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Molecular identification of bacteria associated with Anopheles mosquitoes for prevention and control of Malaria in the Environment

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Abstract

Every year, hundreds of thousands of people die of malaria in African countries especially Nigeria. Current control measures against malaria are based on drugs against the parasites (*Plasmodium* spp) and the vector (mosquitoes) using insecticides. Additional areas of malaria control must be explored because of the development of resistance, one of such areas involves the use of bacteria associated with the vector mosquitoes. This study investigated the molecular characterization of bacteria associated with anopheles' mosquito for the prevention and control of malaria fever in the environment. Twenty (20) samples were collected from five different mosquito breeding places including water lodged areas, drainages, stagnant water, domestic wastes and stored rain water. Isolation of bacteria was carried out by pour plate method, identification of bacteria, mass rearing of mosquito's larvae, larvicidal activity, optimization of the bacterial isolates for the best lavarcidal activity and molecular characterization of the isolated bacteria were carried out by standard methods. The isolated bacteria include *Proteus vulgaris*, *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Bacterial isolates with the best larvicidal activity were *Staphylococcus aureus* (WL3), *Bacillus subtilis* (S4), *Pseudomonas aeruginosa* (R5) and *Proteus vulgaris* (D1). Optimum temperature, pH and appropriate carbon source play an important role in determining the larvicidal effectiveness of bacterial isolates**.** Optimum temperature of 37^{o}C and near-neutral pH conditions (6.5–7.0) were more favorable for larvicidal metabolite production while glucose and sucrose were more effective than soluble starch in enhancing larvicidal activity. The study revealed naturally occurring pathogenic bacteria associated with mosquitoes, these bacterial isolates and their metabolites may be used to reduce transmission of malaria fever by reducing population size, shorten life span and inhibit the ability of mosquitoes to transmit malaria parasites by eliminating the parasites in the vectors.

Keywords: Bacteria; Mosquitoes; Malaria; Larvicidal Activity; Vectors

1. Introduction

Malaria fever is a significant environmental problem that poses a potential health risk to human. It is spread by mosquitoes which breed in open water and spend most of their larva stage feeding on fungi and microorganisms on water surface (CDC, 2024). In 2015, malaria transmission occurred in 95 countries and territories placing about 3.2 billion people- almost half of the world's population, are at risk of the disease (WHO, 2015). There were an estimated 229 million cases of malaria worldwide in 2019, resulting in approximately 409,000 deaths, making malaria one of the most significant vector-borne diseases globally (WHO, 2020). Each year, more than 400 000 people die of malaria and an estimated two thirds of deaths are among children under the age of five, malaria fever is therefore a life-threatening disease caused by parasites that are transmitted to people through the bites of infected female Anopheles mosquitoes (WHO, 2025).

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Malaria is preventable and curable, and increased efforts are dramatically reducing the malaria burden in many places. The use of pesticides to control mosquitoes in the various stages of the life cycle of mosquitoes have been shown to have weaknesses and limitations as most of the mosquito populations have grown resistance to them and many people are concerned about their harmful effects on the environments, animals, plants and on human (Coleman *et al*., 2016). Bacterial larvicides have been used with considerable success in nuisance insect and vector control programs for almost two decades. However, current operational cost for larvcides based on isolated bacteria are high even though they have been investigated in some African countries and beyond and considered environmentally friendly for mosquito control (Poopathi and Abidha, 2010). The global burden of mosquito-borne diseases, particularly malaria transmitted by *Anopheles* species, has necessitated a persistent search for novel and sustainable vector control strategies. The rise of insecticide resistance to conventional chemical agents has pivoted research efforts towards biological control, focusing on the identification and application of naturally occurring entomopathogenic microorganisms (Poopathi, 2022). Comprehensive studies correlating culturing of bacteria associated with breeding places of anopheles mosquitoes with its optimization of the growth conditions such as optimum temperature, pH and utilization of different carbon sources are limited. There is therefore, the need to deepen the search for more potent larvicide producing bacteria within Ado Ekiti as a potential alternative strategy for the prevention and control of malaria fever.

