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African Journal of Microbiology Research

Full Length Research Paper

Isolation and Characterization of Bacteria from Lakes Olbolosat and Oloiden, Kenya

Catherine Wachera Kiama[1*], Moses Mucugi Njire[1], Anne Kelly Kambura[2], Julius Ndirangu Mugweru[3], Viviene Njeri Matiru[1] and Eliud Nalianya Wafula[1]

[1]Department of Botany, Jomo Kenyatta University of Agriculture and Technology, P. O. Box 62000-00200 Nairobi, Kenya.
[2]School of Agriculture, Earth and Environmental Sciences, Taita Taveta University, P. O. Box 635-80300 Voi, Kenya.
[3]School of Pure and Applied Sciences, University of Embu, P. O. Box 6 -60100, Embu, Kenya.

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There is still unexplored reservoir of microorganisms from sediments and water within Lakes Olbolosat and Oloiden using culture dependent technique. The current study compares bacterial diversity within Lake Olbolosat a freshwater lake and Lake Oloiden a saline alkaline lake. Out of 60 isolates obtained from sediments and water samples, 35 were from Lake Olbolosat and 25 from Lake Oloiden. Microbial count ranged between 0-1.75× 10^5 cfu/ml from both lakes. There was a significant difference between bacterial density and sampling points ($p<0.001$, $F=6.667$), 58 were Gram-positive and 2 Gram-negative. Fifty-five isolates that were rod-shaped, 3 were cocci and 2 filamentous. There was excellent growth of isolates at an optimum growth pH range of 6-10, a temperature range of 25-30°C and a salinity range of 0-5%. There was significant difference at $p<0.001$ for bacterial growth within physiological parameters. The isolates utilized skimmed milk, starch, olive oil, cellulose powder and xylan, hence the production of extracellular enzymes. There was antimicrobial activity against *Pseudomonas aeruginosa, Bacillus subtilis, Escherichia coli, Staphylococcus aureus* and *Candida albicans* by bacterial isolates. BLAST analysis of partial sequences showed there were 4 different phyla. *Firmicutes* scored 77% closely affiliated with 20 strains*,* *Actinobacteria* scored 15% closely affiliated with 4 strains, *Proteobacteria* and *Bacteroidetes* scored 4% affiliated with 1 strain each. Novel bacteria from this study could provide insights into their diversity and biotechnological applications.

Keywords: Lakes, bacteria, sediment, water, culture-dependent.

INTRODUCTION

Lake Olbolosat which means a marshy area in the *Maasai* language is a freshwater marine ecosystem and is endangered. The gradual drying up of this lake is probably due to human activities (Wafula and Murunga, 2020). Lake Oloiden, which means salty in the *Maasai* language, was also considered for its saline alkaline. The lake lacks water inflow resulting in saline-alkaline conditions (Maina et al., 2018). It is separated from its

*Corresponding author. E-mail: kiamac65@gmail.com. Tel: +254712878757.

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west shore by a peninsula. Studies in Kenya have so much focused on salty lakes like Lake Magadi, Lake Elementaita, Lake Nakuru and scanty on fresh and saline alkaline lakes. Microbes are ubiquitous (Laxma-Reddy et al., 2017). Saline alkaline and freshwater lakes are economically and ecologically important ecosystems due to their high productivity and nutrient recycling capacities. The input of nutrients and fast recycling is due to active anaerobes and aerobes microorganisms. Microbes are essential to the functioning and major biogeochemical cycles within lakes (Krivtsov et al., 2020). Culture-dependent technique is important for industrial application of the microbial isolates (Spini et al., 2018). Fresh and saline-alkaline lakes are ecosystems that can serve as models for studying microbial diversity. There is still unexplored reservoir of microorganisms from sediments and water within Lakes Olbolosat and Oloiden. Microbial communities have been mostly studied using culture-dependent techniques and due to the uncultivability of most microbes, very few organisms can be isolated from these lakes. However, culture-dependent technique helps in better understanding of microbial in physiology for industrial application (Spini et al., 2018). Culture-dependent technique cannot be used solely for the analysis of populations within microbial communities. Metagenomics, a culture independent technique, provides detailed information on the metabolic and functional capacity of a microbial community (Yadav et al., 2019). Culture-dependent technique studies from Lake Oloiden of the Kenyan Rift Valley revealed the presence of diverse populations of high G+C content belonging to the genus *Artrobacter, Dietzia* and *Terrabacter* (Duckworth et al., 1998). Bacteria of the genera, *Pseudomonas Paenibacillus*, *Arthrobacter, Bacillus, Fictibacillus* and *Acinetobacter*, have been isolated from L.Olbolosat (Wafula and Murunga, 2020). The current study is in line with the Kenyan government's big four priority areas within the framework of vision 2030 whereby the department of Regional Development Management supports the conservation of natural resources through sustainable utilization and conservation of river basins and large water bodies (Kiunjuri, 2017).The study is in line with African Commission priority areas of Agenda 2063 framework on the use of indigenous knowledge in Science and technology and innovation for sustainable development that acts with a sense of urgency on climate change and environment (AU, 2015). Environmental conservation will contribute towards reaching the United Nations Millennium development goals to conserve marine resources for sustainable development, protect and restore the terrestrial ecosystem, hold and reverse land degradation, halt biodiversity loss and finally combat desertification (United Nations General Assembly, 2015). This study aims at isolating and characterizing novel bacteria from Lakes Olbolosat and Oloiden to provide insights into their diversity and biotechnological applications.

MATERIALS AND METHODS

Research authorization

Research authorization was obtained from the National Commission for Science, Technology and Innovation (NACOSTI) (Research Permit Number NACOSTI/P/20/3808) and permission to obtain samples for research from Lakes Olbolosat and Oloiden (Reference Number KWS/BRM/5001) was obtained from the Kenya Wildlife Service (KWS).

Study site

Lake Oloiden is about 4-7.5 km^2. The lake is situated at a latitude of 0° 48'S and 36° 16'E (Maina et al., 2018). The lake lies at an average altitude of 1995 meters. The lake is a saline-alkaline lake that becomes fresh during rainy season caused by the overflow of Lake Naivasha (Maina et al., 2018). The lake records pH of 9 and temperature of 25°C. It is separated from its west shore by a peninsula. The distance between Lake Naivasha and Lake Oloiden are about 200 m. Lake Olbolosat is a freshwater body and is about 43 Km^2 (Wafula and Murunga, 2020). The lake records pH of 7 and temp of 22°C. The lake is situated at a latitude of 0° 09'S and longitude of 36° 26'E in Nyandarua County in the central part of Kenya. It lies at an average altitude of about 2340 m in a wedge-shaped rift valley floor, known as Ongata Pusi Pusi, sloping in the eastward – northward direction. There is an underground inlet seeping under and emanates into the lake. From the Aberdare ranges, water from the basin flows northwards seeping to Thomson's Falls into the northern part of Ewaso Nyiro River. It is formed by down warping and it is among the lakes in Kenya outside the rift valley (Figure 1).

Measurement of physico-chemical parameters

The geographical position of the sampling sites in terms of longitude, latitude and elevation were taken using Global Positioning System (GARMIN eTrex 20). The on-site metadata for temperature, electrical conductivity (EC), total dissolved solids (TDS) and dissolved oxygen (DO) of each sampling point were measured using Electrical Chemical Analyzer (Jenway - 3405) The pH was measured with a portable pH-meter (Oakton pH 110, Eutech Instruments Pty. Ltd) and confirmed with indicator strips (Merck, range 5-10) (Table 1).

Sample collection

Nine sampling points were selected randomly. Four from Lake Oloiden and 5 from Lake Olbolosat. Wet, dry sediments and water samples were randomly collected in triplicates. The experimental design used in the current study was purposive. The sample size was determined based on the unique features of the optimum coverage. There were three biological replicates for all water, wet and dry sediments. This was done by scooping wet and dry sediments with a hand shovel into sterile 250 ml plastic containers. The sterile plastic containers were used to fetch water from both lakes. All samples were transported on dry ice to the laboratory at Jomo Kenyatta University of Agriculture and Technology (Table 1).

Isolation and enumeration of bacterial isolate

Ten milliliter of water was suspended in 90ml of ringer salt solution powder (RSSP Himedia-M525) consisting of Sodium Chloride 8.50 g,

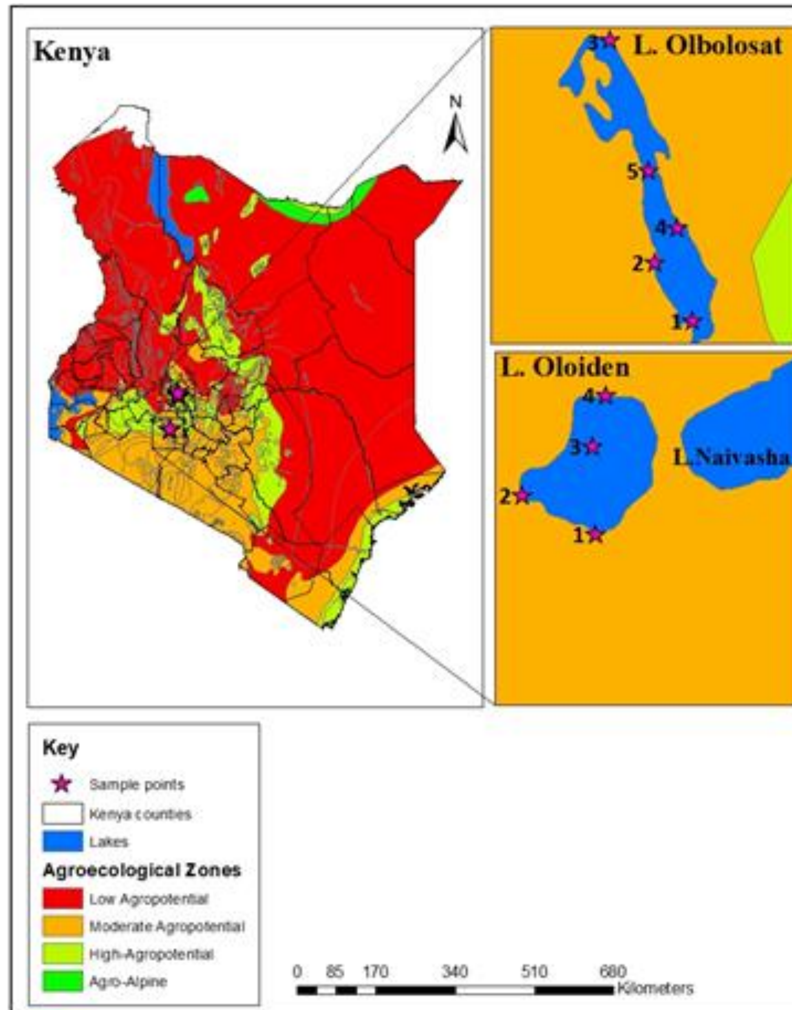

Figure 1. Lakes Olbolosat and Oloiden sampling points in (Nyandarua and Nakuru counties respectively), Kenya.

Potassium Chloride 0.20 g, Calcium chloride anhydrous 0.20 g and Sodium bicarbonate 0.01g. Ten grams of the dry and wet sediments were also suspended separately in 90 ml of ringer solution. This was followed by filtration through sterile 125mm (Whatman ®) qualitative filter paper, Grade 1(Merck). One ml of the filtrate was transferred to 90ml of ringer solution to make 10^{-2} and10^{-3}. The inoculation mixture with serial dilution was then spread in triplicate on the plates containing Plate count agar (PCA) Himedia- M091S) for the bacterial diversity. The medium consisted of Casein enzymic hydrolysate 5 g, Yeast extract 2.50 g, Dextrose 1.00 g and agar 15 g in 1 litre of water from the lakes to mimic the lake conditions. This was followed by incubation at 30°C for 24 to 72 h. To measure survival efficiency, colonies were counted using the following formula (Das and Dutta, 2018).

$$\text{Viable cell count (CFU per g sample)} = \frac{\text{Number of colonies (25-300 CFUs)}}{\text{The volume of inoculum (100 µl)}} \times \text{Dilution factor}$$

To obtain pure cultures, distinctive colonies were picked, transferred to fresh media and incubated at 30°C for 24 to 72 h. Purified colonies were grown on nutrient broth (Difco) and stored in 20% glycerol at -75°C.

Morphological and cultural characterization of bacterial isolates

Morphological and cultural characterization of the isolates was done under the dissecting and compound×100 microscope to observe pigmentation, form, elevation, margin, cell size, shape and arrangements as described by Cappuccino and Sherman, 2014). Classical Gram-staining and catalase tests were performed and the Gram-reaction confirmed by 3% (w/v) KOH test according to Moyes et al. (2009).

Physiological and biochemical characterization of bacterial isolates

The isolates were determined for their ability to grow at different temperatures, pH and also at different salt concentrations using PCA medium**.** The ability of the isolates to grow at different temperatures ranges were determined by growing the isolates at 25, 30, 35, 40, 45 and 50°C. The ability of isolates to grow at different pH ranges was determined by growing the isolates at 30°C at pH4, pH6, pH8, pH10, pH12 and pH14 adjusting each pH using

Table 1. Summary of samples collected from Lakes Olbolosat and Oloiden and their parameters.

Lake	Sampling point	Sample label	Sample type	Number of isolates	Latitude (°S)	Longitude (°E)	Elevation (m)	Temp (°C)	pH	Ec (mS/cm)	TDS (mg/L)	DO (mg/L)
L. Oloiden	1	EBP	Wet sediment	10	0° 47' 59.496'' S	36° 16' 45.444'' E	1885	23	9.787	852	196.5	3.45
	2	ECP	Wet sediment	3	0° 49' 6.744'' S	36° 15' 49.392'' E	1890	23	9.752	922	124.5	3.61
	3	EAP	Water	4	0° 48' 33.66'' S	36° 16' 36.624'' E	1884	23	9.845	695	57.6	3.79
	4	EBP	Dry sediment	9	0° 49' 32.772'' S	36° 16' 38.748'' E	1895	23	9.101	623	122	3.34
L.Olbolosat	1	ZBP	Dry sediment	2	0° 8' 43.008'' S	36° 26' 26.664'' E	2338	22	7.634	655	215	4.19
	2	ZCP	Wet sediment	10	0° 10' 45.264'' S	36° 26' 46.392'' E	2335	22	7.257	1413	110.5	3.74
	3	ZAP	Water	9	0° 4' 38.352'' S	36° 24' 58.068'' E	2336	22	7.985	562	105.2	3.83
	4	ZBP	Dry sediment	10	0° 9' 28.98'' S	36° 25' 56.712'' E	2347	22	7.229	270	105.3	4.87
	5	ZCP	Wet sediment	3	0° 7' 26.976'' S	36° 25' 49.224'' E	2339	22	7.688	566	103.3	3.66

1M of HCL or NaOH. Salt tolerance was also determined by growing the cultures at 30°C with the media supplemented with 0, 5, 10, 15, 20, 25 and 30% NaCl concentrations (Vinet and Zhedanov, 2010). Intracellular and extracellular enzyme activities were determined according to (Cappuccino and Sherman, 2014). The biochemical tests included sugar utilization, catalase, urease, gelatin liquefaction, motility, starch and IMVIC.

Screening of bacterial isolates for hydrolytic enzyme production

The bacterial isolates were screened qualitatively for the production of five important enzymes such as xylanase, amylase, lipase, cellulase and protease. The bacterial isolates were cultured separately on the substrates such as xylan, starch, olive oil, cellulose powder and skimmed milk amended agar plates respectively (Mohammad et al., 2017). The isolates were then incubated at 30°C for 24-48 h. After growth Petri dishes were flooded with indicator solution. The negative control consisted of the uninoculated plate.

Screening the bacterial isolates that produce antimicrobial activity

Sixty bacterial isolates were screened for their ability to inhibit the growth of bacterial test organisms; *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (NCTC 10418), *Staphylococcus aureus* (NCTC 10788), *Bacillus subtilis* (ATCC 55732) and fungal test organism *Candida albicans* (ATCC 90028) obtained from Kenya Medical Research Institute- Centre for Microbiology research. The bacterial isolates were cultured in nutrient broth and incubated at 30°C for 24 h. The cultured bacterial isolates were centrifuged at 10,000x *g* for 1min and the supernatant sieved using sterile micro membrane filters to remove any bacterial cells. The impregnated sterile Whatman ® qualitative filter papers, Grade 1(Merck) discs measuring 1centimeter paper discs were aseptically placed on Mueller Hinton agar (Himedia-M173). The media was swabbed with 0.1 ml per Petri dish of the test organisms following Kirby-Bauer diffusion protocol followed by incubation for 24-48 h at 30°C after which the results were recorded while negative control consisted of the uninoculated plate (Hudzicki, 2012).

Molecular characterization of bacterial isolates using partial 16S rRNA genes

Bacterial isolates were grown in nutrient broth media (Himedia-M002) consisting of Peptone, 5g, sodium chloride, 5 g, HM peptone B# 1.5 g, Yeast extract, 1.5 g in 1 L of distilled water. The overnight cultures were centrifuged at 10,000× g for 1 min and the supernatant discarded remaining with the pellet. DNA was extracted using QIAamp DNA Mini Kit (Qiagen, Germany) extraction kit according to manufacturer's instructions. Bacterial universal primers 27F forward (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R reverse (5′-GGT TAC CTT GTT ACG ACT T-3′) were used for the amplification of 16S rRNA gene. PCR was carried out using PEQLAB, Erlangen, Germany, 96 PCR thermocycler machine. The PCR was carried out in a 50 µl mixture containing 25 µl 3X *Taq* PCR Master Mix (Qiagen, Germany), 2.5 µl of each primer, 10 µl of DNA template (50 ng) and 10 µl RNase free water. The reaction mixtures were subjected to the following PCR conditions: Initial activation of the enzyme at 95°C for 5 min followed by 32 cycles consisting of 1-min denaturation at 95°C for 1-min, primer annealing at 55°C for 2 min, chain extension at 72°C for 1.5 min and a final extension at 72°C for 10 min (Roux, 1995). The amplified PCR products were checked by gel electrophoresis using 1.2% (w/v) agarose gels stained with ethidium bromide (1 µg/ml) and visualized using Biotec-Fischer Felix6050 gel documentation system (ProfiLab24, Germany) according to the manufacturer's instructions and stored at -20°C. Purification and Sanger sequencing of PCR products of the 60 bacterial isolates were carried out at Human Genomics Macrogen Europe (Macrogen Europe B.V, Amsterdam, Netherlands).

Statistical analysis

Data on bacterial density was noted and recorded in an Excel sheet. Two Way Analysis of Variance was used to

Table 2. Morphological and cellular characterization for 60 bacterial isolates from Lakes Olbolosat and Oloiden.

	Colony Characterization				Cell Characterization	
Isolate	**Pigment**	**Form**	**Elevation**	**Margin**	**Cell shape**	**Gram reaction**
EBP 8.2	Cream	Circular	Raised	Entire	Rod	+
ZCP 6.3	Cream white	Circular	Umbonate	Entire	Rod	+
ZCP 6.1	White	Circular	Umbonate	Entire	Rod	+
ZCP 6.8	Cream white	Circular	Umbonate	Entire	Rod	+
ZCP 1.2	Cream white	Circular	Flat	Entire	Rod	+
EBP 2.2	Cream	Circular	Umbonate	Entire	Rod	+
ZCP 6.7	Cream yellow	Circular	Flat	Entire	Cocci	+
ZCP 17.4	Cream	Irregular	Flat	Serrated	Cocci	+
ECP 3.1	Cream white	Circular	Flat	Entire	Rod	+
ZAP 9.6	Cream white	Irregular	Flat	Undulate	Rod	+
ZCP 1.7	White	Circular	Umbonate	Entire	Rod	+
ZCP 6.2	White	Circular	Flat	Entire	Rod	+
ZCP 1.3	White	Circular	Flat	Entire	Rod	+
EBP 2.1	Cream	Circular	Flat	Entire	Rod	+
ZAP 16.3	Cream	Irregular	Pulvinate	Entire	Rod	-
ECP 3.4	Cream white	Circular	Pulvinate	Undulate	Filamentous	+
EBP 8.1	Cream	Filamentous	Raised	Entire	Rod	+
EBP 10.1	Cream white	Filamentous	Umbonate	Entire	Rod	+
ZCP 17.2	Cream white	Irregular	Flat	Undulate	Rod	+
ZAP 16.2	Cream white	Circular	Flat	Entire	Rod	+
ZAP 10.1	Orange	Circular	Flat	Entire	Rod	+
EBP 3.9	Cream white	Circular	Flat	Entire	Rod	+
EBP 5.1	Yellow	Circular	Flat	Entire	Rod	+
EBP 8.8	Orange	Irregular	Flat	Serrated	Cocci	+
ZAP 16.1	White	Circular	Pulvinate	Entire	Filamentous	+
EBP 2.5	Watery	Concentric	Flat	Entire	Rod	+
ZAP 9.1	Reddish	Circular	Flat	Entire	Rod	-
ZAP 9.2	Cream white	Irregular	Flat	Serrated	Rod	+
ZCP 6.4	Cream white	Circular	Flat	Entire	Rod	+
ZCP 17.1	Cream white	Circular	Umbonate	Entire	Rod	+
ZCP 1.1	Cream white	Circular	Flat	Entire	Rod	+
ZCP 6.5	White	Circular	Raised	Entire	Rod	+
ZCP 17.5	White	Circular	Flat	Entire	Rod	+
ZBP 9.7	White	Irregular	Flat	Undulate	Rod	+
EBP 8.4	Cream white	Irregular	Flat	Undulate	Rod	+
EBP 8.5	Cream white	Filamentous	Pulvinate	Lobate	Rod	+
ZAP 10.2	Brownish	Circular	Flat	Entire	Rod	+
EBP 10.5	Cream white	Circular	Umbonate	Entire	Rod	+
EBP 5.3	Cream white	Circular	Flat	Entire	Rod	+
EBP 10.4	White	Circular	Raised	Entire	Rod	+
EAP 3.7	White	Circular	Flat	Entire	Rod	+
ECP 3.2	Cream white	Irregular	Flat	Serrated	Rod	+
EBP 10.2	Cream white	Circular	Flat	Entire	Rod	+
EBP 2.4	Cream white	Circular	Umbonate	Entire	Rod	+
EBP 8.3	Cream white	Circular	Flat	Entire	Rod	+
ZBP 9.2	White	Circular	Umbonate	Entire	Rod	+
ZCP 13.2	Cream white	Circular	Umbonate	Entire	Rod	+
ZCP 1.11	Cream white	Circular	Flat	Entire	Rod	+
ZCP 1.10	Cream	Circular	Umbonate	Entire	Rod	+
ZCP 1.9	Cream yellow	Circular	Flat	Entire	Rod	+

Table 2. Contd.

