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Original Research Article

Isolation and Characterization of *Bacillus thuringiensis* Strains and their Toxicity against *Anopheles* Mosquito Larvae from Amhara Region, Ethiopia

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Abstract: ***Background:*** Malaria is one of the most prevalent public health problems worldwide. Globally, nearly half the world's population remains at risk of malaria transmitted by the Anopheles mosquito vector. Chemical insecticides used to control the malaria vector are toxic and cause environmental deprivation. Therefore, safe, environmentally friendly, and effective alternative methods are needed to control malaria vectors. The study objective was to isolate and characterize Bacillus thuringiensis (BT) strains and evaluate their insecticidal activity against malaria vectors.

Methods: Soil samples were collected from different sites in Amhara Regional State, Ethiopia. Serial dilution was conducted in normal saline; Cutured Coommasie Brilliant Blue staining was performed. BT strains were isolated and characterized based on morphological, microscopic parasporal crystal staining, and biochemical characteristics. Toxicity was assessed by bioassay against Anopheles mosquito larvae.

Results/Discussion: Thirteen isolates of Bacillus thuringiensis were identified and characterized based on their morphological, microscopic parasporal crystal staining, and biochemical characteristics. From resuts found that some of the BT strains were 75% effective and while others demonstrate 100% larvicidal potential similar with the reference strain Bti and better than Btk NCIM 2514 within 24 hrs.

Conclusions: Five Bacillus thuringiensis isolates showed significantly higher larvicidal efficacy against Anopheles mosquito larvae. Among them, three isolates were promising candidates for future applications in mosquito biocontrol.

Keywords: Anopheles Mosquito, Bacillus thuringiensis, Bio-control, Insecticidal proteins, Malaria.

1. BACKGROUND

Malaria is one of the major public health problems worldwide causing a significant burden of illness and mortality (Haile *et al*., 2020). Nearly half of the world's population, or over 4 billion people, are still at risk of contracting malaria. Pregnant women and children are especially vulnerable to the disease due to weaker immune systems (WHO, 2023). World Health Organization (WHO, 2020) estimated 229 million malaria cases from 87 malaria-endemic countries. From these Nigeria accounts (27 %), followed by the Democratic Republic of Congo (12 %), Uganda (5 %), Mozambique (4 %), and Niger (3 %) which is more than 51 % of all malaria cases worldwide (WHO, 2020).

Ethiopia accounts for 6% of malaria cases worldwide. As per the report of the Ethiopian Federal Ministry of Health (FMoH) (2020), malaria affects 75% of the country's population, with 68% of them residing below 2000 meters altitude, which is considered malaria-prone. The Amhara region accounts for 31% of Ethiopia's malaria burden and is targeted for malaria elimination by the FmoH. Thus, malaria prevalence is highly seasonal throughout the region, depending on altitude and climatic variations and the causative agent

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for malaria is *Plasmodium* parasite. Malaria prevalence peaks in most parts of Ethiopia during September to December, after the principal rainy seasons (June - September). The lowest pervalence was during March to May, following autumn rains (FMoH, 2017). However, the malaria rate has fluctuated, and drug resistance concerns have arisen, but the Ethiopian government still maintains its commitment to malaria prevention and control activities to contain morbidity and mortality.

All human malaria is transmitted by female mosquitoes of the genus *Anopheles*, but not all anophelines are vectors of malaria. So, far, 537 *Anopheles* species have been identified, and more than 30 of them can spread malaria to humans (Lempang *et al*., 2023). The *Anopheles arabiensis* is the primary vector responsible for malaria transmission in Ethiopia; however, *A. pharoensis, A. funestus,* and *A. nili* are all implicated in the disease's transmission (Solomon *et al*., 2020).

The Global Technical Strategy for Malaria 2016–2030 sets the most ambitious targets for reductions of malaria cases and deaths by reducing malaria mortality rates globally compared with 2015 at least 40% in 2020, at least 75% in 2025, and at least 90% in 2030 (WHO, 2016). This plan states vector control as the principal method to achieve the target. Insecticide-treated mosquito nets and indoor residual spraying are two methods for controlling vectors; other approaches include chemoprevention, which shields humans from blood-stage illnesses, and case management, which handles infection diagnosis and treatment (WHO, 2014). In Ethiopia, the main vector control activities implemented are Indoor residual spraying, long-lasting insecticide nets, and mosquito larval source minimization. The insecticides still used in the country include dichloro-diphenyl-trichloroethane (DDT), malathion, and deltamethrin (FMoH, 2014). Those who utilized mosquito nets and lived in homes treated with indoor residual spraying had a very low risk of contracting malaria (Ayele *et al*., 2012).

