($p=0.24$), lower mother educational rates ($p=0.20$), behaviorally such as defecation outdoors when at home and contact with water flows ($p=0.09$) (Table 3). Of the 510 school children examined, 10 (2.0%) were infected with *Trichiuris trichiura*, 10 (2.0%) with hookworm eggs, and 4 (0.8 %) housed *Ascaris lumbricoides* eggs.

Clinically, a large proportion of school children stated they had stomach pains (30.0%), diarrhea (16.7%), and constipation (16.7%) at the time of the investigation. However, only two cases of *S. mansoni* were detected in children reporting stomach pains, and one case was found in each of the group of school children reporting diarrhea and constipation (Table 4).

DISCUSSION

The prevalence of *S. mansoni* identified in this study (10.2%) was lower than that of two prior studies carried out in western Côte d'Ivoire. The first was performed in the city of Man between 2004 and 2005, and reported 51.4% prevalence (Matthys et al., 2007), although the study focused on farmers. The second took place in 2007 in Biankouman and reported a prevalence of 35.5% among school children (Adoubryn et al., 2012). Despite the lower prevalence reported in this study, it is still a moderate level that represents a serious morbidity risk to schoolchildren of the region. This morbidity is largely due

Table 1. Socio-demographic characteristic of school children in Danane, western Côte d'Ivoire, 2016.

Variable	Characteristics	Frequency	Percentage
Gender	Male	393	57.5
Age (in years)	5-9	332	65.1
	10-15	178	34.9
Deworming history (in months)	< 6	472	92.5
	> 6	38	7.5
Home	Rural area	362	71.0
	Urban area	148	29.0
Mother's school level	Not in school	325	63.7
	In school	185	36.3
Mother's activity	Housewife	366	71.8
	Liberal activity	110	21.6
	Farmer	26	5.1
	Civil servant	8	1.6
Household drinking water	Faucets	109	22.4
	Wells	377	77.5

Table 2. *Schistosoma mansoni* infection prevalence per school, department of Danané, Western, Côte d'Ivoire, 2016.

Area	*PPS surveyed	Examined	Positive	Prevalence (%)
Rural	Protestante 1	72	9	12.5
	Gouezepleu	74	3	4.1
	Gopoupleu 2	72	12	16.7
	Gniampleu 2	71	3	4.2
	Kedere	72	0	0.00
Urban	Gningleu 1	30	8	26.7
	Blessaleu 3	31	8	25.8
	Commerce 1	27	5	18.5
	Houphouet ville 3	31	3	9.8
	Moribadougou 2	30	1	3.3
Total		510	52	10.2

*PPS, Primary Public School.

to diarrhea, appetite suppression, weight loss, growth delay, malnutrition, anemia, cognitive issues, hepatomegaly, and even death in severe cases (King and Dangerfield-Cha, 2008; Stephenson et al., 2000). The lack of treatment may explain the higher prevalence rates observed in earlier studies as mass distribution of praziquantel has only been used in schools since 2012. During this study, various school directors stated that deworming days are that are normally organized by teachers but under the guidance of the Ministry of Health. The most recent mass treatment in Danané was just one month prior to the beginning of the study. However, teaching staff also stated that they are often faced with treatment refusal by many school childrens, perhaps explaining the continued elevated prevalence in the city. Typically, the potential side effects of praziquantel were provided as justification for refusing treatment (Adoubryn et al., 2012). A high *S. mansoni* prevalence was also demonstrated in a study in Senegal that found 21.8% prevalence rate in the delta of the Senegal River. This was also despite several mass treatments programs using 600 mg praziquantel, suggesting the phenomenon

Table 3. Relation between socio-demographic characteristics and prevalence of intestinal schistosomiasis (%) among primary school children in Danané, western Côte d'Ivoire, 2016.

Variable	Prevalence of intestinal schistosomiasis			p-value
	Yes (%)	No (%)	Total (%)	
Child gender				
Male	30 (10.2)	263 (89.8)	293 (57.5)	0.97
Age (in years)				
5-9	26 (7.8)	306 (92.2)	332 (65.1)	0.015*
10-15	26 (14.6)	152 (85.4)	178 (34.9)	
Deworming history (in months)				
< 6	46 (9.8)	425 (90.2)	471 (92.4)	0.24
> 6	6 (15.8)	32 (84.2)	38 (7.5)	
Home				
Rural area	27 (7.5)	335 (92.5)	362 (71.0)	0.001*
Urban area	25 (16.9)	123 (83.1)	148 (29.0)	
Mother's school level				
Illiterate	29 (8.9)	296 (91.1)	325 (63.7)	0.20
Litterate	23 (12.4)	162 (87.6)	383 (75.1)	
Faecal matter evacuation / houshold				
Outdoors	8 (6.3)	119 (93.7)	127 (24.9)	0.09
Latrine	44 (11.5)	339 (88.5)	383 (75.1)	
Faecal matter evacuation/school				
Outdoors	6 (2.8)	212 (97.2)	218 (42.7)	< 0.001*
Latrine	46 (15.8)	246 (84.2)	292 (57.3)	
Contact with water flows				
Yes	23 (8.2)	259 (91.8)	282 (55.3)	0.09
No	29 (12.7)	199 (87.3)	228 (44.7)	

*Significant difference.

Table 4. Declared clinical symptoms, Danané, West of Côte d'Ivoire, 2016.

Symptom	Frequency (n)	Proportion (%)	*S. Mansoni* cases n (%)
Stomach pain	9	30.0	2 (22.2)
Nausea	7	23.3	1 (14.3)
Diarrheas	5	16.7	1 (20)
Constipation	5	16.7	1 (20)
Vomiting	4	13.3	1 (25)
Total	30	100	6 (20)

is not unique to Côte d'Ivoire (Abdellahi et al., 2016).The present study noted that there was variation in *S. mansoni* prevalence between schools, confirming the heterogeneous distribution of the disease in the region. This could be due to the focal repartition of schistosomiasis (Booth et al., 2004) that depends on local hydrography (Beck-Wörner et al., 2007; Mugono et al., 2014). Hydrography may be particularly relevant in Danané as the city is crossed by many rivers. The proximity of these waterways to homes would favor

contact between people and water, exposing children to intestinal schistosomiasis. However the present study revealed that intestinal schistosomiasis is not highest among school children who have high level contact rate with permanent water. This finding could be due to activities such as water collection usually causes a relatively small fraction of the total exposure to schistosomes, while other activities such as recreational swimming, bathing, and laundry are often more important (Grimes et al., 2015; Khonde et al., 2016; Nyati-Jokomo and Chimbari, 2017). Several studies have noted that intestinal schistosomiasis is highest among children whose parents have high levels of contact with water (Alemu et al., 2016; Ekpo et al., 2010; Odogwu et al., 2006). An alternative explanation of this finding is periodic deworming effect. Differences in the prevalence of *S. mansoni* have also been noted between regions. For example, further south of Danané in the district of Taabo, a study was carried out in 2011 among the inhabitants of two villages and seven camps (Schmidlin et al., 2013). This surveillance project found a much lower prevalence than our study (1.3%) that was hypothesized to be due to the launch of an integrated intestinal parasite control approach that worked within the framework of the health surveillance system of southern Côte d'Ivoire health demographic surveillance system (HDSS) (Schmidlin et al., 2013). Two other studies have also noted a lower prevalence *S. mansoni* in Côte d'Ivoire compared to ours. The first examined six cities across the south-west of the country between 1999 and 2001 and found a prevalence of 2.6% in schools (Evi et al., 2007). The second study was carried out in 2005 in urban schools in Divo and reported a prevalence of 6.1%. However, this study results are broadly consistent with data from rural Divo gathered in 2005 (12.0% prevalence) (Kassi et al., 2008) and Tiassalé (9.3% prevalence) collected in 2007 (Menan et al., 2008). Some studies have also reported higher *S. mansoni* prevalence. For example, in the Agneby region in the south-east of Côte d'Ivoire (Adoubryn et al., 2006), the prevalence among school children was 20.6% in 2001. Further afield, *S. mansoni* prevalence in Kenya has been found to be much higher than Côte d'Ivoire (60.5%). This large difference has been linked to the lack of anti-helminth control programs in this region of Kenya (Odiere et al., 2012). In the present study older children are significantly more infected than younger at the $p<0.05$ threshold; we did observe a general trend of an increasing likelihood of intestinal *S. mansoni* infection with age, supporting several studies (Adoubryn et al., 2006; Alemu et al., 2016).

In this study, the lack of significantly association between *S. mansoni* infection and school children gender may be explained by the fact that males and females are subject to the same infestation risk. Males were not slightly more in contact with water than females. Additionally, hygiene school levels in Danané remain poor, leading to increased rates of infection. This appears magnified by the fact that schistosomiasis significantly associated with various hygiene-related habits, such as defecating outdoors at school ($p<0.001$). These school children would therefore be closely involved with maintaining contaminated areas and this factor will likely need to be addressed to improve control measures (Schmidlin et al., 2013).

Finally, the prevalence of *S. mansoni* in many schools was roughly comparable to that of other soil-transmitted helminths. For example, the prevalence of various helminths in China in 2002 were 0.20% *Ascaris lumbricoides*, 1.08% *T. trichura*, and 2.5% ankylostomes. This may be due to similar control programs aiming to combat intestinal parasite infections via sanitary education (Jia et al., 2012). However, during the study in Danané, a general inadequacy in the control measures being applied was noticed. This included the distribution of praziquantel and access to clean water and latrines. In addition, the latrines that are available are typically poorly maintained, unhygienic, and regularly not functional. Due to this, school children often prefer to defecate outdoors, resulting in further contamination and the generation of permanent biotopes. This may be a contributing factor in explaining the continuing moderate prevalence of *S. mansoni* despite control efforts and should be considered when establishing policies going forward. Increased use of sanitation should reduce open defecation in and around the school compound, and might therefore be expected to reduce transmission of schistosomiasis (Grimes et al., 2016).

Conclusion

This study found moderate prevalence of *S mansoni* infection among scholar children aged 4 to 15 years old in Danané. Age, living areas, and certain hygienic behaviors all are associated with higher intestinal schistosomiasis prevalence. Stomach pains and diarrhea were not found to be pathognomonic signs of schistosomiasis but could constitute orientation elements. Therefore, control efforts targeting school-aged children should be based on a program of sensitization, sanitary education, and periodic assessment of prevalence to control the transmission of schistosomiasis in the study area. The distribution and monitored administration of praziquantel is also recommended.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

The authors extend their thanks to the school principals and teachers for their availability. Their thanks also go to

to all school children for their participation in the study.

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Vol. 13(27), pp. 488-499, September, 2019
DOI: 10.5897/AJMR2019.9181
Article Number: 68623CA61924
ISSN: 1996-0808
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Construction of a genetically engineered strain of nattokinase and assessment of its fibrinolytic activity

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Received 25 July, 2019; Accepted 3 September, 2019.

A strain of *Bacillus natto* was isolated from natto and found to have a high yield of nattokinase. The aprN and pro-aprN gene fragments, encoding nattokinase from *Bacillus natto*, were amplified using two pair of primers and were expressed in *Bacillus subtilis* WB800N host cells using the pHT43 plasmid as a vector. In this system, the effect of leader peptides on nattokinase activity, revealing that these leader peptides mediate the folding function of nattokinase was explored. After optimizing the expression conditions (1 mmol/L IPTG inducer at 37°C, pH 7.5, cell culture OD_{600} of 0.6), maximal nattokinase enzymatic activity of 848.52 IU/mL after induction of fermentation for 4 h, was achieved, at which time maximal extracellular protein had been produced. The fermentation medium of the engineering strain was optimized, and purified nattokinase via salt precipitation and ultrafiltration was isolated. Relative to fermentation supernatants, the purification ratio of nattokinase reached 6.63, with a total recovery of 80%, and a specific enzyme activity of 11507.92 IU/mg. These results indicate that the nattokinase overexpression using the pHT43 vector in WB800N cells is an effective means of achieving efficient nattokinase production, and the engineered strains constructed herein have great promise as potential industrial strains for nattokinase production.

Key words: Nattokinase, *aprN* gene, *Bacillus subtilis* WB800N, Fibrinolytic activity, engineered strain, fermentation.

INTRODUCTION

Sumi et al. (1987) first demonstrated the presence of a strong fibrinolytic enzyme in natto which they named nattokinase (NK) (Sumi et al., 1987; Sumi et al., 1988). Subsequently, researchers from various countries have conducted research on nattokinase, determining that the open reading frame encoding of the nattokinase gene consists of 381 amino acids, of which the 29 N-terminal amino acids form a signal peptide, the next 77 constitutes a leader peptide, and the following 275 amino acids form a mature peptide (Nakamura et al., 1992). Nattokinase is an alkaline protease with a molecular weight of about 27.7 kDa and the protein isoelectric point（PI）of 8.6 (Fujita et al., 1993).

In both animal and human studies, NK has exhibited significant and potent fibrinolytic and antithrombotic activity (Fujita et al., 1995; Nagata et al., 2017; Chen et

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al., 2018). NK not only directly dissolves fibrin, but also promotes the release of tissue-type plasminogen activator (tPA) from cells and catalyzes the release of prourokinase from the liver, thereby increasing the formation of plasminogen (Dabbagh et al., 2014). In clinical trials, oral NK increased tPA, significantly reduced ELT, and significantly reduced levels of factor VII and factor VIII in human blood, suggesting that NK can be used as a fibrinolytic/anticoagulant agent with the potential to reduce the risk of thrombosis and CVD in humans (Sumi et al., 1990; Hsia et al., 2009; Kurosawa et al., 2015). In addition, NK has been found to possess antihypertensive (Lee et al., 2015), anti-atherosclerotic, lipid-lowering, anticoagulant (Park et al., 2012) and neuroprotective effects (Metkar et al., 2017). Recent toxicological studies (both *in vivo* and *in vitro*) provide strong evidence for the safety of NK oral consumption (Lampe et al., 2016). Therefore, studies have shown that NK is safe, economical, has a long half-life, is easy to produce in large quantities, and has a variety of beneficial cardiovascular effects, making it a feasible drug for the treatment of cardiovascular diseases (Dabbagh et al., 2014; Ren et al., 2017).

To enhance nattokinase activity and simplify downstream operations, heterologous expression of nattokinase has been studied in several microbial expression systems, including in *Escherichia coli*, Pichia pastoris, *Bacillus subtilis* and *Lactococcus lactis* (Wei et al., 2015; Wu et al., 2011). Nattokinase achieves high levels of expression in *E. coli* model systems, but most recombinant proteins in them are in the form of inactive inclusion bodies (Ni et al., 2016). Although active nattokinase can be expressed in recombinant *P. pastoris* and *L. lactis*, expression is relatively low and product purification is difficult (Dabbagh et al., 2014). As a Gram-positive bacterium, *B. subtilis* is an attractive host for the production of heterologous secreted proteins, as it is non-pathogenic and capable of secreting functional extracellular proteins directly into the culture medium (Nishito et al., 2010).

In this study, we used the pHT43 shuttle plasmid to construct two genetically engineered strains of *B. subtilis* WB800N cells, and we then optimized the expression conditions of these genetically engineered strains. Finally, we established optimal protocols for the downstream separation and purification of NK through salt precipitation and ultrafiltration.

MATERIALS AND METHODS

Bacterial strains, plasmids, and reagents

B. *subtilis* natto and *E. coli* DH5α are laboratory-preserved strains. *B. subtilis* WB800N and the pHT43 vector were purchased from Wuhan Miaoling Biotech. All enzymes used for DNA manipulations were purchased from Takara (Dalian). Isopropyl-β-D thiogalactopyranoside (IPTG) was obtained from Bio Basic Inc. Urokinase standard (1240 IU) was purchased from Beijing Zhongke Quality Biotech. Fibrinogen and thrombin were purchased from Shanghai Yuanye Biotech.

Amplification of the nattokinase gene

The reference nattokinase gene sequence from *B. subtilis* YF038 (GenBank accession number AY219901) was used to design specific primers (Table 1) using the biological software Clone Manager, and these primers were synthesized by Sangon Biotech (Shanghai). PCR amplification of the gene sequence was carried out using genomic DNA from *B. subtilis* natto as a template. The optimized PCR program for amplification of the gene was as follows: denaturation at 94°C for 5 min, then 30 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C, followed by a final extension for 5 min at 72°C.