ZCP 1.8	Cream white	Irregular	Flat	Undulate	Rod	+
ZCP 1.6	White	Circular	Umbonate	Entire	Rod	+
ZCP 6.6	White	Circular	Flat	Entire	Rod	+
ZAP 9.4	White	Circular	Flat	Entire	Rod	+
ZAP 9.3	Cream white	Circular	Flat	Entire	Rod	+
EBP 6.6	Cream	Irregular	Pulvinate	Entire	Rod	+
EAP 8.9	Cream white	Circular	Pulvinate	Undulate	Rod	+
EAP 6.4	White	Circular	Flat	Entire	Rod	+
EBP 8.7	Cream white	Irregular	Flat	Serrated	Rod	+
EAP 6.6	Cream white	Circular	Flat	Entire	Rod	+

Key: (+) denotes positive activity while (–) denotes negative reaction.

analyze all measured data. Normality Test (Shapiro- Wilk) was used to compare means using Sigma Plot 12 v 5.0 for bacterial density. Correlation profiles of zones of hydrolysis and bacterial isolates zone of clearance were visualized as heatmaps generated by a hierarchical clustering R script using R v 4.0.2. Sequencing was conducted in one direction using the forward primer (27 F). The Chromas pro program was used to remove ambiguity and comparisons were done with the NCBI GenBank databases using Basic Local Alignment Search Tool (BLAST) and EZBio Cloud algorithms. Sequences were submitted to the GenBank database and were assigned the accession numbers. The differences in the nucleotides were converted into distance matrices using the Maximum Likelihood method (Saitou and Nei, 1987). A phylogenetic tree was constructed using MEGA 7 (Engeset et al., 2003).

RESULTS

Sampling

Sediments and water samples were randomly collected in triplicates. The metadata collected before sampling included the geographical positions of each site in terms of latitude, longitude, elevation, temperature, pH, electrical conductivity, total dissolved solids and dissolved oxygen. Samples were collected from the two lakes and parameters summarized in Table 1.

Isolation of bacterial isolates obtained from Lakes Olbolosat and Oloiden

A total of 60 bacterial isolates were obtained from lakes Olbolosat and Oloiden. Lake Olbolosat recorded 35 while Lake Oloiden recorded 25 bacterial isolates (Table 2).

Enumeration of bacterial isolates obtained from Lakes Olbolosat and Oloiden

The microbial counts for dry sediments ranged between 0 to 1.75×10^5 cfu/ml respectively. The microbial counts for wet sediments ranged between 7.63×10^4 to1.16×10^5 cfu/ml respectively while the microbial count for water samples ranged between 5.3×10^4 to 1.22×10^5 cfu/ml. Bacterial density significantly varied ($p<0.001$, $F=6.667$) between the sampling points (Figure 2 and Plate 1a and b).

Cultural characterization

The isolates had varying colony characteristics. Most bacterial isolates were cream white, some were white, and a few were cream; while the rest were cream yellow, orange, watery, reddish and brown. The highest percentage of isolates were circular, a few were irregular, filamentous and only one was concentric in form. The margin of most isolates was entire while a few were undulate, serrated or lobate. The highest percentage of the isolates had a flat elevation while others were umbonate, pulvinate, or raised (Table 2 and Plate 2a -d). A dendrogram for morphology and cellular characteristics based on Ward D method and the distance between characters measured using Euclidean metric for the hierarchical clustering (Figure 3).

Cellular characterization

Cellular characterization revealed two isolates that were gram-negative out of sixty isolates. Fifty-seven isolates were rod-shaped, two were cocci and one was filamentous in shape (Table 2 and Plate 3a-d). A dendrogram for morphology and cellular characteristics based on Ward D method and the distance between characters measured using Euclidean metric for the hierarchical clustering (Figure 3).

Biochemical characteristics of bacterial isolates

Biochemical tests for the bacterial isolates recorded the following positive results for intracellular and extracellular

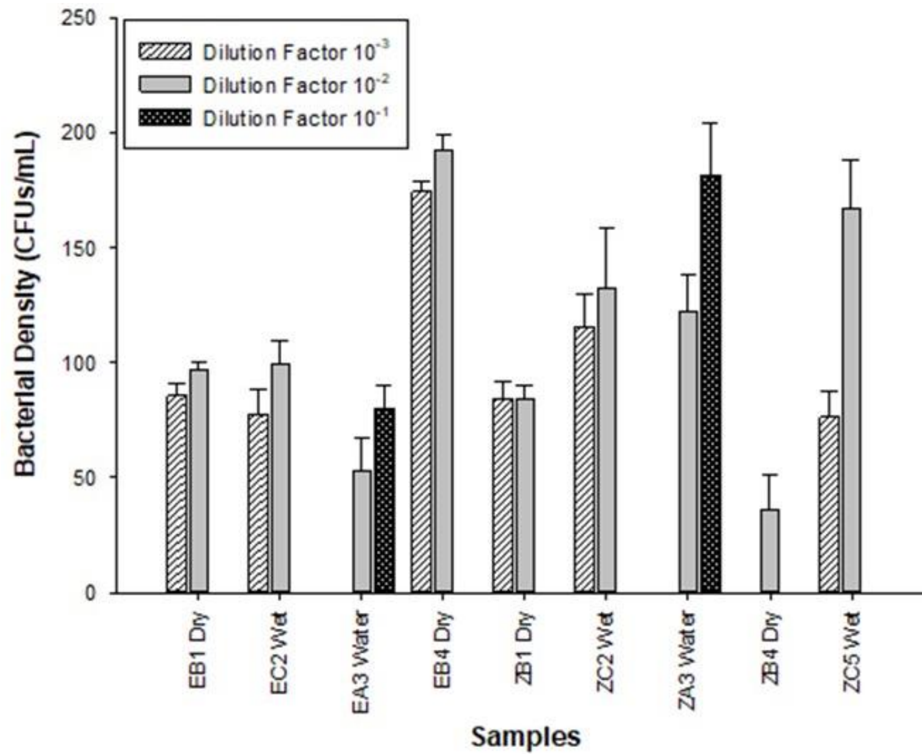

Figure 2. Bacterial density from lakes Olbolosat is indicated with prefix E and Oloiden with prefix Z.

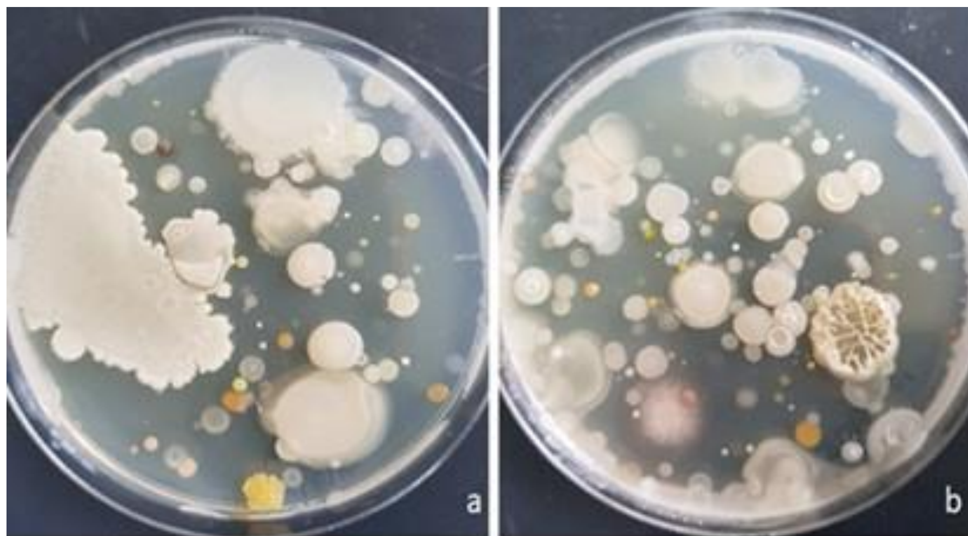

Plate 1. Diversity of mixed colonies before isolation to pure colonies from Lake Olbolosat a) and b) from Lake Oloiden.

enzyme activities; all the 60 isolates were positive for catalase, twenty-six for citrase. forty-six for Methyl Red, seventeen for Voges-Proskauer, ten for urea, twelve for indole, twenty-nine for gelatin liquefaction, thirty-seven for motility, and forty-four for starch. Triple Sugar Iron (TSI) test had twenty-four that were positive for the production of acid and twenty-one for the production of alkaline and seven for hydrogen gas production (Table 3).

Plate 2. A pure culture plate showing a) irregular form and serrated margin for EBP 8.8 b) circular and entire margin for EBP 8.2 isolate c) circular and entire margin for ZCP 6.3 d) circular and entire margin for ZCP 6.8.

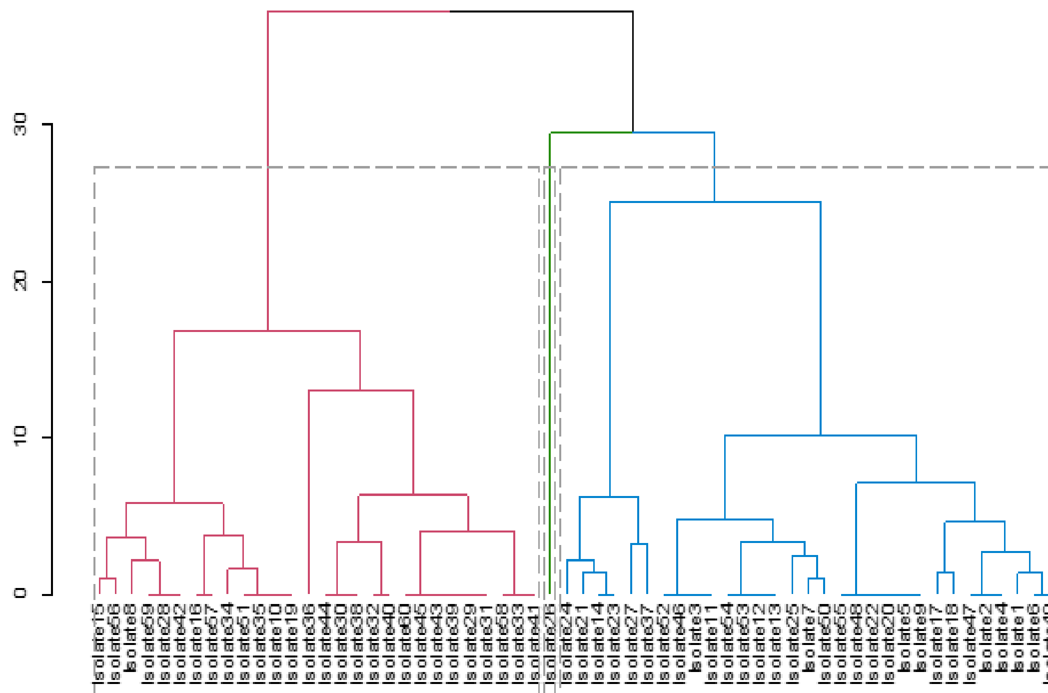

Figure 3. A dendrogram for morphology and cellular characteristics.

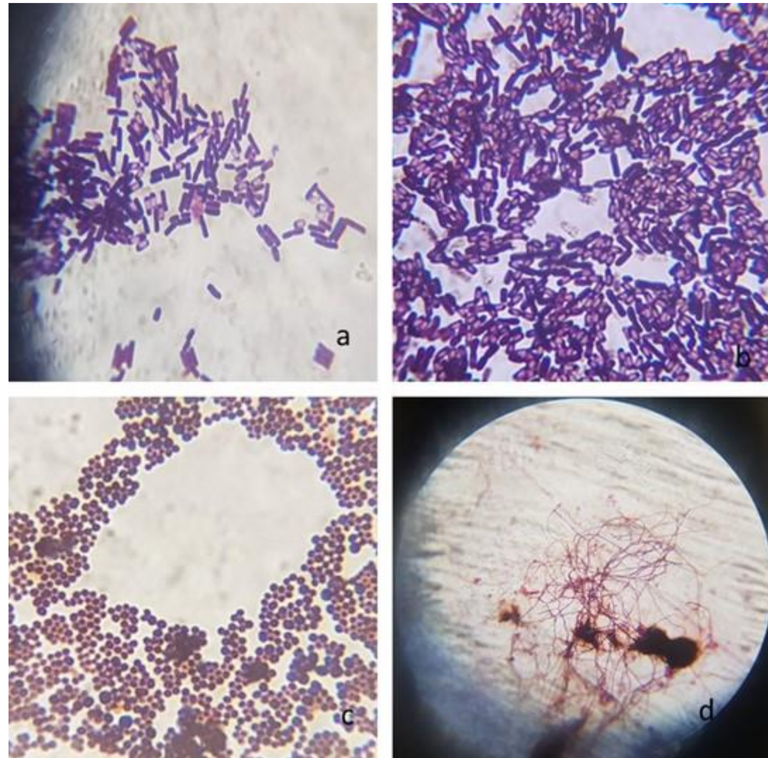

Plate 3. (a) A pure culture plate showing Gram-positive rods for ZCP 1.2; (b) Gram-positive rods for ZCP 17.2; (c) Gram-positive coccus cells for ZCP 6.7 (d) filamentous hyphae for ECP 3.4.

Table 3. Biochemical characteristics of bacterial isolates from lakes Olbolosat and Oloiden.

	Catalase	Citrase	TSI			MR	VP	Urea	Indole	Gelatin	Motility	Starch
Isolate			**Butt**	**Slant**	**Gas**							
ZCP 9.6	+	-	+	-	-	+	-	-	-	-	-	+
ZCP 6.3	+	+	-	-	-	+	-	-	-	+	-	+
ZCP 6.2	+	+	+	-	-	+	-	-	-	+	-	+
ZCP 1.3	+	-	+	-	-	+	-	-	-	-	+	+
EBP 2.2	+	-	+	-	-	+	+	-	-	-	+	+
ZCP 1.7	+	+	+	-	-	+	-	-	-	-	+	-
ZCP 6.1	+	-	-	-	-	+	-	-	-	-	+	+
ZCP 6.7	+	-	+	+	-	-	-	-	-	-	-	+
ZCP 17.4	+	-	+	+	-	-	-	-	-	-	-	+
ECP 3.1	+	+	-	-	-	+	+	-	-	-	+	+
EBP 8.2	+	+	+	-	-	+	+	-	-	+	+	+
ZCP 1.2	+	+	+	-	-	+	-	-	-	-	-	+
ZCP 6.8	+	+	-	-	-	+	-	-	-	+	-	+
EBP 8.1	+	-	-	-	-	+	-	-	-	+	+	+
EBP 10.1	+	-	-	+	-	+	-	-	-	+	-	-
EBP 2.1	+	+	-	-	-	-	-	+	-	+	-	+
ECP 3.4	+	+	+	+	-	+	-	-	-	+	-	+
ZAP 16.3	+	+	-	-	-	+	-	-	-	-	+	+
ZCP 17.2	+	-	+	-	-	+	-	-	-	-	-	+
EBP 8.8	+	-	+	+	-	+	-	-	-	-	-	-
ZAP 10.1	+	-	+	+	-	+	-	-	-	+	-	+

Table 3. Contd.

ZAP 16.1	+	+	–	–	+	+	–	–	–	+	–	+
ZAP 16.2	+	–	+	–	–	+	+	+	–	–	–	–
EBP 3.9	+	–	+	–	–	–	–	–	–	+	–	+
ZAP 9.1	+	–	–	–	–	+	–	–	–	+	–	+
EBP 2.5	+	–	–	–	–	+	–	–	–	–	–	+
EBP 10.5	+	+	–	+	–	+	–	–	–	–	–	+
EBP 8.4	+	+	+	–	–	+	–	–	–	–	–	+
EBP 5.3	+	+	+	–	+	+	–	–	–	–	–	+
EBP 5.1	+	–	–	–	+	+	+	–	–	–	+	+
EBP 10.4	+	–	–	+	–	+	+	–	+	–	+	+
EAP 3.7	+	–	–	+	–	+	+	–	+	–	+	–
ECP 3.2	+	–	–	+	–	+	+	–	–	+	–	+
EBP 10.2	+	–	–	+	–	+	+	–	–	+	–	+
EBP 2.4	+	–	–	+	–	–	–	–	+	–	+	–
EBP 8.3	+	+	+	–	–	+	–	–	–	+	+	+
EBP 8.5	+	+	+	–	–	+	–	–	–	+	+	+
ZBP 9.2	+	–	–	–	–	+	–	–	+	+	+	–
ZCP 1.8	+	–	–	–	–	+	–	–	+	+	+	–
ZCP 17.1	+	–	–	–	–	+	–	–	+	+	+	–
ZCP 1.1	+	+	+	+	–	+	–	–	–	–	+	–
ZCP 6.5	+	–	–	–	–	–	–	+	–	–	+	–
ZAP 10.2	+	+	+	–	–	+	–	+	–	+	+	+
ZBP 9.7	+	+	+	–	–	+	–	+	–	+	+	+
ZAP 9.2	+	+	+	–	–	+	–	+	–	+	+	+
ZCP 13.2	+	+	–	+	–	+	–	+	–	+	+	+
ZCP 1.11	+	+	–	+	–	+	–	+	–	+	+	+
ZCP 1.10	+	–	–	+	–	–	+	–	–	–	+	+
ZCP 1.9	+	–	–	+	–	–	+	–	–	–	+	+
ZCP 1.8	+	+	–	+	–	–	+	–	–	+	+	+
ZCP 1.6	+	+	–	+	–	–	+	–	+	+	+	–
ZCP 17.5	+	+	–	–	+	+	–	–	–	–	+	+
ZCP 6.6	+	–	–	–	+	+	–	–	+	–	+	+
ZAP 9.4	+	–	–	–	+	+	–	+	–	+	+	–
ZAP 9.3	+	–	–	–	+	+	–	–	–	+	+	–
ZCP 6.4	+	–	–	–	–	–	+	–	+	–	+	+
EAP 8.9	+	–	–	–	–	–	+	–	+	–	+	+
EAP 6.4	+	–	–	–	–	–	+	–	+	–	+	+
EBP 8.7	+	+	+	–	+	–	–	+	–	+	+	–
EAP 6.6	+	–	–	+	–	+	+	–	+	–	+	–

Key: TSI denotes Triple Sugar Phosphate, MR-Methyl Red, VP-Voges-Proskauer, (+) denotes positive activity while (–) denotes negative reaction or no observable activity.

Physiological characterization

The isolates were able to grow at a wide range of pH including acidic, neutral and alkaline. There was poor growth at pH 4 and 12, while there was good growth at pH 6, 8 and 10. Salinity that favored the growth was 0% followed by 5%. The best temp for the microbes was at 30° while 25° followed. There was significant difference at $p<0.001$ for bacterial growth in all parameters; Kruskal-Wallis One Way Analysis of Variance on Ranks followed by Tukey's HSD post-hoc analysis (Figure 4).