However, some of the evidence indicates that malaria mosquitoes are resistant to the usual insecticides that are used to treat bed nets (Yewhalaw *et al.,* 2011). Insecticide resistance among malaria vectors is an increasingly important challenge to malaria control programs. Though deltamethrin has been employed as a temporary alternative to DDT in IRS activities, pyrethroid resistance-including that of deltamethrin and lambda-cyhalothrin-and resistance to organophosphates like malathion have been reported from several areas of the world (Riveron *et al*., 2018). Mostly insecticide-treated nets are still used as the main malaria control strategy in the country (FMoH, 2020). Therefore, an alternative to chemical control is needed, which is a crucial part of integrated pest management (IPM) (Aramideh *et al*., 2010).

The *Bacillus thuringiensis* is a Gram-positive, rod-shaped bacterium with the ability to create resistant spores for insecticide that were first identified from diseased larvae of Silkworm *Bombyx mori* larvae in Japan (Valtierra-de-Luis *et al*., 2020). *Bacillus thuringiensis* consistitutes the majority of commercial bio-insecticides which has been effectively used for the past 60 years and is safe, effective, and has a specificity in targeting inscts for controlling a wide range of human disease vectors (Valtierra-de-Luis *et al*., 2020). One of the main characteristics of Bt is to form intracellular parasporal crystal-shaped δ-endotoxins having insecticidal properties during sporulation at the stationary phase. This process starts only after the sporulation phase when growth and nucleic acid synthesis cease (Schnepf *et al*., 1998).

Two varieties of δ-endotoxins are produced by *Bacillus thuringiensis* strains: the Crystal (Cry) toxins, which are encoded with distinct *cry* genes, and the Cytolytic (Cyt) toxins. One of the main causes of the diversity in cry genes amongst Bt strains is plasmid transfer (Thomas *et al*., 2001). Currently, more than 700 cry gene sequences have been identified, and around 80 primary subgroups of Cry toxins, with different primary ranks in the nomenclature (Cry1, Cry2, Cry3, etc.), and their lengths vary from 369 (Cry34) to 1344 amino acids (Cry43). Cyt proteins of δ- endotoxins differ in amino acid composition, protein structure, and mechanism of action from Cry toxins which act as hemolytic activity. In addition to δ-endotoxins, Bt produces other toxic proteins called vegetative insecticidal proteins (Vips) that do not share sequence homology with the known delta-endotoxins and the gene codes for a 791 amino acid (88.5 kDa) protein (Adang *et al.,* 2014).

These crystal proteins dissolve in the alkaline midgut of insects, where they are subsequently activated by intrinsic proteases into an active toxin that binds to a particular receptor in the cell membrane with specificity, creating pores that let in water and ions and ultimately kill the insect. Although many Bt commercial products have been commonly used and various Cry proteins have been identified, identification of the Bt strain is still ongoing (Eswarapriya *et al*., 2010). Earlier, the Cry genes were classified into four; Cry I, Cry II, Cry III, and Cry IV proteins based on their insecticidal activities and two classes of cytolytic genes (Cyt1 and Cyt 2) that govern production of the toxins that kill the susceptible insects. Cry I and Cry II are active against lepidopterans, Cry III is active against coleopterans and Cry III and Cry IV are active against dipterans (Schnepf *et al.,* 1998). A large number of laboratory and field studies indicated that Bt has no adverse effect on human, animals, birds, fish, or many other non-target aquatic vertebrates (WHO, 2020). Therefore, this study was needed to Isolate and characterize *Bacillus thuringiensis* strains and their toxicity against *Anopheles mosquito* larvae for malaria control.

2. MATERIALS AND METHODS

2.1 Study Area

Table 1: Descriptions of study Area

No.	Study area	Region	Elevation	Annual average Temperature	Latitude	Longitude
1	Jawi	Amhara	2131 to 2500m	20-30^{0}C	11°49'12.13"N	36°20'59.26"E
2	Enjibara	Amhara	2560m	22-28^{0}C	10°57'23.98"N	36°55'48.70"E
3	Tara monastery	Amhara	2 142 to 2 484 m	21-28^{0}C	12° 9'17.91"N	37°42'47.53"E
4	Debre Birhan	Amhara	2,840 m	15-18^{0}C	9°40'33.23"N	39°31'58.48"E

Four sites were selected in Amhara regional state, Ethiopia; Jawi, Enjibara, Tara monastery, and Debre Birhan, which are located in Gojjam, Gondar, and Shewa respectively as shown in Table 1. Study sites were selected purposively, Jawi was considered a hot area, Tara Gedam was moderately temperate, and Debre Birhan and Enjibara were considered cold areas laboratory work was conducted in the Institute of Biotechnology, Cellular, Microbial, and Molecular Biology laboratory, University of Gondar, Ethiopia.

2.2 Study Design and Period

Completly randomized experimental design (CRD) was used to conduct this study. Thirteen Bt isolates were tested for their toxicity in triplicate. This study was conducted from October 2020 to October 2021.