Construction of engineered bacterial strains

The amplified aprN (containing only the mature NK peptide sequence), pro-aprN (containing both the leader and mature NK peptide sequence) gene products and the pHT43 plasmid were digested using BamH I and Sma I, and were then ligated using T4 DNA ligase. The ligated pHT43-aprN and pHT43-pro-aprN plasmids were then transformed individually into *E. coli* DH5a cells and propagated in the medium supplemented with 100 μg/mL ampicillin (Tu et al., 2016; Zhou et al., 2018). The recombinant plasmid DNA of pHT43-aprN or pHT43-pro-aprN from single colonies was extracted and confirmed by restriction enzyme digestion and sequencing. The DNA sequences of these pHT43-aprN and pHT43-pro-aprN inserts were analysed by Sangon Biotech (Shanghai).

B. subtilis WB800N cells prepared from glycerol stocks were next streaked onto neomycin-containing agar plates and incubated overnight at 37°C. Competent WB800N cells were then prepared as previously described by Chityala et al. (2015), and the sequence-verified recombinant pHT43-aprN or pHT43-pro-aprN plasmids were introduced into the cells via electroporation (Vojcic et al., 2012). These electrotransformed cells were quickly added into 500 μL of resuscitation medium and incubated for 40 min. These cells were then added to fresh sterile Luria–Bertani medium (LB) (1 mL) and incubated for another 45 min. Cells were the centrifuged at 3000 rpm for 2 min, and the cell pellets were re-suspended in 100 μL LB medium and spread over LB agar plates containing chloramphenicol, which were then incubated at 37°C overnight. The transformed colonies were further selected using a dual-antibiotic plate. The recombinant plasmid DNA from single colonies was then extracted and confirmed by PCR amplification. The verified engineered strains, named WB800N/pHT43-aprN and WB800N/pHT43-pro-aprN, were then stored in glycerol at -80°C.

Induced expression of engineered nattokinase

Single colonies of WB800N/pHT43-aprN and WB800N/pHT43-pro-aprN were inoculated in 25 mL LB containing chloramphenicol (5 μg/mL) and incubated at 37°C, with 200 rpm shanking overnight. The pH was maintained at a fixed value of 7.5 during subsequent culturing. Fresh LB media (25 mL) was inoculated with this overnight culture as a 2% inoculum, and was then incubated at 37°C with 200 rpm shaking. NK expression in these cells was induced using 0.1 mM IPTG after the cell density reached 0.6–0.8 OD_{600} (optical density at 600 nm), and cells were then incubated at 30°C for 24 h. Samples were then collected and centrifuged at 8000 rpm for 2 min at 4°C, and supernatants were stored at 4°C for subsequent SDS-PAGE analysis and determination of NK enzymatic activity.

Table 1. Primers used for PCR amplification.

Primer	Sequence (5' to 3')	Restriction site
aprN-F	CGGGATCCATGGCGCAATCTGTTCCTTATGG	BamH I
aprN-R	TCCCCCGGGTTATTGTGCAGCTGCTTGTA	Sma I
pro-aprN-F	CGGGATCCATGGCCGGAAAAAGCAGTACAGA	BamH I
pro-aprN-R	TCCCCCGGGTTATTGTGCAGCTGCTTGTA	Sma I

Determination of the molecular weight of engineered nattokinase

SDS-PAGE was performed as previously described (Laemmli et al., 1970). To evaluate the expression levels of NK in samples, the same amount of supernatant from each culture was taken to prepare identically diluted loading samples, and these samples were run on a gel along with a molecular weight ladder. The molecular weight of engineered NK proteins was then determined with a standard protein ladder, and the expression levels of this protein were semi-quantitatively measured.

Fibrinolytic activity assay

Engineered NK enzymatic activity was evaluated using the fibrin plate method as previously described (Man et al., 2018) with some minor adjustments. Urokinase, thrombin, agarose, and fibrinogen solutions were all prepared in 10 mmol/L phosphate buffer (pH 7.5). 5 mL agarose solution (10 g/L), 5 ml fibrinogen solution (2.2 g/L) and 100 µL (10 IU) thrombin were all mixed in a 50 mL Erlenmeyer flask, and then poured into sterile petri dishes. The solution in the plate was left undisturbed for 1 h to form fibrin clots, and then 2 mm diameter wells were made in the plate to allow for sample application. 1 mL of fermentation broth was centrifuged at 8000 r/min for 2 min at 4°C, and the supernatant was stored at 4°C for analysis of enzymatic activity. To observe fibrinolytic activity, 4 µL of this sample solution was carefully dropped into each well, plates were incubated at 37°C for 18 h, and the diameter of the fibril transparent circle was then measured. Eight different concentrations of a urokinase standard solution were diluted to prepare a nattokinase activity standard curve, allowing for the quantification of the NK fibrinolytic activity.

Optimizing fermentation parameters for engineered nattokinase expression

A test of fermentation parameters was performed in order to maximize engineered nattokinase production. Test parameters, which were modulated one by one, included: culture temperatures (18, 25, 30, 37, and 40°C), media pH (4, 5, 6, 7, 7.5, 8, 9, 10, 11, and 12), IPTG concentrations for induction of NK expression (0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, and 1.6 mmol/L) and cell growth stage at time of IPTG induction (cell culture OD_{600} of 0.2, 0.6, 1.0, 1.4, and 1.8). There was one control sample and three parallel replicates in each group of experiments. Each sample was measured 3 times on average, and the average value was analyzed as statistical data.

Isolation and purification of nattokinase

The genetically engineered seed solution was inoculated with 3% inoculum into 100 mL of optimized fermentation media (containing 26.05 g/L peptone, 29.29 g/L glucose, 1.5 g/L $MgSO_4$, 0.74 g/L $CaCl_2$, 1.5 g / L KH_2PO_4, 10 g / L NaCl, pH 7.2 ~ 7.5), in a 200 r / min, 37°C shaker and grown to a cell density of OD_{600nm} = 0.6, after which 1 mM IPTG was added to induce fermentation for 4 h. After fermentation, samples were spun at 8000 r/min for 10min to collect the fermentation supernatant. Ammonium sulfate was added to the supernatant to 30%, and samples were allowed to stand at 4°C overnight, followed by centrifugation at 12000 r / min for 20 min to remove the supernatant. Ammonium sulfate was then added to a 60% saturation, after which it was allowed to stand at 4°C overnight, followed by spinning at 12000 r / min After 20 min, the precipitate was dissolved in 5 mL of 0.04 mol/L pH 8.0 barbital sodium-HCl buffer, and the crude enzyme solution was obtained after dialysis.

Appropriate amounts of crude enzyme solution after salting out were added to a 30 kDa ultrafiltration centrifuge tube and spun at low temperature for 20 min at 8000 r/min. The ultrafiltrate was then transferred to a 10 kDa ultrafiltration centrifuge tube and centrifuged at 8000 r/min for 20 min. The ultrafiltered solution was stored at 4 ℃ and subjected to subsequent analysis.

RESULTS

Generation of recombinant plasmids and engineered strains

Two sets of DNA fragments encoding the nattokinase gene were amplified by PCR from the genomic DNA of *B. subtilis* natto. The single bands in Figure 1 confirmed that the PCR products of aprN (828 bp in size) and pro-aprN (1059 bp in size) are of the expected size. The pHT43-aprN and pHT43-pro-aprN recombinant plasmids were then extracted from *E. coli* DH5a cells and verified by restriction enzyme digestion using BamH I and Sma I (Figure 2), and the DNA sequence of the pHT43-aprN and pHT43-pro-aprN inserts were analyzed. The results revealed that the DNA sequence of cloned genes were identical to that of nattokinase from the precursor *B. subtilis* YF038 strain (GenBank accession number AY219901), confirming successful construct generation.

Engineered nattokinase expression and assessment of fibrinolytic activity

As the cloned gene was under the control of lac operon, which is induced by IPTG, induction of engineered nattokinase expression was evaluated via the supplementation of 0.1 mM IPTG in the media of the transformed *B. subtilis* WB800N cells. The IPTG was added to the fermentation broth when the culture OD_{600}

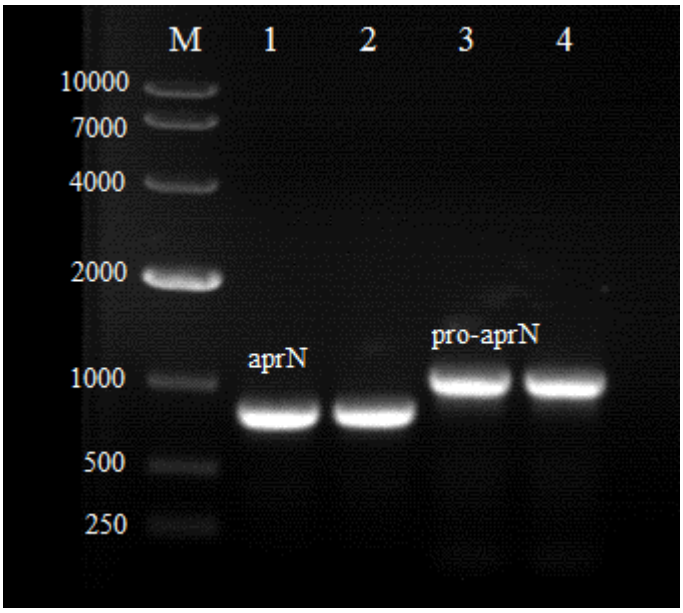

Figure 1. Amplification of the nattokinase gene by PCR from the genomic DNA of *B.subtilis* natto. Lane M, DNA maker 10000; Lane 1-2, aprN fragment of the nattokinase gene; Lane 3-4, pro-aprN fragment of the nattokinase gene.

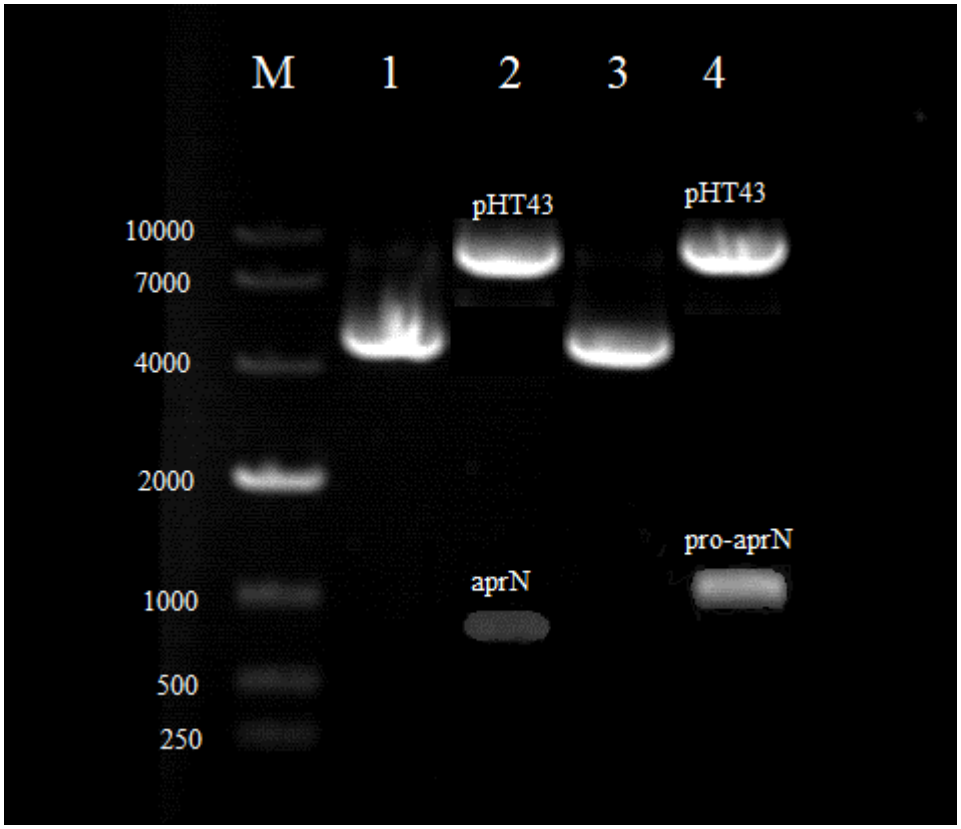

Figure 2. Verification of the recombinant pHT43-aprN and pHT43-pro-aprN plasmids by restriction enzyme digestion with BamH I and Sma I. Lane M, DNA maker 10000; Lane 1, intact pHT43-aprN; Lane 2, digested pHT43-aprN; Lane 3, intact pHT43-pro-aprN; Lane 4, digested pHT43-pro-aprN.

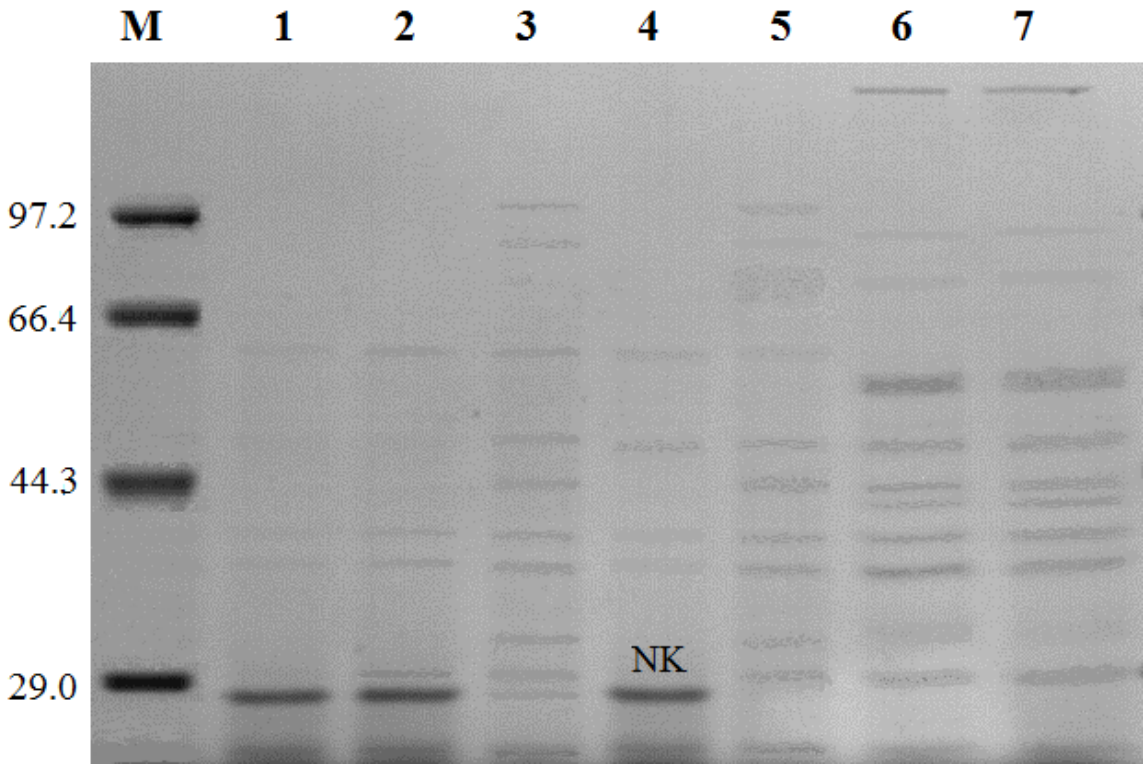

Figure 3. SDS-PAGE revealing that a 28 kDa protein for in the *B.subtilis* WB800N cells engineered to express nattokinase. Lane M, protein molecular weight standards; Lane 1-2, IPTG-induced WB800N/pHT43-pro-aprN; Lane 3, Non-induced WB800N/pHT43-pro-aprN; Lane 4, IPTG-induced WB800N/pHT43-aprN; Lane 5, Non-induced WB800N/pHT43-aprN; Lane 6, IPTG-induced *B.subtilis* WB800N/pHT43; Lane 7, Non-induced *B.subtilis* WB800N/pHT43.

reached 0.6 to 0.8. Extracellular protein produced at 30°C within 24 h post-induction was then collected for SDS-PAGE and for a fibrinolytic activity assay, with *B. subtilis* WB800N cells transformed with an empty pHT43 vector as a control.

The presence of engineered nattokinase protein in culture supernatants was first verified by SDS-PAGE analysis. A major protein band of approximately 28 kDa, similar to the molecular mass of nattokinase from *B. subtilis* natto, was observed in the pHT43-aprN and pHT43-pro-aprN samples, while no corresponding band was observed in the pHT43 samples (Figure 3), indicating that both the WB800N/pHT43-aprN and WB800N/pHT43-pro-aprN strains successfully expressed nattokinase.