Hydrolase activity

The ability of bacterial isolates to produce extracellular

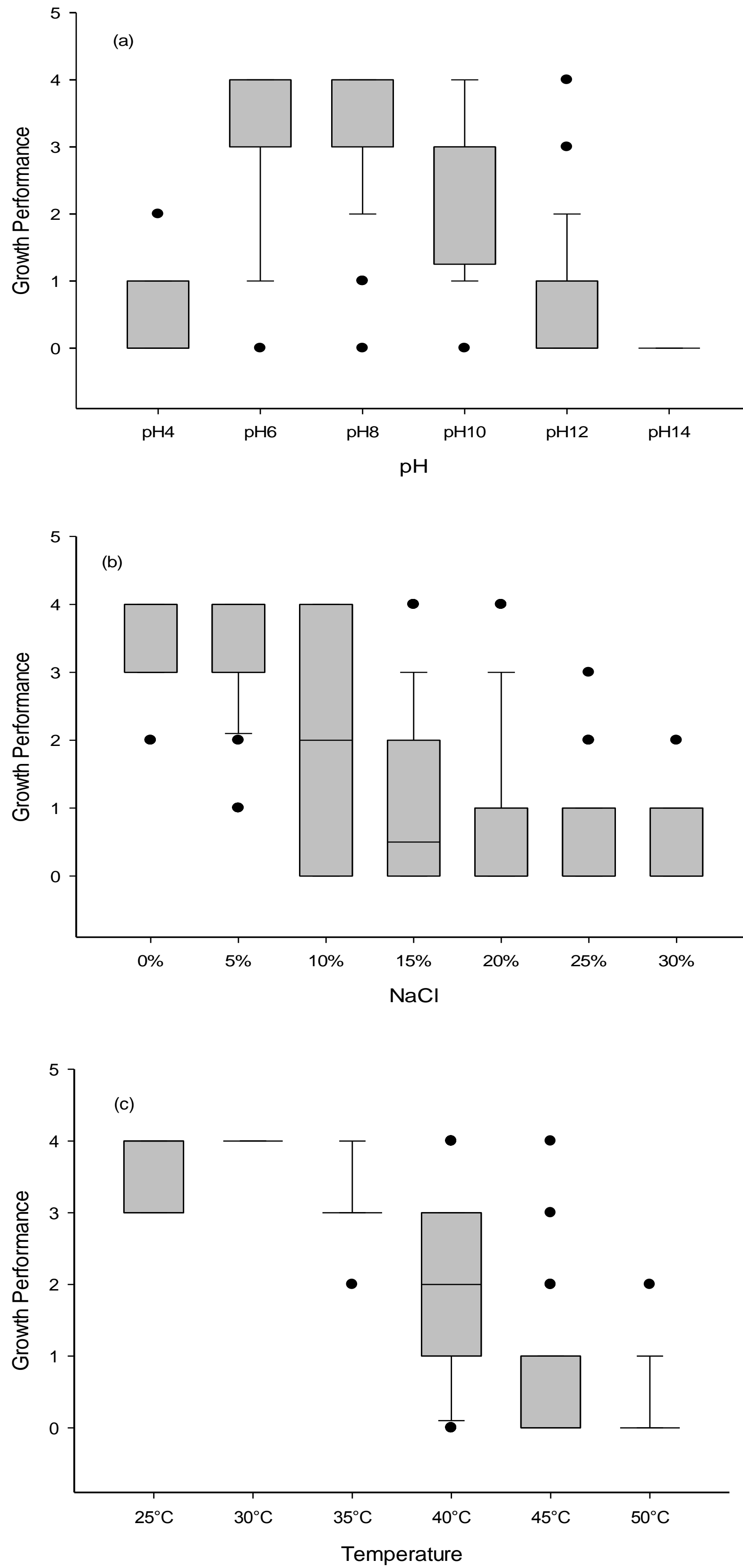

Figure 4. Growth performance of bacteria isolates from various sampling locations in Lake Oloiden and Olbolosat at different (a) pH; (b) Sodium chloride concentration and (c) temperature.

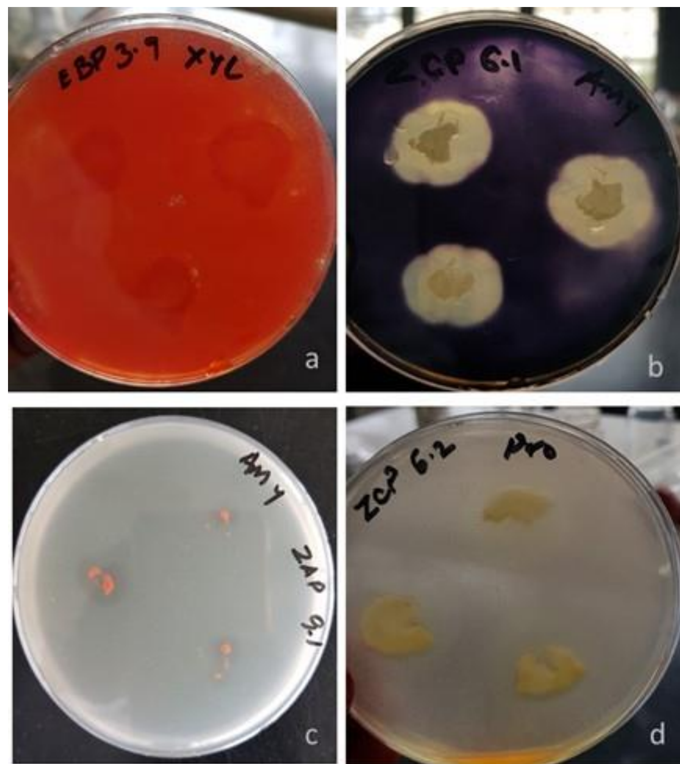

Plate 4. A pure culture plate showing clear zone for positive hydrolysis of a) xylan by EBP 3.9 b) starch by ZCP 6.1 c) starch by ZAP 9.1 c) and d) skimmed milk hydrolysis by ZCP 6.2.

enzymes was studied. Clustering from the heatmap shows that most bacterial isolates were able to utilize different substrates indicating their ability to produce different enzymes. Correlation between enzyme hydrolysis activity and bacterial isolates revealed that there were two functional clusters. Fifty-nine isolates formed a single cluster while ECP 3.1 formed a solitary cluster. Among the five enzymes assayed, all others formed a common cluster while xylanase formed a solitary cluster. This shows that some of the isolates were not able to utilize xylan as a substrate. Hydrolase activity that recorded positive result was indicated by the clear zone around the colony (Plate 4a-d and Figure 5).

Antimicrobial activity

Antagonistic activity recorded positive results that were indicated by a zone of inhibition around the colony. Zone sizes for inhibitions were looked up on a standardized chart by following Kirby-Bauer diffusion protocol for the sensitivity measuring above 18 mm, resistant 13 mm or less and intermediate measuring 14-16 mm. Clustering from the heatmap shows that most bacterial isolates were sensitive (measuring >18 mm) to test organisms. Fifty-seven isolates formed one cluster, two isolates EBP 2.1 and ZCP 1.9 formed their cluster, while ZCP 17.1 formed a solitary cluster. Among the five test organisms assayed *C. albicans* formed a single cluster. Correlation between antagonistic activity estimates and bacterial isolates revealed that there were two functional clusters (Figure 6).

16S rRNA analysis

Genomic DNA was extracted from all the 60 bacterial isolates. Partial sequencing for 16S rRNA gene using bacterial specific primers yielded an amplification product of approximately 1500 base pairs. The 60 amplified PCR products were sequenced and only 26 were unambiguous, and their sequences were selected for phylogenetic analyses. The sequences that were >320 base pairs were edited using Chromas pro software and were compared into public databases using BLAST program (http://blast.ncbi.nlm.nih.gov/). Out of 26 isolates submitted to the NCBI database, only 18 were assigned accession numbers MT801052-MT801069 (Table 4). The pairwise alignment was done using MEGA 7 software and the affiliation of the 26 isolates to closest reference strains were determined (Table 4). The phylogenetic relationship of all the partial sequences was determined

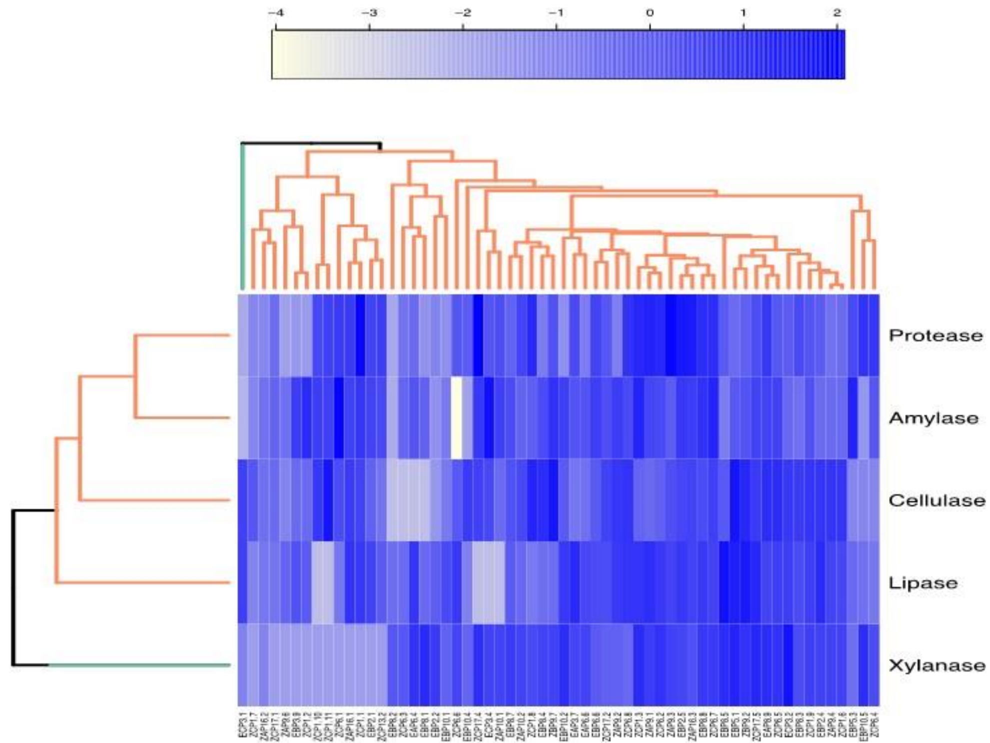

Figure 5. Hierarchical clustering based on Euclidean metric for bacterial isolates that produced enzymes.

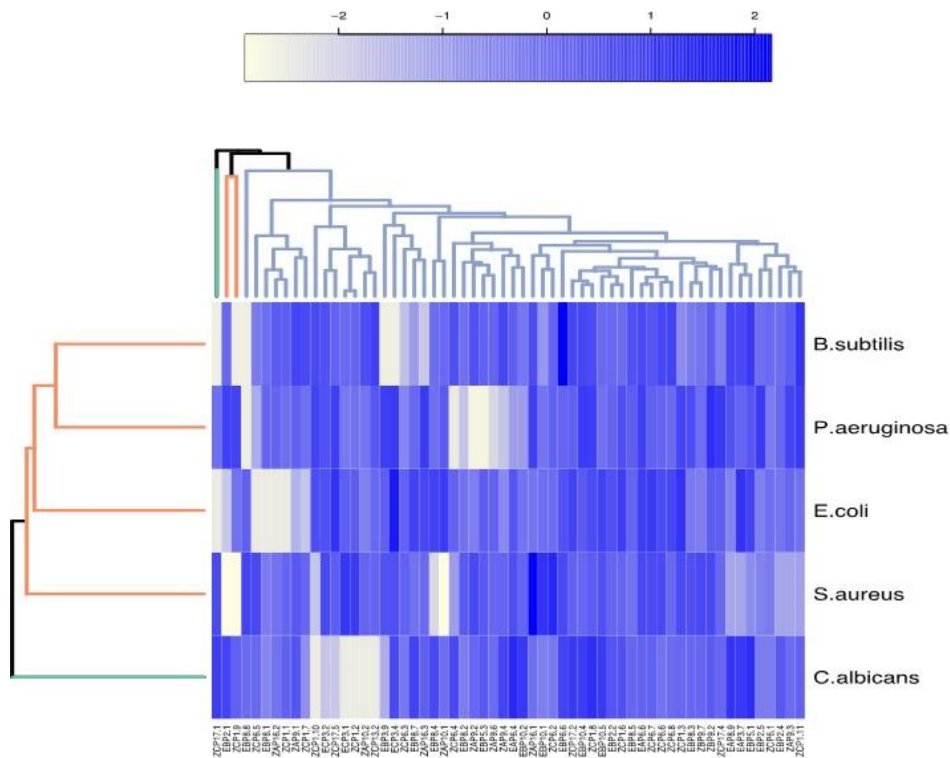

Figure 6. Hierarchical clustering based on Euclidean metric for bacterial isolates that produced antimicrobial activity against test organisms.

Table 4. Taxonomic affiliation of partial sequences of 26 bacterial isolates from both lakes with closest relatives from the GenBank database.

Isolate	Sampling site	Isolation Source	Accession number	Closest taxonomic match in BLAST	Query cover	E-value	ID %	Affiliated to	G+C %
ZAP 16.3	Lake Olbolosat	Water	MT801059	*Pseudomonas sp.* strain LC128	100	0.0	99.59	JQ014351.1	53
ZBP 9.6	Lake Olbolosat	Dry sediment	MT801060	*Bacillus sp.* strain SC134	100	0.0	99.5	MN192133.1	53
ZCP 1.2	Lake Olbolosat	Wet sediment	MT801061	*Bacillus sp.* strain K1	100	0.0	100	MH628021.1	54
ZCP 1.3	Lake Olbolosat	Wet sediment	MT801062	*Paenibacillus oryzisoli* strain 1ZS3-15	100	0.0	99.28	NR_164873.1	55
ZCP 1.7	Lake Olbolosat	Wet sediment	MT801063	*Bacillus megaterium* strain ICMM1	100	0.0	99.48	MN889411.1	55
ZCP 17.4	Lake Olbolosat	Wet sediment	MT801069	*Staphylococcus succinus* strain cqsM8	100	0.0	99.79	MN826566.1	51
ZCP 6.1	Lake Olbolosat	Wet sediment	MT801064	*Bacillus sp.* strain ME76	100	0.0	**97.43**	LR861557.1	54
ZCP 6.2	Lake Olbolosat	Wet sediment	MT801065	*Bacterium* strain TPMX-4	100	0.0	99.54	KY427680.1	53
ZCP 6.3	Lake Olbolosat	Wet sediment	MT801066	*Bacillus aryabhattai* strain A6-P	100	0.0	99.89	MT588720.1	54
ZCP 6.7	Lake Olbolosat	Wet sediment	MT801067	*Staphylococcus arlettae* strain KTSMBNL-77	100	0.0	99.78	KM200327.1	52
ZCP 6.8	Lake Olbolosat	Wet sediment	MT801068	*Bacillus simplex* strain ER20	99	0.0	99.6	MT124545.1	54
EBP 10.1	Lake Oloiden	Dry sediment	MT801056	*Bacillus safensis subsp. safensis* strain EGI17	99	0.0	99.78	MN704393.1	55
EBP 2.1	Lake Oloiden	Dry sediment	MT801052	*Microbacterium oxydans* strain I-S-R2-2	100	0.0	99.47	MK398050.1	57
EBP 2.2	Lake Oloiden	Dry sediment	MT801053	*Bacillus pumilus* strain J8R13LARS	100	0.0	99.69	MT378474.1	55
EBP 8.1	Lake Oloiden	Dry sediment	MT801054	*Bacillus altitudinis* strain SR1-56	100	0.0	98.79	LN995455.1	55
EBP 8.2	Lake Oloiden	Dry sediment	MT801055	*Bacillus pumilus* strain PK3	100	0.0	**97.26**	MH428223.1	55
ECP 3.1	Lake Oloiden	Wet sediment	MT801057	*Bacillus sp.* strain HBUM207125	100	0.0	99.9	MT598008.1	55
ECP 3.4	Lake Oloiden	Wet sediment		*Streptomyces sp.* strain SL37	100	0.0	**98.56**	MN812679.1	57
EBP 2.5	Lake Oloiden	Dry sediment		*Aeromicrobium sp. strain Bt13*	41	0.0	**97.79**	KP195230.1	63
EBP 3.9	Lake Oloiden	Dry sediment		*Bacillus cereus* strain PR37	78	0.0	**96.45**	MN232152.1	51
EBP 8.8	Lake Oloiden	Dry sediment		*Staphylococcus xylosus* strain JM41	100	0.0	99.59	MN758801.1	55
ZAP 9.1	Lake Olbolosat	Water		*Hymenobacter sp* strain R2A-W5	99	0.0	**98.56**	FJ627043.1	55
ZAP 10.1	Lake Olbolosat	Water		*Exiguobacterium sp.* strain Mong-10	99	0.0	98.73	KY962739.1	55
ZAP 16.1	Lake Olbolosat	Water	MT801058	*Streptomyces hawaiiensis* strain HDJZ-ZWM-20	99	0.0	**98.33**	GU227347.1	59
ZAP 16.2	Lake Olbolosat	Water		*Bacillus subtilis* strain MA6	78	0.0	98.84	KT758735.1	55
ZCP 17.2	Lake Olbolosat	Wet sediment		*Bacillus toyonensis* strain HRT5	99	0.0	**97.28**	MH197375.1	54

in MEGA 7 using Maximum-Likelihood analyses. The evolutionary pairwise distances were estimated using the Maximum Composite Likelihood approach (Engeset et al., 2003). The bacterial isolates were identified based on the sequence comparison with the GenBank, NCBI and reference strain. Bacterial isolates were clustered into three different Phyla belonging to *Firmicutes, Actinobacteria* and *Proteobacteria* and *Bacteriodetes* (Figures 7 and 8; Supplementary Table 1). *Firmicutes* scored 77% closely affiliated with twenty strains, *Actinobacteria* scored 15% closely affiliated with four strains while *Proteobacteria* and *Bacteriodetes* each scored 4% closely affiliated with each strain from both lakes. BLAST analysis of the partial sequences showed there were fourteen isolates (54%) that were closely affiliated with the members of the genus *Bacillus* with >96 sequence identity from both lakes. Among these were *Bacillus* group from Lake Olbolosat scoring >97% sequence identity;

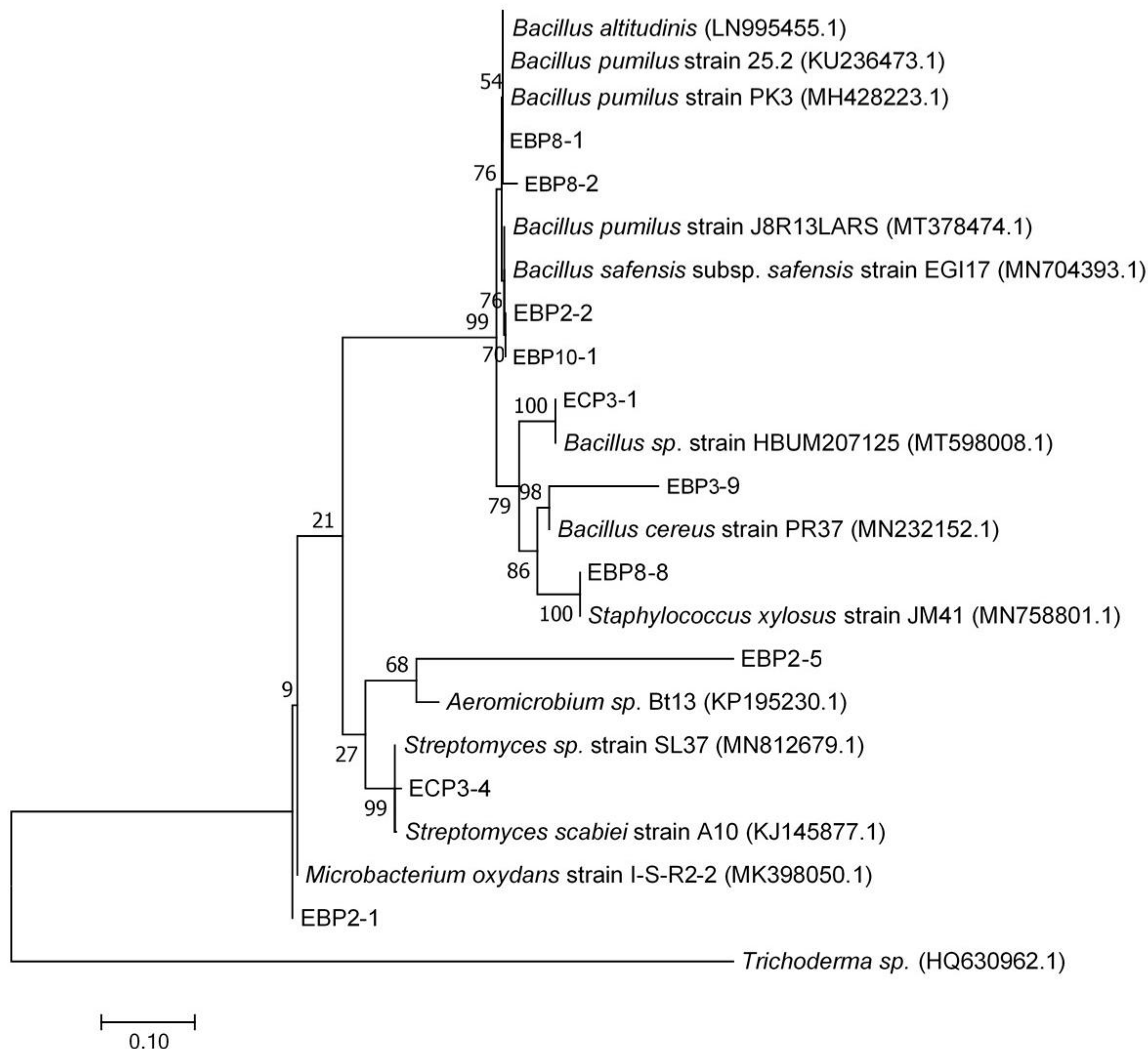

Figure 7. Phylogenetic tree displaying the evolutionary relationship between partial 16S rRNA gene sequences from Lake Oloiden and closest neighbor strain. *Trichorderma* sp. (HQ630962.1) was used to root the tree. Only bootstrap values above 9 are shown. The scale bar indicates approximately 0.01 the sequence difference. Lakes Olbolosat is indicated with prefix E and Oloiden prefix Z.

Bacillus sp recorded three different strains scoring 99.59,100 and 97.43%, *B. megaterium* scored 99.48%, *B. aryabhattai* scored 99.89%, *B. simplex* scored 99.6%, *B. subtilis* scored 98.84% while *B. toyoniensis* scored 97.28%. *Bacillus* group from Lake Oloiden scored >96% sequence identity; two strains of *B. pumilus* scored 99.69 and 97.26%. *Bacillus safensis* subsp *safensis* scored 99.78%, *B. altitudinis* scored 98.79, *Bacillus sp*. scored 99.9%, while *B. cereus* scored 96.45%. Among other *Firmicutes* were three isolates from the genus *Staphylococcus* scoring 99.79, 99.78 and 99.59% sequence identities *Staphylococcus succinus, S.arlettae* and *S. xylosus* respectively. The other three isolates from the *Firmicutes* phylum were isolates (ZCP 1.3, ZCP 6.2 and ZAP 10.1) scoring 99.28, 99.54 and 98.84%, sequence identities with known members of the genera *Paenibacillus oryzisoli, Bacterium* sp and *Exiguobacterium* sp respectively. Phylum *Actinobacteria* was affiliated to four different genera *Microbacterium oxydans* with a score of 99.47%, *Streptomyces* sp scored 98.56%, *Aeromicrobium* scored 97.79% while *Streptomyces hawaiensis* scored 98.73 sequence identities. The Phylum *Proteobacteria* was closely affiliated with only one strain of *Pseudomonas* sp. scoring 99.59% sequence identity. There was one strain from the phylum *Bacteroidetes* closely affiliated to *Hymenobacter* sp scoring 98.56% (Table 4). There were 10 isolates from Lake Oloiden revealing two clusters. One cluster had strains from *Firmicutes* with a bootstrap value of 99, while the other one had strains belonging to *Actinobacteria* with a bootstrap value of 27*:* one strain formed a node from the latter cluster with a bootstrap value of 9 (Figure 6).