2.3 Sampling Techniques

From each study area, a total of 18, 200g weighed soil samples were collected by scraping the soil surface 10-15cm deep, using a clean, dried, and sterile spatula and polythene tube (in ethanol sterilized polyethylene bags). Soil samples were particularly taken from forest areas. All of the collected samples were transported to the Institute of Biotechnology, microbiology laboratory under sterile conditions for Microbiology activities and further analysis.

2.4 Processing of Soil Samples and Isolation and Identification of Microbial Isolates

One gram of collected soil samples was taken separately and dissolved in 9 ml of sterile normal saline (NaCl) solution of 0.85%: w/v to make 1%: w/v soil sample solution. The solution was then homogenized vigorously with a vortex mixer for 2 min. Sample solutions were heat shocked at 80 °C for 10 min, in a water bath to destroy non-spore forming and vegetative *Bacillus* cells. Then homogenized and heat-treated soil sample solutions were serially diluted in sterile normal saline, to prepare 10^{-1} - 10^{-5} concentrated solutions. Then 100 µl volume aliquots of 10^{-1} - 10^{-5} were separately plated on nutrient agar medium and incubated aerobically at 28°C for 24-48 hrs. as described in the method of Chilcott and Wigley (1988). After 24-48 hrs. of incubation, Bt-like isolates were obtained and characterized using various morphological (Microscopic and colony morphology) and biochemical tests as described by Fawole and Oso (2001). A single individual colony above 10^{-3} agar culture was picked and transferred to the nutrient agar medium and then grown for 24 hrs at 28°C. The purity of each isolate was checked through subculturing on a nutrient agar medium for 24 hrs at 28°C as described by Reyaz *et al*., (2017). *Bacillus thuringiensis subspecies kurstaki* (Btk) and *Bacillus thuringiensis var. israelensis* (Bti) were used as references, which were kindly provided by Dr. Meera Indracanti from India (personal communication).

2.5 Morphological identifications of Bt isolate

Colonies exhibiting morphological traits typical of the Bt group, such as being large, dry, flat, or sticky were classified into four groups, denoted as follows: Group A (white, round, flat, and with wavy/scalloped edged margin); Group B (white, round, slightly raised center, and with fried egg appearance); Group C (white, shiny, round, little raised center, and with irregular but entire margin); and Group D (white, round, mucoid, slightly raised center, and spread out with irregular spike-like margin) (El-kersh *et al.,* 2012).

2.5.1 Gram Staining

Gram staining procedure was performed to distinguish the isolated bacterial samples as rod-shaped and Gram-positive bacilli as described by Bartholomew and Mittwer (1952).

2.5.2 Coomassie Brilliant Blue (CBB) Staining

The existence of crystal proteins, as described by Ammons *et al*., (2002), was investigated in the isolated bacterial samples using the Coomassie Brilliant Blue (CBB R-250) staining method. In conclusion, the bacterial samples were cultured for 96 hours at 28°C, then smeared onto a drop of 0.85% saline solution on the microscopic slide, allowed to air dry, and then heated to fix them. The slides were first stained for one minute using 0.133% Coomassie Brilliant Blue stain, and then for twenty seconds, they were left uncolored using distilled water. After allowing the slides to air dry, they were examined under a compound light microscope with an oil immersion magnification of 1000X. Biochemical typing was done on the crystal-positive Bt isolates that were obtained.

2.6 Biochemical Typing

From the sub-cultured colonies, thirteen Bt isolates with gram-positive rods having a parasporal body were selected and subjected to biochemical tests

including the catalase test, Oxidase test, TSI, Citrate utilization, Urease test, motility test, and spore test to know further nature of isolates as described by Jyothi and Priya (2018).

2.7 Antibiotic Susceptibility Test

Drug susceptibility tests were performed in response to commercial antibiotics such as Ampicillin, Bacitracin, Cotrimoxazole, and Ciprofloxacin using the disk diffusion method as described by Hudzicki, J. (2009). The test results of antibiotic sensitivity were determined according to the inhibition zone diameter. According to Sarker *et al*., (2010), the presence of a clear zone indicated that the collected isolates were sensitive to those antibiotics.

2.8 Spore-Crystal Complex Isolation

Pure sub-cultured bacterial isolates were inoculated into sterilized Luria Bertani (LB) broth enriched with salts (g. L-1 in distilled water) 0.002g FeSO4, 0.02g ZnSO4, 0.02g MnSO4, 0.3g MgSO4 and 2g glucose to aid sporulation in 250 ml flasks at pH 7.5. Liquid cultures were incubated in an orbital shaker at 28^0C for five days until sporulation was observed and the broth culture containing spore-crystal complex was centrifuged according to the method of Lobo *et al*., (2018). At each sampling the density of sporulation and crystal formation was monitored using the Smirnoff stain, the stained slides were viewed under a light microscope with an oil immersion objective as described by Smirnoff (1962).

