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Isolation, identification and evaluation of mosquito entomopathogenic *Bacillus* species and related genera from randomly selected sites in Kenya

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This study was carried out to identify specific mosquitocidal *Bacillus* species and related genera for future development of biopesticides in local mosquito control program in Kenya. Bacterial isolation was conducted from 100 soil samples through pasteurization method and preliminary identification conducted through phenotypical analysis. Toxicity analysis was performed through bioassays and lethal concentrations (LC) were determined using probit analysis. Toxic isolates were further identified through analysis of the 16s rRNA and screening of toxin genes through PCR. Expression of toxin proteins was performed using SDS-PAGE. Out of 453 isolates, 7 of them were found to yield highly potent toxicity (>50% mortality) against *Culex quinquefasciatus* during the initial toxicity assays. Among them, two isolates KDHa3 and SKDHb5, with LC50 values of 0.007mg/L and 0.008mg/L, respectively, were the most toxic against the target. Phylogenetic analysis based on 16s rRNA showed high homology to *Lysinibacillus sphaericus* (six isolates) and *Bacillus thuringiensis* (one isolate). Various toxin genes encoding BinA, BinB, Mtx (1, 2 and 3), Cry48A, Cry49A, Cry4A, Cry11A and Cyt1A were detected among the isolates. The protein profiles using SDS-PAGE were consistent with the standard strains *Lysinibacillus sphaericus* C3-41 and *B. thuringiensis* var. *israelensis*. Native toxic *Bacillus* species and related genera were identified with this study being the first to report highly toxic strains of *L. sphaericus* and *B. thuringiensis* strains from Kenyan soil samples.

Key words: *Bacillus* species, *Bacillus thuringiensis, Culex quinquefasciatus*, Entomopathogenic, *Lysinibacillus sphaericus*, toxin.

INTRODUCTION

Mosquito related diseases such as West Nile fever, chikungunya, filariasis, dengue fever, malaria and yellow fever cause a high mortality rate that leads to a major economic constraint within countries endemic to these

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diseases (Poopathi and Abidha, 2010).

Environmental changes caused by human migration, deforestation and poor management of urban centers have led to mosquitogenic conditions and hence a significant increase of the mosquito populations (Robert et al., 2003; Keating et al., 2004). The incidence of mosquito-borne diseases in Kenya is similar to most parts of the sub Saharan Africa which continues to increase with an estimated 6.7 million new clinical cases and approximately 4,000 deaths being reported each year (CDC, 2015). It is considered that mosquito control is one of the effective approaches for minimizing the threat of these mosquito-borne diseases.

During the past decades, synthetic insecticides such as organophosphate insecticides have been used to control mosquito populations, but their wide application has been greatly obstructed due to environmental pollution, resistance in the vectors and harmful effects on beneficial non-target animals (González et al., 2013; Irungu et al., 2016; Peralta and Palma, 2017).

Bacillus species and related genera especially entomopathogenic microorganisms such as Lysini*bacillus* sphaericus previously and *Bacillus* thuringiensis var. israelensis are currently the most commonly and widely used mosquito control agents due to their specific toxicity against mosquitoes and the compatibility in the environment (Pei et al., 2002; Yuan et al., 2003; González et al., 2013; Suryadi et al., 2015). The pathogenicity of *L. sphaericus* and *B. thuringiensis* var. israelensis on mosquito larvae is attributed to toxic proteins' expression after ingestion. This causes destruction of the larval mid gut due to binding of the active toxins to apical microvilli at specific membrane receptors of midgut cells resulting to larval death (Regis et al., 2001). A recent study has also shown a soil isolated strain *Aneurinibacillus aneurinilyticus* to harbor considerable toxicity against mosquito larvae although its use has not been commercialized yet (Das et al., 2016).

The biopesticides based on *L. sphaericus* and *B. thuringiensis* var. *israelensis* have mosquitocidal activity, environmental-friendly and kill mosquitoes at their larval stage rather than adult stage. This plays an advantageous role in that mosquitoes are eliminated within the control programmes before they can disperse to human habitations (Fillinger and Lindsay, 2006; Yohannes et al., 2005). However various studies have reported development of resistance among some commercial toxic strains (Pei et al., 2002; Wirth et al., 2005; Yuan et al., 2003) creating a necessity for better alternatives. Little is known about entomopathogenic strains in Kenya and previous studies have only applied the imported *L. sphaericus* and *B. thuringiensis* var. israelensis formulations in large scale field trials (Fillinger et al., 2003; Mwangangi et al., 2011). Development of mosquitocidal formulations based on indigenous *Bacillus* and related Genera will promote local capability of biopesticide production in Kenya as well as reducing the over dependency on imported biocontrol products.