Fibrinolytic activity of the engineered nattokinase protein was next detected via a fibrin plate-based method. Samples from the aprN-expressing cells did exhibit any enzymatic activity, while those from the pro-aprN-expressing cells exhibited robust activity, indicating that the WB800N/pHT43-pro-aprN strain successfully expressed active nattokinase (Figure 4). The apparent difference in enzymatic activity of the nattokinase expressed by the WB800N/pHT43-aprN and WB800N/pHT43-pro-aprN strains confirmed that the leader peptide sequence of nattokinase may direct the proper folding of the protein in order to facilitate normal enzymatic activity.

The effect of IPTG concentrations on nattokinase activity

IPTG was added to the fermentation flask when the WB800N/pHT43-pro-aprN cell density reached 0.6-0.8 OD_{600}. No effects of different IPTG concentrations on cells growth within the first 26 h of fermentation at 30°C were observed (Figure 5a). An evaluation of enzymatic activity 24 h post-induction with IPTG revealed that increasing of the IPTG concentration up to 1.0 mM improved the engineered nattokinase production, which peaked at 701.59 IU/mL at an IPTG concentration of 1.0 mM (Figure 5b). These data are similar to a previous report in which 1.0 mM of IPTG was found to be optimal for inducing the expression of recombinant enzymes, such as procerain B and the antimicrobial peptide cathelicidin-BF (Nandana et al., 2014). Further increases in IPTG concentrations to 1.6 mM resulted in a decrease of NK expression to a level of 504.82 IU/mL (Figure 5b).

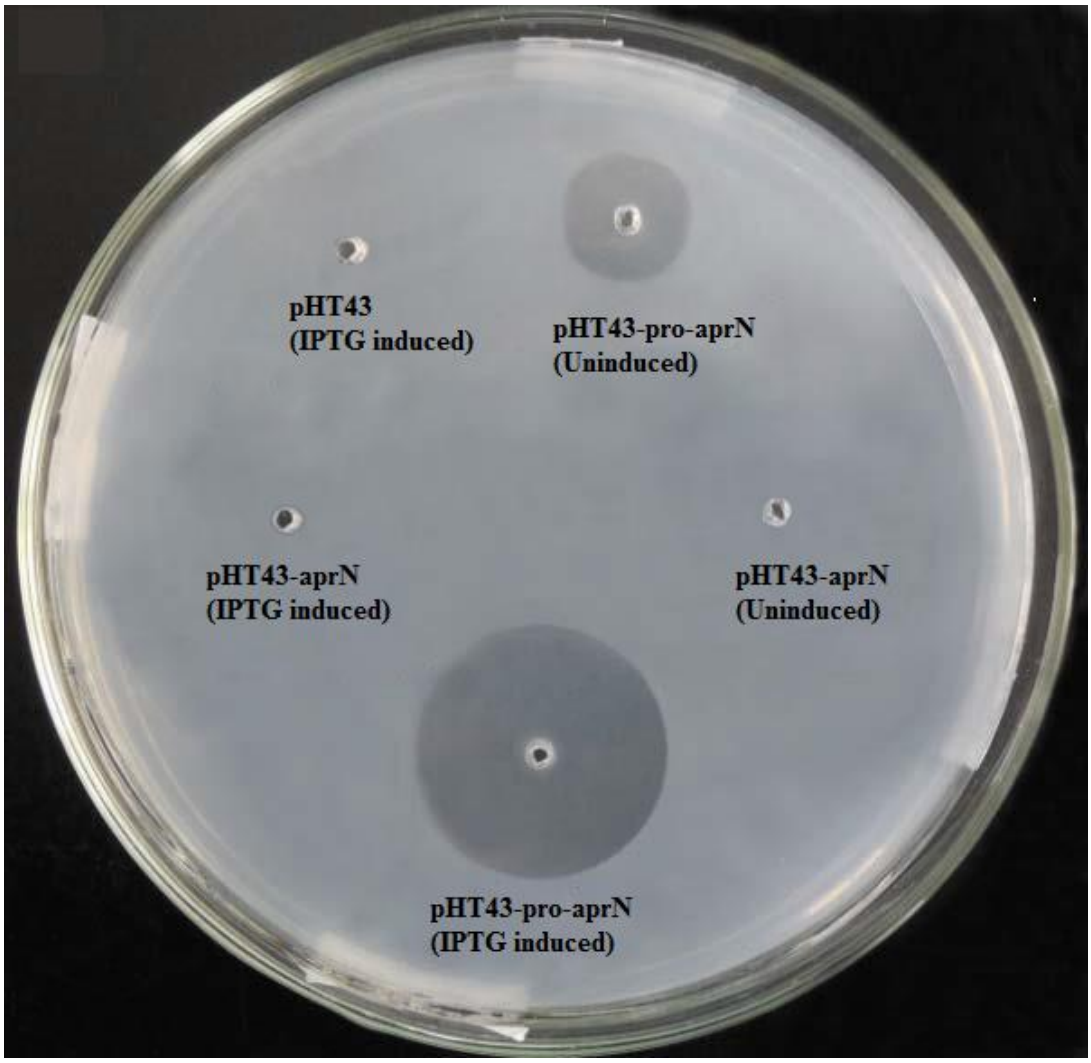

Figure 4. Engineered nattokinase enzymatic activity was evaluated using the fibrin plate method in strains induced with 0.1 mM IPTG at 30°C for 24 h, using *B. subtilis* WB800N cells transformed with an emply pHT43 vector as a control. The right one of each pair of samples was induced using IPTG, while the other was not.

This reduction of enzyme yield at higher concentrations of IPTG may be attributable to an IPTG-induced metabolic burden, as previously suggested (Glick et al., 1995). Examination of the supernatants from these samples by semi-quantitative SDS-PAGE further confirmed that the optimal IPTG concentration was 1.0 mM (Figure 5c).

Effect of temperature on nattokinase activity

The yield of NK from the WB800N/pHT43-pro-aprN cells was investigated at a range of different incubation temperatures from 18 to 40°C. The engineered nattokinase production at these temperatures ranged from 46.78-848.52 IU/mL. It was found that enzyme yield rose with increases in incubation temperature, up to a maximal enzyme production (848.52 IU/mL) that was achieved at 37°C. Further increases in temperature up to 40°C decreased the enzyme production to a level of 691.23 IU/mL (Figure 6a). These results were consistent with a previous finding that 37°C is an optimal temperature for the production of nattokinase from *B. subtilis* WB800 (Nguyen et al., 2013). In addition, different temperatures have a significant effect on cell growth, and consistent with this, it was found that cells grew more slowly at temperatures below 25°C (Figure 6b).

The kinetics of nattokinase production

In order to investigate the kinetics of NK production in

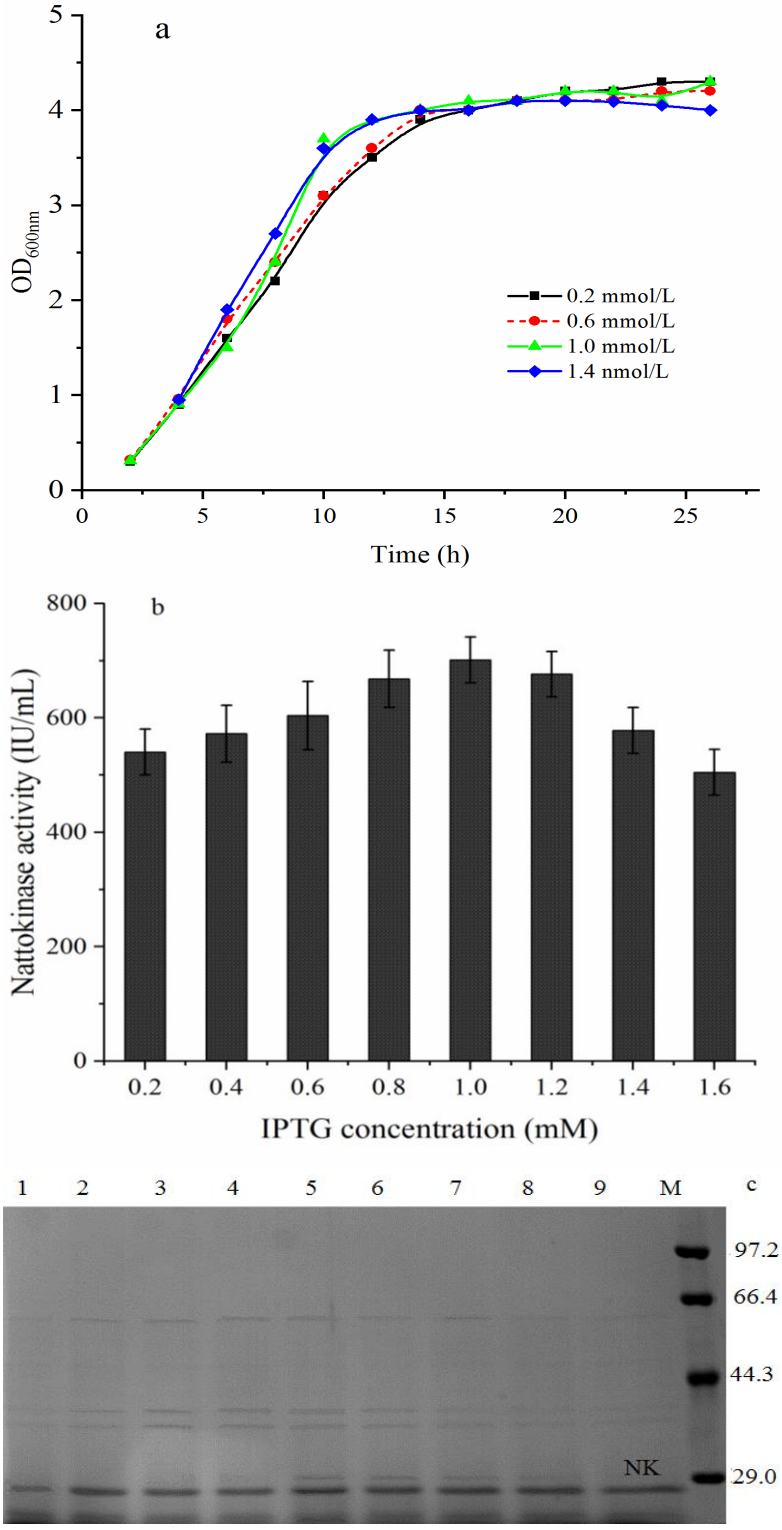

Figure 5. The effect of different IPTG concentrations on WB800N/pHT43-pro-aprN cell growth (a), on nattokinase activity in WB800N/pHT43-pro-aprN cells induced with IPTG for 24 h (b), and on nattokinase production (analyzed by semi-quantitative SDS-PAGE) in WB800N/pHT43-pro-aprN cells induced with IPTG for 24 h (c). All samples were cultured at 30°C.. Lane M, protein molecular weight standards; Lane 1-9 , cell samples induced with IPTG concentrations of 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, and 1.6 mM in that order.

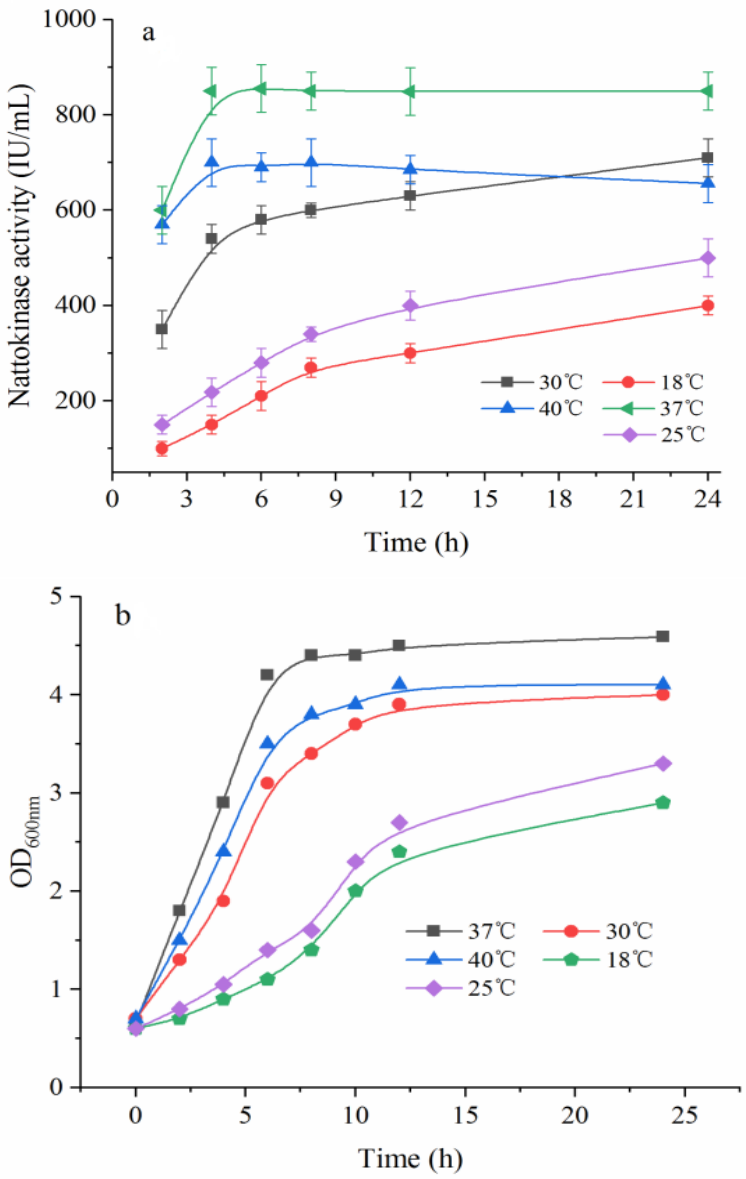

Figure 6. Effect of temperature on nattokinase activity changes (a) and on cell growth dynamics (b) of WB800N/pHT43-pro-aprN cells within 8 h after induction with IPTG.

WB800N/pHT43-pro-aprN cells, changes in the NK activity over the course of fermentation time were assessed. As shown in Figure 7a, NK activity increased rapidly with fermentation time within the first 4 h after IPTG induction before plateauing for the next 4 h. At 4 h post induction with an IPTG concentration of 1.0 mM, the NK expression level (as quantified based on enzyme activity per unit of biomass) reached a maximum of 835.83 IU/mL. Extracellular protein from different fermentation time points by semi-quantitative SDS-PAGE analysis, maximal NK production within 4 h of induction was further assessed (Figure 7b). Further examination of kinetics data revealed that NK production was associated with cell growth only during this first 4 h after induction, and as such these cultures could be harvested before entering into the stationary phase of growth while still ensuring maximal NK production.