2.9 Vector Larvae Collection

Anopheles mosquito larvae were collected from Kola Diba in Amhara Regional state, from selected standing water bodies of ponds, ditches, and irrigation pools depending on proximity to human settlement and quality of water. Standard larval dippers were used for random sampling at several points during early morning and evening hours. The larvae were placed in containers with water from the site and transported in cool conditions to prevent desiccation. The larvae were reared in trays of clean water and fed single-cell protein. Identification was done by ocular inspection and under the microscope by an expert entomologist (Wondmeneh Jemberie).

2.10 Bioassay Activity

The insecticidal efficiency of 13 isolates was tested and the tests were carried out in triplicate with less than 5% significance level value. The biological activity of spore-crystal inclusion complexes of Bt was tested against *Anopheles mosquito* larvae as described by WHO (2020). Five milliliters (ug/ml) of each Bt isolate lyophilized powder of spore crystal suspension was introduced to each triplicate cup containing 10 larvae of third instar stages in 100 ml distilled water and left at room temperature, as described by Hassan *et al.* (2021). Due to its more effectiveness than the crystal alone, the spore-crystal complex was prepared for each isolate to test insecticidal efficacy as stated by Keswani *et al*., (2016). *Bt* subspecies *israelensis and kurstaki* NCIM 2514 were used as positive control and larvae in distilled water without spore crystal suspension served as negative control. Results of scoring mortality were recorded after 24hrs and 48hrs for each treatment. Larvae that could not move were considered as dead.

2.11 Data Analysis

The data were analyzed using the SPSS software package version 25. Means and standard deviations of the triplicate data analysis were calculated via one-way analysis of variance (ANOVA) as described by Gomez and Gomez (1984) to determine the list significance differences (LSD) test, at $p < 0.05$ between the means. Moreover, the insecticidal activity and other data obtained are also presented in multiple drawn charts.

3. RESULTS

3.1 Isolation and Geographical Distribution of Bt Isolates

A total of 18 soil samples were collected from four different sites in the Amhara region (Table 2) under sterile conditions and screened for Bt presence. After heat shock, sixty-seven Bt-like colonies were isolated by nutrient agar medium, out of which thirteen isolates were identified as Bt based on gram-positive rod shape and the presence of parasporal bodies (Figure 3). Since the Bt index is defined as the number of identified Bt colonies divided by the total number of *Bacillus-like* colonies examined, a Bt index of 0.19 was obtained (Table 2). The highest percentage of Bt colonies (69.2%) were found in Jawi, followed by Tara monastery and Debre Birhan (15.3%).

Table 2: Locations and Bt isolates from the soil sample

Sites	Sample type	No. of the samples processed	No. of Bt like colonies	No- of Bt isolate obtained	Bt index
Debre Birhan	Soil	3	12	2	0.17
Jawi	Soil	9	27	9	0.33
Enjibara	Soil	3	7	0	0.0
Tara Monastry	Soil	3	21	2	0.1
Total		**18**	**67**	**13**	**0.19**

Bt index: No. of identified Bt colonies divided by the total number of Bacillus-like colonies examined.

3.2 Colony Morphology, Gram Stain, and parasporal Body Characterizations

Serial dilutions of 10^{-3} to 10^{-5} were used to obtain individual colonies from soil samples. Out of 67 total Bt-like colonies, 13 Bt isolates were having a white, golden and/or milky, round, long rod shape, raised, slightly raised, or flat center as indicated in Figure 1. All isolates were subjected to Coomassie brilliant blue staining for further characterization based on crystal morphology (figure 1). Thirty Bt isolates showed the presence of crystal protein and indicated rod-shaped gram-positive bacterial cells under a light microscope (table 3).

Table 3: Colony morphological, parasporal body, and gram staining characterization of Bt isolates

Isolates	Colony color	Margin	Size	Elevation	Shape	Gram staining	Crystal staining
TGwRLF	White	Round	Large	Flat	Long rod	+	+
TGGRmsr	Golden	Round	Medium	Slightly raised	Long rod	+	+
JW4wRLsr	White	Round	Large	Slightly round	Long rod	+	+
JW2Wrlf	White	Round	Medium	Flat	Long rod	+	+
JW7wrlf	White	Round	Large	Flat	Long rod	+	+
JW3wrlf	White	Round	Large	Flat	Long rod	+	+
JW5wrlf	White	Round	Large	Flat	Long rod	+	+
JW4wrlf	White	Round	Large	Flat	Long rod	+	+
JW9wzlf	White	Zigizag	Large	Flat	Long rod	+	+
JW8wrlf	White	Round	Large	Flat	Long rod	+	+
JW2wzlr	White	Zigizag	Large	Raised	Long rod	+	+
DB2wrlf	White	Round	Large	Flat	Long rod	+	+
DB3wrlf	White	Round	Large	Flat	Long rod	+	+

Key: In DB2wrlf and DB3wrlf= DB represents isolates from Debre Birhan district, numbers represent sites of the isolate; wrlf, sr, r, z= represents colony type like white, round, large, flate, slightly raised, raised, zigizag, JWrepresents isolates from Jawi district, TG represents Tara Gedam isolate district, + = represents positive and - =represents negative.