The study was conducted to isolate, identify and evaluate indigenous mosquitocidal *Bacillus* species and related genera for the development of biopesticide to substitute chemical pesticides and the expensive imported biopesticides. Furthermore this programme once implemented in large scale will be a great enhancement to Kenya in the fight against many mosquito related diseases.

MATERIALS AND METHODS

Study sites and soil sample collection

The study sites were based in Kenya across three randomly selected and geo referenced regions which included: Kwale (4.1744° S, 39.4519° E) located in the Coastal part of Kenya, Murang'a (0.7957° S, 37.1322° E) and Kiambu (1.1462° S, 36.9665° E) located in the Central region of Kenya. The sampling process was conducted in February, 2017 in which a total of 100 soil samples were collected as follows: Kwale-50, Murang'a -24 and Kiambu- 26. The sampling sites which had not been previously sprayed with mosquito biolarvicide formulations were chosen based on closeness to settlements, dams/ water pools and shaded areas creating an appropriate habitat for mosquito breeding (Suryadi et al., 2015). The soil samples were 10 g each taken at depths ranging from 5 to 10 cm, stored in sterile zip-lock bags. The collection and transport of samples was authorized by Kenya Plant Health Inspectorate Service (KEPHIS), Certificate No. KEPHIS/7408/2017.

Isolation and phenotypic characterization of the spore-forming *Bacillus*

1 g of each soil sample was thoroughly mixed with sterile salt solution forming 10% w/v suspension. The soil suspensions were pasteurized at 80°C for 20 min then serial dilutions (10-1 to 10-5) were prepared using sterile physiological saline solution (8.5 g/L NaCl) (Jensen et al., 2002). About 0.1 ml of the diluted suspension was spread on Luria-Bertani Agar (LB) (Tryptone 10 g/L, Yeast 5 g/L, NaCl 10 g/L, pH 7.4) and incubated at 30°C for 48 h. For selective isolation of Lysini*bacillus* species, LB media supplemented with 100 mg/ml streptomycin was used (Yousten et al., 1985). Morphological observations were conducted on single colonies for determination of colour, form, surface and texture. Microscopic observation was also conducted for confirmation of gram stain test and visualization of the endospores. Physiological tests were conducted based on temperature (4°C, 30°C and 48°C), pH (4, 7 and 9) and NaCl tolerance (7 and 10%). Based on the morphology, *Bacillus* like colonies were sub-cultured on new LB media until pure colonies were attained. The resultant pure colonies were stored at 4°C as slants and 20% glycerol at -80°C until further identification was conducted.

Mosquito culture preparations and initial toxicity assay

The susceptible Culex quinquefasciatus was obtained from a laboratory maintained colony. The larvae were reared in enamel pans filled with dechlorinated tap water and fed with a mixture of yeast powder, and wheat mill and cat chow. All larvae and adults were held under controlled conditions of 26°C±2 temperature, relative humidity of 60%±5 and 12:12 (light-dark) photophase. Freshly prepared colonies of the isolated *Bacillus* strains were

Table 1. Primers used in the study.