Effect of growth phase at time of induction on nattokinase activity

To test whether the cell growth phase at time of IPTG induction has any effect on engineered nattokinase activity

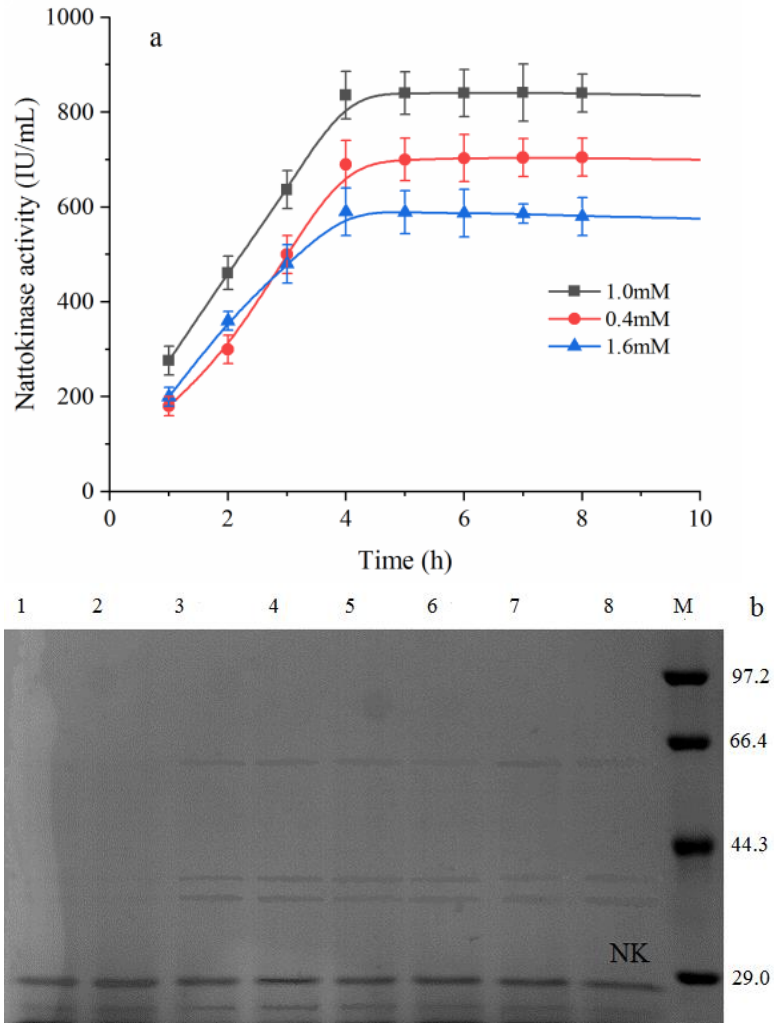

Figure 7. (a) Changes in nattokinase activity/production at 37°C in WB800N/pHT43-pro-aprN cells over a fermentation period of 8 h following 1.0 mmol/L IPTG induction by IPTG, with a corresponding growth curve. **(b)** Semi-quantitative SDS-PAGE analysis of nattokinase production at 37 ℃ in WB800N/pHT43-pro-aprN cells at different fermentation time points after induction with 1.0 mmol/L IPTG. Lane M, protein molecular weight standards; Lane 1-8, cell samples from 1 to 8 h post-induction at 1 h intervals.

in WB800N/pHT43-pro-aprN cells, IPTG induction was carried out at different cell densities reflecting different cell growth phases. Initially, 2% of the seed solution culture was inoculated into fresh media, and then IPTG (1.0 mM) was added at selected cell densities (0.2–1.8 OD_{600}) followed by measurement of enzyme expression 4 h post-induction. When the IPTG induction was carried out from a lower cell density phase to a higher one, the expression of the engineered protein increased, achieving a maximal induction of 835.39 IU/mL at a 0.6 OD_{600}, after which the yield decreased (Figure 8). This finding indicated that early in the logarithmic phase of cell growth, the addition of IPTG can promote the expression of NK, while later in the logarithmic phase of growth IPTG-induced expression gradually decreased. These data indicated that the optimal cell growth phase for IPTG induction of nattokinase expression in these engineered strains is early in the logarithmic phase of cell growth, consistent with a report regarding the production of the antimicrobial peptide cathelicidin-BF expressed in engineered *B. subtilis* WB800N cells (Luan et al., 2014).

Effect of media pH on enzyme activity

The optimal pH for nattokinase expression in WB800N/pHT43-pro-aprN cells was determined to be 7.5, and at this pH the NK enzyme activity reached a

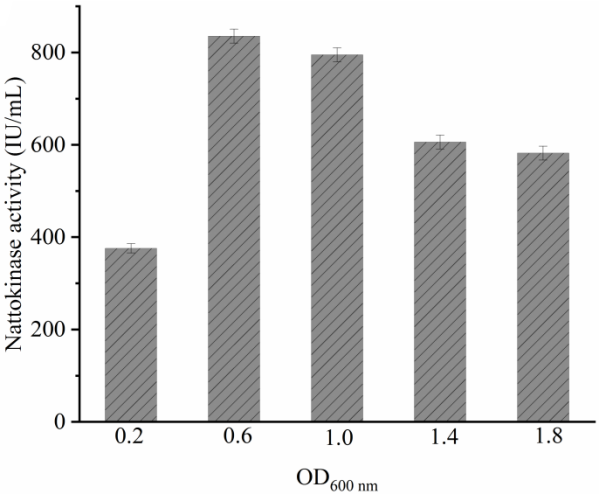

Figure 8. Effect of cell growth stage at time of induction (as determined by OD_{600}) on nattokinase activity in WB800N/pHT43-pro-aprN cells induced with IPTG for 4 h at 37°C.

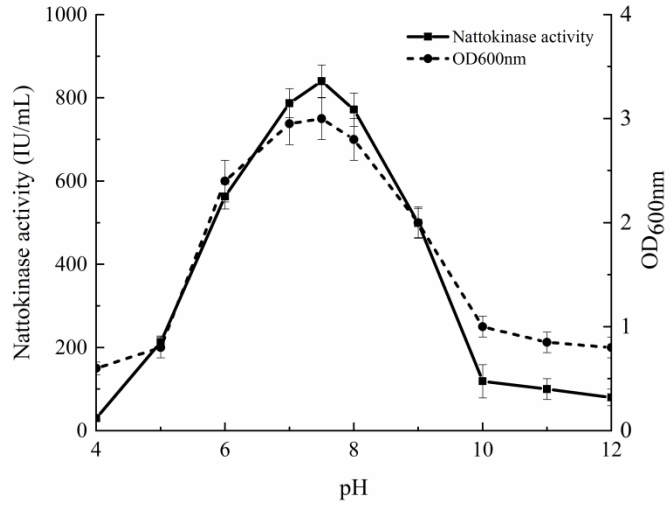

Figure 9. Effect of media pH on nattokinase activity change (solid line) and on cell growth curve (dotted line) of WB800N/pHT43-pro-aprN cells induced with IPTG for 4 h at 37°C.

maximum of 839.17 IU/mL. When the pH was less than 6 or greater than 9, the cell density and the NK enzyme activity decreased dramatically (Figure 9).

Isolation and purification of nattokinase

Compared with the fermentation supernatant, the purification ratio of nattokinase reached 6.63 and the total recovery was 80% (Table 2). The heteroproteins in the fermentation broth were initially removed via salt precipitation, and then nattokinase was isolated and purified via ultrafiltration. An SDS-PAGE analysis revealed that the molecular weight of a single band was about 28 kDa. The purified nattokinase was detected via fibrinolytic plate assay and the results showed good fibrinolytic activity. As such, nattokinase with a high degree of recovery can be obtained via salt precipitation and ultrafiltration.

DISCUSSION

In this study, aprN and pro-aprN encoding nattokinase in

Table 2. Separation and purification results of nattokinase.

Purification step	Protein/mg	Total activity/IU	Specific activity (IU/mg)	Degree of purification	Recovery rate
Fermentation liquid	122.95	213472	1736.25	-	100
Precipitation with ammonium sulfate	56.17	201859.12	3593.72	2.07	94.56
Ultrafiltration	14.84	170777.6	11507.92	6.63	80

Bacillus natto using novel primers was amplified. Two engineered cell lines using the pHT43 plasmid vector, which was expressed in *Bacillus subtilis* was constructed WB800N allowing us to assess enzymatic activity and optimize culture conditions. Although the leader peptide is not part of the functional domain of the NK protein and does not contribute to protein function, it does contribute to the formation of an active 3D structure (Jia et al., 2010). Yabuta et al. (2001) studied the refolding pathway of *B. subtilis* protease E *in vitro*, demonstrating that the leader peptide confers correct folding information for the domain during the folding process. Studies have also shown that specific interactions between the leader peptide and the *B. subtilis* protease domain are important for precursor folding (Weng et al, 2009; Sone et al, 2005). As nattokinase is a serine protease produced by *B. subtilis*, these studies further provide insights into NK folding. In this study, engineered strains expressing NK leader peptide sequences yielded NK protein with high enzymatic activity, while engineered strains expressing only the mature peptide without the leader sequence produced inactive NK protein, consistent with previous studies on the importance of leader peptides.

Man et al. (2018) constructed a *B. subtilis* MX-6 strain expressing nattokinase to maximize nattokinase production 72 h after induction of fermentation, achieving a clear zone diameter on the plasminogen-free fibrin plate of 21.60 mm. Guan et al. (2016) constructed a *B. subtilis* strain expressing nattokinase by optimizing the gene promoter, achieving maximal nattokinase production 36 h after induction of fermentation. Several studies have found that nattokinase production can reach a maximum after 24 h of fermentation. Unlike in these previous studies, in the present study the engineered bacterial strains which we produced achieved maximal nattokinase production just 4 h after induction and initiation of fermentation, with a maximum crude enzymatic activity as high as 848.52 IU/mL, representing a clear improvement in industrialization efficiency. SDS-PAGE based analyses indicated that our engineered strains also produce relatively low levels of heteroprotein species, which will reduce the need for downstream purification processing and will therefore reduce the cost of industrial NK production. We were able to determine that optimal culture conditions for NK production with this bacterial expression system were as follows: media pH at 7.5, 1 mmol/L IPTG induction for 4 h, and fermentation at 37°C, achieving a maximum crude enzyme activity yield of 848.52 IU/mL. These results indicate that overexpression of the pHT43 vector may be an effective and useful strategy for NK production, while the host strain WB800N demonstrates high efficiency and high purity of NK secretion.

In follow-up experiments, the fermentation medium of the engineering strain and isolated and purified nattokinase via salt precipitation and ultrafiltration was optimized. Compared with fermentation supernatants, the purification ratio of nattokinase reached 6.63, with a total recovery of 80%, and a specific enzyme activity of 11507.92 IU/mg. In summary, the engineered strains constructed in this study have great promise as potential strains useful for industrial-scale NK production. Further efforts to optimize and improve NK production are ongoing, with the goal of further improving protein yields and lowering the cost of the industrial production of nattokinase.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

This study was supported by the key projects of Nature Science Foundation of Hubei, China (2013CFA101).

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Vol. 13(27), pp. 500-509, September, 2019
DOI: 10.5897/AJMR2019.9096
Article Number: 8A7D66161930
ISSN: 1996-0808
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African Journal of Microbiology Research

Full Length Research Paper

Evaluation of inactivated vaccine against fowl cholera developed from local isolates of *Pasteurella multocida* in Ethiopia

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Received 14 March, 2019; Accepted 14 May, 2019

Fowl cholera caused by *Pasteurella multocida* is among the serious infectious diseases of poultry in Ethiopia. This study was conducted to develop a vaccine from local strains of *P. multocida* and evaluate its performance. Inactivated vaccine was prepared following the OIE standards in three adjuvant formulations (oil, alum and gel). The performance of the different formulations was evaluated at different dose rates (0.5 and 1 mL) and routes (subcutaneous, SC and interamuscular, IM) in vaccination-challenge experiment in a total of 160 (six weeks old) chicken. The vaccinated groups showed significantly higher (P<0.05) mean antibody titer at day 21 (1365.49±376.97) and day 35 (1707±193.95) post-vaccination compared to the mean value at day 0 (200.01±4.91) and that of the unvaccinated group (196.72±10.51.147). The highest antibody titer obtained was for group vaccinated with 0.5 mL of alum-adjuvanted vaccine given IM (2472.96±603.47). The differences in antibody titer among vaccinated groups with respect to types of adjuvant and dose rates were insignificant. All vaccine formulations provided significant protection with survival rates ranging from 80 to 100% with alum-adjuvanted vaccine given IM being superior both in protective efficacy (100%) and in the absence of clinical signs post-challenge indicating its potential application in the control of fowl cholera.

Key words: Cholera, *Pasteurella multocida*, vaccines, poultry, Ethiopia.

INTRODUCTION

Poultry production contributes approximately 20% of the protein consumed in developing countries (Jenssen and Dolberg, 2003). Intensive poultry farming has become an important economic activity in Ethiopia particularly in the suburbs of major cities. Besides to its economic and social values, it occupies a unique position in terms of high quality protein source to rural smallholder farming families in Africa including Ethiopia (Dessie and Ogle,

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2001). The growing demand for poultry products associated with the rapid increase in urban population in Ethiopia has led to a rapid increase in total chicken population from 50.38 million in 2007 (CSA, 2007) to 56.87 million in 2015 (CSA, 2015).

The poultry industry is growing fast in Ethiopia and is facing many constraints especially infectious diseases causing significant setback to the development of this sector. Most of the infectious diseases are endemic, but some are emerging and re-emerging diseases. Poultry diseases are responsible for a number of adverse economic effects due to mortality and morbidity of chickens, cost of medication, loss in production and ban on international trade and public health significance (Dana et al., 2008).

In Ethiopia, poultry mortalities due to disease are estimated to range from 20 to 50%, but they can rise as high as 80% during epidemics. Fowl cholera has been among the major problems limiting chicken production in Ethiopia (Gebre-Egziabher, 2007). Despite frequent complaints and the reports from public and private poultry farms associated with outbreaks of fowl cholera, no interventions were taken to address the problem (Molalign et al., 2009). Owing to its significant impact on poultry production, control of the disease is essential. Of all the control strategies in areas where the disease is endemic, the use of vaccines is the most efficient from both practical and economical point of view (OIE, 2012). This is due to the fact that the onset of the disease is very rapid, and therefore, high mortality occurs before the disease is diagnosed for subsequent treatment. Antibiotic treatment and prophylaxis are of limited value owing to the emergence of multidrug-resistant strains. Globally, two types of vaccines are being used to immunize birds against fowl cholera namely, live and inactivated vaccines (Rhoades and Rimler, 1991). Live attenuated vaccines provide good protection with long duration of immunity and cross-protection against *P. multocida of* different serotypes or surface lipopolysaccharide (LPS) structures unlike killed vaccines which give protection only against strains with identical or nearly identical surface LPS structures (Harper and Boyce, 2017). However, the use of attenuated (live) vaccine is limited due to the lack of regular maintainable and sustainable attenuation methods and/or vaccine instability problems which may lead to risk of regaining its virulence. In this regard, inactivated vaccines or bacterins have comparative advantages over the attenuated live vaccines and are thus preferred to protect chicken against the disease caused by homologous strains (OIE, 2012).

Poultry enterprises in Ethiopia have experienced problems with the disease due to unavailability of locally produced vaccine. Although these vaccines have been produced in other countries, serotype variations among the pathogen may limit the use of the widely available conventional vaccines, making vaccines developed from locally circulating strains essential and more preferable (et al, 2016). There have been efforts to manufacture the vaccine at the National Veterinary Institute in Ethiopia. The first trial inactivated fowl cholera vaccine (Molalign et al., 2009) developed from local isolates was found to be effective in protecting experimentally challenged layer chickens. This work, however, need to be further validated using different adjuvants and dose rates and moreover there is a need to develop optimized standard operating procedure (SOP) for large scale (industrial) production of the vaccine. This work was aimed at developing inactivated Fowl Cholera vaccine from local *P. multocida* isolates and subsequently evaluate its safety, immunogenicity and protective efficacy in three different adjuvant formulations (Oil, Alum and Gel), at different dose rates and routes of administration.

MATERIALS AND METHODS

Study area and experimental animals

The current experimental study was conducted from November 2016 to May 2017 at the National Veterinary Institute (NVI), Bishoftu/Debrezeit, Ethiopia, located 45 km South East of Addis Ababa. NVI is a center for livestock vaccine research, development and production, and currently produce over 22 vaccines against major bacterial and viral diseases of livestock and poultry.

A total of two hundred nine (four weeks old, Bovans brown, layer chickens) obtained from brood stock farm and four rabbits obtained from NVI's laboratory animal facility were used for the current study. Chicken screened negative for antibody against fowl cholera by Enzyme Linked Immunosorbent Assay (ELISA) were included in the experiment. The chickens were vaccinated against Marek's, Gumboro, Newcastle disease and Fowl pox as per the scheduled time. The chickens were fed with formulated pullet and layer feed and water *ad libitum*. The chickens were kept at the NVI animal experiment facility. All animal experiments were approved by animal ethics committee of NVI and College of Veterinary Medicine and Agriculture (CVMA) of Addis Ababa University.

Experimental design

The safety of vaccines, prepared from three different adjuvants (Alum, Oil and Gel), were tested in three rabbits followed by a safety test in target animal (chicken). A total of 40 chickens randomly assigned into four groups containing 10 chickens each was used for the safety test; three of the groups for each vaccine type while one group was used as control. The immunogenicity and protective efficacy of the trial inactivated fowl cholera vaccine was done in vaccination-challenge experiment using a total of 160 chickens randomly assigned into eight groups of 20 chickens each. Six of the groups were assigned to each vaccine type at two dose rates (0.5 and 1 ml) given subcutaneously while one group was assigned for alum adjuvated vaccine given intramuscularly (0.5 ml) and the remaining as unvaccinated control.

Bacteriological media

Tryptose soya broth, TSB (Oxoid, Hampshire, UK) and Tryptose

soya agar, TSA (Difco, Sparks, USA) supplemented with 10% serum was used for culturing *P. multocida* isolates. *P.multocida* type A inoculum media (PA media), containing peptone (10 g), NaCl (5 g), Na_2HPO_4(3 g), H_2K (2.5 g), $MgSO_4$(1 g), yeast extract (2.5 g), glucose (5 g) and horse serum (5 mL) in 1L of distilled water, was used for growing *P. multocida* vaccine seed strain. Culturing for large scale vaccine preparation was done in *P.multocida* production media containing meat and liver digest broth (1L), yeast extract (2 mL), glucose (5 g) and horse serum(6.25 mL) with PH adjusted to 7.6.