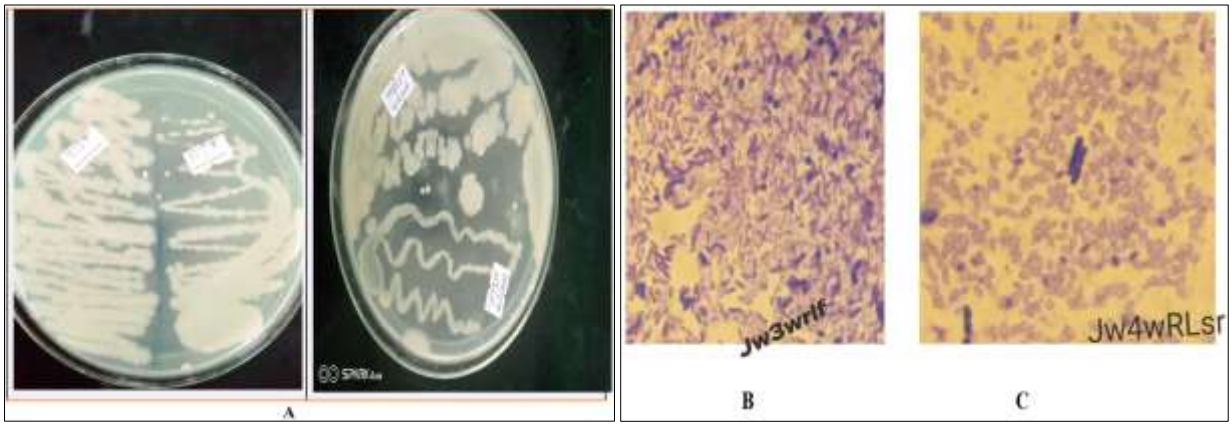

Figure 1: A purified single colony of Bacillus thuringiensis, B. Gram positive Bt isolate and C. Images of parasporal Bodies

3.3 Biochemical Typing

The various biochemical tests for 13 Bt isolates were conducted and the results obtained were presented in Table 4 and Fig. 2. All isolated Bt were positive for catalase activity, urease test, and glucose fermentation, 69.23% lactose or sucrose fermenter, 53.85% of Bt could utilize citrate, 61.54% oxidase positive and 69.23% of Bt were motile.

Table 4: Biochemical tests

No	Isolates	Catalase test	Oxidase test	Urease test	Citrate test	Motility test	Lactose ferm	Glucose ferm	Sucrose ferm
1	JW7wrlf	+	+	+	-	+	+	+	+
2	JW2wRLF	+	+	+	+	+	+	+	+
3	JW3wrlf	+	+	+	+	+	-	+	-
4	JW5wrlf	+	-	+	+	-	-	+	-
5	JW4wrlf	+	-	+	+	+	+	+	+
6	TGwrlf	+	+	+	-	-	+	+	+
7	TGGrmsr	+	-	+	-	-	+	+	+

8	JW9wzlf	+	+	+	-	+	-	+	-
9	JW4wRLsr	+	-	+	+	+	-	+	-
10	JW8wrlf	+	+	+	+	-	+	+	+
11	JW2wzlr	+	-	+	-	+	+	+	+
12	DB2wrlf	+	+	+	+	+	+	+	+
13	DB3wrlf	+	+	+	-	+	+	+	+

Jw=Jawi, w-white,r=round,l=large,m=medium,mfirst=milky, r=raised, sr= slightly raised, f=flat,+=positive,-=negative result, DB=Debre Birhan

3.4 Antibiotic Susceptibility of Potential Bt Isolates

Antibiotic susceptibility testing was conducted on the five potentials of Bt isolates having insecticidal activities, including reference strains (Bti and Btk). As shown in Figure 3, the test indicated that all Bt isolates, including Bti and Btk (reference strains), were resistant to Ampicillin and sensitive to Ciprofloxacin. Whereas 71.43% of Bt isolates, including Bti and Btk (reference strains), were sensitive to Cotrimoxazole and bacitracin except for JW2wzlr and Btk isolates (Table 4 and Figure 3).