Target	Primer name	Sequence (5'- 3')	Amplicon (bp)	Annealing temp (°C)	References
16s rRNA	27F 1492R	AGAGTTTGATCMTGGCTCAG CGGTTACCTTGTTACGACTT	1500	55	Weisburg et al. (1991)
binA	binA-F binA-R	CACTTCCAGAAAACGAGCAATAC TAACCTGAGTTCCATCACTACGA	522	51	Ge et al. (2011)
binB	binB-F binB-R	GCAGGTAGGTAGTGGAGATT CTGAGTGGTCGTTTGGGATA	622	56	Ge et al. (2011)
mtx1	mtx1-F mtx1-R	TGGAACATCAAATACGATAGCA CCCAAGCCAATGAATAGTTAGG	512	52	Ge et al. (2011)
mtx2	mtx2-F mtx2-R	TGTCGTTCCACTGTTTATTGGTTCA AAATCTGCCCCATGAATTAAGTTA	523	52	Ge et al. (2011)
mtx3	mtx3-F mtx3-R	CGAAATGATACCGATAGGGATC AATCAGGGTTATTGACACTTCTTG	502	51	Ge et al. (2011)
cry48Aa	cry48Aa-F cry48Aa-R	GTGCTTCCACMAACTTTCAATCAT TCTTCTTCGGTTAGTAATCGCTCTT	1025	52	Ge et al. (2011)
cry49Aa	cry49Aa-F cry49Aa-R	TACTTTCGCTACTGTCTGCT AATCCATTTCCTTACGGTCT	704	56	Ge et al. (2011)
cry4A	Dip2A -F Dip2B-R	GGTGCTTCCTATTCTTTGGC TGACCAGGTCCCTTGATTAC	1293	55	Carozzi et al. (1991)
cry11A	EE11A-F EE11A-R	CCGAACCTACTATTGCGCCA CTCCCTGCTAGGATTCCGTC	445	55	Ben-Dov et al. (1997)
cyt1A	Cyt1A-F Cyt1A-R	AACCCCTCAATCAACAGCAAGG GGTACACAATACATAACGCCACC	522	55	Bravo et al. (1998)

Degenerate bases: W = A/T, M = A/C, R = A/G, Y = C/T.

inoculated into 20 ml test tubes containing 5 ml sporulating broth (Minimal Basal Salts, MBS- 0.68% KH2PO4, 0.03% $MgSO_4 \cdot 7H_2O$, 0.002% $MnSO_4$, 0.002% $Fe_2(SO_4)_3$, 0.002% $ZnSO_4 \cdot 7H_2O$, 0.002%$CaCl_2$, 1% tryptone, and 0.2% yeast extract , pH 7.2) (Zhao et al., 2014). The tubes were then incubated at 30°C with shaking (200 rpm) for 48 h to allow sporulation and crystal formation. In the initial toxicity bioassays, 1 ml culture of each isolate was added in 200 ml plastic containers containing 100 ml deionized water and twenty 3rd instar larvae of *C. quinquefasciatus*. The tests were performed in triplicates along positive controls (L. s C3-41 and B.t.i) and negative controls with no bacterial culture included. Mortality was recorded after 24 and 48h respectively by calculating the mean value of the larval death.

Spore prevalence and determination of the lethal concentration

The selected high toxic isolates (>50% mortality) were cultured in 500 ml Erlenmeyer flasks containing 100 ml MBS broth and incubated at 30°C with shaking (200 rpm) for 48 h. The total viable cells (VC) and spores (SC) were determined from the culture. Prevalence of spores was calculated as the number of live spores/ number of live cells (100%) (Jensen et al., 2002). The toxicity of selected high toxic isolates was evaluated by Standard Bioassay procedure (WHO, 1985). The bioassays were conducted by placing 20 *C. quinquefasciatus* 3rd instar larvae into 200 ml plastic containers containing 100 ml deionized water with the desired concentrations of the bacterial suspensions. Five concentrations were used giving the mortalities between 2 to 98%, and mortality was recorded after 24 and 48h (Pei et al., 2002). Both positive and negative controls were included, and the tests were conducted in triplicates. Negative controls that recorded 20% mortality were corrected using Abbott's correction formula (Abbott, 1925). The lethal concentrations (LC50 and LC90) expressed in mg/L were identified using probit analysis (Finney, 1971).

Analysis of the 16s rRNA region

The 16s rRNA universal primers 27F and 1492R (Weisburg et al., 1991) were used for amplification of the genetic DNA (Table 1). The PCR amplification was performed in a 50-µL reaction mixture containing 25 µL of 1×PCR Master Mix (Tsingke) (1.25 U Taq polymerase, 5 mM $MgCl_2$, and 2.5 mM of each dNTP), 1 µL of each primer (10 µM) and 2 µL DNA template and toped up with deionized water to the final volume. PCR was performed using T Professional thermocycler, Biometra and the cycling conditions were as follows: Initial denaturation at 94oC for 5min, followed by 30 cycles of 30 s

at 94°C for denaturation, primer annealing at 55°C for 30 s and extension at 72°C for 90 s, and a final extension step at 72°C for 10 min. The PCR products were separated by electrophoresis on a 1% agarose gel stained with 0.1% ethidium bromide and visualized in a transilluminator. The total DNA of each isolate was extracted using TIANGEN DNA extraction kit (TIANGEN Biotech, Beijing, China) according to the manufacturer's instructions. The resultant PCR products were purified using Omega pure cycle kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's protocol and sequenced directly using the ABI prism sequencer. All the sequences were edited using Bio-edit program and blasted against partial 16s rDNA sequences published in the NCBI GenBank database. Sequence alignment and construction of phylogenetic tree was conducted using MEGA 7 neighbor-joining method (Kumar et al., 2016).