2. Materials and Methods

2.1. Samples collection

Twenty (20) samples were collected from five different mosquito breeding places such as water lodged areas, drainages, stagnant water, domestic wastes and stored rain water. Surface water of *Anopheles* breeding sites was collected by placing a steal mesh on the surface to collect the top layer of the water. *Anopheles* larvae were collected from their breeding domestic water-storage by the use of a hand dipper technique while rain water was obtained using a wide mouth container during rain and kept outside for a few days to simulate the colonization of a potential, newly formed breeding site.

2.2. Isolation of Bacteria

The samples collected were diluted serially and bacteria was isolated using pour plate method according to the method of Tito *et al*. (2023).The agar plates were incubated at 37^{o}C for 24h. The bacteria isolates that appeared on the plates were differentiated by colony morphology.

2.3. Identification of isolates

Pure colonies were stocked and characterized by Gram staining and different biochemical tests including catalase, oxidase, citrate utilization, MR-VP, indole production, nitrate reduction, urease activity, starch hydrolysis, gelatin hydrolysis, motility and sugar fermentation according to modified method of Olutilola (2000). The identity of the isolates was confirmed by molecular characterization.

2.4. Mass Rearing of Mosquito Larvae

Mosquito larvae were collected from different water bodies such as water logged areas, drainage and stagnant rain water. Species identification was carried out in Control Research Laboratory, Federal Polytechnic Ado Ekiti. The mosquito larvae were identified as female Anopheles mosquitoes. The collected larvae were acclimatized and maintained under proper temperature and humidity according to the method of Anjali *et al*. (2011).

2.5. Larvacidal bioassay

The larvicidal bioassay was carried out to screen the bacterial isolates for the ability to control mosquito larvae. Each of the bacteria isolate was used to screen for mosquito larvicidal activity, bacterial cells from the nutrient agar slant was inoculated into 10 mL of nutrient broth and incubated at 35°C on a rotary shaker (200 rpm) for 24h. 2mL of each of the bacterial suspension was tested against 20 larvae of anopheles mosquito in 100 ml of distilled water contained in transparent glass jars. Another transparent glass jar containing 100mL of distill water and 20 larvae of anopheles mosquitoes but without the addition of the bacteria culture was used as the control. The larvicidal activity of each bacterial isolate was monitored for a period of 7 days. Counting of the viable larvae was done every day for the period of the screening. A bacterial isolate was considered potent if it both delayed metamorphosis beyond 6 days and caused death of the tested larvae.

2.6. Optimization of bacteria with the best larvicidal activity

Four isolates with the best larvicidal activity were selected for optimization, these isolates include; *Bacillus subtilis (*S4), *Pseudomonas aeruginosa* (R5), *P. vulgaris* (*D1*) and *Staphylococcus. aureus* (WL3). The optimized conditions includes studying the effects of temperature, pH and different carbon sources on the larvicidal compounds produced by each isolate. The influence of pH and temperature and different carbon sources on the production of bioactive secondary metabolites was investigated because these environmental factors significantly affect bacterial growth, sporulation, toxin synthesis, and larvicidal activity (González-Rizo *et al*., 2019).