Table 5: Antibiotic susceptibility of potential Bt isolates, including reference strain Bti and Btk

Isolates	JW2wRLF	JW7wrlf	JW4wRLsr	JW2wzlr	JW3wrlf	Bti	Btk
Ampicillin (Amp)	R	R	R	R	R	R	R
Bacitracin (Bac)	S	S	S	R	S	S	R
Cotrimoxazol (Cot)	R	S	S	R	S	S	S
Ciprofloxacin (Cip)	S	S	S	S	S	S	S

key: R=resistant, S= sensitive by Mueller Hinton Broth with agar

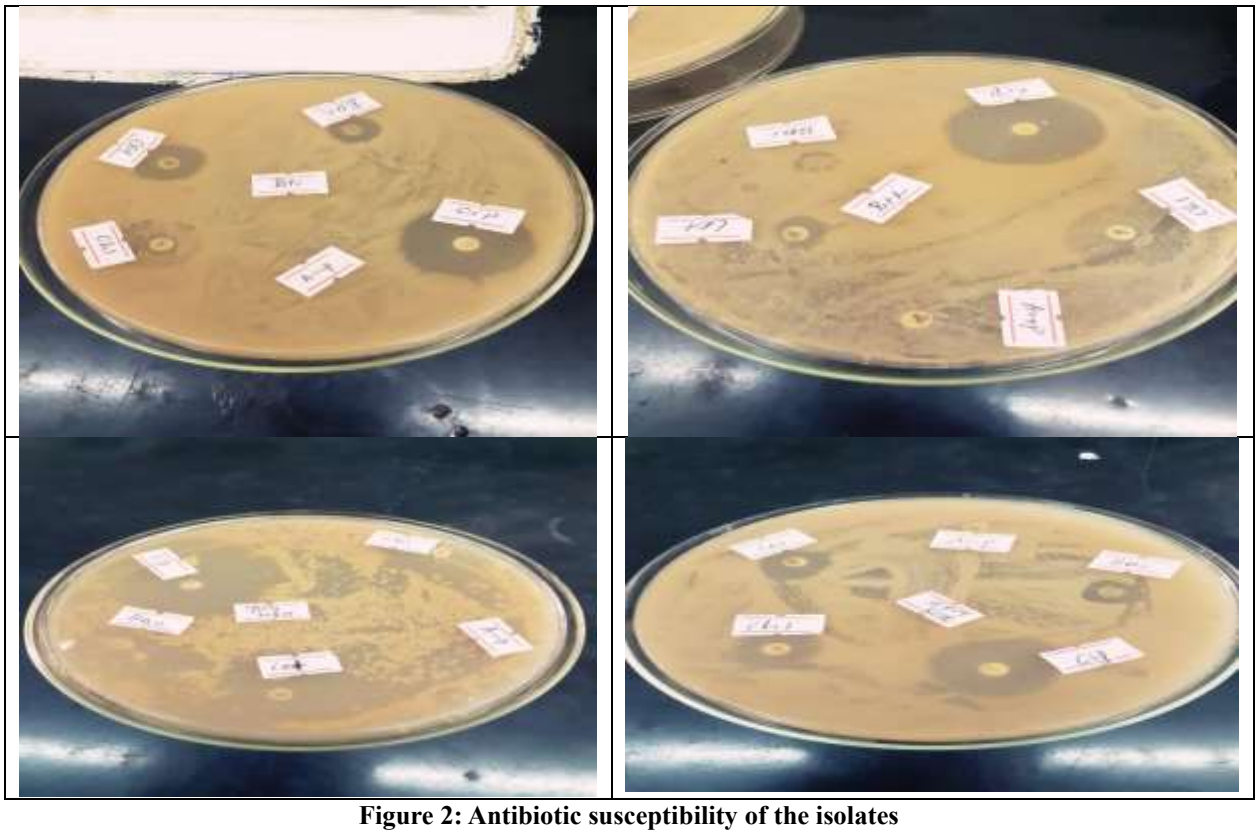

Figure 2: Antibiotic susceptibility of the isolates

3.5 Toxicity Assay of Bt Isolates Against *Anopheles Mosquito* Larvae

The film method of bioassay was used for the evaluation of the insecticidal activity of the different isolates of Bt on *Anopheles* mosquito's thired instar larvae. Thirty isolates were tested with a triplicate treatment and among them, only five were potentially effective against *Anopheles* mosquito larvae. The percent mortality was recorded after 24 hrs and 48 hrs. As shown below in Table 6 and Figure 2, the mortality of larvae was observed in all of the 5 potential Bt isolates immediately after 24 hrs of the Bt treatment. There was 100% mortality for three isolates (JW4wRLsr, JW3wrlf, JW7wrlf) after 24 hrs and also after 48 hrs, there was relatively high mortality for the rest isolates.