Detection of toxin genes

Detection of toxin genes (binA, binB, mtx1, mtx2, mtx3, cry48Aa, and cry49Aa) for L. sphaericus and cry4A, cry11A and cyt1A for B. thuringiensis were conducted using PCR (Table 1). Cycling conditions were as follows: Initial denaturation was at 94°C for 5 min, followed by denaturation at 94°C for 30 s, primer annealing at 50°C for 30 s and elongation at 72°C for 60 s. There were 30 cycles with final elongation at 72°C for 10 min. The PCR products were analyzed using agarose gel electrophoresis in 1% gel.

Protein analysis

The selected toxic isolates were cultured in 20 ml Luria Bertani (LB) broth at 30°C with shaking at 150 rpm with shaking for 48 h. Spore-crystal mixtures were then washed twice with deionized water (Yuan et al., 2003). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as previously described by Laemmli (1970) where proteins were separated on a 12% gel and later stained with Coomassie Brilliant Blue R250.

RESULTS

Isolation of *Bacillus* species

A total of 453 endospore forming isolates were obtained from 100 soil samples collected in Kenya, and analyzed for presence of *Bacillus* species and related genera with an occurrence rate of 46, 29 and 25% in Kwale, Murang'a and Kiambu, respectively. The isolated strains were heat-resistant, Gram positive, spore-forming and various morphological appearances (Table 2)

Toxicity analysis

During initial toxicity analysis, seven isolates were found to yield high toxicity (>50% mortality) against *C. quinquefasciatus* 3rd instar larvae. Further standard bioassay procedure showed that the isolates KDHa3 and SKDHb5 recorded the highest toxicity, with LC50 of 0.007 and 0.008 mg/L, respectively against *C. quinquefasciatus* and this was comparable with the reference strains of *L. sphaericus* C3-41 and *B. thuringiensis* var. *israelensis*. Other isolates SKMb3, SCNa2, SVGd5, SCNd4 and SGTa1 showed moderate toxicities, with the LC50 values ranging from 0.01 to 1.326 mg/L. Isolate KDHa3 however stood out in that it recorded maximum mortalities after 24 h of exposure unlike the other six strains that showed maximum mortality after 48 h (Table 3).

Phenotypical and molecular characterization of isolates

Maximum spore prevalence was recorded at about 30 to 42 h for six of the isolates followed by a plateau similar to reference strain L.s C3-41. However for isolate KDHa3, maximum spore prevalence was recorded at 48 h similar to reference strain and *B. thuringiensis* var. *israelensis*. After 48 h, isolate KDHa3 showed the highest spore prevalence (78%) while isolate SCNd4 had the lowest spore prevalence (46%) (Table 3). Amplification of the isolates' 16s rRNA gene region resulted to formation of 1500 bp bands on 1% agarose gel (Table 4). The sequence analysis results showed that the isolates were highly homologous (>99%) to the members of *Lysinibacillus* group (6 isolates) and *Bacillus cereus* group (1 isolate). Phylogenetic tree was inferred in MEGA7 using the neighbor-joining method (Saitou and Nei, 1987) as shown in Figure 1.

The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1981) in the analysis that involved 25 nucleotide sequences. Evolutionary analysis grouped the isolates and the reference strains into two major clusters; *Lysinibacillus* group and *B. cereus* group. However, six of the isolated strains showed high homology to the toxic reference strains of L. sphaericus while one showed high homology to B. thuringiesis vr israelensis (Figure 1)