Bacterial strains and growth conditions

Three isolates of *P. multocida* isolated from three different outbreaks (farm A, B and C) and stored at NVI bacterial culture collection were used as vaccine strains for the study. The lyophilized isolates were initially reconstituted with 2ml of TSB, which was then inoculated into the TSB supplemented with 10% horse serum and incubated at 37ºC for 18 h. Bacterial growth was checked by measuring the turbidity and pH of the media.

Identification and characterization of candidate vaccine seed bacterial strains

The three isolates of *P. multocida* strains were cultured on TSA, blood agar and MacConkey agar (Oxoid, Hampshire, UK) after which purity and colony characteristics was observed and recorded. Primary and biochemical (secondary) identifications were done employing Gram's staining and biochemical tests such as oxidase, catalase tests were performed according to standard methods described previously (Quinn et al., 2002).

Identity of the isolates to serotype level was done by *P. multocida* serotype specific multiplex PCR assay using the 5 primer sets specific to each capsular type (A, B, D, E and F) that target capsular biosynthesis gene cluster (*cap)* described previously (Townsend et al., 2001).

Bacterial DNA extraction

An overnight pure culture of the bacterial isolate in the log phase was used for DNA extraction. Genomic DNA was extracted using DNeasy Blood and Tissue kit (Qiagen, Germany) following the manufacturer's instructions. The eluted DNA was labeled and stored at -20ºC until analysis.

Serotype specific multiplex PCR assay

Briefly, 50 µL PCR reaction mixture contained 1 U *Taq* DNA polymerase (Qiagen), 3.2 mM of each of the forward and reverse primers (Eurofins Genomics, Austria), 200µM of each dNTP (Qiagen), 1×PCR buffer, and 2 mM $MgCl_2$ (Fermentas, Germany). Amplification protocol used was initial denaturation at 95°C for 5 min, followed by 35 cycles 95°C for 30s, 55°C for 30s, 72°C for 30s and final extension at 72°C for 7 min (Townsend et al., 2001). Each PCR product was detected in electrophoresis after running on a 1.5% agarose gel stained with GelRed for 1h at 100V. PCR products were visualized under UV illuminator and image taken in gel documentation system (UVI TEC, UK).

Sequencing and phylogenetic analysis

The PCR products were purified using the Wizard SV Gel and PCR clean-up system kit (Promega, Germany). The purified PCR products concentration were determined using the NanoDrop 2000c spectrophotometer (Thermo Scientific, USA) and then sequenced through a sequencing service (LGC genomics, Germany) using both primers (forward and reverse).

The sequences were edited using Vector NTI Advances™ 10 software (Invitrogen, Carlsbad, CA, USA) and consensus sequences were generated using BioEdit. For comparative multiple sequence analysis, blastn was used to retrieve *P. multocida HyaD* gene sequences data from the GenBank database. The sequences of the current isolates together with the homologous gene sequences retrieved from GenBank were aligned using BioEdit version 7.1.3.0 (Hall, 1999). Multiple sequence alignment of the nucleotide sequences were performed using the ClustalW and phylogenetic tree was constructed using the Neighbor-Joining method with the maximum composite likelihood nucleotide substitution model and the pairwise deletion. All analysis was done using MEGA version 6 software (Tamura et al., 2013). The accession number and details of the isolates' sequences included in the study is presented in Table 1.

Preparation of experimental vaccine

Of the three identified isolates, the isolate from farm A was considered as a master seed strain for the current trial vaccine due to the highest titer (CFU/mL) it had compared to the other two isolates (Farm B and C) grown under the same conditions. Formalin inactivated fowl cholera vaccine was prepared in three 500 mL flask each containing 300 mL media. One vial of the lyophilized isolate seed was initially reconstituted with 2 ml of TSB, homogenized well and then inoculated into sterile TSA supplemented with10% horse serum and incubated at 37ºC overnight.

The colonies were examined visually and microscopically after gram staining for their purity and cellular morphology, cocco-bacilli organisms and Gram negative. A single colony was transferred to 2 mL tube containing *P. multocida* type A inoculum media and incubated for 7 h at 37°C after which the purity was checked again by Gram staining. Half mL of the broth culture was transferred into 30 mL of *P. multocida* type A (PA) inoculum media and incubated overnight.

The purity of the PA inoculum was checked as described above and inoculated into PA production media at the ratio of 7 mL of *P. multocida* Type A (PA) inoculum, 7 mL of glucose and 3 mL of serum per 300 mL of *P. multocida* production media then incubated for 48 h with slow agitation. After two days, the purity was evaluated by Gram staining while the titer was determined by measuring the pH (5.2 to 5.8) and Optical Density (OD) of the culture medium as well as by titration using streak plate method. At the pH and/or OD value that corresponded to the desired titer (10^8 CFU/mL and above), the culture was inactivated by adding sterile formaldehyde at a proportion of 0.05% followed by incubation at 37°C with slow agitation for 6 days.

In process vaccine quality control tests

Purity and sterility test

The inactivated cultures (the vaccines) were checked for purity using gram stain while sterility was tested by culturing on media such as TSA, TSB, VF broth and Sabouraud agar media. All the test media were incubated at 37ºC except Sabouraud agar which was incubated at room temperature. Un-inoculated media from each type was also incubated as a negative control. All these inoculated media were observed for two weeks for any microbial growth (OIE, 2015).

Table 1. Nucleotide sequences of the local isolates and sequences retrieved from the GenBank included in the present study.

Strain	Accession number	Source	Reference
P. multocida	AY225345	Iran	Jabbari and Esmaelized (2003a)
P. multocida	AY225346	Iran	Jabbari and Esmaelized (2003b)
P. multocida	AY225347	Iran	Jabbari and Esmaelized (2003c)
P. multocida	AF036004	USA	De Angelis et al. (1998)
P. multocida	AF237926	USA	Fuller et al. (2000)
P. multocida	JF922885	USA	Tahmtan et al. (2011)
P. multocida	KP036621	China	Yang et al. (2014)
*P.multocida (*NVI-01-2017)	MK802880	Ethiopia	This study
P. multocida (NVI-02-2017)	MK802881	Ethiopia	This study

Table 2. Experimental layout of chickens used for immunization and challenge test.

Group	Dose (ml)	Adjuvant	Route of injection
A	0.5	Alum	SC
B	1	Alum	SC
C	0.5	Alum	IM
D	0.5	Oil	SC
E	1	Oil	SC
F	0.5	Gel ($Al(OH)_3$)	SC
G	1	Gel ($Al(OH)_3$)	SC
Control	-	-	-

Safety test of inactivated vaccine

The safety of inactivated vaccine was done in laboratory animals. Three rabbits were injected with 1mL of inactivated culture intramuscularly from each batch (flask) of vaccine, and observed for 14 days for any adverse reaction (OIE, 2015).

Vaccine adjuvant formulation

In this study, sterile Montanide oil, aluminum hydroxide (gel) and Aluminum potassium sulphate (Alum) were used as adjuvants. Oil adjuvant vaccine was used in 1:1 proportion while Aluminum hydroxide gel and Aluminum potassium sulphate (alum) were used at the rate of 1.2 and 1% of the vaccine, respectively. After the addition of each adjuvant, the mixture is continuously agitated using magnetic stirrer and the pH was adjusted to 7.0 before dispensing into 50 mL sterile polypropylene vials.

Quality control of the final prototype vaccine product

Sterility of the final product was evaluated employing same method described for inactivated culture while the safety was done in target animals (Chickens). For safety test, 40 chickens were grouped randomly in to four groups (each with 10 chickens), three of the groups assigned for each vaccine formulation while one was used as control. Chicken in group I, II and III were injected with 1mL each of the vaccine formulations (oil, alum and gel adjuvanted), respectively, through subcutaneous route at the back of the neck and the fourth control group was injected with 1 mL of sterile TSB media. The chickens were observed for 14 days for any adverse effects (OIE, 2015).

Evaluation of vaccine efficacy

Experimental layout

The protective efficacy of the newly prepared vaccine formulations was determined in a vaccination-challenge experiment in chicken. A total of 160 layer pullets (13 weeks old) randomly divided into eight groups each with 20 birds were used for vaccine efficacy trial. Six of the groups were assigned for Oil, gel and Alum adjuvated vaccines and 2 groups per vaccine type for two different doses rates (1 mL and 0.5 mL) subcutaneously. An additional one group was assigned for alum adjuvant vaccine for vaccination using IM route at dose rate of 0.5ml while the remaining group was used as unvaccinated control. The experimental layout is presented in Table 2.

Immunization

The chickens were acclimatized for a week in the experimental facility. At day 21 after the first vaccination, booster vaccination was administered (OIE, 2015). Blood sample was collected from the wing vein from vaccinated and control birds at days 0, 21 and 35 post-vaccination to determine antibody titer. Sera samples were stored at -20°C until analysis.

Determination of immune response and test interpretation

The immune response was evaluated based on the relative level of antibody to *P. multocida* in chicken serum using IDEXX PM ELISA Kit (IDEXX Laboratories, Inc. UK) according to the manufacturer's instruction. Briefly, the test was performed on a 96-well ELISA plate coated with *P. multocida* antigen. Test sera were diluted five hundred fold (1:500) and dispensed in duplicates (100 uL) on coated wells while undiluted negative and positive controls (each 100 uL) were also dispensed on the coated wells. The plate was incubated for 30 min at 20^{o}C so that antibodies specific to *P. multocida* form a complex with the coated antigens. The plate was washed with distilled water to remove any unbound material from the wells followed by addition of a conjugate (100 uL) and incubation at 30 min at 20°C to enable binding to any attached chicken antibody in the wells. Unbound conjugate was washed away as above and enzyme substrate (100 uL) was added. The

Table 3. Phenotypic characteristics the *P. multocida* type A isolates.

Test	Isolates		
	1	2	3
Hemolysis on blood agar	No-hemolytic	No-hemolytic	No-hemolytic
Growth on MacConkey's agar	No growth	No growth	No growth
Gram's reaction	-	-	-
Cellular morphology	Coccobacilli	Coccobacilli	Coccobacilli
Indole production	+	+	+
Catalase test	+	+	+
Oxidase test	+	+	+
MP test	-	-	-
VP test	-	-	-
TSI test	Y/Y	Y/Y	Y/Y
Citrate test	+	+	+
Urease test	-	-	-
Interpretation	*P. multocida*	*P. multocida*	*P. multocida*

+, Positive; -, negative; Y/Y=yellow slant and yellow butt

absorbance or optical density (OD) of the subsequent color development was measured by spectrophotometer at 650nm and the corresponding OD value was directly related to the amount of antibody to *P. multocida* present in the test sample. The corresponding antibody titer was determined from the OD values using the method stated in the test kit instruction which was given as:

$$\text{Titre} = \text{antilog}\,(1.09(\log_{10} S/P) + 3.36$$

Where:

$$S/P = \frac{\text{Mean OD of test sample} - \text{Mean OD of negative control}}{\text{Mean OD of positive control} - \text{Mean OD of negative control}}$$

The value 3.36 relates S/P at a 1:500 dilution as endpoint titre. The test result was interpreted as S/P ≤ 0.20 as negative and S/P ≥ 0.20 (titer greater than 396) as positive.

Challenge experiment

P. multocida Kombolcha strain used as seed for vaccine preparation was also used as a challenge strain. Before the actual challenge test, a pilot challenge experiment was carried out to determine LD_{50} of the challenge strain and to optimize the pathogenicity and the challenge dose, which was found to be $5x10^8$ CFU/mL. At day 35, chicken in all groups (vaccinated and controls) were challenged with a suspension of 6 hr culture of *P. multocida* containing $5x10^8$ CFU/mL IM on breast muscle.

The chickens were then followed-up for two weeks for any development of clinical signs and mortality and the results were recorded. All dead birds were necropsied and specimen from organs was cultured for re-isolation of *P. multocida* by streaking on TSA with 10% serum. Surviving chickens were slaughtered at the end of experiment and specimen collected from internal organs for re-isolation of *P. multocida*. Isolation was done by culturing on blood agar followed by identification through morphology, staining, cultural, biochemical test, and finally by species-specific PCR.

Ethical declaration

All animal experiment has been approved by the Animal Research Ethics Committee of the National Veterinary Institute regarding its conformity to the ethical standards set in international guiding principles for animal experiment research.

Data analysis

Raw data were entered in to Microsoft Excel spreadsheet and transferred to SPSS 23.0 for analysis. Descriptive statistics such as proportions, averages and frequencies were used in summarizing quantitative data as required. The Analysis of Variance (ANOVA) was used to find out the differences in the mean antibody titers among immunized groups vaccinated with the different vaccine formulations and dose rates. The desired level of precision and confidence level used in the analysis was 5 and 95%, respectively (Thrusfield, 2005).

RESULTS

Identification of isolates

The three isolates obtained from three different outbreaks suspected of fowl cholera showed similar phenotypic characteristics consistent with *P. multocida*. The results of phenotypic characteristics of the isolates are presented in Table 3. Molecular identification of the isolates confirmed that all belong to *P. multocida* with the same capsular type, that is, capsular type A.

Molecular identification

Molecular identification in capsular typing multiplex PCR assay showed that all the three isolates were positive for capsular type A with PCR product of 1044 bp similar to the positive control (Figure 1).

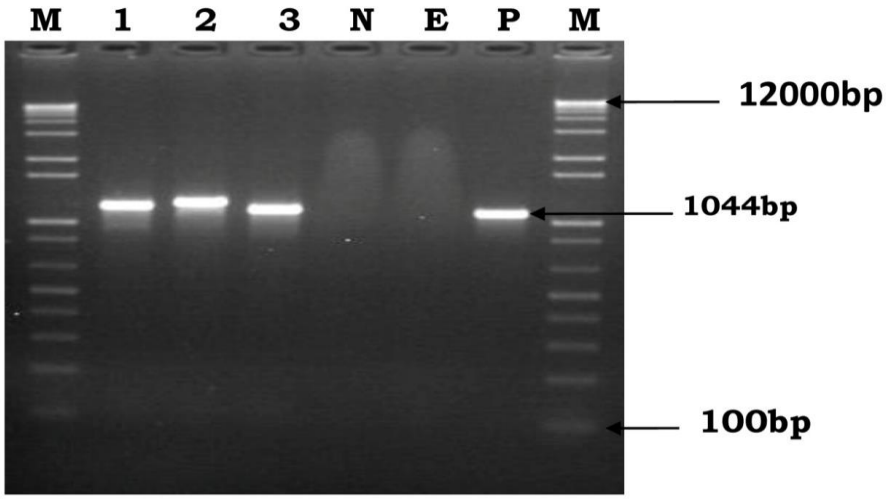

Figure 1. Multiplex PCR using four sets of specific primers targeting capsular biosynthesis genes (*capA, capB, capD* and *capF*) of *P. multocida*. Lanes: M, Molecular marker (started 100bp 1kb plus, Invitrogen); 1, *P. multocida* Kombolcha isolate; 2, *P. multocida* Genesis isolate; 3, *P. multocida* Tadesse farm isolate; N, Negative control; E, extraction internal control; P, positive control (*P. multocida* capsular

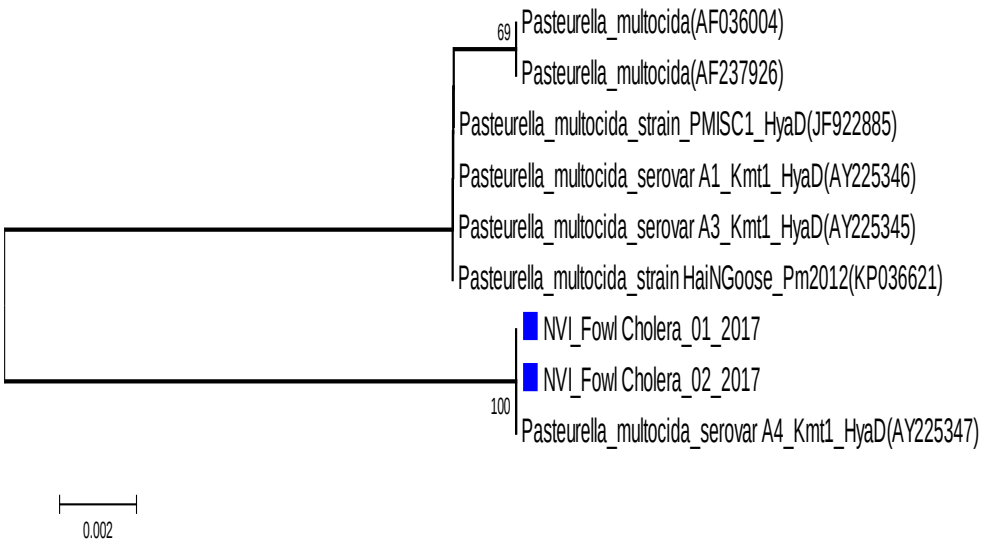

Figure 2. Phylogenetic analysis of the *HyaD* gene coding sequence of *Pasteurella multocida* Isolates.