Table 6: Mean Percentage of Mortalities

Mean Percentage of Mortality (%) ± SD		
Isolates	24 hrs	48 hrs
JW2Wrlf	50.00 ±0.00^{d}	83.33 ±5.77^{d}
JW4wRLsr	100.00 ±0.00^{y}	--
JW3wrlf	100.00 ±0.00^{y}	--
JW2wzlr	90.00 ±10.00^{d}	93.33 ±5.77^{yd}
JW7wrlf	100.00 ±0.00^{y}	--
BtK	70.00 ±0.00^{d}	90.00 ±0.00^{yd}
Bti	100.00 ±0.00^{y}	--
	P<0.001	p<0.001

Key: Mortality is expressed as mean ± standard deviation; P values were less than 0.001 both at 24 hrs and 48 hrs. Mean followed by letters **y** and **yd** in each column are not statistically significant differences and mean followed by **d** are significant.

Figure 3: The insecticidal activity of Bt isolates A. Larvae before died B. Larvae after died C. Larvae control

4. DISCUSSION

Gram-positive Bacillus thuringiensis is a significant pest management agent due to its high specificity for the target organism and lack of toxicity to non-target organisms (Sahin *et al*., 2018). They are ubiquitously found in soil, water, compost, mammalian feces, dead larvae, and leaves (Afriani *et al*., 2018). Research on this bacterium was insufficient, despite Ishiwata initiating the first isolate in 1902 (Melo *et al*., 2016). A selected agroecological sites—Debre Birhan, Injibara, Jawi, and Tara Monastery soils—were investigated for the first time in this study.

In this study, a total of 67 Bt-like colonies were isolated from 18 soil samples 13 of which were successfully identified as Bt after parasporal body microscopy. These had similar characteristics to the reference Bacteria (Bti and Btk). From four sites, jawi 9 (69.2 %), Tara Gedam, and Debre Birhan 2 each (15.4 %) Bt isolates were found. This is indicative that Jawi could be a hotspot for the existence of Bt possibly because of suitable environmental conditions. The results thus demonstrate the possibility of local search for Bt strains for a biocontrol perspective. But in the Enjibara soil sample, no Bt isolate existed, this may be because of contamination, staining error, or sample handling problem. This study shows potential malaria vector -controlling Bt were isolated from soil samples in line with the Birhan *et al.*, (2021) report. The results show that the Jawi soil sample had the highest Bt index, 0.33, which makes it a more promising source for the isolation of Bacillus thuringiensis, followed by Debre Birhan, 0.17, and Tara Gedam, 0.1, with an overall average of 0.19. This is in line with El-Kersh *et al*., (2012) but lower than the Bt index reported by Shishir *et al*., (2012) (0.6). This may be because of the variation in soil characteristics or sampling methods. It is, however higher than Gebremariam *et al*., (2021) (0.1) and shows that these soils from Ethiopia have a better presence of Bt. These variations bring out the role of geographical and environmental factors on the distribution of Bt.

In this work, different isolates showed variations among one another in their colony color, margin, surface, and elevation. The different colony morphologies were white/milky/ or golden, round, circular or zigzag shape, and flat, raised, or slightly raised center. This is similar to Chai *et al*., (2016) and Padole *et al*. (2017) colony morphology characterization. Morphologically characterized isolates were further subjected to microscopic studies based on gram staining and Coomassie brilliant blue staining as the suited parameters to distinguish Bt isolates from the B. cereus group. All isolates were Gram-positive, sporulating rod-shaped soil-inhabiting bacteria. The Coomassie brilliant

blue staining analysis showed that from the total of 67 isolates, only 13 isolates were of parasporal inclusion and classified as Bt whereas other isolates failed to have parasporal inclusion. Studies reported that crystal protein inclusions were the unique characteristic of Bt that distinguished Bt from other *Bacillus* species and this result was in line with Hassan *et al., (*2021). The Bt gram staining result and Das *et al.,* (2015) also reported similar results under a light microscope.

In this study, Bt isolates that had positive results for Gram staining and Coomassie brilliant blue staining were further characterized by their biochemical typing. From biochemical characterization, the nature of the 13 Bt isolates was similar in being catalase and urease positive and glucose fermenters, as reported by Padole *et al*., (2017). In addition, 61.5% were oxidase-positive and 53.8% utilized citrate, indicating metabolic diversity among these isolates. Most (69.2%) were motile and fermented lactose and sucrose, indicating their possible use in varied ecological niches. This study result was in agreement with Rajashekhar and Kalia (2017), for the lactose/sucrose test and motility test. The Citrate utilization of Bt isolate was in contrast with Padole *et al.,* (2017) report, as it had 100% negative utility. Oxidase test results were similar to Gorashi *et al.,* (2014) report. From this biochemical characterization, we have different Bt isolates across the world that may express different results.