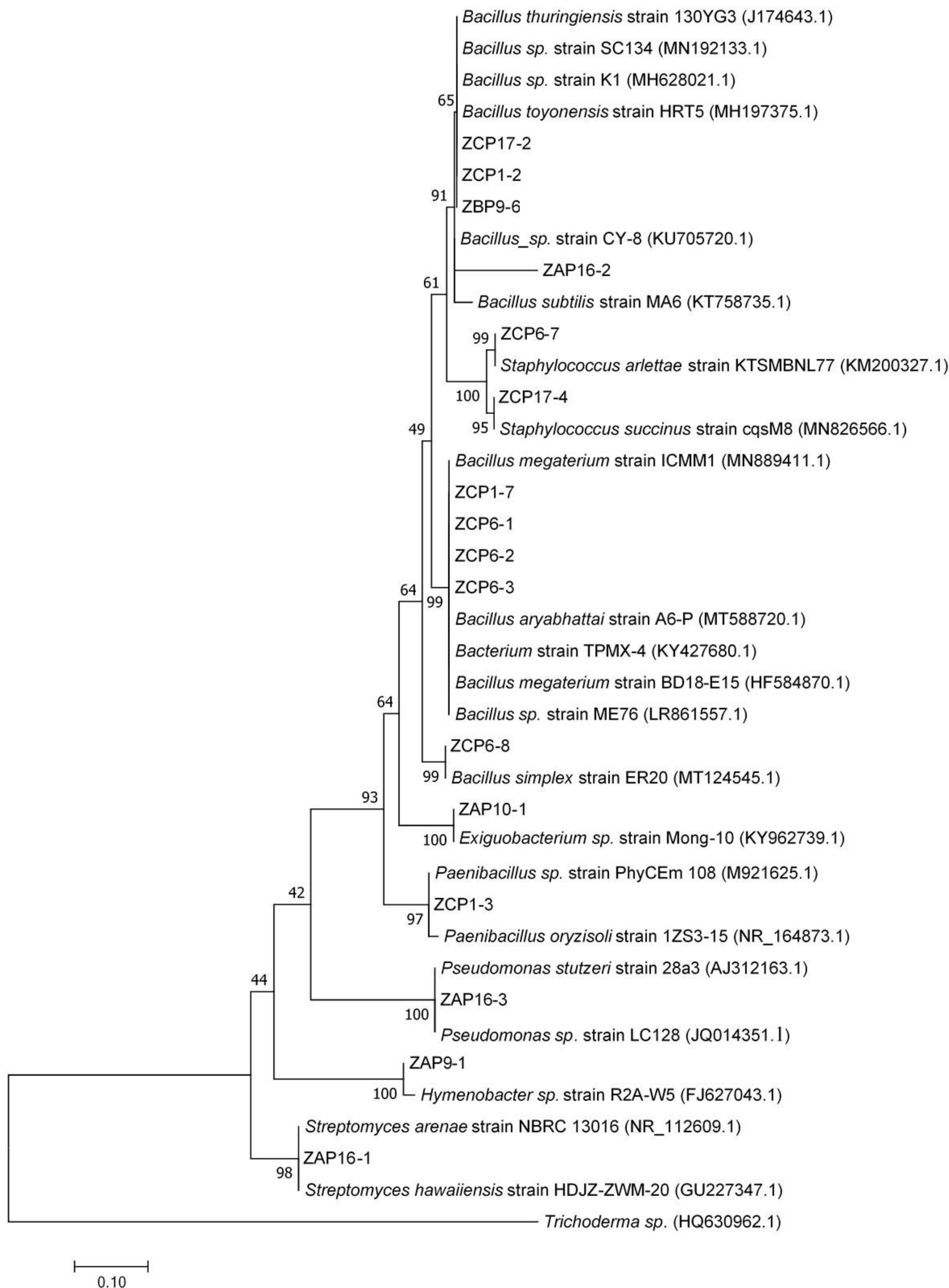

Figure 8. Phylogenetic tree displaying the evolutionary relationship between partial 16S rRNA gene sequences from Lake Olbolosat and closest neighbor strain. *Trichorderma* sp. (HQ630962.1) was used to root the tree. Only bootstrap values above 42 are shown. The scale bar indicates approximately 0.01 of the sequence differences. Lakes Olbolosat is indicated with prefix E and Oloiden prefix Z.

The phylogenetic tree of the 16S rRNA partial sequences of the 16 isolates from Lake Olbolosat revealed two major clusters. One cluster had strains belonging to *Firmicutes*, *proteobacteria* and *Bacteroidetes* with bootstraps values 64, 100 and 100 respectively while the other one had strains belonging to *Actinobacteria* Phyla with bootstraps

values of 98 (Figure 7).

DISCUSSION

A total of 60 isolates were obtained from lakes Olbolosat and Oloiden. The cfu counts per ml ranged between 0-1.75×10^5 cfu/ml. The highest cfu counts were obtained from dry sediments within Lake Oloiden while the lowest was from dry sediments from Lake Olbolosat. Growth of isolates in culture medium at different salinity, pH, and temperature ranges indicates that they can tolerate and can adapt to adverse growth conditions in the marine ecosystem. The pH, temperature and salinity are indicators of environmental setting that shapes microbial communities according to O'Brien et al. (2019) and also could affect the activities of extracellular enzymes and breakdown of organic matter (Li et al., 2019). The physiological conditions (pH, temperature, and sodium chloride) are important in the current study if the isolates are to be cultured in the laboratory and be exploited for industrial use (O'Brien et al., 2019).Eight strains from the current study; ZCP 6.1, EBP 8.2, EBP 3.9, ZCP 17.2, ECP 3.4, EBP 2.5, ZAP 9.1 and ZAP 16.1 had sequence similarity of 97.43, 97.26, 96.45, 97.28, 98.56, 97.79, 98.56 and 98.33% sequence similarity respectively representing novel genera of organisms within the lake ecosystem according to Kim et al. (2014) who reported that a bacteria organism could be considered novel if the sequence similarity is <98.65%. The presence of bacteria in the lake ecosystem could be involved in the biodegradation of contaminants such as polycyclic aromatic hydrocarbons through the use of their extracellular enzymes (Yadav et al., 2019). Production of extracellular enzymes by bacterial isolates in this study such as amylases, lipases, proteases, xylanases and cellulases and intracellular enzymes which include starch, catalase, gelatinase and citrase, indicates their biotechnological potential in agriculture, food industries, detergent, medicinal formulations and wastewater management (Yadav et al., 2019). Out of the 60 sequences for bacterial isolates from both lakes, 26 were without ambiguities. The 26 bacterial isolates identified in the current study belonged to the domain bacteria and four different Phyla: *Firmicutes, Actinobacteria*, *Proteobacteria and Bacteroidetes*. *Firmicutes* were predominance within the two lakes. They are known to produce spores that are highly resistant to environmental stress. Formation of spores explains why they may be able to easily outgrow other microorganisms after transfer to microbiological media with their repeated isolation from sediments (Vos et al., 2009). *Firmicutes* biodegrade complex compounds therefore breaking down macromolecules entering the lake ecosystem such as plants and dead animals providing energy and carbon sources for microbial communities (Vos et al., 2009). There were 13 strains of *firmicutes* belonging to the family *Bacillaceae* identified in this study; among these were *Bacillus safensis* subsp *safensis, Bacillus pumilus, B. altitudinis, B. simplex, B. aryabhattai*, *Bacillus megaterium, B. simplex, B. cereus, B. subtilis* and *Bacillus toyoniensis.* *Bacillus pumilus* is known to play a good role in the biodegradation of macromolecules in the ecosystem (Mishra et al., 2017). *Bacillus altitudinis* utilizes various kinds of carbon sources in the lake ecosystem according to Mishra et al. (2017). *Bacillus megaterium* could be used as an industrial organism since it produces proteins and has been used in bioremediation. Proteins are commonly used in agriculture as plant promoting bacteria and in health sectors (Wafula and Murunga, 2020). *Bacillus subtilis*, *B. cereus, B. pumilus*, *B. aryabhattai, Bacillus safesis* subp *safensis* and *B. simplex* have been used as plant promoting bacteria to fix nitrogen, secrete plant hormones or antibiotics, solubilize phosphates, inhibit pathogenic microbes and modify insoluble iron to soluble iron (Chiboub et al., 2018). This is because they are resistant to adverse environmental conditions through the production of spores, they replicate rapidly, and they have a broad-spectrum to biocontrol ability (Chiboub et al., 2018). Plant promoting bacteria are important in enhancing seedling vigor, leaf area, shoot and root growth. Plant promoting hormones like GA3 and IAA are enhanced by the different species of bacteria. The hormone GA3, together with auxin play an important role in the elongation of plant and leaf bud formation (Chiboub et al., 2018). The hormone IAA helps in the emergence and origination of adventitious roots and enhancement of shoot development. Plant promoting hormones also enhances the availability of nutrient uptake to plants helping them against abiotic and biotic stresses (Shafi et al., 2017). The production of antibiotics by bacteria may help them in colonization. Both pathogenic and nonpathogenic organisms compete for space and nutrients with other organisms around them. This is because the soil has a limited amount of nutrients available to sustain them (Shafi et al., 2017). *Bacillus cereus* occurs naturally and is responsible for most food poisoning (Bartoszewicz and Czyzewska, 2017). *Bacillus toyoniensis* was isolated from South Africa marine sediment by Ugbenyen et al. (2017) for the production of flocculant used in the biodegrading of pollutants. *Staphylococcus* spp belonging to *Firmicutes* occurs ubiquitously in nature and have been isolated from various animals such as birds and mammals (Rossi et al., 2020). *Staphylococcus xylosus* and *S. succinus* identified in this study have been used in Italy for the fermentation of traditional sausages (Ratsimba et al., 2017). *Staphylococcus xylosus* produces biosurfactant an important bioactive compound used in food, cosmetic, petroleum, medicine and pharmaceuticals industries (Ratsimba et al., 2017). *Exiguobacterium* genus is another *Firmicute* that was identified in this study and has been isolated earlier from different environmental niches

such as sediments, seawater, soils glaciers, hydrothermal vents and industrial effluents (Kasana and Pandey, 2018). Isolates from *Exiguobacterium* genus can grow under extreme environment with temperature ranging from 12-50°C and under low nutrients conditions (Vishnivetskaya et al., 2009). Different strains from *Exiguobacterium* genus has been used in industries, in agriculture as a plant promoting bacteria and in biodegradation of pollutants (Kasana and Pandey, 2018). *Actinobacteria* are known to produce extracellular enzymes and secondary metabolite products. Members of this group are known to have high *mol%* G+C because of their triple hydrogen bond of the chromosomal DNA content (Hamid et al., 2020). *Streptomyces sp*, *Microbacterium oxydans* and *Aeromicrobium* identified in this study having a high *mol%* G+C content could make them to adapt to the unfavorable environment, with low mutation rate and tolerant to antagonism factors (Hamid et al*.,* 2020). *Streptomyces* spp is known to produce 80% of the antibiotic compounds according to Hamid et al. (2020) which are the most important secondary metabolites of the bacterial isolate. *M. oxydans* and most species in this genus inhabit diverse environments and are associated with the aquatic plants as symbionts according to (Mishra et al., 2017) *M. oxydans* are also used in a commercial application such as food colorants, dietary supplements, cosmetics and pharmaceuticals purposes (Meddeb-Mouelhi et al., 2016). *Bacteroidetes* and *Proteobacteria* are abundant during or following an algal bloom (Meddeb-Mouelhi et al., 2016). *Hymenobacter* sp. belongs to phylum *Bactroidetes* was also identified in this study by Royo-Llonch et al. (2017) who reported that *Hymenobacter* spp, inhabit different environmental niches like marine, fresh water, air, soil, and glacier. *Pseudomonas* sp a *Proteobacteria* identified currently is common in the aquatic environment according to (Mishra et al., 2017) and most strains are known to be phosphate solubilizing bacteria and also produce antagonism to other pathogens (Paul and Sinha, 2016).

Conclusion

The study shows that both lakes harbor diverse and novel bacterial species: *Firmicutes* (*Bacillus, Stapylococcus* and *Exiguobacterium*), *Proteobacteria* (*Pseudomonas*), *Actinobacteria* (*Streptomyces, Microbacterium*, *Aeromicrobium*) *Bacteroidetes* (*Hymenobacter*). The above- mentioned species have the potential for industrial application based on the enzyme, physiological, biochemical, antimicrobial and molecular properties. The study also showed some isolates could be novel strains; ZCP 6.1, EBP 8.2, EBP 3.9, ZCP 17.2, ECP 3.4, EBP 2.5, ZAP 9.1and ZAP 16.1 according to Kim et al. (2014) who reported that a bacteria organism could be considered novel if the sequence similarity is <98.65%. DNA-DNA hybridization could be done to establish the novel strains. An upscale for isolates with the industrial application could also be done as a way forward.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Review

Viruses, coronaviruses and COVID-19: A note for non-virology specialists

Yakubu Egigogo Raji[1*], Yahaya Muhammad Sanusi[2] and Zamberi Bin Sekawi[3]

[1]Faculty of Natural and Applied Sciences, Ibrahim Badamasi Babangida University, Niger State, Nigeria.
[2]Department of Theriogenology and Animal Production, Faculty of Veterinary Medicine, Usmanu Danfodiyo University, Sokoto, Nigeria.
[3]Department of Medical Microbiology and Parasitology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Selangor, Malaysia.

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An outbreak of a respiratory disease with severe acute respiratory syndrome (SARS) – like manifestations emerged in late December 2019 in the Wuhan city of China. The causative agent of this disease was later identified to be a novel Coronavirus. Subsequently, the disease was named coronavirus disease 2019 (COVID-19) by the World Health Organisation and later declared as a pandemic. The outbreak of COVID-19 came with many misinformation and misconceptions about viruses and the COVID-19 disease. Questions have been asked by non - virologists and the general public about what viruses are. Some wondered if viruses are living organisms or not. While some asked how big viruses are or if all viruses and the COVID-19 virus is artificially created. Yet others attributed the outbreak to the new quantum leap in the electromagnet field; the latest 5G technology. There are also concerns raised about the new virus being a bioweapon or an act of biowarfare. These numerous questions needed to be clarified concisely with available scientific knowledge. Thus, this review is providing answers to the 10 frequently asked questions aimed at informing the non - virologist in the academic field and beyond.

Key words: Virus size, virus origin, giant viruses, synthetic viruses, SARS-Cov-2, bioweapon.

INTRODUCTION

Virology and viruses are an important and fascinating area of biology. The nature of viruses, their behaviour, diversity and complexity make the area of virology a bit difficult for many specialists to understand adequately. These include health personnel, teachers and other biologists who do not major in virology. These personnel often need to explain to people a lot about viruses. However, lack of adequate understanding of the subject matter constrains their ability to offer enough and convincing explanation to satisfy the ever-growing public curiosity about viruses.

The advent of COVID-19 has been an eye opener, it exposes how little people know about viruses and their mechanisms of action. It has attracted many conspiracy

*Corresponding author. E-mail: rajiegigogoy@ibbu.edu.ng. Tel: +2348028702332.

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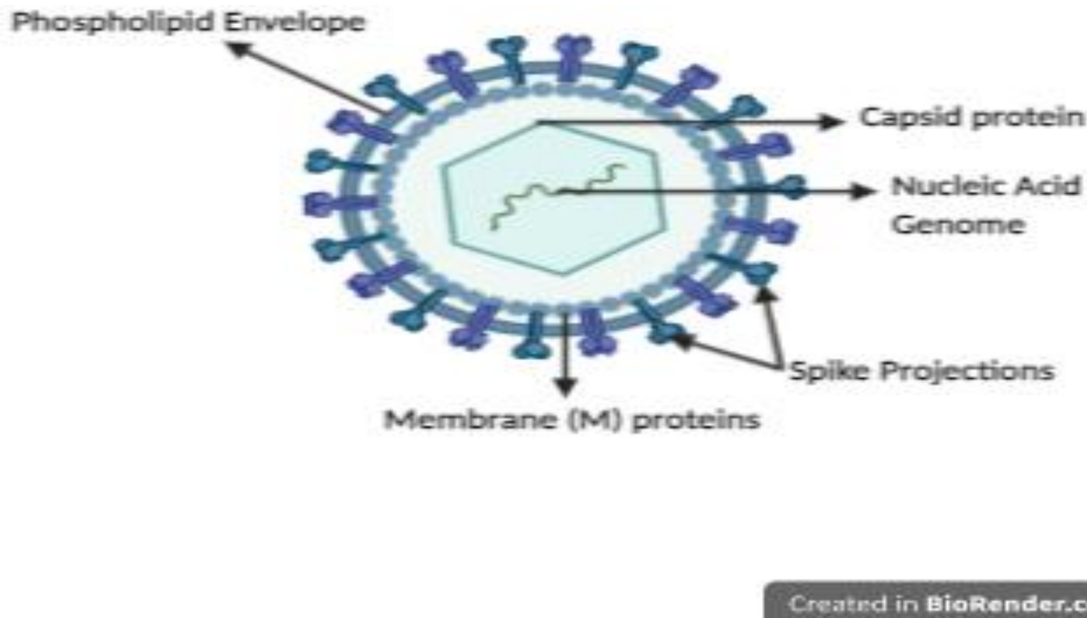

Figure 1. Schematic of a typical enveloped virus (BioRender.com: Generic Viral Life Cycle, 2020)

theories and misconceptions about viruses generally, and COVID-19 in particular. Researchers and healthcare professionals across the world have made several attempts to address some of these misconceptions (Okunlola et al., 2020; Khalid et al., 2020). However, many of these myths are yet to be fully addressed. Thus, now more than ever before is the time to empower the (unprofessional) personnel with suitable but simple enough information about viruses to adequately deal with current misinformation. This short (opinion) review aims to make information about viruses available to academics outside virology specialization and who may need the information but do not have the time for extensive study of virology now. The review answered a few questions regarding viruses and COVID-19; what has been established through scientific methods and what has not.

(1) Questions regarding what a virus is
(2) Questions regarding the origin of viruses
(3) Questions regarding whether viruses are living organisms or not
(4) Questions regarding the size of viruses
(5) Questions regarding viral replication process
(6) Questions regarding the difference between Coronavirus and COVID–19
(7) Questions regarding the available diagnostic kits for COVID -19 and whether they are produced only by China
(8) Questions regarding whether viruses in general and SARS-CoV-2 in particular are man-made
(9) Questions regarding the relationship between SARS-CoV-2 and biological warfare?
(10) Questions regarding the relationship between Electromagnetic waves (EMF) and virus epidemics.

VIRUSES WHAT ARE THEY?

A virus is an infectious agent (sub-microscopic) that can replicate only in living cells or organisms (obligate intracellular parasites). A virus can infect all forms of life (the three supper-kingdoms of life); bacteria, archaea, and eukaryotes (Sandaa and Bratbak, 2018). An infectious virus particle is called a virion; it consists of a genetic material (viral genome) which is enclosed by a protein coat (outer shell) (Chaitanya, 2019). The protein shell is known as capsid; in some viruses, a lipid membrane called an envelope surrounds the capsid (Figure 1). Therefore, viruses can be enveloped viruses (with capsid enclosed by a lipid membrane) or non-enveloped viruses. The viral genome can be DNA or RNA (nucleic acids) and varies in length (the number of DNA or RNA molecules). The viral genome can be double or single-stranded and circular or linear. The viral genetic material (DNA or RNA) encodes the protein(s) that surrounds it, thus conferring protection to the genetic material (Chaitanya, 2019).

THE ORIGIN OF VIRUSES

The origin of viruses had been, for a long time, a topic of debate, because viruses do not form fossils. The debate on viral origin is yet to be resolved completely thus the concept of viral origin is still under re-evaluation by virologists (Krupovic et al., 2019). Scientists believed that viruses have multiple evolutionary origins (Krupovic et al., 2019). The current stand is that viruses are ancient particles whose origin pre-dates the divergence of life into

the three domains (super-kingdoms) (Thomas, 2019). However, earlier, Paleovirologists have proposed three major theories to explain the origin of viruses; (i) Virus–first hypothesis or co–evolution theory (Mughal et al., 2020). This theory proposes viruses as primitive precursors of a cellular system. This simply means viruses are the earliest phase of life's evolution, evolving from the complex molecules of nucleic acids and proteins. The principle assumes that viruses existed first before cells as self-replicating particles. (ii) Degeneracy hypothesis (Mughal et al., 2020). This hypothesis proposes that viruses originate as small parasitic cells that depend on larger cells for survival, and due to extreme parasitism over a long time, these cells lost those genes that are not required for parasitic life (degenerate). As a result, they became obligate parasites that are not capable of survival outside cells. This hypothesis is also known as the regressive or reduction hypothesis. (iii) Escape hypothesis (Mughal et al., 2020). This hypothesis suggests a cellular origin of viruses. According to this theory, viruses emerged from genes of larger organisms as break–away bits of RNA or DNA due to cell destruction (damage) of larger cellular systems. They acquired the ability to move from one gene to another, as mobile genetic elements, thereby surviving as minimal parasitic replicons (Domingo, 2020). This hypothesis is also known as cellular origin hypothesis or Vagrancy hypothesis. Each of the aforementioned hypotheses has its limitations, which makes it difficult to single out one that is correct. However, viruses might have evolved from different mechanisms. The mechanism of virus evolution might be any one of the above, a combination of two or more of them (Mughal et al., 2020) or others yet to be discovered.