Toxin gene and protein analysis

The toxic isolates were further analyzed for presence of specific toxin genes using PCR and compared to the reference strains *L. sphaericus* C3-41 and *B. thuringiensis* var. *israelensis* Binary toxins (binA-522bp, binB-622bp) and mosquitocidal toxin (mtx3 -502bp) were detected in all six isolates that clustered in the *Lysinibacillus* group. Isolate SGTa1 showed negative results for mtx1 -512bp and mtx2-523bp but the only positive for gene cry48A-1025bp and cry49A-704bp (Table 4, Figure S1). For *B. thuringiesis* toxin gene detection, isolate KDHa3 showed amplified dipteran specific cry (cry4A- 1293bp and cry11A-445bp) and cyt (cyt1A-522bp) genes similar to the reference strain *B. thuringiensis* var. *israelensis*. This explains the high toxicity recorded against mosquito larvae. SDS-PAGE revealed that the isolates SCNa2, SKMb3, SVGd5, SGta1, SCNd4 and SKDHa5 produced binary toxin profiles (binA-41.9kDa and binB-51.4kDa) similar to the

Table 2. Phenotypic characterization of the toxic isolates.

Isolate			*L.s* C3-41	SCNa2	SKMb3	SVGd5	SGta1	SCNd4	SKDHa5	KDHa3	*B.t.i*
Colony characterization	Color	Cream	+	+	+	+	+	+	+	-	-
		Pale white	-	-	-	-	-	-	-	+	+
	Form	Circular	+	+	+	+	+	+	+	-	-
		Irregular	-	-	-	-	-	-	-	+	+
	Elevation	Raised	+	+	+	+	+	+	+	-	-
		Flat	-	-	-	-	-	-	-	+	+
	Margin	Entire	+	+	+	+	+	+	+	-	-
		Undulate	-	-	-	-	-	-	-	+	+
	Texture	Smooth	+	+	+	+	+	+	+	-	-
		Rough	-	-	-	-	-	-	-	+	+
	Size	Large	-	-	-	-	-	-	-	+	+
		Medium	+	+	-	+	+	-	-	-	-
		small	-	-	+	-	-	+	-	-	-
Cell characterization	Shape	Rods	+	+	+	+	+	+	+	+	+
	Gram test	+/-	+	+	+	+	+	+	+	+	+
	Endo-spores	Sub terminal	-	-	-	-	-	-	-	+	+
		Terminal	+	+	+	+	+	+	+	-	-
	Swollen spore	+/-	+	+	+	+	+	+	+	-	-
	Crystals	+/-	-	-	-	-	-	-	-	+	+
Physiological characterization	4^oC	+/-	-	-	-	-	-	-	-	-	-
	30^oC	+/-	+	+	+	+	+	+	+	+	+
	48^oC	+/-	-	+	-	+	-	-	+	+	+
	pH(4)	+/-	-	-	-	-	-	-	-	-	-
	pH(7)	+/-	+	+	+	+	+	+	+	+	+
	pH(9)	+/-	+	+	+	+	+	+	+	+	+
	7% NaCl	+/-	+	+	+	+	+	+	+	+	+
	10% NaCl	+/-	-	-	-	-	-	-	-	-	-
Region Isolated			Ls	Kb	Mr	Kw	Mr	Kb	Kw	Kw	Ls

Ls-Lab strain, Kb- Kiambu, Mr-Murang'a, Kw-Kwale; - negative, + positive.

reference strain L.s C3-41 while KDHa3 showed similar protein pattern as the reference strain *B. thuringiensis* var. *israelensis* in expressing Cry4A-128kDa, Cry11Aa-72kDa and Cyt 1Aa-27kDa. However, the degree of toxin gene expression varied among the isolates (Figure 2).

DISCUSSION

Previous studies have shown isolation of entomopathogenic *Bacillus* species from various diverse habitats such as dried plant leaves, dried animal dung, sewage water, excreta of arid birds, soil among others (Poopathi et al ., 2014; El-kersh et al., 2016; Soares-da-Silva et al., 2015). However soil samples have shown greater richness in the diversity of *Bacillus* species and related genera compared to other sources making it the most preferred substrate for isolation (González et al., 2013; Lobo et al., 2017; Suryadi et al., 2016). In this study, 453 *Bacillus* strains and related genera were isolated from 100 soil samples collected from random

Table 3. Growth parameters and lethal concentrations of the 48 h cultures.