2.7. Effect of pH and temperature on the larvicidal compounds production

The effect of pH and temperature on the production of the secondary metabolite (bioactive compounds was carried out by using modified nutrient broth medium as described by Gonzalez-Rizo *et al*. (2019) The varying pH and temperature used were pH (6, 6.5 and 7) and incubation temperature (30^{o}C, 35^{o}C and 45^{o}C). Twenty four hours old culture of isolates was inoculated and incubated in a digital gas bath thermostats oscillator (THZ-82B) at 200 ± 5 rpm for 5 days after which the broth was centrifuged at 4000rpm for 10 minutes and filtered using filter paper (Whatman No. 1) and tested for best larvicidal activity against mosquito larvae

Effect of different carbon source on larvicidal compounds production: Effects of different carbon source was studied on Modified Nutrient broth as describe by Patil *et al*. (2014). The different carbon sources used were: (10g/L): Soluble Starch, Glucose and Sucrose. The tests medium was inoculated with 24 hours old culture of the best isolates and incubated at 37^{o}C in a digital gas bath thermostats oscillator (THZ-82B) at 200 ± 5 rpm for 5 days. After incubation, the broth was centrifuged at 4000rpm for 10 minutes and filtered using filter paper (Whatman No. 1). The filtrate was tested for larvicidal activity against mosquito larvae.

2.8. Molecular Identification of the isolates with the best larvicidal activity

Molecular identification was carried out according to the method of Sadiqi *et al*. (2022). Molecular characterization of the bacterial isolates was based on 16S rRNA conserved gene sequences using universal bacterial primers. The targeted gene sequence was amplified using the conventional PCR method, and the size of the amplified fragments was confirmed by running the final product by 1% gel electrophoresis. The amplified samples and appropriate sequencing fragments were sent for sequencing, and the retrieved nucleotide sequences were studied using MEGA software (MEGA-11). Bacterial isolates were further classified at the species level by BLAST search using GenBank NCBI (National Center for Biotechnology Information).

2.9. Statistical Analysis

Data obtained from this study were analyzed by using descriptive statistical method such as means, tables and bar chat. Spearman's correlation was also used to assess the relationship between variables using Microsoft Excel.

3. Results and Discussion

The occurrence of bacteria isolates from the samples is shown in Figure 1, 38 bacterial isolates belonging to six distinct genera were isolated. These include *Proteus vulgaris*, *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa and Klebsiella pneumoniae.* The isolation of these specific bacteria from the breeding environment of anopheles mosquitoes is highly consistent with recent literature on mosquito microbiome. The mosquito gut and its larval aquatic habitat form a complex ecosystem where the mosquito ingests and harbours a diverse bacterial community (Tainch *et al*., 2023). Genera from the family Enterobacteriaceae, such as *Proteus* vulgaris and *Escherichia coli*, along with *Pseudomonas* aeruginosa and *Bacillus subtilis*, are consistently reported as dominant and core members of the *Anopheles* gut microbiota (Dieme *et al*., 2022; Tainch *et al*., 2023). The presence of *Staphylococcus aureus* is also documented, though often less prevalent than Gram-negative bacteria (Dada *et al*., 2023). This validates that the isolates from the present study are not laboratory contaminants but ecologically relevant organisms that naturally interact with the *Anopheles* vector, making them ideal candidates for bioprospecting.