VIRUSES, ARE THEY LIVING OR NOT?

Another age-long debate, among scientist, about viruses is whether they are living organisms or not. Some biologists believe that viruses are organisms at the edge of life while some consider them as non-living (Kaján et al., 2020; Shwetha, 2018). The debate is anchored on what it means to be alive. Is life about survival, growth, and reproduction? Is cell a prerequisite for life? To that end, some biologists declared that viruses are non-living because they lack cell and are not capable of independent survival, growth, and reproduction. This line of thinking became official in the year 2000 following the declaration by the International Committee on Taxonomy of Viruses (ICTV) that "viruses are not living organisms" (Zimmer, 2015). However, as years went by and with more discoveries about viruses, some scientists questioned this declaration, while some rejected it outrightly (Claverie, 2006). These group of virologists believed that classifying viruses as non–living is an old–fashioned notion. Asserting that discoveries have overtaken this old rule. They said viruses have some characteristics of life. Viruses have genetic materials (genes), reproduce (replicate) and evolve through natural selection. In addition, viruses such as giant viruses can expand their genome to acquire the ability for independent metabolism, growth, survival, and reproduction. Giant viruses also have viral factories' that act as cells (Mougari et al., 2019). As a result, some of these Microbiologists have proposed reclassification of living organisms into two groups; ribosome-encoding organisms (Eukaryotes, Archaea and Bacteria) and capsid-encoding organisms (Viruses). They, therefore, defined viruses as capsid-encoding organism that are composed of proteins and nucleic acids, capable of self-assembling in a nucleocapsid and uses a ribosome-encoding organism for the completion of its life cycle (Raoult and Forterre, 2008).

THE SIZE OF VIRUSES

One of the most remarkable features of viruses is their size. It was the uniqueness of their size that led to their discovery; viruses were able to be filtered through filters used for pathogenic bacterial filtration. Viruses are largely sub-microscopic, and their size was elucidated with the help of electron and fluorescence microscopy. There are two aspects when it comes to understanding virus size; the viral (structural) size and the genome (length of nucleic acid) size. The structural size of viruses is measured in nanometres (nm); 1×10^{-9} or 1 billionth of a metre that is, 1 billion nanometres equal 1 m. So, the size of most viruses is within the range of 5- 300 nm. However, recently discovered giant viruses are in the diameter range of 500- 1000 nm (Koonin and Yutin, 2018). Virus genome size is measured either in terms of the number of bases in the genome or their mass. The virus genome size is in the range of a few thousand bases to several hundred kilobases (O'Carroll and Rein, 2016). A few examples of viruses are listed in Table 1.

Replication of viruses

Viral replication is the process of forming new viruses. As obligate parasites, viruses need cellular systems to replicate. To facilitate understanding, the viral replication process can be explained under the following steps; adhesion, entry, uncoating, replication, assembly, and release. The process explained here is the general rule that applies to many viruses but there are exceptions. Adsorption (adhesion); a virus attaches to a susceptible host's cell membrane at a specific position known as the receptor site using the viral attachment proteins. Entry; following attachment, the virus is engulfed into the cytoplasm of the cell through the process known as endocytosis. Uncoating; once the virus gets inside the

Table 1. Different viruses, size of their genomes and their genetic materials.

S/N	Virus name	Virus size (diameter) (nm)	Genome size	Type of genetic material
1	Poliovirus	30	7.5 Kb	RNA
2	HBV	42	3.2-3.3 Kbp	DNA
3	HCV	50	9.6- 12.3 Kb	RNA
4	HIV	120	9.2- 9.6 Kb	RNA
5	Measles virus	150	16 Kb	RNA
6	Coronaviruses	80-200	26 – 33 Kb	RNA
7	Paramyxoviruses	150 -300	13 – 19 Kb	RNA
8	Ebola virus	80 × 970	18 -19 Kb	RNA
9	Mimi virus	500	1.2 Mbp	DNA
10	Pandora virus	500 × 1000	2.8Mbp	DNA

HBV, Hepatitis B Virus; HCV, Hepatitis C Virus; HIV, Human Immunodeficiency Virus; RNA, Ribonucleic acid; DNA, Deoxyribonucleic acid; Kbp, Kilo base pairs; Kb, Kilo bases; Mbp, Mega base pairs.

cell, the viral genome is released by degradation of the capsid. Replication; this implies making a copy of the viral genome and the mechanism is dependent on genome type. For viruses with a DNA genome, the host cell's machinery is used to make a copy of the DNA, from the DNA a messenger RNA (mRNA) copy is made in a process called transcription. The mRNA is then used (translation process) for protein synthesis to make new viral capsids. While for RNA viruses, copies of viral (genomic) RNA and mRNA are synthesised. The mRNA is then translated to viral enzymes and capsid proteins. Assembly; the viral particles (viral genome and capsid proteins) are in turn packaged to form new virions. Release; this is the last step of the viral replication process and it is achieved through different means depending on the type of virus. New viruses can be released into the extracellular environment from the cell by any of these processes, apoptosis (programmed cell death), cell lysis (bursting) or budding. The newly released viruses then infect new cells and the cycle continues (Virus Replication, in Fenner's Veterinary Virology (Fifth Edition), 2017) (Figure 2).

CORONAVIRUS AND COVID – 19, WHAT IS THE DIFFERENCE?

Understanding the classification of viruses will assist in answering this question. There are two major classification systems for viruses; the ICTV and Baltimore classification systems. The ICTV classification system of viruses is similar to the classification of other cellular organisms. The ICTV system classified viruses into 15 levels starting from Realm to species. In between these levels are the Family and Genus levels. So, viruses are grouped under these levels as such viruses have 'Family', 'Genus' and 'Species' names. Names of viruses thus end with affixes 'viridae' and 'virus' for family and genus respectively. Therefore, 'Coronavirus' is a genus name of viruses under the 'Coronaviridae' family. The viruses under this family are of two distinguished genera (plural of genus) – the Coronavirus (Alpha, Beta and Gamma) that infects both humans and animals and the 'Torovirus' that infects mostly animals. The Coronavirus was discovered in 1931 and named Corona due to its spiky crown – like architecture or reminiscent of the solar corona as revealed by electron microscopy (Lalchhandama, 2020) (Figure 3). Coronaviruses are large RNA viruses with genome size ranging between 27-33 kilobases and a structural diameter of 80 – 200nm. Notable Human Coronaviruses include Human Coronavirus 229E (HCoV-$_{229}$E), HCoV-OC$_{43}$, HCoV-HKU$_1$, Severe Acute Respiratory Syndrome Coronaviruses (SARS-CoVs) and Middle Eastern Respiratory Syndrome Coronavirus (MERS-Cov). The HCoVs species usually cause common cold in humans second to Rhinoviruses with global distribution (Burrell et al., 2017; Shah et al., 2020). Common symptoms include mild fever, sore throat, rhinitis, and cough. While the SARS-CoV and MERS-CoV are known for causing severe acute respiratory diseases (Burrell et al., 2017; Shah et al., 2020). So, in late December 2019, a new SARS – like disease emerged in Wuhan City of Hubei province in China. The causative agent of this respiratory disease was later identified to be a novel Coronavirus (nCoV) after its genome was sequenced. The nCoV was not among the previously known Coronaviruses causing human infection. The virus was subsequently named Severe Acute Respiratory Syndrome Coronavirus – 2 (SARS-CoV-2) by the ICTV. On the 11th of February 2020, the World Health Organisation (WHO) named the disease caused by the SARS-CoV-2 as Coronavirus disease-2019 (COVID-19) (Vashist, 2020). Therefore, COVID-19 is the respiratory disease caused by the Coronavirus SARS-CoV-2.

Diagnostic assays for COVID -19 and where they are produced

There are many available diagnostic assays for COVID-19

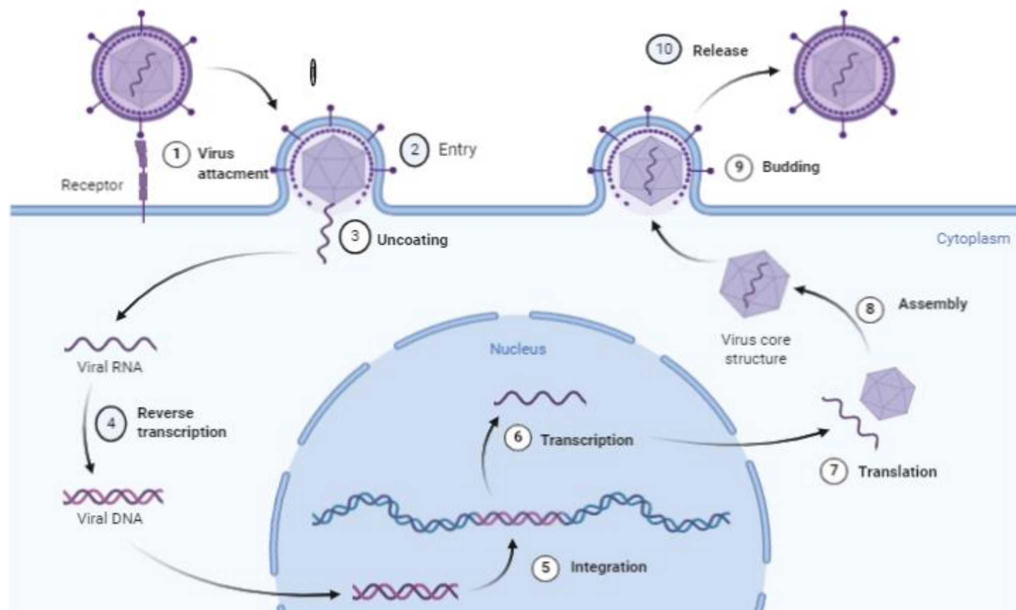

Figure 2. Diagram of viral replication process (BioRender.com: Coronavirus Replication Cycle, 2020a)

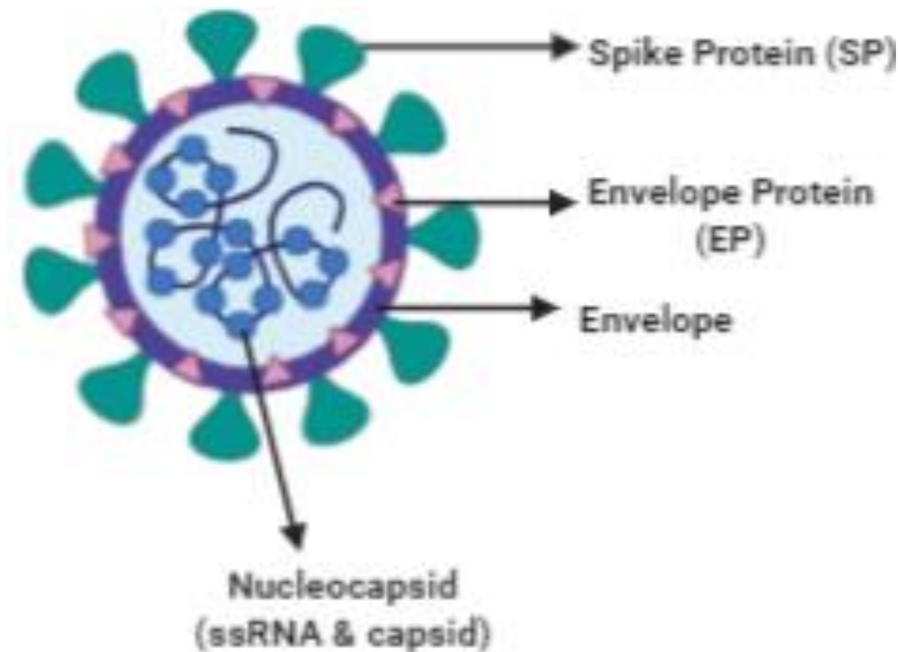

Figure 3. Diagram of a typical Coronavirus (BioRendercom: Coronavirus Replication Cycle, 2020b).

which are either molecular or serological based. Serological methods are those that detect antibodies (IgM and IgG) against SARS-CoV-2 while molecular methods tend to detect viral RNA. However, it is only the molecular method that is recommended by the world health organisation (WHO) as a confirmatory test for COVID-19 (WHO, 2020). It is also worth mentioning that molecular (PCR) methods have been used for the diagnosis of respiratory viruses including other HCoVs with remarkable outcomes (Etemadi et al., 2019). Thus,

Table 2. List of selected different manufacturers of real-time RT-PCR Kits for COVID-19 diagnosis.

S/N	Manufacturer	Viral gene target
1	China, CDC	ORF1b, N
2	India, TruPCR	E, RdRP, N
3	Thailand, NIH	N
4	Japan, NIID	SP, Pancorona & Multiple targets
5	Hong Kong SAR, HKU	ORF1b-nsp14, N
6	Germany, Charité	RdRP, E, N
7	France, IIP	RdRP (2 targets)
8	USA, CDC	N gene (3 targets), RP

CDC; centre for disease control and prevention, E; envelop protein, HKU; the university of Hong Kong, IIP; institut pasteur paris, N; nucleocapsid protein, NIH; national institute of health, NIID; national institute of infectious disease, nsp; non-structural protein, ORF; open reading frame, RdRP; RNA dependent RNA polymerase, SP; spike protein, USA; United States of America.

the confirmatory test approved for COVID-19 is based on the real–time reverse transcriptase polymerase chain reaction (real–time RT-PCR). Real–time RT-PCR for COVID-19 diagnosis is a quantitative test that amplifies targeted viral genes for RNA virus detection. Numerous real–time RT-PCR kits developed by different countries, institutions, and companies are available (FIND, 2020). A few examples of the available kits are given in Table 2.

IS SARS-COV-2 AND OTHER VIRUSES MAN-MADE?

To answer this question, we need to understand the magnitude of virus diversity. Viruses are the most numerous life forms on planet earth. Viruses are found in virtually every single ecosystem on earth. Viruses are present in oceans, lakes, rivers, under the Antarctic ice and in other life forms (Zimmer, 2015). To estimate the numbers of viruses in existence, “try multiplying a billion by a billion then, multiply the product by ten trillion”. The result: 10 to the 31st power “is the mind boggling–number estimate of how many individual viral particles are estimated to populate the planet” (Bruce, 2020). Viruses are also diverse in their composition; they are either RNA or DNA, single stranded or double stranded, linear or circular, and enveloped or naked (non-enveloped). Viruses are of different sizes and structures, different hosts range, mode of transmission and mechanism of replication. Scientists can indeed manipulate microbes and studies have shown how viruses can be chemically synthesised without an existing template. So, improvement in biotechnology and synthetic virology has made it possible to modify and artificially create viruses (Thiel, 2018). Certainly, the process involved in chemical synthesis of viruses is not perfect but successful infectious viruses have been produced using the technique (Thiel, 2018). However, the same advancement in biotechnology has means of verifying modified or artificially created viruses. Comparative genome analysis is a means by which gene sequences are examined and compared between organisms. With comparative genomics, unique and conserved genes among species are identified. This technique can be used to scientifically determine the origin of different organisms. So, to establish whether SARS-CoV-2 was artificially created, Kristian Andersen and colleagues conducted a comparative analysis of available Coronavirus genomic data. Their analysis revealed that SARS-CoV-2 was neither artificially constructed nor was it purposefully modified (Andersen et al., 2020). At this stage, considering the innumerability, diversity and architectural complexity of viruses, it is only rational to say that though it is true that viruses can be man–made, but all the viruses cannot be man-made. For SARS-CoV-2, to date there is no proven evidence that the virus is man-made but it has been proven that the virus is not man–made.

SARS-COV-2 AND BIOLOGICAL WARFARE

To answer this question effectively, there is a need for basic knowledge of biological weapons, the history of biological warfare and biotechnology. Biological warfare (BW), is the use of pathogenic agents or microbes to kill humans, animals or plants (Rao, 2011; Oliveira et al., 2020). The agents that can be used in BW include bacteria, fungi, viruses, insects or biological toxins (Berger et al., 2016; Pal et al., 2017). The BW agents are known as bio–agents or bio–weapons and can be used during war or for bioterrorism. Bio-weapons can be naturally occurring highly infectious agents or artificially created (Berger et al., 2016; Pal et al., 2017). History has shown evidence of BW right from antiquity to the modern ages. Arrowheads, swords, and wells have been poisoned with biological toxins during wars in ancient times. Also, ancient means of BW include sending plague

victims or their dead bodies into the enemy's territories to cause plague epidemics or the use of beasts to attack the opponent (Rao, 2011; Oliveira et al., 2020). In modern history, there have been reports of attempted or alleged use of smallpox by the British army on Native Americans and the Aboriginal population in the USA and Australia respectively (Carus, 2017). During world war II, nations such as the USA, Russia, UK, Canada, Japan, and France established BW centres and successfully weaponised different highly infectious agents for war purposes (Rao, 2011; Carus, 2017). Although some countries never had to use these weapons, some did with some level of success. Japan conducted human experiments of these weapons on prisoners and launched it against Chinese soldiers and civilians (Rao, 2011). Biosecurity concerns were heightened even more with the advent of novel advancements in biotechnology. Advancement in biotechnology has enabled Scientists to manipulate the genetic materials of microbes for different purposes. Improved understanding of genomics and genetic engineering has led to the development of novel vaccines, drugs, modified foods, and beverages. Newer technologies for genome editing such as the Clustered, Regularly Interspaced, Short Palindromic Repeat (CRISPR) system, has made manipulation of micro-organisms even faster and easier (Adli, 2018). Therefore, there is the potential of using the knowledge of these technologies to enhance pathogens for BW. Although natural pathogens can indeed be used for BW, it is equally possible to enhance the efficiency of these pathogens using genetic engineering (Berger et al., 2016; Pal et al., 2017). Also, as stated above, viruses can be chemically synthesised (Thiel, 2018). However, for any pathogen to qualify as an ideal bioweapon it must be highly infectious, lethal, efficiently transmissible, environmentally robust, and massively produced with sustained pathogenicity (Clark and Pazdernik, 2016). Additionally, treatment or vaccine for the agent must have been established by the developers of the bio-agent for their protection. These features thus, make a bioweapon or BW a highly dangerous concept with unimaginable consequences. In view of the dare consequences of Bio-agents and BW, a proposal for banning of bioweapons was presented to the United Nations (UN) by selected countries. Accordingly, in 1969, the then US president Richard Nixon made a statement that bioagents are "repugnant to the conscience of mankind" and it has the potential to "cause massive, unpredictable and potential uncontrollable consequences" (Rao, 2011). This proclamation by Nixon, led to the termination of the US BW programme and the destruction of all its existing bioweapons. Accordingly, the proposal presented to the UN led to the Biological Weapon Convention (BWC); a treaty that came into force in 1975 and so far, over 180 nations have either ratified or acceded to it (Geneva, 2020). The BWC bans the development, creation and stockpiling of all forms of bioweapons and provided for the destruction of the existing ones (Rao, 2011). Finally, bioweapons and BW are possibilities, but SARS-CoV-2 couldn't have been a bio-agent or an act of BW due to three reasons. Firstly, BW contravenes international laws as stipulated by the BWC and ratified by over 180 nations. Secondly, the devastating outcome of a BW on a global scale is unimaginable and is too much of a risk for any country to contemplate. Thirdly, SARS-CoV-2 does not meet the requirement of an ideal bioweapon. Although it is highly infectious, the disease mortality is less than 4% and no country seems to have any available cure or vaccine for the disease.

VIRUS EPIDEMICS AND ELECTROMAGNETIC FIELD (EMF), IS THERE A RELATIONSHIP?

EMF is simply defined as waves propagated or radiating through space carrying radiant energy (Funk et al., 2016). The EMF is thus, a spectrum of waves (fields) that includes gamma rays, X-rays, ultraviolet radiations, visible light, infrared, microwaves, radio waves and extremely low frequencies (ELF) in decreasing order of frequency and increasing wavelength (Funk et al., 2016). The energy emitted by each field is directly proportional to its frequency. This implies that high frequency waves have greater energy while low frequency fields have less energy (Funk et al., 2016). EMFs with high frequencies thus can directly cause cellular damage. Remember that biological molecules present in cells include DNA and RNA. The high energy EMFs achieve cellular damage by breaking water molecules to produce reactive oxygen species (ROS) known as free radicals. As a result, they are called ionizing radiations. These waves can damage DNA molecules causing mutations that can lead to cancers. On the other hand, the low frequency waves are non–ionizing because they do not lead to the production of free radicals. However, since all EMFs are packets of energy, they can generate heat at sufficient levels (intensity). A perfect example of this is the heat generated by the microwave oven. Therefore, low frequency waves can generate heat leading to cell damage. Now, the critical issue to be resolved is to establish whether non–ionizing radiation at low intensities can cause cell damage or lead to health hazards. Over the years, scientists have conducted association studies to link low frequency EMFs to various health risks including cancers (Carpenter, 2013; Miller et al., 2019). Also, there are correlation studies linking quantum leap in EMF usage in history to virus epidemics (Lauer, 2015). These correlation studies and the escape theory of virus origin explained earlier are the reason given for linking non–ionizing radiation to virus disease outbreaks. However, it is important to note that correlation or association does not mean causality. Proving causality effect needs empirical evidence and a strong mechanism of causation. On this note, it is safe to say that there is no

identified mechanism by which low energy EMFs at sub-thermal intensities induce cellular damage resulting to cancer or ill health. Nevertheless, there is the need for more research to decipher this lingering theory.