Antibiotic studies revealed that all strains showed variation in susceptibility to antibiotics. In this study, five potential isolates including reference strains were tested for antibiotic susceptibility. All isolates were resistant for Ampicillin and sensitive for Ciprofloxacin. This result was in agreement with Bouba *et al.,* (2014) and Rajashekhar and Kalia (2017). Insecticidal Bt isolates of JW2wRLf and JW2wzlr were resistant to Bacitracin and except JW2wzlr all isolates were sensitive to Cotrimoxazol antibiotics, which was also similar to Rajashekhar and Kalia (2017) report.

Bioassay was conducted to detect the toxicity nature of 13 Bt isolates from soil samples against the 3rd larvae stage of the *Anopheles* mosquito. Out of 13 Bt isolates JW2wRLF, JW4wRLsr, JW3wrlf, JW2wzlr, and JW7wrlf potentially kill Anopheles mosquitos larvae within 48 hrs. This is in parallel with Hassan *et al.,* (2021), in which out of 12 isolates, four were potentially toxic. All potential isolates were motile, catalase and urease positive, glucose fermenter, and also exhibited oxidase activity except JW2wzlr. This was in agreement with Hassan *et al.,* (2021) and El-krsh *et al*., (2012). These results were in accordance with commercially available Bti which was toxic for *Anopheles mosquitoes* (Faiz and Bukhari, 2018). The motile activity of the isolates was also an indicator of their toxicity nature against the Anopheles mosquito's larvae (Bouillaut *et al*., 2005).

Out of 13 Bt isolates JW2wRLF, JW4wRLsr, JW3wrlf, JW2wzlr, and JW7wrlf potentially kill *Anopheles mosquitos* larvae within 48 hrs. The mortality of JW3wrlf, JW4wRLsr and JW7wrlf were 100% effective within 24 hrs, which was similar to the commercially available Bti strain. Even though JW2wRLF and JW2wzlr effectively kill above 50% within 24 hrs, they exhibited a relatively higher percentage of mortality in 48 hrs. This may be due to the toxic nature (mode of action) of the Bt isolates. Our result was better than Lobo *et al.,* (2018) report, only 12 (4%) out of 300 Bt isolates were toxic for *Anopheles mosquito* larvae, and only 3 of them were 100% effective within 24 hrs. This may be due to concentration and larvae differences. Out of the five Bt isolates, three isolates displayed significantly higher larvicidal bioactivity than Btk, but similar to Bti reference strains within 24 hrs. There was a significant difference among isolates at 24 and 48 hrs ($p<0.001$) (table 5). The isolates JW3wrlf, 4wRLsr, and JW7wrlf would be used for further experiments and commercially because they had best effect on *Anopheles mosquito* larvae.

In this study, we had 23.1% of Bt isolates 100% effective within 24 hrs 15.4% of them were effective within 48 hrs and in total 38.5% of them were effective within two days. This is in agreement with the report described by Zeleke *et al.,* (2009); in which out of 43 isolates 12 were 100% effective within 24 hrs and 12 killed 100% in the second day 48 hrs and in total 55.8% were killed within two days. According to El-Kersh *et al*., (2012), out of 68 Bt isolates, 23 (33.8%) were potentially toxic against the *Anopheles mosquito* of 3rd stage larvae. The isolates were comparable with several available commercial larvicide products showing 99% killing efficacy according to (Bellini *et al*., 2009), Efficacy, and lasting activity of four IGR formulations against mosquitoes in catch basins. The isolates have better potential for the control of mosquito populations, although it will be necessary to identify their active components, given that the molecular analysis using specific primers did not detect any of the expected cry and cyt genes of the isolates.

5. CONCLUSION

In conclusion, this study demonstrates the effective larvicidal of Bt isolates against Anopheles mosquito larvae. A total of 67 Bt-like isolates were obtained from soil samples collected from different sites, and out of these 13 isolates were confirmed as Bt based on the presence of crystal proteins. Five isolates showed potential larvicidal effects against larvae of the Anopheles mosquito. The highest percentage of mortality (100%) was observed for JW4wRLsr, JW3wrlf, and JW7wrlf isolates and this suggests that locally isolated Bt is highly effective in controlling *Anopheles* mosquito. All of the effective isolates were motile, oxidase, and urease positive, antibiotic-susceptible or resistant, and contained crystal protein. Although there have been many commercial products

and have identified various crystal proteins, isolation and identification of Bt is underway. These new strains of Bt could provide alternatives when insect resistance appears for certain Bt strains. Therefore, Screening Bt isolates from different sources and habitats could be useful to obtain Bt isolates with high potential insecticidal activity.

DECLARATION

We declare that the manuscript entitled: Evaluation of Insecticidal Activities of Soil *Bacillus thuringiensis* Toxin against Malaria Causing *Anopheles mosquito* Larvae from Amhara Region, Ethiopia is our original work at the University of Gondar, institute of biotechnology.

This manuscript contains no material that has been submitted previously, in whole or in part, which signifies the manuscript is our own work only. All sources of material used for the manuscript have been duly acknowledged.

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