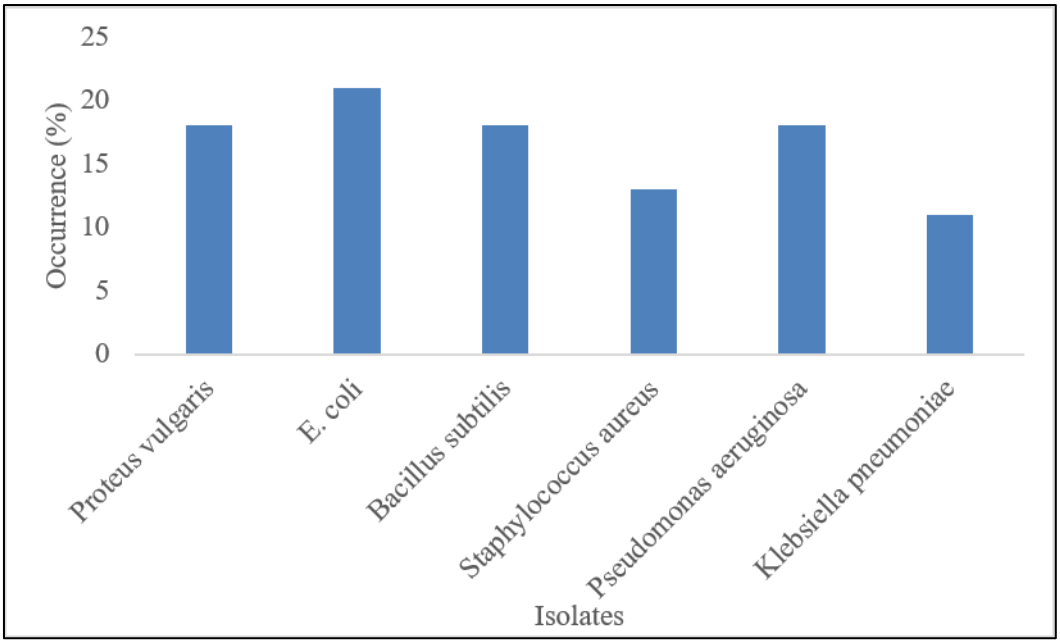

Figure 1 Occurrence of Bacterial isolates from the samples

Table 1 Larvacidal activity of the isolates

	Days **No of dead larvae**							**Total No of Dead larvae**	**Percentage (%)**
Isolates	1	2	3	4	5	6	7		
E. coli (S1)	3	2	2	2	2	2	2	15	75
P. vulgaris (D1)	5	4	2	3	3	2	0	19	95
E. coli (WL3)	4	2	2	5	4	0	1	18	90
B. subtilis (S2)	2	3	2	1	2	2	3	15	75
B. subtilis (R2)	0	4	2	3	4	2	1	16	80
S. aureus (WL3)	5	2	3	2	3	3	2	20	100
P. vulgaris (DW4)	4	3	2	4	1	2	1	18	90
B. subtilis (S4)	2	6	1	2	2	4	2	19	95
P. vulgaris (R3)	2	2	1	1	1	1	2	10	50
P. aeruginosa (S2)	5	1	2	2	4	1	2	17	85
P. aeruginosa (R5)	5	3	1	2	4	2	3	20	100

Keys: *E. coli* = *Escherichia coli*, *P. vulgaris*= *Proteus vulgaris*, *S. aureus*= *Staphylococcus aureus*, *P. aeruginosa*= *Pseudomonas aeruginosa*, *B. subtilis* = *Bacillus subtilis,* S = stagnant water, D = Drainage, WL = water lodged area, R = Stored rain water, DW = Domestic waste

Table 1 shows the larvicidal activity demonstrated by the isolates. Two isolates, *Staphylococcus aureus* (WL3) and *Pseudomonas aeruginosa* (R5) exhibited 100% mortality against *Anopheles* larvae. *P. aeruginosa* is a well-established entomopathogen known to produce a suite of insecticidal compounds, including proteases, chitinases, and rhamnolipids (Tuno *et al*., 2023). A recent study by Jamil *et al*. (2022) isolated *P. aeruginosa* strains from mosquito breeding sites and demonstrated 100% mortality against *Anopheles stephensi* larvae within 24 hours, a result that mirrors the high toxicity observed in the present study. Tuno *et al*. (2023) further detailed the mode of action, showing that *P. aeruginosa* toxins, particularly rhamnolipids, effectively disrupt the larval gut epithelium, leading to rapid mortality. The 100% mortality induced by *Staphylococcus aureus* (WL3) is a particularly novel and compelling finding. While *S. aureus* is extensively studied as a human pathogen, its role as a primary insect pathogen is less defined. However, research into Gram-positive

mosquito pathogens is expanding. Lall *et al*. (2021), for instance, identified marine-derived *Staphylococcus* species (e.g., *S. xylosus*) that exhibited significant larvicidal activity. Dada *et al*. (2023) also identified *S. aureus* within the midgut of wild *Anopheles* mosquitoes, confirming its natural association.