Strain	Growth parameters			Toxicity (mg/L)	
	VC (*10^9 CFU/mL)	SC (*10^9 CFU/mL)	% spore prevalence	LC_{50} (95% CI)	LC_{90} (95% CI)
Ls C3-41	4.2	2.7	64	0.009 (0.003-0.020)	0.173 (0.067-0.932)
SCNa2	5.1	3.0	59	0.014 (0.004-0.037)	0.910 (0.272-7.761)
SKMb3	3.4	1.9	56	0.085 (0.033-0.220)	1.091 (0.390- 5.588)
SVGd5	4.5	2.2	49	1.299 (0.476-5.973)	3.315 (1.077-22.253)
SGTa1	4.0	2.4	60	0.010 (0.040-0.022)	0.221 (0.083-1.260)
SCNd4	2.8	1.3	46	1.326 (0.564-6.763)	2.688 (1.004-7.202)
SKDHb5	5.6	3.7	66	0.008 (0.004-0.050)	0.204 (0.076-1.179)
KDHa3	6.0	4.7	78	0.007 (0.003-0.014)	0.080 (0.034-0.398)
Bti	5.7	4.3	75	0.006 (0.003-0.011)	0.048 (0.022-0.221)

VC-Viable cell count; SC- Spore count; CI- Confidence Interval. Lethal concentration average values calculated according to three bioassay results.

Table 4. Amplification results of the isolates and reference strains.

Strain	PCR results										
	16s rRNA	***binA***	***binB***	***mtx1***	***mtx2***	***mtx3***	***cry48A***	***cry49A***	***cry4A***	***cry11A***	***cyt1A***
Ls C3-41	+	+	+	+	+	+	-	-	-	-	-
SCNa2	+	+	+	+	+	+	-	-	-	-	-
SKMb3	+	+	+	+	+	+	-	-	-	-	-
SVGd5	+	+	+	+	+	+	-	-	-	-	-
SGTa1	+	+	+	+	+	+	-	-	-	-	-
SCNd4	+	+	+	-	-	+	+	+	-	-	-
SKDHb5	+	+	+	+	+	+	-	-	-	-	-
KDHa3	+	-	-	-	-	-	-	-	+	+	+
Bti	+	-	-	-	-	-	-	-	+	+	+

+, Positive; -, Negative.

sites in Kenya.

Initial bioassay results showed that only 7 (1.5%) isolates yielded considerable toxicity (>50% mortality) against *C. quinquefasciatus* 3rd instar larvae. This low occurrence rate of mosquitocidal strains in soil was comparable with other reported results (Lukenge et al., 2017; Pereira et al., 2013) and this shows that mosquitocidal strains are rare to find as compared to other toxic strains against members of orders coleoptera and lepidoptera (Silva et al., 2012; Silva et al., 2010).

Notably, only one mosquito species (*C. quinquefasciatus*) was applied for mosquitocidal analysis in this study and this could have contributed to the low percentage of toxic isolates due to the smaller number of described toxins known to affect this group of insects (Lobo et al., 2017). Phenotypic characterization of the toxic isolates marked the basic identification procedure where six of the isolates showed high similarity to the reference strain L.s C3-41 while one isolate showed high similarity to the reference strain *B. thuringiensis* var. *israelensis* (Table 2). Physiologically, all the isolates grew at 30°C with no growth being recorded at 4°C and only the isolates SCNa2, SVGd5, SKDHa5 and KDHa3 showed growth at 48°C temperature. PH conditions 7 and 9 recorded growth for all the isolates while pH 4 recorded no growth. All the isolates also showed NaCl tolerance of up to 7% but no growth was recorded at 10% (Table 2). These physiological variations among some isolates might have been attributed to the fact that the strains were isolated from varying ecological regions. Generally the optimum conditions were concluded to be 30°C temp, pH 7 and ≤ 7% NaCl concentration similar to what was described by Gama et al. (2013).This data can be applied during the biopesticide production to promote maximum toxin yield.

The toxic strains exhibited different levels of toxicities against *C. quinquefasciatus*. Isolates KDHa3 and SKDHb5, classified as *B. thuringiensis* and *L. sphaericus* based on 16s rRNA sequences and phylogenetic analysis (Figure 1) respectively, had the highest toxicities comparable with the standard strains L.s C3-41 and B.t.i while the isolates SGTa1, SCNa2, SKMb3, SVGd5 and