CONCLUSION

As the world battles against SARS-CoV-2, the need to clear arising misconceptions cannot be underestimated. Understanding the fascinating world of viruses will certainly help in the fight against the COVID-19 pandemic. However, it is worth stating that within the last four decades, the world has witnessed outbreaks of viral infectious diseases. From HIV-AIDS, SARS, MERS, Zika, H1N1 influenza to Ebola and now the COVID-19 pandemic. This goes to show that viruses and particularly zoonotic RNA viruses are major sources of emerging and re–emerging infectious diseases. Therefore, it is of utmost importance for the world to unite with a common goal of fighting the scourge of these emerging infectious diseases and prepare for subsequent pandemics.

Data availability statement

Data sharing not applicable to this article as no data were used to support this study.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Evaluation of epidemiological factors of the bacterial pharyngitis in children in Shouk Al-Kameesh, Al-Khoms, Libya

Ishtaiwi Y. M.[1], Alghazal M. A.[2], Nouh A. M.[3*], Abousittash H. B.[4] and Tamalli M.[5]

[1]School of Basic Sciences, Libyan Academy, Misrata, Libya.
[2]Faculty of Medical Technolgy, Libyan Academy, Misrata, Libya.
[3]Faculty of Natural Science, University of Western Cape, South Africa.
[5]Faculty of Science, University of Elmergib, Khoms, Libya.

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Pharyngitis is an inflammation or irritation of the pharynx or palatine tonsil. The etiology is usually viruses which cause the majority of the pharyngitis but most bacterial cases are attributed to group A *Streptococci* (GAS). This study aimed to identify the frequency of bacterial pharyngitis especially GAS of children, according to epidemiological factors. Symptoms present and signs for each patient were recorded, following which a throat swab was collected. There were 237 cases of children aged between 1 and 14 years with inflammation; out of 394 cases that visited the ENT primary care at Souk Al-Kameess during the 12 months study period. From the incidence of 394 sore throat cases, GAS was observed in 237 cases (60.15%). Of these 110 (46.41%) were girls and 127 (53.59%) were boys. A higher proportion of children were found with GAS pharyngitis aged 5-9 and the lowest incidence of sore throat occurrence was 3-4 years. The highest incidence of children pharyngitis was recorded in January (Winter) and September (Autmn). The following factors showed independent positive correlation with GAS sore throat infection; patients visiting, months, year season, tonsillectomy, culture result, catalase test result and bacitracin test result with significant value of $P<0.01$, whereas sex, age and gram stain results with $P> 0.01$. In conclusion, the results of this study found that cases of bacterial sore throat were higher than non-bacterial sore throat. The main cause of bacterial pharyngitis was GAS. Therefore, this study suggested that the use of the effectiveness of antibiotics and recommended anti-bacterial pharyngitis therapy to prevent initial rheumatic fever and its complications.

Key words: Pharyngitis, tonsillitis, epidemiological factors, *Streptococcus,* children.

INTRODUCTION

The most common etiology for Pharyngitis is by viral upper respiratory transmission. However, inflammatory processes of the nasopharynx are pharyngitis and tonsillitis can be caused by bacteria (Lin et al., 2003). The bacteria that cause pharyngitis and tonsillitis are beta-hemolytic *Streptococcus* and *Staphylococcus aureus*

*Corresponding author. E-mail: alinouh12@yahoo.com.

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(Bhalla et al., 2019). Bacteria cause erythema of the nasopharynx mucosa along with an exudative membrane (Leung and Kellner, 2004).

Most bacterial cases are due to group A *Streptococci* (GAS) infection (Van Schoor, 2013). Other causes include allergic reaction, trauma, toxin and neoplastic (Hildreth et al., 2015). Symptoms may include a sore throat, fever, headache and a hoarse voice (Rutter and Newb, 2015). G*onorrhea*, fungus, irritants such as smoke, allergies and gastro esophageal reflux cause Pharyngitis. Pharyngitis occurs mostly by people contact with nasal discharge, rather than by oral contacts (Kirkpatrick, 1996). Approximately 7.5% of people have a sore throat in any full three-months (Principi et al., 2013).

Identifying the cause of pharyngitis, especially group A Beta-Hemolytic *Streptococcus* (GABHS), is important to prevent the potentially life-threatening complications (Singer, 2001). Strep throat is a bacterial infection, which is the cause in about 25% of children and 10% of adults (Hildreth et al., 2015). An estimated 616 million cases of GABHS pharyngitis occur annually worldwide (Carapetis et al., 2005).

Rapid diagnosis and treatment of pharyngitis and tonsillitis patients with unnecessary antibiotic prescriptions in primary health care center lead to high costs and increased risk of antibiotic-resistance. Pharyngitis and its epidemiological factors are a public health problem. Since there is no such study in this area, this research was done in Souk Al-kamees, Libya. The aim of this study was: (i) to identify the frequency of childrens' pharyngitis in Souk Al-kamees, Libya, (ii) to study the epidemiological factors of pharyngitis according to gender, age, seasonal variation, history of tonsillectomy and associated disease among the survey population, and (iii) to identify the bacterial pharyngitis especially GABH among the study population.

METHODOLOGY

Study design

A study designed to be prospective of suspected cases of pharyngitis. The preliminary data collected by questionnaire from Souk Al-Kamees outpatient clinic, Alkhoms, Libya. The study was conducted for 12 month (February 2018 - January 2019). The study population age ranged between 1 and 14 years of those attending Souk Al-Kamees outpatient clinic with a suspected case of pharyngitis with throat-related complaints (sore throat).

Moreover, patient demographics clinical data include age, sex, race, blood sample, and patient symptoms were recorded. The samples were collected by the throat swab from just above the tonsils of an infected throat of the patients. The sample was immediately placed in a nutrient broth and incubated at 37°C for 24 h. The samples were transported to the laboratory for culture and identification.

Methods

The collected swabs from the patients were cultured on the Blood agar and incubated at 37°C in an anaerobic incubator for 24 to 48 h. The colonies were tested with Gram stain for morphology and identification (Biomaghret, Tunisia). Biochemical tests like catalase test were done with the slide test for the identification of Group A beta hemolytic *Streptococcus*. A bacitracin disc was added to a plate of blood agar to find the GABH sensitive to bacitracin (huge inhibition occurred around bacitracin disk). *Streptococcus pyogenes* are sensitive to bacitracin (Pires et al., 2009). The ASO serological methods like latex agglutination for the qualitative and semi-quantitative measurement of antibody to anti streptolysin-O in human serum were done (Biomaghret, Tunisia). The collected data were analysed by SPSS software version 1.3 and significance level of $P< 0.01$.

RESULTS

This study was done for a period of 12 months from February 2018 to January 2019 on 394 children. But 237 were found with acute pharyngitis in targeted-age (1-14 years). Samples were taken in Souk Al-Kamees Primary Clinic, throat swab were collected from patients for blood culture and biochemical tests and the blood samples were taken for Anti Streptolysin-O titre.

Frequency of sore throat cases from the total number of patients

Total number of patients who visited the clinic with age bracket of 1 to 14 years were 394. Table 1 shows the percent of sore throats from the total number of visited patients was 60.15%. Significant relationship between sore throat cases and the number of patients visiting primary health care units by $P<0.01$. Observed and expected counts of infected patients are as shown in Figure 1. The result indicates that sore throat is one of the most common reasons for visits to the clinic.

Frequency of sex and age (as age interval)

Table 2 shows age range 5-9 years, is the most affected by sore throat with 43.46% and age range 10-14 years is less affected by 14.77%. In females, the most affected by pharyngitis is age range 1-4 years by 44.55% and in male the most affected by pharyngitis is age range 5-9 years with 44.09%. The results also show that the cases of sore throat in males by 53.59% were higher than females by 46.41%. Figure 2 shows the shape of the distribution of the age by sex of the normal curve. No significant relation was found between age and sex and occurrence of pharyngitis in children by $P>0.01$ (Table 3).

Occurrence pharyngitis in children according to months

Table 4 shows that during the different months of this study; January and September have the highest rate of

Table 1. Frequency of sore throat with total number.

Infection	Count	Percent	Chi-Square	P-Value
No (absent)	157	39.85		
Present	237	60.15		
Total	394	100	16.2437	0.000

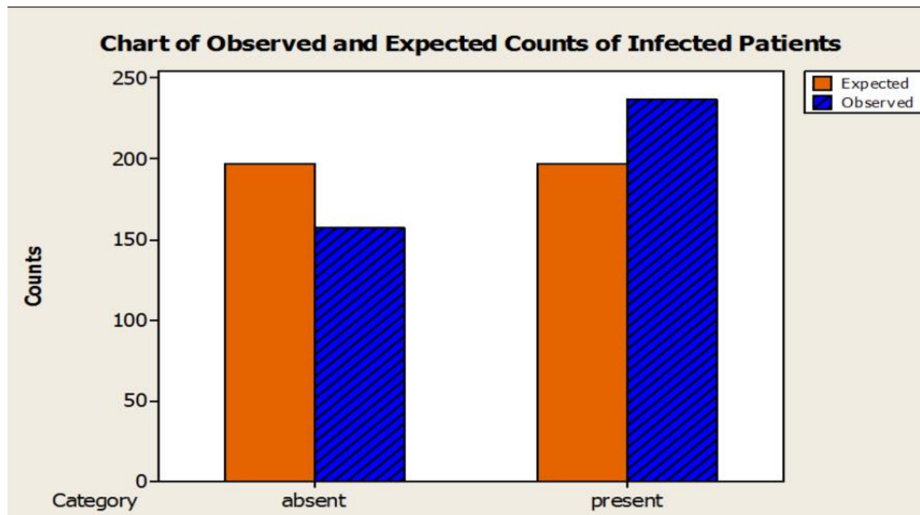

Figure 1. Observed and expected counts of infected patients.

Table 2. Frequency of sex and age (as age interval)

Sex	Age(1-4)	Age(5-9)	Age(10-14)	All
Female	49 (44.55%)	47(19.88%)	14(5.91%)	110 (46.41%)
Male	50(21.10%)	56(44.09%)	21(8.86%)	127 (53.59%)
All	99(41.77%)	103 (43.46%)	35 (14.77%)	237 (100%)

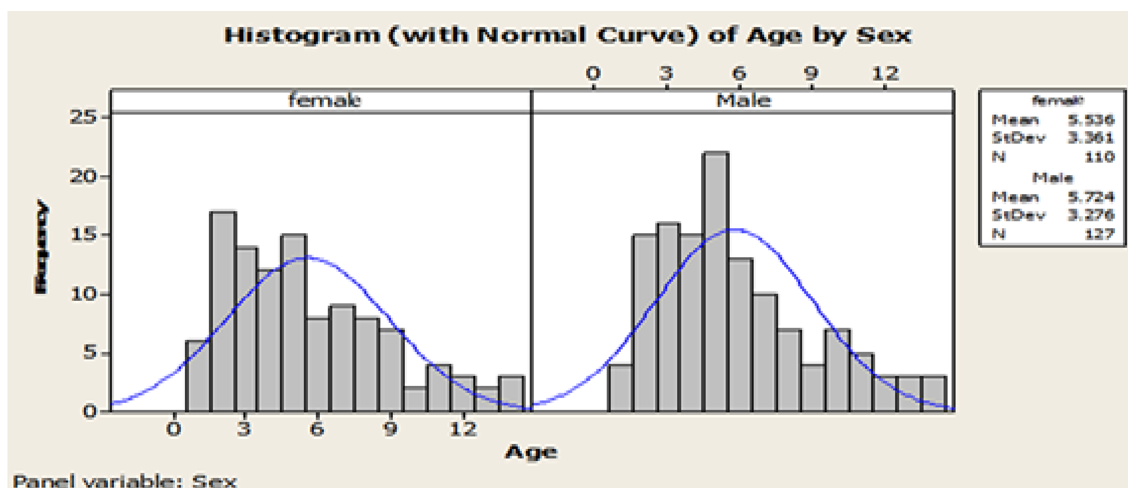

Figure 2. Normal curve of age by sex

Table 3. chi-square goodness-of-fit test for categorical variable, sex and age (as age interval).

Patient	Chi-Square	P-Value
Pearson	0.982	0.612
Likelihood Ratio	0.987	0.611

Table 4. Occurrence and frequency of pharyngitis in children according to months.

Month	Frequency	Percent	Chi-Square	P-Value
Jan	39	16.5		
Feb	14	5.9		
Mar	7	3		
Apr	9	3.8		
May	19	8%		
Jun	10	4.2		
Jul	10	4.2		
Aug	20	8.4		
Sep	33	13.9		
Oct	30	12.7		
Nov	26	11		
Dec	20	8.4	60.3671	0.000

Table 5. Occurrence of pharyngitis in children according to season.

Season	Frequency	Percent	Chi-Square	P-Value
Winter	73	30.8		
Spring	35	14.8		
Summer	40	16.9		
Autumn	89	37.5		
Total	237	100.0	34.308	0.000

acute pharyngitis in children by 16.5% and 13.9% respectively. While the lowest rate of acute pharyngitis in children in March and April by 3 and 3.8%, respectively, showing significant relation between occurrence pharyngitis in children and months by P- < 0.01.

Occurrence of pharyngitis in children according to season

Table 5 shows that acute pharyngitis in children peaked twice during autumn by 37.5% (November, October and September) and winter by 30.8% (December, January and February), showing a significant relation between occurrence pharyngitis in children and season by P- < 0.01.

Occurrence of pharyngitis in children according to symptoms

Table 6 shows that fever, cough, and running nose were the highest symptoms among children with acute pharyngitis (64% of fever, 47.7% of cough and 43% of running nose).

Relationship of occurrence of acute pharyngitis in children and tonsillectomy

Table 7 shows that only 23 patients from total of 237 patients had tonsillectomy. Most cases had viral sore throat; this means the tonsillectomy operation reduced GAS pharyngitis episodes in children. Showing significant relationship between occurrence of pharyngitis in children and operation of tonsillectomy by P < 0.01.

Occurrence of pharyngitis in children according to ASO titer and culture result

Table 8 shows that the ASO titer positive result associated with pharyngitis (GAS) by 95.42% more than anther causes of pharyngitis (SPP) by 0.92%. It also

Table 6. Occurrence of pharyngitis in children according to symptoms.

Symptoms	Frequency	Percent
Cough	113	47.7
Running nose	102	43.0
Fever	152	64.1
Enlarged LN	81	34.2
Nausea vomiting-abdominal pain	69	29.1
Diarrhea	69	29.1
Odynophagia	68	28.7
Hoarseness of voice	61	25.7
Enlarged tonsils	92	38.8

Table 7. Relationship of occurrence and frequency of acute pharyngitis in children and tonsillectomy.

After tonsillectomy	Frequency	Percent	Chi-Square	P-Value
Absent	214	90.3		
Present	23	9.7		
Total	237	100	153.928	0.000

Table 8. Occurrence and frequency of pharyngitis in children according to ASO titer and culture result.

ASO titer	GAB	SPP	No bacteria	All	Chi-Square	P-Value
Positive	104 (95.42%)	1 (0.92%)	4(3.67%)	109		
Negative	4(3.13%)	10(7.81%)	114(89.10%)	128		
All	108(45.57%)	11(4.64%)	118(49.79%)	237	202.275	0.000

Table 9. Occurrence and frequency of pharyngitis in children according gram stain result.

Gram stain	Frequency	Percent	Chi-Square	P-Value
Nigative	118	49.8		
Positive	119	50.2		
Total	237	100.0	0.0042194	0.948

shows positive result with no bacteria by 3.67% in some carrier cases. Showing significant relation (P- < 0.01) between positive result of ASO titer in this study and pharyngitis by GAB.

Occurrence pharyngitis in children according to gram stain

Table 9 shows Gram positive bacteria as a cause of acute pharyngitis in children such as GAS that is most common then SPP. Showing No significant relationship between bacterial pharyngitis frequency in children and nonbacterial pharyngitis according to Gram stain results by P > 0.01.

Occurrence pharyngitis in children according to catalase test

Table 10. shows catalase tests, more negative than positive results, which means that bacterial pharyngitis affects children with GAS type more than SPP. Non-bacterial samples were not tested. Showing significant relation (P- < 0.01) between bacterial pharyngitis and catalase test results. This result of catalase test can be used to differentiate between two types of Gram-positive bacteria in this study.

Table 10. Occurrence and frequency of pharyngitis in children according to catalase test result.

Catalase test	Frequency	Percent	Chi-Square	P-Value
Negative	106	44.7		
Not done	119	50.2		
Positive	12	5.1		
Total	237	100.0	74.9831	0.000

Table 11: Occurrence pharyngitis in children according bacitracin test result.

Bacitracin test	Frequency	Percent	Chi-Square	P-Value
Not done	119	50.2		
Not sensitive	11	4.6		
Sensitive	107	45.1		
Total	237	100.0	263.329	0.000

Occurrence pharyngitis in children according to bacitracin test

Table 11 shows the bacitracin test result indicating that the sensitive results are more than non-sensitive tests. This result shows bacterial pharyngitis in children by GAS is more sensitive than SPP in this study. Non-bacterial samples were not tested. Showing significant relation (P-< 0.01) between bacterial pharyngitis and bacitracin test results. So bacitracin test can be used to differentiate between two types of Gram-positive bacteria in this study.

DISCUSSION

Sore throat is still the most common reason for visits to physicians. In the present study the percent of sore throat from total number of visitor equals 60.15%. Similar result was found by Bisno et al. (2002) in America and also in another study by Linder and Stafford (2001).

In this study, the culture result shows the occurrence of bacterial pharyngitis in children was more than nonbacterial pharyngitis by 50.2%. Results have shown that the pharyngitis by GAS is the most common as a bacterial cause of pharyngitis in children by 45.6%. Previous studies have shown exudative pharyngitis in children with acute *Streptococcal* infection 86% in Spain (Baquero et al., 1999) and 54% in Latvia (Zavadska, 2010). Another study showed that the GAS disease prevalence ranged from 21.4 to 27% among children aged 1-15 years in northern Taiwan (Lin et al., 2003). In contrast, a study carried out by Chi et al. (2003) showed that virus caused most acute pharyngitis and that GAS pharyngitis was uncommon in Taipei and Jos, Nigeria. Previous reports have also shown that *Streptococcal* throat carriage rate was 9.78% amongst primary school pupils in Benin, Nigeria (Sadoh, 2001).

The highest occurrence rate was in the 5-9 years age group. This might be due to high exposure to the outer environment and lack of awareness. Several previous studies show similar results as pharyngitis is the highest at age 5-15 year by 37% in England (Bisno, 1996; Pichichero, 1997; Pickering et al., 2000). Evidence suggests that the prevalence of infection in this age-group may be due to the increased activity of children, giving a higher chance for the exposure to infection (Saleh, 2009). Besides, this school-age children may be making contact with each other in classrooms leading to increased infections. In the current study, an occurrence of pharyngitis in children among boys was 53.6%, compared to 46.4% in girls. A study in Sabha, Libya done by Soliman (2012) showed the pharyngitis infection rates for males was 64.3% and that of females was 35.7%. In contrast, a study in Syria found that distribution of pharyngitis in male was 47.4% and female was 53.6% (Cherith et al., 2005).

A monthly comparison showed that January (16.5%) and September (13.9%) contain the highest rate of occurrence of acute pharyngitis in children. While the lowest rate of occurrence of acute pharyngitis in children occurred in March (3%) and April (3.8%). In addition, the acute pharyngitis in children peaked twice during year in autumn 37.6% (November, October and September) and winter by 30.8% (December, January and February). This results may be due to children staying mostly indoors in crowded conditions, which may increase the rate of transmission of infection. In a similar study in Benghazi by Alhouni (2005), the peak incidence of infection was reported in winter and autumn. In contrast, previous studies in Taiz/Yemen by Saleh (2009), found the highest prevalence during winter season (November and December) as well as rainy season (July and August). In north India, a study by Nandi et al. (2001) showed that

the relative incidence was higher during the winter (November to January) and rainy (August) months.

In the current study, results have shown that fever, cough, and running nose were the highest symptom among children with acute pharyngitis (64% of fever, 47.7% of cough and 43% of running nose). A study in Benin city showed fever and inflamed tonsils/pharynx were the most frequent signs or symptoms occurring in 89.04 and 87.67%, respectively (Wilson, 2008). Another study in Latvia showed pharyngeal erythema and fever were prevalent up to 90 and 85%, respectively (Zavadska, 2010).

The results of the study showed that only 23 patients out of the total number of patients have tonsillectomy, usually a viral sore throat, which means that the operation of tonsillectomy reduces the occurrence of pharyngitis in children. Similar to a study in Finland by Alho et al, (2007) reported that the tonsillectomy reduces the number of acute pharyngitis and symptoms.

Antistreptolysin-O (ASO) test is an accurate diagnosis of pharyngitis and, is necessary to rule out group A streptococcus (Vintilescu et al., 2020). In this study, the positive result of ASO titer is 46%; this percent represents previous infection by GAS and negative result by 54%. In similar study by Stollerman and Dale (2008), the ASO titer significantly elevated over normal levels during a *Streptococcal* infection. Therefore, the ASO titer is the standard serological assay for confirmation of Group A *Streptococcal* infection.

Conclusion

The study indicated both female and male have occurrence of pharyngitis in this age range (1-14 years), but no significant difference between the gender. The age range 5-9 years is the most affected with sore throat in patients. The January and September months contained the highest rate of occurrence of acute pharyngitis in children while the lowest rate of occurrence of acute pharyngitis in children was in March. Occurrence of acute pharyngitis in children peaked twice during the year in autumn and winter.