The study also identified high larvicidal activity from *Proteus vulgaris* (D1) which showed 95% mortality, and *B. subtilis* (S4) which exhibited 90% mortality. Recent research supports the pathogenic potential of *Proteus* species against insects. Wei *et al*. (2021) identified *Proteus* sp. as a larvicide against *Aedes* mosquitoes and attributed the toxicity to secondary metabolites and cell-wall components like lipopolysaccharide (LPS). Similarly, Senthilkumar and Murugesan (2022) reported that *Proteus mirabilis* caused over 80% mortality in *Aedes aegypti* larvae.

The 90% and 70% mortality from the two strains of *E. coli* () strain is another unusual and promising result. *E. coli* is typically used as a non-pathogenic control or food source in larvicidal assays. However, the field of paratransgenesis often uses genetically engineered *E. coli* to *express* insect-specific toxins (Carballar-Lejarazú *et al*., 2020). The high mortality from the wild-type isolate suggests it may be a native entomopathogenic strain, perhaps producing toxins or compounds that are cross-toxic to mosquito larvae. This finding highlights the potential of bioprospecting for "wild" pathogenic strains rather than relying solely on genetic engineering.

The effects of temperature and pH on the larvicidal activity of the isolates are shown in Tables 2 and 3. The optimum temperature for the growth of all the bacteria isolates was 37^{o}C at day 3, day 5 and day 7, with the highest larvacidal activity at this temperature. *B. subtilis* (S4) and *P. vulgaris* (D1) had optimum pH of 6.5 while the optimum pH for *P. aeruginosa (R5)* and S. aureus (WL3) at day 3, day 5 and day 7 was 7.0. Most of the isolates showed their best larvicidal activity at 37°C, this is an indication that 37°C is their optimum temperature for growth and for the production of the larvicidal compound (secondary metabolites) in the isolates. This is the ideal temperature for bacterial growth, sporulation, and the synthesis of insecticidal crystal proteins (Cry and Cyt toxins) (Schnepf *et al*., 1998). The isolates showed reduced larvicidal activity at 30°C and 45°C, indicating that this temperature was less favorable to them. At 45°C when there is excessive heat, this can cause protein denaturation whereby the toxic crystal proteins lose their stable structures, drastically reducing larvicidal efficacy (Aminu *et al*., 2025). This result is in line with Noor *et al*. (2019), where the authors observed that a temperature of 37°C was the optimum temperature required by most of the enthomopathogens such as *P. aeruginosa*, and *Bacillus* spp.

The results indicate that pH significantly influenced the larvicidal effectiveness of the isolates. pH of 6.5 was the most favorable condition for the growth and production of larvicidal compounds of most of the isolates including *B. subtilis* S4 and *P. vulgaris* (D1) while the most favourable pH for *P. aeruginosa* (R5) and S. aureus (WL3) was 7.0. pH 6.5 and 7.0 favour high larvicidal activity, because in this range, the bacterial crystal proteins remain highly stable in the aquatic environment. All bacterial isolates exhibited considerable larvicidal activity across the tested pH values, although the degree of activity varied depending on the isolate and pH condition. The results suggest that near-neutral pH conditions (6.5–7.0) were more favorable for larvicidal metabolite production than the slightly acidic pH of 6.0. *P. aeruginosa* R5 and *Proteus vulgaris* D1 were the most effective isolates, each attaining a maximum larvicidal activity of 95% under optimal pH conditions. *P. aeruginosa* R5 performed best at pH 7.0, whereas *P. vulgaris* D1 showed optimum activity at pH 6.5. These differences may be attributed to species-specific variations in enzyme activity and secondary metabolite production under different pH conditions.