Phylogenetic analysis

Phylogenetic analysis based on the sequence data of *HyaD* gene indicated that the local *P. multocida* isolates were closely genetically related to one of a previously characterized reference isolate *P. multocida* serovar A4 of Iran origin while divergent from the remaining strains included in the analysis as shown in Figure 2.

The tree was constructed using neighbor-Joining method with the pairwise deletion option in MEGA6.

Vaccine titre and safety

The titre of the current vaccine was $5x10^8$ CFU/mL and with pH and OD (optical density) value of 5.62 and 0.738, respectively. Follow-up of rabbits and chicken injected with formalin inactivated ana-culture and final vaccine

Table 4. Mean serum antibody titer of chickens in different groups at day 21 after primary vaccination with inactivated Fowl Cholera vaccine.

Group	Dose (ml)	Adjuvant	Route	Mean±SE of Ab titer	S/P ratio	F value	Significance level (P value)
A	0.5	Alum	SC	599.35±114.9689	0.285		
B	1	Alum	SC	648.54±233.88	0.298		
C	0.5	Alum	IM	1205.28±296.59	0.535		
D	0.5	Oil	SC	761±193.30	0.350	1.18	0.321*
E	1	Oil	SC	2035.43±763.24	0.836		
F	0.5	$Al(OH)_3$	SC	775.12±255.39	0.349		
G	1	$Al(OH)_3$	SC	3533.33±2463.47	1.304		

* P>0.05, no significant difference among the means of antibody titers.

Table 5. Mean serum antibody titer of chickens in different groups 2 weeks after booster vaccination (day 35) with inactivated fowl Cholera vaccine.

Group	Dose (ml)	Adjuvant	Route	Mean±SE of Ab titer	S/P ratio	F-value	Significance level (P value)
A	0.5	Alum	SC	1852.24±431.06	0.797		
B	1	Alum	SC	1500.76±772.43	0.611		
C	0.5	Alum	IM	2472.97±603.47	1.034		
D	0.5	Oil	SC	1177.59±276.07	0.52	0.71	0.642*
E	1	Oil	SC	1471.37±331.67	0.642		
F	0.5	$Al(OH)_3$	SC	1466.23±345.20	0.640		
G	1	$Al(OH)_3$	SC	2009.93±643.84	0.85		

* P>0.05, no significant difference among the means of antibody titers.

with adjuvant, respectively, did not show any adverse effect or clinical signs of infection during the 14 days of period indicating that the product was safe.

Evaluation of immunogenicity

In immunization test, the pre-vaccination mean serum antibody (Ab) titer in chickens of all groups was 200.01±4.91(S/P ratio, 0.11) while that of unvaccinated control group was 196.72±10.51.147 (S/P ratio, 0.13). The Mean serum Ab titers at day 21 and 35 were 1365.49±376.97 and 1707±193.95, respectively which were both significantly higher (P<0.05) than the control group and background titer at day 0 of the respective groups. The highest serum Ab titer was obtained for chicken groups vaccinated with 0.5 mL of alum adjuvanted vaccine given IM (2472.96±603.47). However, no significant differences were observed in antibody titers between the respective groups of birds vaccinated with vaccine formulations of different adjuvants and dose rates. The mean serum antibody titer of chicken's in the different groups at day 21 and 35 after primary vaccination is summarized in Tables 4 and 5.

Evaluation of protective efficacy

Protective efficacy test showed that some chicken in all vaccinated groups, except 0.5 mL Alum IM group, showed signs of depression, ruffled feathers, loss of appetite and drop in egg production a day after challenge which lasted from 2 to 5 days. None of the chicken vaccinated with 0.5 mL alum adjuvant vaccine given through IM route showed any signs and all were active after challenge. Chicken in non-vaccinated control group showed clinical signs including depression, loss of appetite, greenish diarrhea, conjunctivitis, cloudiness of the eye with unilateral or bilateral blindness, labor breathing, lameness with swollen joint in some cases with death starting from the second day of challenge. Number of Chickens showing clinical signs indicative of fowl cholera is summarized in Table 6.

The protective efficacy evaluation showed that alum ($AlK(SO_4)_2$) adjuvant vaccine given intramuscularly provided 100% protection with none of the birds showing clinical signs after challenge. Although 1 mL of Gel ($Al(OH)_3$) adjuvated inactivated vaccine given SC provided 100% protection, two of the birds showed clinical signs consistent to Fowl cholera from which later *P. multocida* was re-isolated (Table 7). In all chicken that

Table 6. Number of chicken showing different clinical signs indicative of fowl cholera starting a day after the challenge experiment.

Group	Adjuvant and route of injection	Labor breathing	Depression	Greenish diarrhea	Swelling of joints and lameness	Conjunctivitis	Ruffled feathers	**
A	0.5 mL Alum, SC	3	3	2	3	2	3	3
B	1 mL Alum, SC	3	3	2	2		2	3
C	0.5 mL Alum IM	-	-	-	-	-	-	0
D	0.5 mL Oil, SC	2	2	3	2	2	4	4
E	1mL Oil, SC	3	3	1	3	-	3	3
F	0.5 mL Gel, SC	3	3	2	3	1	3	3
G	1 mL Gel, SC	2	2	1	2	1	2	2
H	Control, 1mL SC	20	20	16	20	20	20	20

** = Total No of Chickens which showed clinical sign.

Table 7. Protective efficacy of inactivated fowl cholera vaccine formulated with three types of adjuvants.

Group	Birds (no)	Dose (ml)	Adjuvant	Route	No of chicken Survived (%)	No of chicken died (%)
A	20	0.5	Alum	SC	19 (95)	1 (5)
B	20	1	Alum	SC	20 (100)	0 (0)
C	20	0.5	Alum	IM	20 (100)	0 (0)
D	20	0.5	Oil	SC	19 (95)	1 (5)
E	20	1	Oil	SC	19 (95)	1 (5)
F	20	0.5	$Al(OH)_3$	SC	19 (95)	1 (5)
G	20	1	$Al(OH)_3$	SC	18 (90)	2 (10)
H	20	NA	NA	NA	4 (20)	16 (80)

died during the course of the experiment, *P. multocida* was re-isolated from blood, joint lesion and internal organs (liver, spleen and heart) and subsequently identified using both phenotypic and molecular methods.

P. multocida was isolated from the liver, spleen, heart, blood and lung of the control group in 4 randomly selected birds from each group at the end of the experiment (14 days after challenge), whereas none from vaccinated groups.

DISCUSSION

Fowl cholera caused by *P. multocida* is a highly contagious disease of poultry presenting one of the major challenges of the poultry industry worldwide (Singh et al., 2014). The fast growing poultry sector in Ethiopia has been confronted by a number of infectious diseases among which one is fowl cholera. However, there is no information on the genotypes of *P. multocida* causing fowl cholera and neither locally available vaccine. The current studies is aimed at characterizing P. multocida strains, from cases of fowl cholera and develop vaccine from local strains, which is pertinent strategy to address the problem associated with the disease.

The identification of capsular biotype A *P. multocida* isolates from an outbreak in the current study was supported by the results of phylogenetic characterization where both isolates

were found to be of same genotype. However, this may not reflect nationwide distributions of genotypes that may be involved in causing fowl cholera thus requiring further studies to elucidate its molecular epidemiology. The phylogenetic relationships of the current isolates from Ethiopia with the Iranian isolate (Jabbari and Esmaelized, 2003a) suggest the strains may have global distribution and are common causes of fowl cholera elsewhere.

The significantly higher immune response as seen from antibody titre between vaccinated and non-vaccinated category, despite the high variation within a group, shows that the vaccine is highly immunogenic. The variation within a group observed may be due inherent individual differences since precautions were taken during the study design to minimize 'within group effects' where same breed and age group of birds were included. The significantly higher immune response was further substantiated by the results of the protective efficacy where all the three adjuvant formulations of the current vaccine conferred significant protection of chicken. The findings of significantly higher immune response in groups vaccinated through IM route than the SC groups unlike the absence of significant differences among the vaccines with three adjuvant formulations (alum, oil and gel) may suggest that the route of administration is more important than the type of adjuvant used. This is in agreement with previous reports where IM delivery of inactivated fowl cholera vaccine provided better antibody response and protective efficacy than the SC route of administration (Rahman et al., 2004). Previous study by Molalign et al. (2009) also found that alum ($AlK(SO_4)_2$) adjuvant fowl cholera vaccine given IM provided better HAI titer at bacterial dose of 10^{12} CFU/mL than the Oil based (Montanide ISA 50) and $Al(OH)_3$ adjuvants.

The absence of significant differences in immune response with respect to vaccine formulations prepared with three adjuvants, however, contradicts the fact that oil adjuvant evokes an enhanced and better immune response due to delayed absorption from site of injection providing prolonged source of antigen. It has been demonstrated that the antibody response to a protein antigen in oil adjuvant vaccine remained constant for over 300 days in contrast to the antibody response to the same antigen in aqueous solution or alum precipitation, which declined after 10 days (Talmage and Maurer, 1953). On the other hand, the current finding is in agreement with a previous similar study where the three adjuvants showed no significant difference in stimulating the immune response against *P. multocida* (Molalign et al., 2009). This may suggest that the differences in immune response among vaccine formulations with respect to the three adjuvants may not be significant for the short term requiring long term evaluation to have conclusive information.

In evaluation of protective efficacy, the clinical pictures observed in un-vaccinated controls in the challenge experiment agree with previous studies where similar clinical signs were also reported (Akhtar et al., 2016). The absence of clinical signs and death in groups vaccinated with 0.5 mL of Alum adjuvated Fowl Cholera vaccine given through IM route substantiates the finding of significantly higher antibody titer of this group than the remaining vaccinated groups. Although all the chicken survived in group vaccinated with 1mL Alum adjuvant vaccine given through SC route, few of them showed the characteristic clinical signs of fowl cholera. The lack of 100% protection (Table 7) and the clinical signs observed in some of the chicken in four of the vaccinated groups all administered subcutaneously (Table 6) may indicate that the route of administration had more effect on the immune response than the type of adjuvant used. The higher protective efficacy of IM administered Fowl cholera vaccine was also documented in previous works (Rahman et al., 2004). The lower mortality (80%) recorded in the unvaccinated group compared to previous similar study in Ethiopia where 86% mortality was recorded (Molalign et al., 2009) may be attributed to the older age of the chickens used in the current study and other intrinsic factors such as breed or differences in individual immune status. Thus, further optimization study of the IM route is required using the different adjuvants not only due to its better immune response but also from practical point of view of its suitability during vaccine administration.

In conclusion, isolates of *P. multocida* obtained from outbreak cases characterized in this study including the isolate used for vaccine preparation fall into the same genotype, which may suggest the important role of these strains in causing fowl cholera in Ethiopia. However, further study on outbreak investigation covering wider areas of the country is necessary to demonstrate the most dominant strains causing fowl cholera which will be important to develop multivalent vaccine. The formalin inactivated alum adjuvant fowl cholera vaccine induced better antibody titer and conferred better protection in a challenge experiment indicating its potential use as a vaccine against Fowl cholera but requires further evaluation of its field performance.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

We acknowledge the Ministry of Science and Technology of Ethiopia for the financial support. The authors would like to thank the National Veterinary Institute (NVI) of Ethiopia for availing the laboratory and animal experimentation facilities. The technical support of the

staff at the Research and Development directorate of NVI is also highly appreciated. The study was supported by a grant from the Ministry of Science and Technology of Ethiopia.

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Vol. 13(27), pp. 510-517, September, 2019
DOI: 10.5897/AJMR2019.9189
Article Number: 3D4402A61948
ISSN: 1996-0808
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http://www.academicjournals.org/AJMR

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African Journal of Microbiology Research

Full Length Research Paper

Molecular characterization of *Acinetobacter baumannii* from patients with prolonged hospital stays in three tertiary hospitals of Kano Metropolis, Northwestern Nigeria

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Received 6 August, 2019; Accepted 10 September, 2019

***Acinetobacter baumannii* is one of the most important opportunistic bacterial pathogens that cause serious health care associated complications in hospitalized patients. This leads to prolong hospital stay which increase cost to both healthcare provider and family of the patients. The study aimed at molecular characterisation of *A. baumannii* from patients with prolonged hospital stays in three tertiary hospitals of Kano Metropolis, Northwestern Nigeria. A total of 401 samples were collected from orthopedic and post-surgical wound infections, urine, urine catheters and nasal intubation. *Acinetobacter* spp was isolated using standard microbiological methods. Identification of *A. baumannii isolates* were done using Phynotypic methods such as culture on Leed *Acinetobacter* medium, conventional biochemical tests and API 20NE. Suspect *Acinetobacter* species were further identified using polymerase chain reaction (PCR) and Sanger sequence typing methods. Out of 401 samples collected 138 (34.4%) were positives by yield suspect bacterial isolates 14 (10.1%) of which were suspect *A. baumannii*. The results of confirmatory sequence typing of isolates showed that 9 (6.5%) of suspect *Acinetobacter* spp were *A. baumannii*. The result of susceptibility test showed that *A. baumannii* isolates were highly resistance to Ampicillin\ salbactam 13 (92.8%) and least resistance to Ciprofloxacin 2 (14.3%) and Amikacin 3 (21.4). The results of this finding showed presence of *A. baumannii* species resistant to conventional antibiotics and associated with prolonged duration of patients admission in the three studied hospitals. There is need for improved sanitary working condition and proper patients management to reduce the spread of this health care associated infection agent.**

Key words: Molecular, characterization, *Acinetobacter baumannii,* patients, prolonged hospitals, Kano, Northwestern Nigeria.

INTRODUCTION

Acinetobacter baumannii is a Gram negative coccobacillus, aerobic, non fermentative and non-motile bacterium that belong to the genus *Acinetobacter*. Current taxonomic classification of this bacterium put it in γ-proteobacteria, family Moraxellaceae and order Pseudomonadales (Nemec et al., 2016). It belongs to *Acinetobacter calcoaceticus-baumannii* complex group which comprises four different Acinetobacteria: *A. baumannii, Acinetobacter pittii, Acinetobacter nosocomialis,* and *Acinetobacter calcoaceticus (*Pourabbas et al., 2016; Nemec et al., 2016; Muhammad et al., 2018). *A. baumannii* repels complete decolorization and can mislead as Gram-positive cocci. It is non-fastidious, and does not produce cytochrome oxidase, urease, citrate, and indole; however, it produces catalase enzyme. Furthermore, *A. baumannii* grow well at 35-37°C; however, some environmental isolates grow well in the temperature range of 20-30°C. *A. baumannii* is the only bacterium in the genus that can grow at 44°C (Bouvet and Grimont, 1987; Muhammad et al., 2018). *A. baumannii* grow well on culture media such as blood agar, chocolate agar, and MacConkey agar. On blood agar, it forms colorless, non-hemolytic, shiny mucoid colonies, smooth in contexture with a diameter of 1-2 mm after 18-24 h of incubation at 37°C. It produces colorless colonies on MacConkey agar which are shiny mucoid and tomb shaped, indicating its non-lactose fermenting ability. On selective media, Leeds Acinetobacter Medium, it gives pink color colonies when grown in the presence of supplement (Almasaudi, 2018; Muhammad et al., 2018).

A. baumannii was reported in health care environments and recently considered one of the important oppoturenistic bacterial pathogens that cause health care associated infections worldwide (Muhammad et al., 2018; Mirnejad et al., 2018). This leads to prolong hospital stay (>14 days) which increase cost to both healthcare provider and family of the patients. *A. baumannii* was the most dangerous among *Acinetobacter calcoaceticus-baumannii* complex, however, *A. pittii*, *A. nosocomialis* was also reported to cause infections (Muhammad et al., 2018).