The clinical features like fever, cough, running nose were the highest symptoms among children with acute pharyngitis. Appearance of bacterial pharyngitis in children is more than non-bacterial pharyngitis and the most common is bacterial pharyngitis (GAS) in children. The positive results of ASO titer associated with pharyngitis by GAB are more than other causes of pharyngitis. However, this study shows two-Gram positive bacteria as a cause of bacterial pharyngitis in children, such as GAS, that is, the most common and *Staphylococcus* species.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Isolation, identification and growth conditions of calcite producing bacteria from urea-rich soil

Noor E. S. Abu Tayyem[1,2*], Khadeja T. M. Elhello[1,2] and Abdelraouf A. Elmanama[1]

[1]Medical Laboratory Science Department, Islamic University in Gaza, P. O. Box 108, Gaza, Gaza Strip, Palestine.
[2]UCAS Technology Incubator (UCASTI), P. O. Box 1415, Gaza, Gaza Strip, Palestine.

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Bacterial chemical reactions, such as urea hydrolysis can induce calcium carbonate precipitation. The induced production of calcium carbonate formed by microorganisms has been widely used in environmental and engineering applications. The present study aimed to isolate, identify and optimize growth conditions of urease positive bacteria from urea rich soil in Gaza Strip. Bacterial isolates, which tolerated ≥10% urea concentration, were selected for the investigation. Eight isolates recovered and identified to be spore forming, urease positive, alkaliphile, halotolerant, and presumptively belonged to *Bacillus* species. All isolates showed best growth at temperature 37°C, and pH 9-9.5. After exposure to UV irradiation, most isolates showed improved tolerance to urea concentration, however, other strains showed a decline in their adaption to urea concentrations. The mutant form of isolate in soil sample #3 showed the highest tolerance to urea concentrations at all exposure intervals, when compared with wild type. Moreover, all isolates precipitated calcium carbonate. The locally recovered isolates are promising contributors in the process of calcite Biomineralizaion and may be utilized in the remediation of concrete cracks, increase of compressive strength of concrete, decrease water permeability, and solve the problems of soil erosions.

Key words: Calcite bio-mineralization, microbial induced calcium carbonate precipitation (MICP), urease, *Bacillus* spp., Gaza strip.

INTRODUCTION

Biological precipitation of minerals (Bio-mineralization) is a widespread phenomenon in the microorganism's world, and is mediated by bacteria, fungi, protists, and even by plants. Calcium carbonate (Calcite) is one of those minerals that naturally precipitate as a by-product of microbial metabolic activities (Seifan and Berenjian, 2019).

Microbial metabolic activities facilitate calcium carbonate (calcite) precipitation, in a well-studied process called microbial induced calcium carbonate precipitation (MICP) (Zambare et al., 2019). MICP usually occurs due to the chemical alteration of the environment induced by the microbial activity (Sarikaya, 1999; Stocks-Fischer et al., 1999; Warren et al., 2001; De Muynck et al., 2010a). Bacteria can be invested as a major player in the MICP phenomenon through various mechanisms. The most significant mechanism is the bacterial ureolytic activity (Stocks-Fischer et al., 1999; Warren et al., 2001; Krajewska, 2018). Urea hydrolysis can be facilitated by

*Corresponding author. E-mail: nooreabutayyem@gmail.com. Tel: 00970594157573.

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bacteria that can produce urease (urea amidohydrolase) enzyme and thus are able to induce $CaCO_3$ precipitation (Stocks-Fischer et al., 1999; Hammes and Verstraete, 2002; Phillips et al., 2013; Bhaduri et al., 2016). Calcium carbonate precipitation is a chemical process controlled mainly by four key factors: (1) calcium ions concentration, (2) dissolved inorganic carbon (DIC) levels, (3) the pH, and (4) the availability of nucleation sites (Hammes and Verstraete, 2002; Seifan and Berenjian, 2018).

Over recent years, MICP has received considerable attention and has been proposed as a potent solution to address many environmental and engineering issues (Seifan and Berenjian, 2019). It has been intensely investigated in bulk systems, sand columns (Dhami et al., 2013a; Seifan et al., 2016; Tziviloglou et al., 2016), and bio-cementation processes (Seifan et al., 2016). It has been found that MICP may drive many potential applications in civil engineering such as enhancing stability of slopes and dams, reducing the liquefaction potential of soil, road construction, prevention of soil erosion, increase durability and compressive strength of concrete, as well as the repair of the cracks in concrete (De Muynck et al., 2010a; Stabnikov et al., 2011, Shashank et al., 2016).

Many bacterial species have been studied to exploit their abilities in the biomineralizing of calcite (MICP). One of the most robust ureolytic bacteria is *Sporosarcina pasteurii* (formerly known as *Bacillus pasteurii*). *S. pasteurii* is facultative anaerobes, spore forming, bacilli bacteria. It utilizes urea as an energy source and eliminates ammonia which increases the pH in the environment and generates carbonate, causing Ca^{2+} and CO_3^{2-} to be precipitated as $CaCO_3$ (Clive, 1990, Stocks-Fischer et al., 1999; Chahal et al., 2011). Other studies showed the role of bacteria that are mostly related to Bacillus spp. in the process of MICP (Stocks-Fischer et al., 1999; Elmanama and Alhour, 2013; Ali et al., 2020). The aim of this study is to isolate, identify, and optimize growth conditions of locally isolated urease-producing bacteria that are able to produce calcite crystals.

MATERIALS AND METHODS

Sample sources and characteristics

This study utilized soil samples varying in urea content from Gaza Strip. About 100 g of each sample were collected during June 2020 from the following sources: (1) Sea coastal sand from Rafah city beach, (2) outlet sewage water (treated sewage), (3) inlet sewage water (untreated sewage), (4) soil sample with cat`s urine from Gaza city, (5) coastal sand with dog`s urine from Rafah city beach, (6) sand with dog`s urine from Rafah city, (7) agricultural soil with dog`s urine from Gaza city, (8) agricultural soil with dog`s urine from Rafah city, (9) urea rich soil from greenhouse in Gaza city, and (10) ammonia rich soil from a greenhouse in Gaza city.

Sample processing and bacterial isolation

Five grams of soil samples were mixed with 20 ml sterile saline (stock suspension) and dilutions 10^{-1} and 10^{-2} were made. A volume of 0.1 ml of the stock suspension as well as the two dilutions were cultured onto 2 and 3% urea containing Nutrient Agar (NA) plates (HiMedia, India). The media were prepared according to HiMedia manufacturer recommendations. Extra pure urea suspensions (Honeywell Riedel-de Haen, Germany) were filtered, then added to media after autoclaving and cooling to 50°C. Cultures were incubated at 37°C, and plates were examined after 24 and again after 48 h.

Bacterial tolerance to high urea concentration

Bacterial isolates were obtained as a pure culture and then cultured on 5, 8, 10, 12, and 15% urea enriched NA media, incubated at 37°C for 48-72 h, and after the incubation period bacteria were harvested to be cultivated in nutrient broth and agar plates. Bacterial isolates tolerated ≥ 10% urea concentration were selected for further testing.

Bacterial identification and biochemical characterization

The selected isolates were Gram stained (Liofilchemm Italy), spore position was identified, growth at basic media under normal conditions and at 45°C has been evaluated, and tested for oxidase, catalase, urease, O-Nitrophenyl-β-D-Galactopyranoside (ONPG), arginine dihydrolase (ADH), lysine decarboxylase (LDC), ornithine decarboxylase (ODC). Isolates were also inoculated into Triple sugar iron agar (TSIA), Citrate agar, Sulfide-Indole-motility media (S.I.M), and starch agar (HiMedia, India). Isolates were also tested for gelatin liquefaction, Voges proskauer (VP), and sugar fermentation tests adopted from API-20E.

pH profile

The isolates were inoculated into 3 ml of Nutrient Broth tubes with pH scale of 1 to 14. A bacterial suspension was made and the turbidity was adjusted to 0.5% McFarland standard, incubated for 24 h at 37°C, and growth has been measured as turbidity at O.D 600 nm using CT-2200 spectrophotometer (Chrom Tech, Taiwan). Results were recorded against a blank of bacterial suspension. 1N HCl (HiMedia, India) and 1N NaOH (Frutarom, Palestine) were used to adjust the pH. An additional nutrient broth tubes at pH 7 were inoculated with the bacterial isolates, incubated at 37°C, and the change in pH was monitored during growth, using a pH meter (Jenway pH Meter 3510 /mV, USA) results were recorded after 30 min, 1 , 2, 4, 8, 24, and 32 h of inoculation.

Effect of sodium chloride concentration on growth

Bacterial isolates were inoculated onto Yeast Extract agar (HiMedia, India), that was supplemented with 0.2, 0.5, 0.8, 1, 1.5, 2, 3 and 5% NaCl (HiMedia, India). Plates were observed for growth after 24 and after 48 h of incubation at 37°C.

Effect of temperature on growth

Bacterial isolates were inoculated into nutrient broth tubes (HiMedia, India), adjusted to 0.5% McFarland standard, incubated for 24 h at 0, 4, 25, 37, 45 and 60°C, and the turbidity was measured using spectrophotometer at O.D 600 nm.

Ultraviolet (UV) induced mutagenesis for bacterial isolates

The selected isolates were grown overnight in NB + 2% urea in a

Table 1. Bacterial isolates and their tolerance to various concentrations of urea.

Isolate no.*	% Urea tolerated				
	5	8	10	12	15
1	+	-	-	-	-
2	+	-	-	-	-
3	+	+	+		
4	+	-	-	-	-
5	+	+	-	-	-
6	+	+	-	-	-
7	+	+	+	+	+
8.1	+	+	+	+	-
8.2	+	+	+	+	-
8.3	+	+	+	+	+
8.4	+	+	+	+	-
9	+	+	+	-	-
10.1	+	+	+	+	+

*Isolate number correspond to the soil sample number. + = Growth and - = No Growth

shaking incubator (Boeco, Germany) at 37°C. The isolates were washed three times with sterile phosphate-buffered saline, re-suspended in urea free and sterile NB. The turbidity of cell suspensions was adjusted to a 0.5% McFarland reagent and exposed to UV light using a Philips 20 W germicidal lamp for 2-20 min with 2 min intervals. From each exposure interval, a loopful of the exposed bacteria was cultured onto urea-based agar (HiMedia, India). After incubation of 24 h at 37°C, a single, well-defined colony was chosen, cultivated on NA plates, and then inoculated onto NA with varying urea concentrations; 5, 8, 10, 12 and 15% respectively. After incubation, bacterial growth was observed and compared to wild type growth on the different urea concentrations.

Mini-scale of calcium carbonate precipitation

Bacterial isolates were subjected to calcium carbonate production test as described previously (Ghosh et al., 2019). Alive bacterial isolates were inoculated into nutrient broth (NB) containing both urea and calcium chloride (NBUC), NB with only urea (NBU), and NB with only calcium chloride (NBC). The same procedures were repeated with autoclave killed bacterial suspension (pellet and supernatant filtrate). To all tubes, a concentration of 0.012 g/L phenol red was used as a pH indicator. NBUC and NBU were prepared to contain 2% of urea. NBUC and NBC were prepared to contain 2.8 g/L of calcium chloride. Urea and calcium chloride solutions were filter sterilized and separately added to phenol red containing NB before bacterial inoculation. A urease enzyme reagent obtained from Blood urea nitrogen kit (Biosystems, Spain) was used as a positive control, while *Escherichia coli* ATCC 25922 (urease negative) was used as a negative control. Non-inoculated tubes were used as a validity control. All tubes were incubated at 37°C for 24 h. The trial was performed in triplicate.

RESULTS

Bacterial isolates characteristics

After the incubation of soil samples cultures, one to four colonies of isolated bacteria were selected from those plates containing few and well isolated colonies. Colonies were creamy white or pale yellow to bright orange colored and slightly convex with an entire margin.

Selection of the suitable urease producing isolates

By culturing all isolates on 5, 8, 10, 12, and 15% urea agar media, those that tolerated ≥ 10% urea concentration has been chosen to proceed with. Table 1 shows the highest concentration of urea that each isolate tolerated. Thus, eight isolates: 3, 7, 8.1, 8.2, 8.3, 8.4, 9, and 10.1 were selected for further testing.

Bacterial Identification and biochemical characterization

All selected isolates were spore-forming, Gram-positive bacilli, catalase and urease positive. Table 2 shows the phenotypic characteristics of the eight isolates and indicates the biochemical tests that have been used in the identification process. ABIS online Software has been used in bacterial identification (Costin and Lonut, 2017). Table 3 shows the presumptive identification of the selected isolates according to the ABIS online Software.

Growth conditions

The optimal pH at which all selected isolates showed the highest turbidity and rapid growth ranged from 7 to 10, with a preference to the pH 9 (Figure 1), thus all tested isolates are moderate alkaliphiles. For most isolates, the

Table 2. Selected biochemical tests for isolates identification.

Test \ Bacterial Isolates		3	7	8.1	8.2	8.3	8.4	9	10.1
Gram stain		+	+	+	+	+	+	+	+
Spore position		C	T/S	T/S	T/S	C	T/S	C	C
Growth on Usual media		+	+	+	+	+	-	+	+
Growth at 45◦ C		+	+	+	+	+	+	+	+
Oxidase		+	+	+	+	+	+	+	-
Catalase		+	+	+	+	+	+	+	+
Urease		+	+	+	+	+	+	+	+
ONPG		-	-	-	-	+	-	-	-
ADH		-	-	-	+	-	-	-	+
LDC		-	-	-	-	-	-	-	-
ODC		-	-	-	-	-	-	-	-
H_2S production		-	-	-	-	-	-	-	+
Motility		+	+	+	+	+	+	+	-
Starch hydrolysis		-	-	-	-	+	-	-	+
Citrate utilization		-	+	-	+	+	+	-	+
Indole test		+	-	-	-	-	-	-	-
VP test		-	-	-	-	+	-	-	+
Gelatin liquefaction		+	+	+	+	+	+	+	+
Nitrate reduction		-	-	+	-	-	-	-	+
Acid production from:	Glucose	+	-	-	-	+	-	+	+
	Mannose	-	-	-	-	+	-	-	-
	Arabinose	-	-	-	-	+	-	-	-
	Inositol	-	-	-	-	+	-	-	-
	Sorbitol	-	-	-	-	+	-	-	-
	Rhamnose	-	-	-	-	-	-	-	-
	Sucrose	-	-	-	-	+	-	-	-
	Melibiose	-	-	-	-	+	-	-	-

C = Central; T= Terminal; S= Subterminal.

Table 3. Presumptive identification of the selected isolates.

Isolate No.	Closely related to	Similarity %
3	*Paenibacillus alvei*	90.3
7	*Lysinibacillus spaericus*	98.1
8.1	*Lysinibacillus spaericus*	98.1
8.2	*Lysinibacillus spaericus*	94.2
8.3	*Bacillus licheniformis*	94
8.4	*Lysinibacillus spaericus*	98.1
9	*Paenibacillus residui*	93.8
10.1	*Bacillus mycoides*	94.3

pH of media has increased during growth to reach the maximum of 9 to 9.5. All isolates showed significant growth at temperature 37°C (Table 4). Most isolates showed halophilic characteristic as they grew at NaCl concentration up to 5%.

Urea tolerance for bacterial isolates after UV exposure

In general, most isolates showed improved tolerance to urea concentration after exposure to UV light when

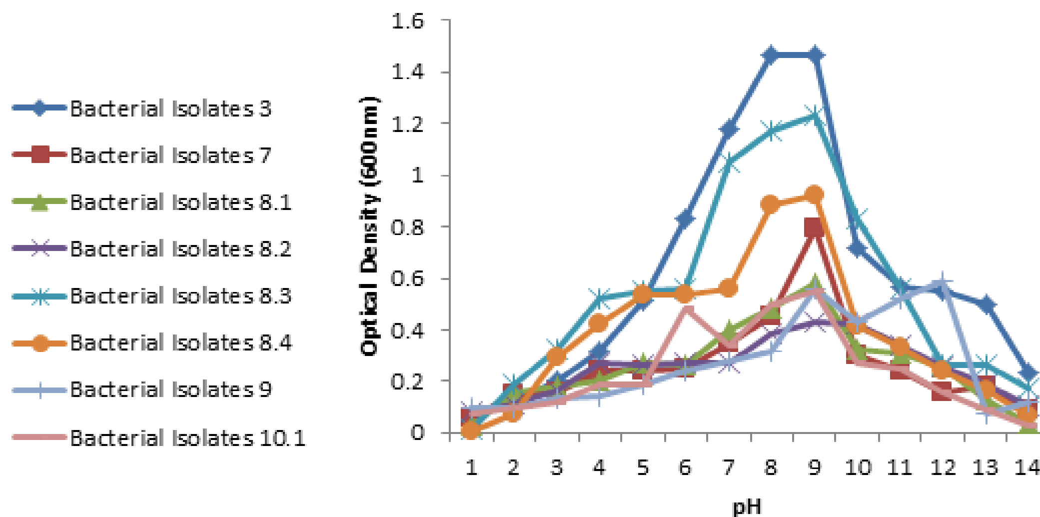

Figure 1. The effect of the varying pH on bacterial growth among the selected isolates.

Table 4. Growth of the isolates at the different temperatures.

Temperature (°C)	Bacterial Isolates (O.D at 600 nm)							
	3	7	8.1	8.2	8.3	8.4	9	10.1
0	0.007	0.014	0.059	0.172	0.098	0.037	0.079	0.142
4	0.014	0.061	0.065	0.178	0.13	0.088	0.057	0.115
25	0.14	0.983	0.708	0.462	0.572	0.567	0.95	0.918
37	1.04	1.3	1.169	1.137	0.774	0.949	1.161	1.131
45	0.72	0.19	0.477	0.831	0.27	0.47	0.57	0.27
60	0.143	0.062	0.192	0.06	0.106	0.048	0.074	0.137

Table 5. The influence of UV light exposure on Urea tolerance of mutant bacteria compared with wild type.

	Bacterial Isolates (tolerance to urea concentrations)							
	3	7	8.1	8.2	8.3	8.4	9	10.1
Wild type	10%	15%	12%	12%	15%	12%	10%	15%
Time of exposure to UV light								
2 min	15	8	12	8	12	15	15	8
4 min	15	15	12	8	10	15	8	15
6 min	12	15	12	8	15	15	8	12
8 min	12	12	12	12	15	12	8	12
10 min	15	15	12	12	12	12	10	12
12 min	15	12	12	10	15	15	8	12
14 min	15	12	12	10	12	15	10	12
16 min	12	12	12	15	15	15	12	15
18 min	15	12	12	15	12	15	12	12
20 min	12	12	12	12	15	15	12	10

compared with the wild type (Table 5). The mutant form of isolate #3 showed the higher tolerance to urea concentrations at all exposure intervals, when compared with wild type. At certain time intervals, some isolates showed decrease tolerance to high urea concentrations in compared to their wild type. Isolate # 8.1 showed no

Table 6. Experimental setup implemented for evaluation of bacterial involvement of calcium carbonate precipitation.

All bacterial isolates: 3, 7, 8.1, 8.2, 8.3, 8.4, 9, 10.1		
Media	**Color change**	**$CaCO_3$ precipitation**
NBUC	+	+
NBU	+	-
NBC	-	-
Autoclave killed bacterial isolates (cells and supernatant)		
NBUC	-	-
NBU	-	-
NBC	-	-
***E. coli* (negative control)**		
NBUC	-	-
NBU	-	-
NBC	-	-
Urease enzyme (positive control)		
NBUC	+	+
NBU	+	-
NBC	-	-
Non-inoculated tubes		
Media	**Color change**	**$CaCO_3$ precipitation**
NBUC	-	-
NBU	-	-
NBC	-	-

NBUC: Nutrient broth with urea and calcium chloride, NBU: Nutrient broth with urea, NBC: Nutrient broth with calcium chloride.

difference of tolerance to urea between wild type and mutant form.

Mini-scale calcium carbonate precipitation experiment

NBUC tubes for all live isolates, and the pure urease enzyme showed change in pH from neutral to alkaline (yellow to pink), and a precipitate of calcium carbonate were noticed at the bottom of the tubes. NBU tubes for all live isolates and urease enzyme showed only change in pH. NBC tubes for all live isolates and urease enzyme showed neither a change in pH nor calcium carbonate precipitation. Changes in color and pH indicate ureolytic activity (Table 6).

A comparison of NBUC of live bacteria versus killed isolates (both killed cells and supernatant) and *E. coli*, all of them were unable to change pH, so there was no urea hydrolytic activity due to the absence of the enzyme. Consequently, there was no calcium carbonate precipitation. Unchanged non-inoculated tubes suggests that results obtained are reproducible and representative (Table 6). Precipitate containing and non-containing tubes were examined under light microscope to confirm the presence of calcium carbonate crystal (Figure 2).

DISCUSSION

The present study was conducted to isolate, characterize, and optimize locally adapted urease-releasing bacteria that inhabits urea rich soils. Microbial activity that involves the cleavage of urea into ammonia and carbon dioxide by the urease enzyme, leading to the precipitation of carbonate ions as calcium carbonates. This potentially useful application explains the need to enhance urease production by various methods among candidate microorganisms (Vempada et al., 2011). The biochemical profile of the selected isolates showed that all isolates belong to the genus *Bacillus* (Table 3). This is similar to a previous study that isolated and characterized urease positive bacteria from urea rich soils, in which several isolates were mostly related to the *Bacillus* group (Ali et al., 2020).