This findings showed that pH is an important factor affecting the larvicidal activity of bacterial isolates. Near-neutral pH values (6.5–7.0) generally enhanced larval mortality compared to pH 6.0. The highest larvicidal activity (95%) was achieved by *P. aeruginosa* R5 at pH 7.0 and *Proteus vulgaris* D1 at pH 6.5, indicating that these isolates have strong potential for biological mosquito control when cultured under optimal pH conditions. This result corroborates the result of Aminu *et al*. (2025) who observed that pH of 6.5 to 7.5 (near- neutral pH) was the optimum pH for growth and larvicidal activity of *Bacillus thuringiensis* while lower pH values resulted in reduced growth and effectiveness, supporting the observation that near-neutral pH conditions enhance mosquito larval mortality.

Table 2 Effects of Temperature on larvicidal activity of the isolates

Isolates	**Temperature (°C)/Larvacidal activity (%)**								
	(Days)								
	3			5			7		
	30	37	45	30	37	45	30	37	45
B. subtilis S4	75	90	68	75	90	70	75	80	70
P. aeruginosa R5	73	80	72	70	100	70	70	95	70
P. vulgaris D1	70	90	78	75	95	70	70	95	70
S. aureus WL 3	70	80	70	75	90	76	70	90	71

Keys: S = stagnant water, R = Stored rain water, D = Drainage, WL = water lodged areaTable 3:

Table 3 Effects of pH on the larvicidal activity of the isolates

Isolates	**pH /Larvacidal activity (%)**								
	(Days)								
	3			5			7		
	6.0	6.5	7.0	6.0	6.5	7.0	6.0	6.5	5
B. subtilis S4	75	90	70	80	90	70	80	85	72
P. aeruginosa R5	73	75	80	70	82	95	87	90	95
P. vulgaris D1	70	90	75	80	95	77	80	95	73
S. aureus WL 3	70	75	84	79	82	90	78	87	90

Keys: S = stagnant water, R = Stored rain water, D = Drainage, WL = water lodged area

Table 4 shows the effects of three different carbon sources (soluble starch, glucose, and sucrose) on the larvicidal activity of the isolates. The result indicates that the type of carbon source significantly influenced the larvicidal activity of the bacterial isolates. Among the carbon sources tested, glucose and sucrose generally supported higher larvicidal activity than soluble starch, suggesting that these readily metabolizable sugars may enhance the production of larvicidal metabolites. *B. subtilis* S4 exhibited high larvicidal activity across all carbon sources with the exception of soluble starch, with the highest value of 90% and 85% on Days 5 and 7 respectively when glucose was used indicating sustained larvicidal potential.

P. aeruginosa R5 also demonstrated strong larvicidal activity, particularly in glucose medium where activity increased from 80% on Day 3 to 90% on Day 5. This trend suggests that glucose enhanced the growth and metabolic activity of the organism, leading to increased production of larvicidal compounds over time. Among all the isolates, *P. vulgaris* D1 showed the highest larvicidal activity, reaching 95% on Days 5 and 7 in sucrose medium. This indicates that sucrose was the most suitable carbon source for maximizing larvicidal metabolite production by this isolate. *S. aureus* WL3 displayed moderate to high larvicidal activity throughout the study. The highest activity (90%) was recorded on Day 7 in glucose medium. Similar to the other isolates, glucose supported greater larvicidal activity than soluble starch, while sucrose produced intermediate results. In all the isolates, soluble starch yielded comparatively lower activity values (40–45%)

The results demonstrate that carbon source plays an important role in determining the larvicidal effectiveness of bacterial isolates. Glucose and sucrose were more effective than soluble starch in enhancing larvicidal activity. *Proteus vulgaris* D1 grown on sucrose showed the highest larvicidal activity (95%), indicating its potential as a promising biological control agent against mosquito larvae. The findings suggest that optimizing culture conditions, particularly carbon source selection, can improve the production of larvicidal compounds by bacterial isolates (Patil *et al*., 2014). The higher larvicidal activities observed in glucose- and sucrose-containing media may be attributed to enhanced production of bioactive metabolites, as optimization of carbon sources has been reported to significantly improve mosquitocidal metabolite production in bacterial isolates (Manonmani *et al*., 2011).