In the hospital enviroments, *A. baumannii* can survive on beds, curtains, walls, roofs, medical devices, equipment, belongings of medical personnel, tap water sinks, telephones, door handles, hand sanitizers, dispensers, trolleys, bins, and even on computers (Muhammad et al., 2018). Furthemore, *A. baumanii* was isolated from different parts of healthy person such as nose, ear, throat, forehead, trachea, conjunctiva, vagina and perineum, axillae, groin, hands, and toe webs. *A. baumannii* was reported to be involved in bloodstream infections and account for about 15% cases due to invasive procedures such as intravascular or respiratory catheters, tubes, or cannulas among others (Muhammad et al., 2018; Mirnejad et al., 2018). The origin of infections (20-70%) caused by *A. baumannii* still remain unknown and the abilty of *A. baumanii* to cause infections in hospitals was linked to its ability to survive in desiccants, resistance to vital antimicrobial drugs and disinfectants (Muhammad et al., 2018).

Literature have shown that global burden of infections cause by *A. baumanii* still remain unknown due the lack of comprehensive data especially from African contries (Egwuenu et al., 2018) but the burden can be up to 35% (Xie et al., 2018) with mortality rate of 26% and this can increase up to 45% in intensive care unit (ICU) (Muhammad et al., 2018). Nigeria like other African countries, the story remain the same, but Egwuenu et al. (2018) reported that, *A. baumanii* was associated with blood stream catheter associated infection from different parts of the country including carbapenem resistant *Acinetobacter* spp (Aibinu et al., 2003; Taiwo et al., 2005; Ngwa et al., 2007; Jido and Garba, 2012; Nwadike et al., 2014; Bashir et al., 2019).

This leads to delay of patients in the hospitals due to treatment failure. More than 90% of the researches done on *A. baumanii* in Nigerian health care settings focused widely on phynotypic identification and antimicrobial resistant profile negelecting molecular aspect which gives more insight on the different types of strans involved in health care associated infections and antimicrobial resistance within a particular community. In our recent study (Bashir et al., 2019) reported superbugs-related prolonged admissions in three tertiary hospitals, Kano State, Nigeria including *Acinetobacter* spp which we lack information about their genetics relatedness with other known *Acinetobacter* spp sequneces stored in global genebanks. Therefore this study aimed at molecular characterization of *A. baumannii* from patients with prolonged hospital stays in three tertiary hospitals of Kano Metropolis, Northwestern Nigeria.

MATERIALS AND METHODS

Study area

The study was conducted in Kano State metropolis located in Northwest geopolitical zone of Nigeria. The state is made up of 44 Local Governments with an estimated population of over 13 million people (NBS, 2018). The study was ccarried out at 3 tertiary hospitals within the state and these were; Aminu Kano Teaching Hospital (AKTH), Murtala Muhammad Specialist Hospital (MMSH) and Muhammad Abdullahi Wase Specialist Hospital (MAWSH). All hospitals were strategically located for access to both rural and

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urban populations throughout Kano State.

Study design

This was a cross sectional descriptive hospital based study which involved molecular characterization of *A. baumannii* isolated from urine, orthopedic and post-surgical wound infections, urine catheter and nasal feed tube from patients who were eighteen years and above of ages and both sexes with prolonged hospital admission admitted in AKTH, MMSH and MAWSH. The isolated *A. baumannii* were characterised using both phynotipic, polymerase chain reaction (PCR) and Sanger sequencing methods.

Sample collection

A total of 401 sample were collected from patients who were admitted for ≥14 days and aged ≥18 years from three study hospitals. The samples collected included orthopedic and post-surgical wound infections, urine, urine catheters and nasal intubation. The swab samples were collected after cleaning the wound with physiological saline (0.85%) as described by Ibrahim et al. (2018) all swabbed samples were transported to Microbiology laboratory in a Stuart media. Urine samples were collected in sterile clean leak proof bottles from each patient according to the method described by Odoki et al. (2019).

Isolation and identification of *A. baumannii*

All samples (both urine and swabs) were inoculated on freshly prepared MacConkey agar (HiMedia Laboratories Pvt Ltd, Mumbai, India, M173) media and incubated for 24 h at 37°C. After incubation, isolates that were non lactose fermenting (shiny mucoid and tomb shaped) on MacConkey agar, Gram negative coccobacilli and oxidase negative were subcultured on Leed *Acinetobacter* Media (HiMedia Laboratories Pvt Ltd, Mumbai, India, M1839) and incubated at 37°C for additional 18-24 h. Suspected *Acinetobacter* spp from Lead Acinetobacter Media (that is, pink color) colonies were further identified using biochemical tests such as, catalase, coagulase, indole, citrate utilization, urea, methyl red, Voges-proskauer, motility and Triple sugar Iron tests (Cheesbrough, 2010).

API 20 Multi test systems

The suspected *Acinetobacter* spp were further subjected to API 20 NE multi test system (Biomeniuex, France) test. These tests were used according to manufactures protocol for identification of non enteric bacteria. Well of the biochemical test were inoculated with bacterial suspension (0.5 McFarland) made from fresh bacterial colony. The inoculated try was incubated at 37°C for 18-24h. The result was read after addition of appropriate reagents as 7-digit number that identify API 20 NE analytical index (API 20 Biomeriux France, 2010).

Molecular identification of *A. baumannii*

DNA extraction

Extraction of DNA was done by ethanol precipitation after phenol:chloroform:isoamyl alcohol (24:25:1 v/v) treatment as previously described (Gumińska et al., 2018). Briefly, 200 µl of 24 h suspected *Acinetobacter* spp culture was transferred into 2 ml sterile tube, to which 500 µl of lysis buffer (eBioscience™ Thermo Fisher Scientific, USA) and 20 µl of proteinase K (200 µg/ml) (Thermo Fisher Scientific, USA) were added. This was then vortexed and incubated at 65°C for 1 h. The lysate was extracted twice with 500 µl of phenol:chloroform:isoamyl alcohol (24:25:1, v/v).The aqueous fraction was transferred to a clean 1.5 ml tube and DNA preciptitated at -20°C for 3 h after the addition of 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol. The DNA was recovered after centrifugation at 15,000 g for 20 min, supernatant discarded and the DNA pellet washed with 70% ethanol. After a final centrifugation step, the supernatant was carefully removed, DNA pellet was allowed to air-dry and finally re-suspended in 100 µl of sterile distilled water. The extracted DNA was stored at -20°C until required for further study.

Polymerase chain reaction

A PCR was performed using the extracted DNA as template. A set of two primers, 16S Fw (5'-GTG CCA GCA GCC GCG CTA-3') and 16S Rev (5'-AGA CCC GGG AAC GTA TTC AC-3'), amplifying a 850 base pair (bp) 16S rRNA genomic region were used. Amplification reactions were run in a 10 µl final volume containing; 2 µl of extracted DNA, 1 µl of 10x standard Taq reaction buffer (Thermo Fisher Scientific, USA), 1 µl of 2.5 mM $MgCl_2$ (Thermo Fisher Scientific, USA), 0.5 µl of 0.25 pMol of each primer (Thermo Fisher Scientific, USA), 1 µl of 10% DMSO (Thermo Fisher Scientific, USA), 0.8 µl of 200 µM dNTPs (Thermo Fisher Scientific, USA) and 0.1 µl of 0.5 units of Taq polymerase (NEB, UK) and 3.1 µl of nuclease free water. The PCR conditions included an initial denaturation step at 94°C (5 min), followed by 36 cycles of 94°C (30 s), 56°C (30 s) and 72°C (45 s). Following amplifification, a final extention at 72°C (7 min) was done. The amplified products were analyzed on a 1.5% agarose gel containing 0.5% ethidium bromide and visualized under U.V. illumination. A band corresponding to 850 bp was gel purified using the QIAquick gel extraction kit (QIAGEN, Thermo Fisher Scientific, USA).

Sequencing

The purified PCR product was subjected to cycle sequencing using the BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, USA). Sequencing reactions were prepared as a 6 µl reaction mix containing; BigDye Direct Sequencing Master mix (2 µl), sequencing primer (MP13Forward/ Reverseprimer) (1 µl) and PCR product (3 µl), and loaded 3 µl of the reaction mix to the appropriate well in the respective forward or reverse reaction plate. Sequencing was performed in a thermocycler using the following conditions; at 96°C (1 min), followed by 25 cycles of 96°C (10 s), 50°C (5 s) and 60°C (75 s). At the end of the reaction, the tubes were briefly centrifuged and samples loaded onto the ABI 3700 gene sequencer. The results obtained were analyzed using MEGA software (version 6.0) and blastn (NCBI). A phylogenetic tree was constructed using Neighbor-Joining method and bootstrapping performed by creating 1000 trials. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 1.11983619 is shown. The confidence probability (multiplied by 100) that the interior branch length is greater than 0, as estimated using the bootstrap test (200 replicates is shown next to the branches (Rzhetsky and Nei, 1992; Dopazo, 1994). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the

Table 1. Socio demographic characteristics distribution of *A. baumannii* in the 3 studied hospitals.

Variable	AKTH	MAWSH	MMSH	
	A. baumannii	***A. baumannii***	***A. baumannii***	
	n (%)	**n (%)**	**n (%)**	**Total (%)**
Ages (years)				
18 – 28	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
29 – 38	0 (0.0)	0 (0.0)	1 (16.7)	1 (7.1)
39 – 48	0 (0.0)	1 (33.3)	0 (0.0)	1 (7.1)
49 – 58	3 (60.0)	1 (33.3)	1 (16.7)	5 (35.7)
59 – 68	1 (20.0)	1 (33.3)	2 (33.3)	4 (28.6)
69 – 78	1 (20.0)	0 (0.0)	2 (33.3)	3 (21.4)
Total	**5 (35.7)**	**3 (21.4)**	**6 (42.9)**	**14 (100.0)**
Sex				
Male	4 (80.0)	2 (66.7)	2 (33.3)	8 (57.1)
Female	1 (20.0)	1 (33.3)	4 (66.7)	6 (42.9)
Total	**5 (35.7)**	**3 (21.4)**	**6 (42.9)**	**14 (100.0)**

units of the number of base substitutions per site. This analysis involved 20 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1610 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

Antimicrobial susceptibility testing

The susceptibility testing of isolates to various antibiotics was carried out by the disk diffusion method using a modified form of the Kirby Bauer method (CLSI, 2015, 2016, 2018). Briefly, freshly prepared Mueller Hinton agar (Oxoid, UK) plates were inoculated with 0.5 McFarland standard of *Acinetobacter* spp suspension and placed the following single antibiotic discs on the inoculated plates: Amoxicillin (AM, 10 µg), Gentamycin (CN, 10 µg), Ceftriaxone (CRO, 30 µg), Ciprofloxacin (CIP, 5 µg), Ceftazidine (CAZ 30 µg), Imipenem (IMP, 10 µg), Tetracycline (TET, 30 µg), Amoxicillin/ Cluvanic (20 µg), Ampicillin/Subactam (SAL, 20 µg), Nitrofurantion (NIT, 300 µg), Amikacin (AK, 30 µg) and Sulfamethoxazole/ trimethoprim (SXT, 1-25/23.75µg). The plates were allowed to stand for 5-10 min at room temperature and then incubated at 37°C for 24 h, after which the zone of inhibition was measured and interpreted according to the method described by (CLSI, 2015, 2016). *Eschrichia coli* ATCC 25922 and *Pseudomona aeruginosa* ATCC was used as quality reference strains.

Ethical permission

Ethical approval for this study was obtained from the Medical advisory committee of Aminu Kano Teaching Hospital and the health service management board of Kano State with numbers NHREC/21/08/2008a/ AKTH/EC/1780 and HMB/GEN/488/VOL respectively.

RESULTS

Out of 401 samples collected, 138/401 (34.4%) bacterial isolates were obtained in which 14/138 (10.1%) were *A. baumannii* using phenotypic methods. The results of prevalence of *A. baumannii* according to studied hospitals showed that, MMSH has the highest prevalence 6/14 (42.9%) of *A. baumannii* followed by AKTH 5/14 (35.7%) and MAWSH had the least prevalence 3/14 (21.4%). The prevalence of *A. baumannii* according to the age of the studied participants showed that, age groups 49-58 has the highest prevalence 5/14 (35.7%). Prevalence of *A. baumannii* according to gender showed that male had the high prevalence 8/14 (57.1%) (Table 1). Furthermore, the prevalence of *A. baumannii* according to the sites of infection showed that, urine samples had the highest prevalence 5/14 (35.7%) while nasal intubation has the least 1/14 (7.1%) (Table 2).

All 14 suspected *A. baumannii* identified using phentyphic methods were subjected to molecular characterization and results showed that, 12 isolates were confirmed to be *A. baumannii* using PCR method (Figure 1). The PCR results showed that a band of about 850 base pairs (bp) of ribosomal RNA gene were obtained. However, the gel electrophoresis result showed that isolates 7 and 9 were not successfully amplified during PCR, this could be due to quality of the extracted DNA of those isolates or the concentration of extracted DNA was very low. Furthermore, sequencing results using Sanger sequencing method were blastered in NCBI database using BLASTn search to confirmed their identities. The blasted results showed that 9 isolates (16SF 1, 16SF 3, 16SF 4, 16SF 6, 16SF 8, 16SF 10, 16SF 12, 16SF 13, and 16SF 14) were found to have 99.5, 99.5, 97.66, 99.01, 99.14, 90.15, 99.75, 99.26 and 98.11% identities respectively with *A. baumannii* strain DSM 30007, which represents 9/138 (6.5%) of the isolates (Figure 2).

The antimicrobial susceptibility profile of the isolated *A.*

Table 2. Distribution of *A. baumannii* according to sampling sites based on three study site.

Samples	Hospitals			Total n (%)
	AKTH n (%)	MAWSH n (%)	MMSH n (%)	
Urine	2 (14.3%)	1 (7.1)	2 ((14.3%))	5 (35.7)
Urine Catheter	2 (14.3%)	1 (7.1)	1 (7.1)	4 (28.5)
Nasal Intubation	0 (0.0)	0 (0.0)	1 (7.1)	1 (7.1)
Wound Swab	1 (7.1)	1 (7.1)	2 (14.3%)	4 (28.5)
Total	**5 (35.7)**	**3 (21.3)**	**6 (42.8)**	**14 (100.0)**

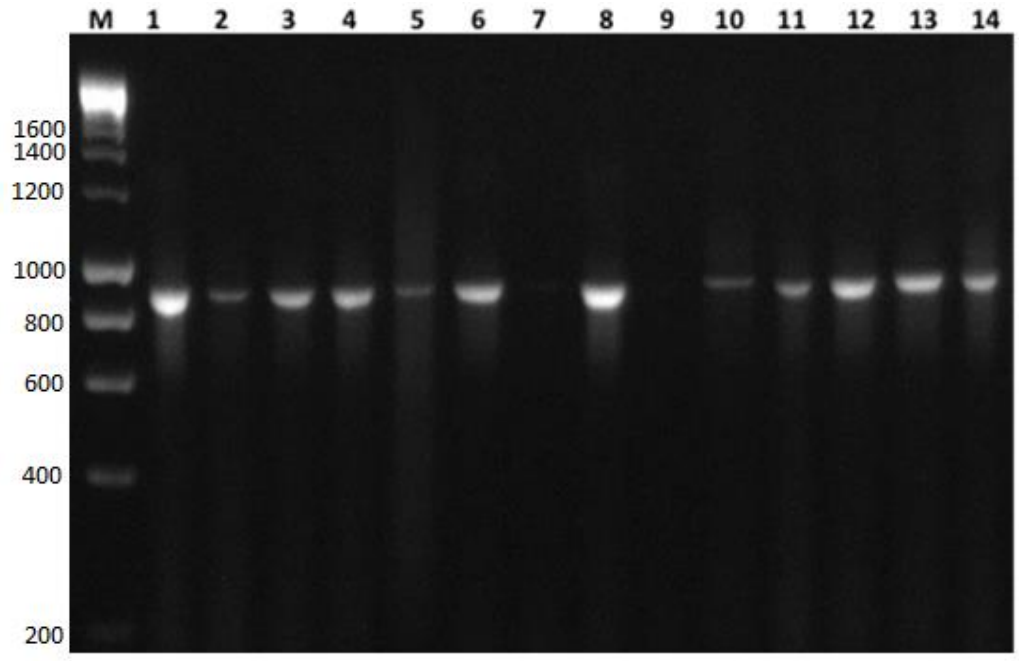

Figure 1. PCR results 1.5% agarose gel showing results of 16S rRNA PCR amplified products from extracted DNA of A. baumannii isolates. The electrophoresis was performed for 1.5 h. Lane M: DNA molecular size marker (2000bp ladder) (manufacturer, city, country); Lanes 1-14: the 14 *A. baumannii* isolates

baumannii in this study showed that, the isolates were highly resistant to Ampicillin salbactam and Perfloxacin 13 (92.9%) each. The least resistant were observed from Ciprofloxacin and Amikacin 2 (14.3%) and 3 (21.4%) respectively (Table 3).