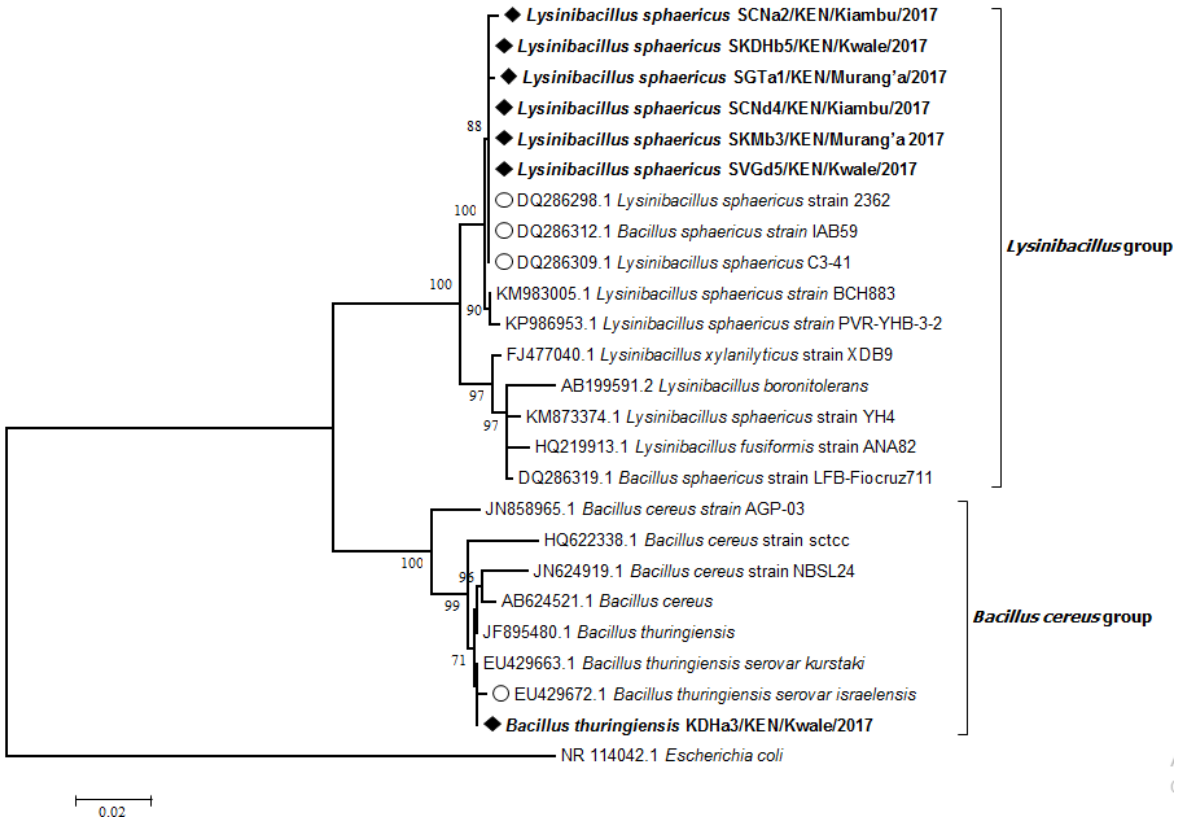

Figure 1. Neighbor-joining cladogram based on 16s rRNA gene sequences of the isolated toxic strains (bold and marked with a black diamond node) in relation to both known toxic (marked with a circular node) and non-toxic (unmarked) members of *Lysinibacillus* group and *Bacillus cereus* group. *Escherichia coli* represents an outgroup species. Toxic isolates from this study are named in the following order: Blast closely related strain and isolate code/Country/Collection site/Year.

SCNd4 classified as *L. sphaericus* showed moderate activities (Table 3). Furthermore, the maximum toxicities were recorded in 24 h for isolate KDHa3 and 48 h for the other six isolates. These findings of high toxic strains has also been reported in previous studies (El-kersh et al., 2016; Ibarra et al., 2003) and suggests that the toxic isolates are promising candidates for the development of new mosquitocidal formulations in Kenya.

Toxicity of entomopathogenic *Bacillus* species and related genera is attributed to various endotoxins they harbor. For example *L. sphaericus* principle toxin is the Binary toxin (bin) composed of two subunits (binA and binB) located in the chromosome and crystallize together into one parasporal body inform of a toxin dormain and a binding dormain (Berry, 2012; Colletier et al., 2016; Kale et al., 2013). They are also known to produce other less toxic proteins called mosquitocidal toxins (mtx1, 2, 3) expressed during the vegetative stage of the bacterial growth (Berry, 2012). Recently new cry toxins (cry48Aa and cry49Aa) were described in some toxic L. sphaericus strains such as L.s IAB59 (Ge et al., 2011; Rezende et al., 2017).