Table 4 Effects of different carbon sources on the larvicidal activity of the isolates

Isolates	Carbon sources /Larvacidal activity (%)								
	(Days)								
	3			5			7		
	S. S	Glu.	Suc.	S. S.	Glu	Suc.	S.S	Glu	Suc.
B. subtilis S4	40	80	70	40	90	70	50	85	68
P. aeruginosa R5	45	80	75	45	82	70	40	90	70
P. vulgaris D1	45	73	80	40	70	95	40	70	95
S. aureus WL 3	45	84	67	40	85	75	45	90	65

Keys: S.S = soluble starch, Glu = glucose, Suc. = sucrose, S = stagnant water, R = Stored rain water, D = Drainage, WL = water lodged area

Figure 2 shows the agarose gel electrophoresis of PCR amplification of the 16S rRNA gene from the bacterial isolates used in the study. The gel contains a molecular marker (MK) and four lanes representing the amplified DNA fragments of the bacterial isolates identified as *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus,* and *Proteus vulgaris*.

The presence of distinct DNA bands in lanes 1, 2, 3, and 4 indicates successful amplification of the 16S rRNA gene from all the bacterial isolates. The amplified products appeared at approximately the expected size range for bacterial 16S rRNA gene fragments, confirming the presence of bacterial genomic DNA suitable for molecular characterization. The identification of these bacterial species is important because they have been reported in previous studies to possess various bioactive compounds, including metabolites with insecticidal and larvicidal properties. Their confirmed identity therefore strengthens the validity of the larvicidal activity results obtained in the study.

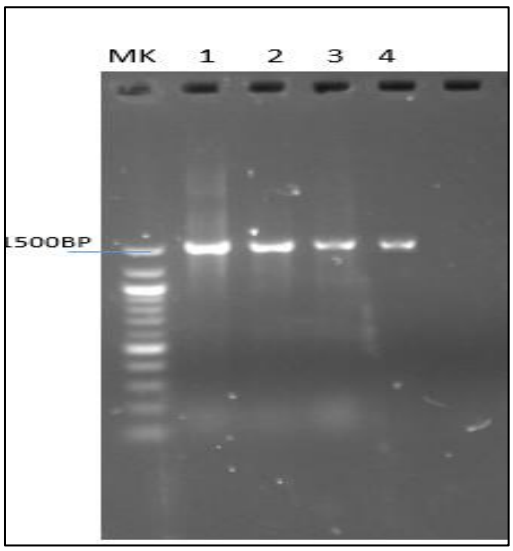

Figure 2 Agarose gel showing positive amplification of the 16S rRNA regions amplified by the bacteria isolates

Keys: MK= molecular marker, 1= *Pseudomonas aeruginosa*, 2 = *Proteus vulgaris*, 3 = *Staphylococcus aureus*, 4 = *Bacillus subtilis*

4. Conclusion and Recommendations

A total of 38 bacterial isolates belonging to six distinct genera including *Proteus vulgaris*, *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa and Klebisiella pneumoniae* were isolated from the samples. All the isolated bacteria exhibited high larvicidal activity with the exception of *P. vulgaris* (R3) which showed 50% larvicidal activity but, *B. subtilis* S4, *P. aeruginosa* R5, *P. vulgaris D1 and S. aureus* WL 3 have the best larvicidal activity. Optimum temperature of 37^{o}C and near-neutral pH conditions (6.5–7.0) were more favorable for larvicidal metabolite production while glucose and sucrose were more effective than soluble starch in enhancing larvicidal activity. It is therefore

recommended that the isolated bacteria and their metabolites may represent a highly viable and effective alternative for *Anopheles* vector control due to their high larvicidal activity, offering a clear path away from chemical insecticide dependency.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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