DISCUSSION

A. baumannii is emerging as a cause of health care associated outbreaks world wide (Villegas and Hartstein, 2003; Kais et al., 2016; Muhammad et al., 2018). Molecular characterization of any bacterial pathogens is important in ruling out the sources of infection, understanding the relationships and distribution patterns of that pathogens (Mirnejad et al.,2018). From a total of 138 isolates obtained in this study, 14/138 (10.1%) were *A. baumannii* which was inline with the findings of Nwadike et al. (2014) who reported prevalence of *Acinetobacter* spp (9.0%) from ICU deparment University College Hospital, Ibadan, Nigeria. However, the prevalence reported in this study was higher compared to the prevalence 1 (0.7%) reported by Heydarpour et al. (2017) from open-heart surgery patients at Imam Ali Hospital in Kermanshah, Iran. The mix in sites swabed including the inherent microbial sub population must be more contaminated compared to theatre in specialzed open heart surgical attention in the place. Prevalence of *A. baumannii* according to the age groups of the studied participant showed that age group 49-58 years had the

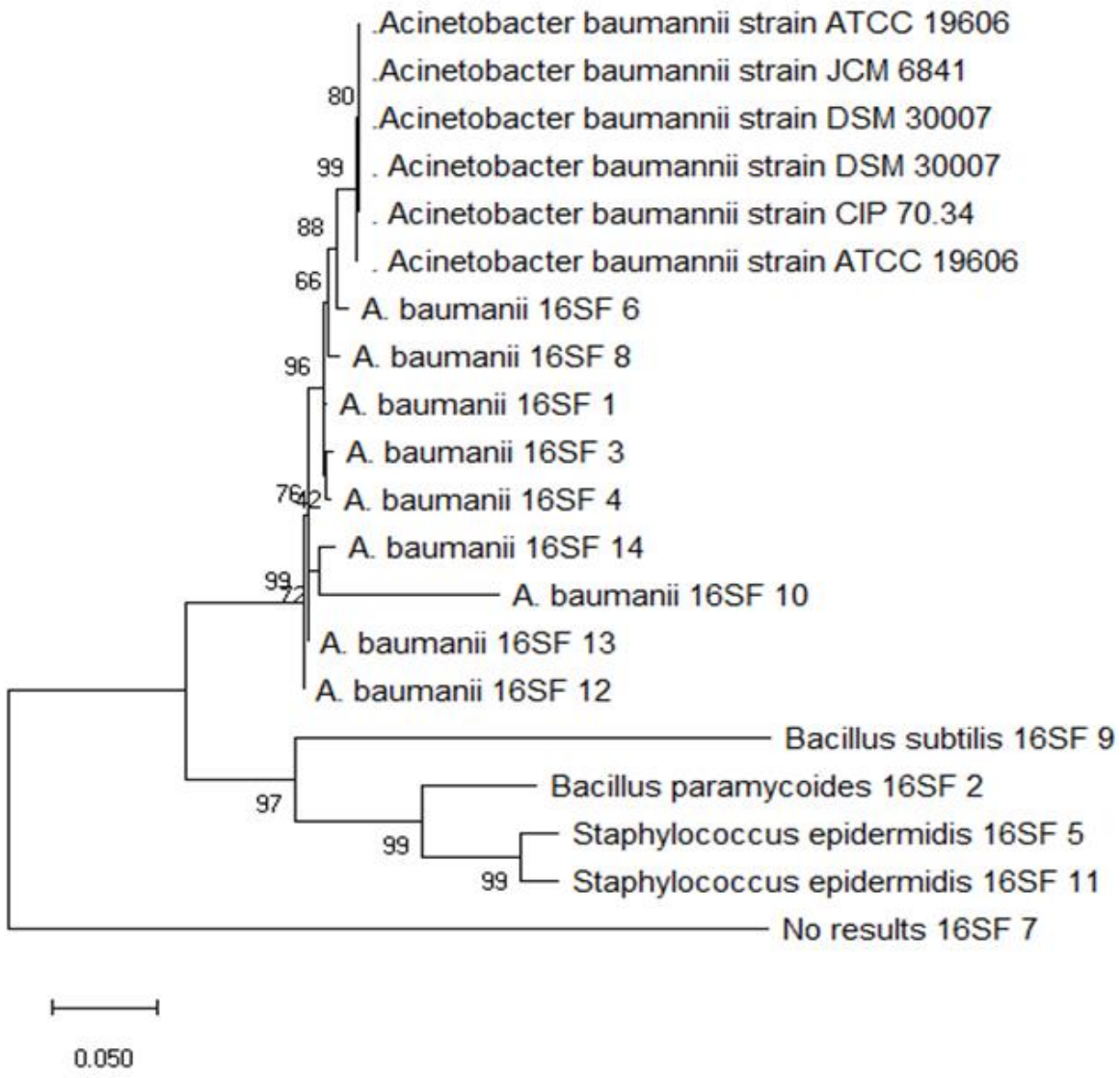

Figure 2. Phylogenetic tree showing evolutionary relationships isolated *A. baumanii* with other known *A. baumanii* sequences from NCBI.

Table 3. Antimicrobial susceptibility profile of the isolated *A. baumannii* from all the three study site.

Antibiotics (µg)	Sensitivity n (%)	Intermediate n (%)	Resistance n (%)
Amikacin (30)	11 (78.6)	0 (0.0)	3 (21.4)
Amoxicillin (10)	1 (7.1)	1 (7.1)	12 (85.7)
Ampicillin sulbactam (10)	1 (7.1)	0 (0.0)	13 (92.8)
Amoxicillin/Cluvanic (20)	3 (21.4)	0 (0.0)	11 (78.4)
Ceftazidine (30)	3 (21.4)	1 (7.1)	10 (71.4)
Ceftriaxone (30)	2 (14.3)	0 (0.0)	12 (85.7)
Ciprofloxacin (5)	11 (78.6)	1 (7.1)	2 (14.3)
Gentamycin (10)	1 (7.1)	1 (7.1)	11 (78.6)
Imipenem (10)	2 (14.3)	0 (0.0)	12 (85.7)
Nitrofurotoin (300)	6 (42.9)	2 (14.3)	6 (42.9)
Tetracycline (30)	3 (21.4)	1 (7.1)	10 (71.4)
Trimetoprim sulfamethoxazole (1.25/23.75)	2 (14.3)	0 (0.0)	12 (85.7)

highest prevalence (35.7%). This could be due to immunity issues, long time hospitalization and use of invasive procedures which can raise the risk of infections by opportunistic pathogens such *A. baumannii*. This was in agreement with Odewale et al. (2016) who reported in their study that age 41- 70 years are more susceptible to *A. baumannii* infection. Prevalence of *A. baumannii* according to the gender showed that, male had the highest prevalence (57.1%) this was in line with report of Pal et al. (2017) who reported higher prevalence in male patients (76.4%). This could be as a result of male patients constantly shifting their locations due to their job placements which make them more prone to accidental traumas since they are the most frequent patients using invasive devices. Prevalence of *A. baumannii* according to the site of infections showed that urine samples had the highest prevalence (35.7%) which correspond with the finding of Zuhair (2011) who reported high prevalence of *A. baumannii* from urine samples. However, this was contrary to the findings of Pal et al. (2017) who reported that frequently isolated *A. baumannii* were from secretions 54.6% and Suction tip 23.5%.

Out of the 14 phenotypically isolated *A. baumannii* obtained in this study, 12 were confirmed to be *A. baumannii* using PCR which represented 12/138 (8.7%) of the isolates. However, the sequencing results and blast search in the NCBI database confirmed only 9 isolates (isolates 16SF 1, 16SF 3, 16SF 4, 16SF 6, 16SF 8, 16SF 10, 16SF 12, 16SF 13, and 16SF 14) to be *A. baumannii*. The prevalence of *A. baumannii* using molecular method reported in this study was inline with finding of Nabil et al. (2001); Namita et al. (2012) and Odewale et al. (2016) who reported the prevalence of 8.8, 9.4 and 8.5% respectively using molecular method. However, these results indicate that we can not completely rely only on biochemical tests for identification of *A. baumannii*, but there is need to also use molecular techniques such as PCR and sequencing for accurate diagnosis.

The unique character of *A. baumannii* in resistance to most antibiotics makes it an organism of high importance especially in hospital setting as a nosocomial pathogen among immune compromised and patients with prolonged hospital stay. Majority of the isolates in our study were resistant to commonly used antibiotics such as ceftazidime, gentamicin, ceftriaxone, amoxicillin/ Cluvanic, cotrimaxole, amoxicillin, imipenem and ampicillin/sulbactam. Sensitivity was only found to be in presence of amikacin, ciprofloxacin, and nitrofurantion. This means Multi drugs resistant (MDR) isolates are increasing, probably due to indiscriminate use of these antibiotics in healthcare settings. *A. baumannii* showed high resistant of 92.8% to Ampicillin salbactam (10 µg) followed by Amoxicillin (85.7%) and Ceftriaxone (85.7%).

Carbapenems have been the drug of choice for treating Acinetobacter infections, but unfortunately, carbapenem resistant *A. baumannii* is becoming common worldwide (Towner, 2009). Of the β-lactamases, those with carbapenemase activity are the most concern for drug resistance and include the serine oxacillinase (belonging to Ambler class D OXA type) and the metallo-b-lactamases (Ambler class B) (Walsh et al., 2005). The present study observed Imipenim (85,7%) lower than 92.2% as reported by Anil and Nirav (2015) but higher than Mostofi et al. (2011) who showed low resistance of 76% (Mostofi et al., 2011). Trimetoprim sulfamethoxole showed 85.7% resistance, the least resistant of *A. baumannii* were obtained in Ciprofloxacin and Amikacin 14.3 and 21.4% respectively. In another study done by Pal et al. (2017) reported high resistance of *A. baumannii* to penicillin and cephalosporin antibiotics while Odewale et al. (2016) reported 100% resistance of Ciprofloxacin and Amikacin which was contrary to the present study.

Conclusion

The result of this finding showed the presence of *A. baumannii* associated with health care associated infection among prolonged hospitalized patients from the studied hospitals. There is needs for the concern management of the studied hospitals to improved sanitary working condition and proper patients management that can reduce the spread of health care associated bacterial pathogens especially *A. baumannii*.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGMENT

The authors appreciate the management of the three studied hospitals for granting us permission to carry out this study. Study participants for participating in this study. Finally the staff of the microbiology laboratories for their kind support during this study.

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Vol. 13(27), pp. 518-519, September, 2019
DOI: 10.5897/AJMR2019.9202
Article Number: 7500E6A61950
ISSN: 1996-0808
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Letter to Editor

Region of acquisition of urinary tract infection (UTI) may be an important parameter while treating UTI cases

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Received 26 August, 2019; Accepted 5 September, 2019

The study recently published on urinary tract infection (UTI) cases at Najran University Hospital, Najran region of Saudi Arabia (Alshabi et al., 2019), though small, only on 19 cases, have been concluded beautifully, "regional clinical data regarding the prevalence and efficacy of antibiotics should be taken into consideration along with the treatment guidelines". The authors reported a study on only 19 cases of UTI, of which 14 patients (73.68%) were positive for *Escherichia coli*, two (10.52%) each for *Staphylococcus aureus* and *Pseudomonas*, and one for *Staphylococcus haemolyticus.* However, in a recently published study, on 217 confirmed human cases of UTI from Rohilkhand, Bareilly region of Northern India (Singh, 2019), the picture was much different, the most common cause of UTI, *Escherichia coli,* was associated with only 22.12% cases, *S. aureus* was a rare (0.98%) cause of UTI but *S. haemolyticus* and other staphylococci caused 5.53 and 13.36% cases of UTI, respectively. In the Bareilly study, pseudomonads caused only 2.76% cases while other less known bacteria were responsible for more than 55% of the cases of UTI in humans. In Ireland too, only 14.3 and 19.4% cases of UTI were associated with *E. coli* infection in male and female, respectively (Tandan et al., 2016). Although in Nepal (Pandey et al., 2017), *E. coli* caused 78.6% and in Iran (Fallah et al., 2018) it caused 69% cases of UTI, similar to the study in Saudi Arabia (Alshabi et al., 2019), level of drug resistance was quite high. In a study in Nigeria (Ekwealor et al., 2016), *E. coli* was associated with only 24.6% cases of UTI and among the other causes *S. aureus* dominated the scene causing 28% of the UTIs, followed by *Staphylococcus saprophyticus* (20%) and streptococci and enterococci caused a sizeable number of cases (7.4%). Though *E. coli* is one of the most common cause of UTIs (Pandey et al., 2017) emergence of new pathogens including *Enterobacter, Enterococcus, Proteus, Klebsiella, Citrobacter, Raoultella, Streptococcus,* etc., are complicating the therapeutics of UTIs (Ekwealor et al., 2016; Howell and Fakhoury, 2017; Abejew et al., 2014).

In the study in Saudi Arabia (Alshabi et al., 2019), the most effective antibiotics on Gram-negative isolates were ceftriaxone (87.5%) followed by amoxycillin + clavulanic acid (81.25%), amikacin (75%), cefuroxime (75%), cefixime (68.75%) and mezlocillin (62.5%) and on Gram-positive isolates ceftriaxone, amikacin and amoxycillin + clavulanic acid were the most effective. The study reported only 35.71% of *E. coli* producing extended-spectrum beta-lactamases (ESBL) and compared similarity with a report from New Delhi (Akhtar et al., 2014). India is a big country with different climatic and socio-cultural regions and societies with different hygienic standards and health-care system in Delhi itself. In another study from Pondicherry in Southern India (Gopichand et al., 2019), of the 326 isolates, 319 (97.8%) were resistant to the third generation cephalosporins and produced ESBL while in a study in Bareilly, Northern India, 75% *E. coli* produced ESBL and the most effective antibiotics on Gram-negative bacteria were tigecycline (85.4%), followed by imipenem (83.9%), meropenem (81.7%), ceftriaxone (53.6%), gentamicin (52.8%), cefixime (34.9%) and amoxicillin + clavulanic acid (21.9%). On Gram-positive bacteria causing UTI in Bareilly (Singh, 2019), the most effective antibiotics were tigecycline (98%) followed by linezolid (96.6%), imipenem (84.9%), amoxycillin + sulbactam (83.9%), amoxycillin +

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clavulanic acid (67%), ceftriaxone (62.4%), gentamicin (44%) and cefixime (34.7%). Not only in India, in other countries too, emergence of antibiotic resistance in UTI causing bacteria towards the commonly prescribed antibiotics is reported (Tuem et al., 2018). In Ethiopia (Abejew et al., 2014), *E. coli* causing UTIs were much less often sensitive to ceftriaxone (53.1%), amoxycillin (15.4%) and gentamicin (66%), while older antimicrobials like nitrofurantoin and nalidixic acid were reported as the best options effective to inhibit >86% *E. coli* isolates (Abejew et al., 2014). The study in Ireland also reported low utility of amoxicillin + clavulanic acid (~5%) and nitrofurantoin was the most prescribed (>50%) and effective antimicrobial (Tandan et al., 2016). In Nigeria, resistance in UTI causing bacteria has been shown to be emerging for amoxycillin-clavulanic acid, cefuroxime, ceftazidime and cefixime (Ekwealor et al., 2016). In Nepal, where *E. coli* was the most common cause of UTIs, amikacin was reported to be effective only on 42.85% isolates and nitrofurntoin inhibited only 28.57% of *E. coli* isolates (Pandey et al., 2017).

In light of observations of the different contemporary studies in India (Gopichand et al., 2019; Singh, 2019), Saudi Arabia (Alshabi et al., 2019) and other countries (Tuem et al., 2018; Tandan et al., 2016; Ekwealor et al., 2016; Abejew et al., 2014) it may be concluded that the regional effect might be one of the most important factors for clinicians while treating UTI cases as suggested by Alshabi et al. (2019). Besides generalized guidelines for antibiotic uses in UTI (Anderson, 2019; Gopichand et al., 2019), considering the wide variation in antimicrobial susceptibility of bacteria causing UTI for an effective treatment of UTI cases guidelines have been issued time to time recommending the urine culture and antibiotic sensitivity before instituting the antimicrobial therapy (Wenzler and Danziger, 2016; Weese et al., 2011). Thus, while treating any case of UTI, clinicians must consider recent visits of UTI patient or region of acquisition or origin of UTI as an important parameter for instituting antimicrobial therapy.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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