Presence of these cry toxins in some *L. sphaericus* strains might be attributed to their co-evolution with other bacteria such as *B. thuringiensis* (Ge et al., 2011). *B. thuringiensis* also forms a group of high toxic strains and this toxicity is primarily as a result of the cyt (cytolysins) and cry (crystal delta endotoxins) toxins they contain (Federici et al., 2003; Xu et al., 2014). This study confirmed the relationship between toxicities and the presence of various toxin genes as well as the production of specific toxins through molecular analysis. All the toxic strains harboring bin, mtx, cry48Aa, cry49Aa, cry4A, cry11A and cyt1A toxin genes were characterized accordingly.

Conclusion

The pursuit for native mosquito entomopathogenic *Bacillus* species and related genera was achieved in that seven isolates (6- *L. sphaericus* and 1-*B. thuringiensis*) identified by both morphological and genetic techniques were found to yield considerable toxicity against *C. quinquefasciatus* larvae. This study marked the first report of highly toxic isolates of *L. sphaericus* and *B. thuringiensis* from Kenyan soil samples. The results from this study will provide key knowledge needed for future

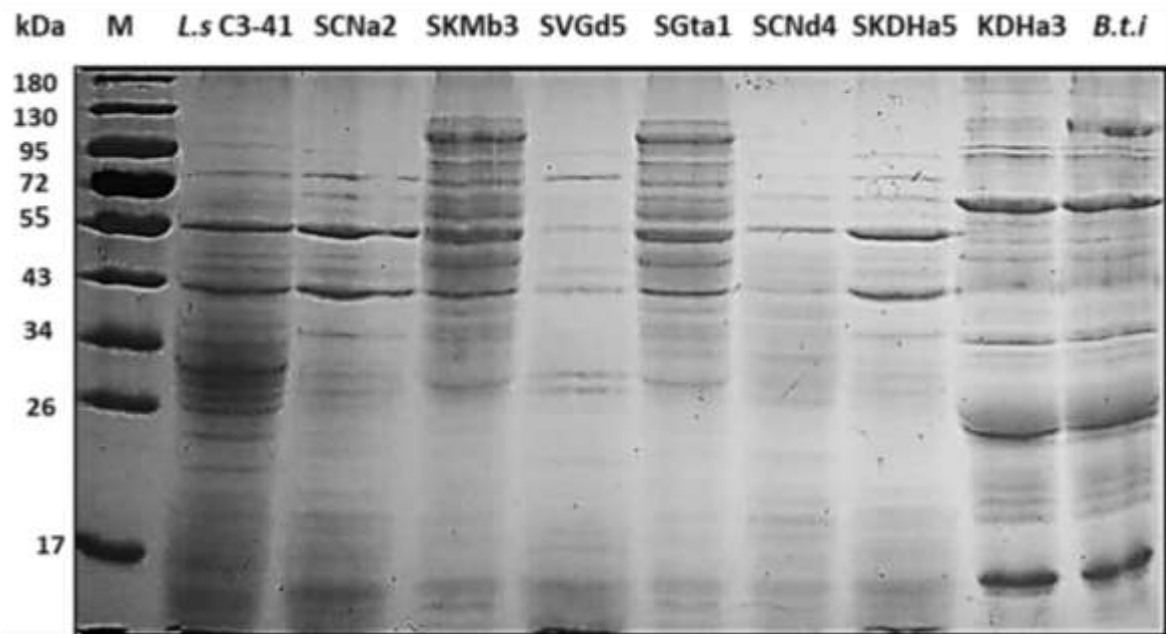

Figure 2. Protein profiles of the toxic isolates and reference strains after separation by 12% SDS-PAGE and Coomassie blue staining. Lanes: M- marker.

application of the native strains against various mosquito species in Kenya. More also, future applications will involve the use of locally available industrial and agro-based by- products as a medium for growing the already identified Kenyan based strains to increase yields as well as cut down the cost of production especially for large scale applications.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Supplementary materials

Figure S1. PCR amplification profiles on 1% gel agarose. **a-h** represents amplifications for 16s rRNA, *binA, binB, mtx1, mtx2, mtx3, cry48A* and *cry49A*, respectively. i (I,II,III) represent positive amplifications for *cry4Aa, cry11Aa* and *cyt1Aa*, respectively.