Despite the differences in their characteristics, the obtained isolates showed similar behavior in their

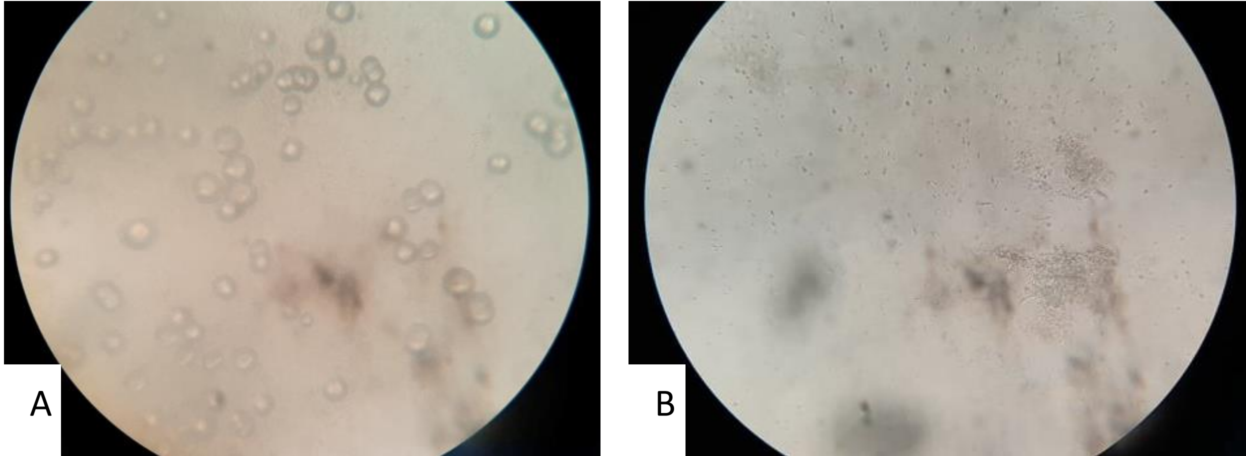

Figure 2. Calcium carbonate crystals precipitation in the mini-scale experiment: A- calcium carbonate precipitation in positive NBUC tubes, B: negative calcium carbonate precipitation in negative tubes.

ureolytic capability. This is in agreement with the findings of Stocks-Fischer et al. (1999); Hammes et al. (2003) and Stabnikov et al. (2011) that reported the same ureolytic *Bacillus* strains that can be isolated and cultivated using the same followed protocols of isolation and cultivation. Phenotypic and biochemical profiles of the isolates were matched to those *Bacillus* species reported previously that proved active in MICP process (Stocks-Fischer et al., 1999; Elmanama and Alhour, 2013).

Ureolysis-driven MICP is a phenomenon that has many applications for biochemical and engineering purposes (Omoregie et al., 2020). It has been widely investigated for soil stabilization, healing of concrete cracks, restoration of limestone surfaces, preventing soil erosions, and treatment of industrial wastewater and removing heavy metals (Whiffin et al., 2007; Sarda et al., 2009; Van paassen, 2009; De Muynck et al., 2010a; De Muynck et al., 2010b; Wu et al., 2019).

All obtained isolates showed ureolytic activity, tolerance to high urea concentrations, as well as calcium carbonate production. This suggests that isolates are potential candidates for the applications of MICP. Isolates 10.1 that was identified as *B. mycoides*, has been previously isolated and showed an efficient role of increased sand consolidation and compressive strength of cement (Elmanama and Alhour, 2013). Isolate 8.3 has been identified as *B. licheniformis,* has been reported in a previous study that it was able to precipitate calcium carbonate by ureolysis (Helmi et al., 2016).

Other studies mentioned the use of *Sporosarcina pasteurii* (the bacteria used in most studies) (Achal et al., 2009, 2010, 2011). *Bacillus sphaericus*, *Bacillus subtilis* (Atkinson and Fisher, 1991; Dhami et al., 2013b; Stabnikov et al., 2013), and *Bacillus cereus* in MICP applications (Wu et al., 2019; Dhami et al., 2013b).

Bacteria are previously known to breakdown urea in order to: (1) elevate the ambient pH (Burne and Marquis, 2000), (2) consume it as a nitrogen source (Burne and Chen, 2001), and (3) use it as a source of energy (Mobley and Hausinger, 1989). The amount and rate of urea that can be cleaved were influenced by the urea and calcium source (Wang et al., 2017). In this system, urea is the source of the carbonate. The more urea is supplied, the more $CaCO_3$ can be produced, if a sufficient amount of calcium ions is available (Wu et al., 2019).

In this study, isolates that were selected tolerated and grew in the presence of 10 - 15% urea concentration. This because urease activity, as well as, calcium carbonate production rate depend on urea concentration. A previous study utilized *S. pasteurii* and emphasized the role of urea containing cultural medium in the proliferation of bacteria. Moreover, it reported that bacteria cultivated with urea displayed a healthier cell surface and more negative surface charge for calcium ion binding than the bacteria have been cultivated without urea (Ma et al., 2020).

Increasingly, it has been reported that the bacterial concentration and ureolytic activity are important contributors in the efficiency of MICP process. The urea hydrolysis is an extremely slow process, whereas the presence of urease enzyme can substantially increase the hydrolysis of urea (De Belie et al., 2018). Therefore, the selection of the bacterial isolates with higher ureolytic activity is desirable for the higher production of calcium carbonate.

However, it has been shown that when the content of urea is excessive, bacterial growth and ureolytic activity are inhibited. For instance, when the urea concentration was greater than 0.75 mol/L, the amount of urea breakdown was decreased and thus appears as an inhibitory component. The reason could be due to too high urea molecule transportation over the cell

membrane into the cell, at elevated urea concentrations, inhibiting other cellular processes. Therefore, a certain amount of bacteria can only metabolize a certain amount of urea hydrolysis (Wu et al., 2019).

In our study, the local isolates were halo-tolerant, and corroborate with the findings of previous studies (Stabnikov et al., 2013). The observation of the pH tolerance profile of bacterial isolates showed a common moderate alkaliphile property. The best growth was at pH range 7-10 with a preference to pH 9. This is in agreement with a previous study that showed the good alkali tolerance of *B. cereus* which was successfully used to heal concrete cracks (Stabnikov et al., 2013; Wu et al., 2019).

Generally, the optimal pH range for bacterial growth is 7 to 8. Under higher alkaline conditions (pH 9 -12) bacteria can still grow but at a much-declined rate. Although the pH is relatively high in fresh concrete, the pH at cracks may drop to 8-11 due to carbonation, exposure, and humidity (De Muynck et al., 2010a). Above pH 11, the bacteria have a limited capacity to precipitate $CaCO_3$, thus limited ability to heal cracks. This implies that bacterial spores will keep dormant after being embedded in the concrete matrix (pH > 12), and only start to become active after cracks appear and crack surface pH drops (Wang et al., 2017; Wu et al., 2019). Therefore, alkaline pH is the primary factor by which bacteria promote calcite precipitation (Castanier et al., 2000; Fujita et al., 2000). Another study showed that the calcium carbonate yield (mg calcium carbonate/CFU) in the presence of *Bacillus* species increases when bacteria grown at a relatively high pH in compared with those bacteria that grown at uncontrolled pH solution (Seifan et al., 2017). Another study investigated the factors affecting the *S. pasteurii* induced biomineralization process, reported that the rise in medium pH to 9.5 accelerate bacterial growth (Ma et al., 2020). This may be promising that *Bacillus* isolates in this study can be used to heal concrete cracks. Especially, in the pH range of 7-11, bacteria will have a remarkable ureolytic activity, which ensures the decomposition of urea and the precipitation of $CaCO_3$. This meets also with (Phang et al., 2018) findings, It has been reported that some bacterial ureases exhibited high activity in alkaline conditions at pH of 9.

In the present study, the effect of temperature on isolates growth showed a temperatures range from 25 to 40°C. Bacterial mediated urea hydrolysis is an enzymatic reaction controlled by many factors including temperature. It has been reported in the literature that temperature affects bacterial activity, urease activity, and therefore reaction rate. Hence, the rate of formation of biogenic $CaCO_3$ and crack healing efficiency will be affected as well.

Urease activity is stable between 15 and 25°C, and an increase in temperature (until 60°C) results in increased urease activity (Whiffin, 2004; Peng and Liu, 2019).

Isolates that were exposed to UV irradiation were compared with their corresponding wild type isolates for the ability to tolerate higher urea concentrations. Most isolates showed improved tolerance to urea concentration. However, other strains showed a decline in their adaption to urea concentrations. This suggests that the mutagenesis process is random and did not correlate to the time of exposure to UV light. This is similar to the findings of a previous study, which used UV irradiation on *S. pasteurii* in order to improve urease activity (Wu et al., 2019).

The established calcium carbonate precipitation process showed that all NBUC tubes containing the viable isolates showed accompanied ureolytic and calcite precipitation activity. On the other hand, NBUC tubes containing the autoclave-killed isolates (pellet or supernatant) showed neither ureolytic nor calcite precipitation activity. This suggests that bacteria activity and urease positivity is a principal contributing to pH change due to urea cleavage, as well as calcium carbonate precipitation. In all NBC tubes inoculated with the viable isolates there was no calcium carbonate precipitation observed. This suggests that calcium carbonate production is enhanced by the change of pH. In NBC tubes (without urea), there was no difference in color change or calcium carbonate precipitation between live bacteria, killed bacteria, or *E. coli*. All NBU tubes inoculated with the viable isolates showed a change in pH as a proof for the ureolytic activity they possess. Negative control (*E. coli*) showed no change in pH or calcite production. These findings matched a previous study that reported the ability of urease producing bacteria *S. pasteurii* to produce calcium carbonate crystals under the same conditions (Ghosh et al., 2019). This is in agreement with the previous studies that reported that *Bacillus* sp. is with high respect in compared with other genus and that this might be due to their physiological ability to adapt to stressed conditions (Helmi et al., 2016).

In conclusion, this study successfully and easily isolated several *Bacillus* species from locally collected soil samples. These strains are alkaliphile, grow well at pH 7-10, and tolerate high urea concentrations. They showed calcite biomineralizing properties and may be employed in bacterial remediation of concrete cracks, increasing the compressive strength of concrete, decreasing water permeability, and solve the problems of soil erosions. Further studies on a larger scale are recommended to confirm the findings.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Antibiotic resistance in food producing Animals in West Africa French speaking countries: A systematic review

Andre Pouwedeou BEDEKELABOU*, Wilfried Délé OYETOLA, Abiezer DINGAM RIGUENODJI and Rianatou BADA ALAMBEDJI

Ecole Inter-Etats des Sciences et Médecine Vétérinaires (EISMV) de Dakar, Sénégal.

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This review aimed to make an inventory of the relevant work carried out on antibiotic resistance in the animal sector during the last two decades in French-speaking countries of the West African sub-region. English and French published articles from 2000 to 2019 indexed in PubMed, Google Scholar, and African Journals Online were reviewed in accordance with an adapted PRISMA guideline. Mean Resistance (MR) and interquartile ranges (IQR) of resistance were calculated for each antibiotic-bacterium combination for each country and globally. 28 articles were eligible for this qualitative review. One third of the countries did not have suitable data on antibiotic resistance in animals. Senegal (11/28) and Ivory Coast (8/28) are at the top of countries where more studies have been carried out. Poultry (17/28), cattle (10/28) and pigs (4/28) are the most investigated species. In poultry, resistance in *E. coli* strains was high to Tetracycline's (MR: 97%; IQR [80.65%- 98.5%]). Resistance in *Salmonella* spp. strains from poultry was high to Erythromycin (MR: 100%; IQR [99%-100%] and Amoxicillin-Clavulanic acid (47.76%; IQR [16.06%-52.52%]). In cattle, resistance of *Staphylococcus* spp. was low in general for all antibiotics with resistance of 16.25% IQR [11.75%-20.58%], 14.63% IQR [13.82%-31.32%], 10% IQR [8.55%-16%] respectively for Tetracycline's, Penicillin, and Gentamicin. More studies deserve to be done in West Africa French speaking countries in order to draw attention of decision-makers, lead to regulations on the correct use of antibiotics in the veterinary sector, and if possible set up a sub-regional network for the monitoring of antibiotic resistance.

Key words: Antibiotic resistance, animals, West Africa, French countries.

INTRODUCTION

Antimicrobial resistance (AMR) both in human and veterinary medicine has reached alarming levels in most parts of the world and has been recognized as a significant emerging threat to global public health and food security. West African countries face, like the rest of the world, this serious problem of the emergence of resistances to antibiotics (Ouédraogo and Sylvain, 2017). To fight against this threat, joint resolutions and actions are promoted by international organizations to combat AMR globally (Wall et al., 2016). Antimicrobial resistance affecting humans and animals is primarily influenced by an increase in using antimicrobials for a variety of

*Corresponding author. E-mail: pouwedeouandre@gmail.com.

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purposes, including therapeutic and non-therapeutic uses in animal production. These practices contribute to the spread of drug-resistant pathogens in both livestock and humans (Van Boeckel et al., 2015). In low- and middle-income countries including African sub-Saharan countries, population growth and rising incomes have driven an unprecedented growth in demand for animal protein. As a result, efforts to meet rising demand are driving a shift in animal production from small holder, mixed crop, and livestock operations to increasingly intensive, large-scale, and specialized commercialization farms (Schar et al., 2018). These intensive production systems are known to employ more and more antimicrobial for therapeutic and non-nontherapeutic use, including mass administration for prevention and control of disease and as growth promoter. Unfortunately, in most African sub-Saharan countries, it is known that there is no or less control over the distribution of veterinary pharmaceuticals and phytosanitary products. Worse still, no appropriate legislation yet exists to guarantee the quality of the various antimicrobial products released onto the market. In West African French Speaking countries for example, there are massive shortcomings in the organization of the veterinary drug market. These include lack of specific legislation following the recent liberalization of veterinary drugs, lack of veterinary drug inspections before marketing drugs, lack of registration because of the existence of parallel channels alongside the official distribution channel of veterinary drugs (Mensah et al., 2014). The consequence of all these shortcomings is, in one side, the large scale of antimicrobial misuse in farms to combat low productivity and high mortality caused by infectious diseases and, in the other side, the development of resistances to antimicrobials. Unfortunately, there is limited data concerning anti-microbial use and antimicrobial resistance in these countries in comparison with English speaking countries of the West African Sub region due to the absence of systematic surveillance systems. Thus, conclusions must be drawn from point-prevalence assessments or research studies. Here, the available information are piece together to build a picture of the situation of resistance development in food producing animals in West African French speaking countries. Food producing animals are linked to humans via the food chain and shared environment (Oloso et al., 2018). Thus, they can play an important role in the dissemination of resistance pathogens or resistance genes to humans (Chantziaras et al., 2014). This review over his importance in animal health will have an importance in public health because it offers the opportunity to see the burden of Antibiotic resistance in the region.

Research questions and objective

Some research questions guide this study, which aimed to establish the situation of ABR in food producing animals in 09 West African French speaking countries. These questions were: (i) what is the status of antibiotic resistance (ABR) in the food producing animals according to previous studies in the two last decades? (ii) what is the pattern of resistance in each state? (iii) what is the status of ABR among the common antibiotics that are used to control pathogens at animal's level?

MATERIALS AND METHODS

Data search design

Free databases (Pub Med, Google Scholar and African Journals Online) were searched using broad terms in English and French, "antimicrobial, resistance, and country name". Where necessary, search terms were stated as strings: Antimicrobial resistance OR Antimicrobial susceptibility AND country name AND animals; "animals" was substituted with different animal names (poultry, goat, sheep, cattle, camel, pig, etc.). References in the identified materials were also searched. Indeed, the reference lists of all included articles were used to carry out a supplementary literature search. Review articles in English were retrieved and assessed for potential relevant studies related to ABR in food producing animals in West Africa French speaking countries. The PRISMA-style flowchart was modified and used for this review (Figure1). Each publication was treated as a study, which contains single or multiple reports.

Exclusion and inclusion criteria

Articles were assessed using predesigned eligibility forms and according to predefined eligibility criteria (Table 1). Briefly, studies on parasites, viruses, and fungi were excluded. Studies dealing with ABR in humans were excluded. Studies reporting data from outside West Africa French speaking countries were not further selected. The selection of French and English published articles was based on clearly defined populations involving food animals at farms and/or processed/freshly slaughtered animals at abattoirs/markets. To be included, studies must have performed antibiotic susceptibility testing with antibiotics using appropriate methods and results interpreted according to appropriate guidelines.

Literature screening and data extraction

Mendeleyev (version 1.19.4) was used for literature management, and relevant data from included articles were extracted. The data were abstracted and analyzed using a framework on an Excel (Microsoft Office Excel 2013) spreadsheet. Each study included Country, first author details, year of publication, aims, study population (such as, pigs, poultry, cattle, sheep, goat), type of sample (such as, nasal swabs, rectal swabs, fecal samples, and meat products), sample size, clinical status (such as, apparently healthy, sick, and dead), study site (slaughterhouse, farm, and market), type of study (cross sectional, longitudinal), bacteria of interest (such as, *Staphylococcus aureus*, *Salmonella* spp., *Campylobacter* spp., *Escherichia coli*, and *Enterococcus* spp.), antibiotics tested, antimicrobial susceptibility testing (AST) methods (disk diffusion, micro-broth dilution, agar dilution, E-test, and automated methods), guidelines of interpretation of AST (such as, CA-SFM, EUCAST, CLSI, and NCCLS), ABR prevalence and molecular investigations.

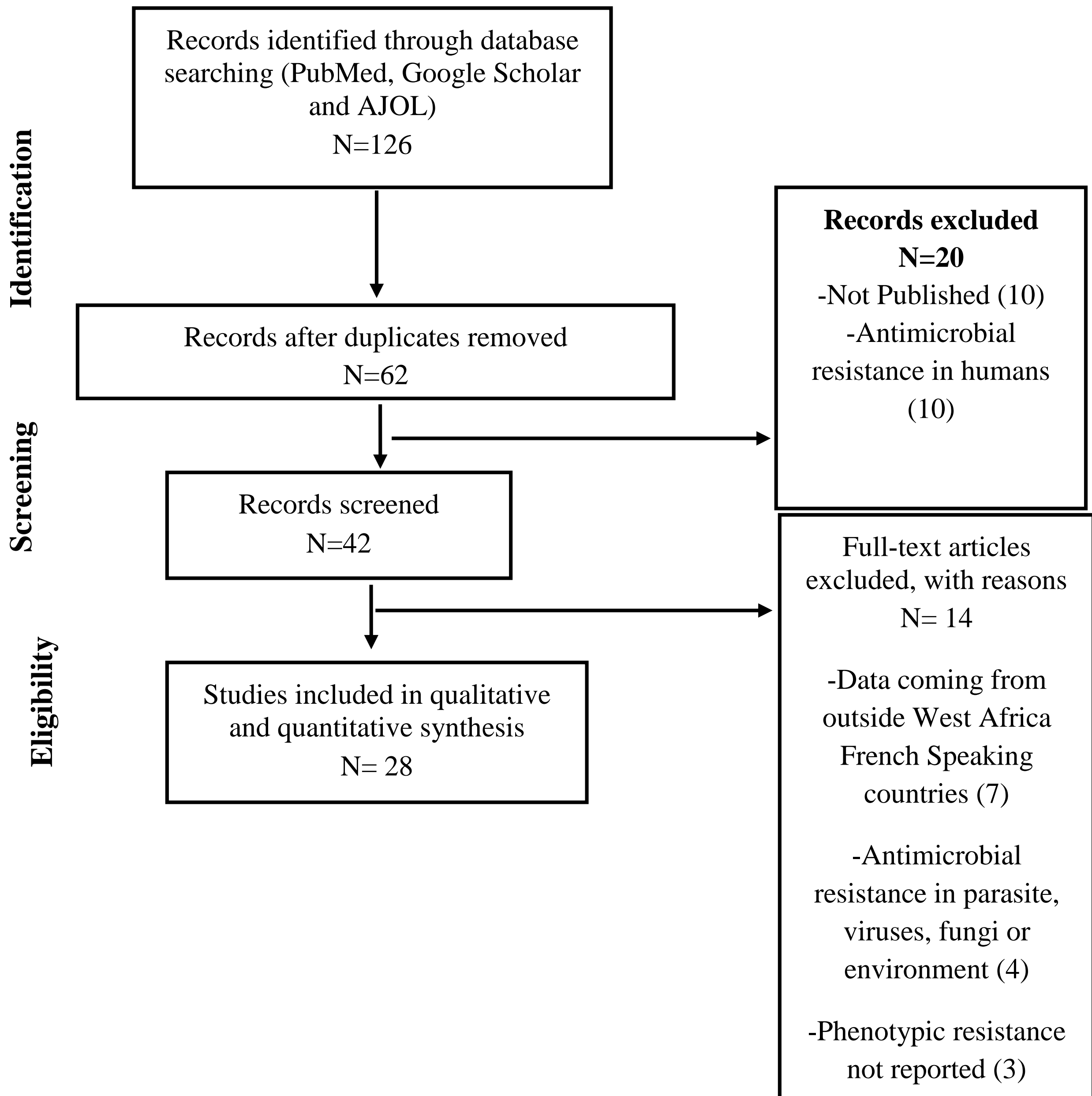

Figure 1. Adapted PRISMA method for data research.

Quality assessment

Preexisting scales were not used to assess study quality. Appraisal tool was used to assess the quality of included studies. So the researcher checked if:

(1) the basic data including: sample type, bacteria of interest, and study site, was provided; (2) samples of the study were collected in an appropriate way; (3) sample size was representative of the target population; (4) the number of strains representative; (5) the Antimicrobial Susceptibility Test perform with a valid method and interpreted according to a valid guideline; (6) all important sub-groups (Animal population or sample) identified and accounted for when reporting resistance rate. If all the criteria were met, the study was ranged according to the quality. If one or more criteria were not fulfilled, the study was ranged as moderate quality.

Data analysis

Microsoft Excel (2013 for Windows) was used to analyze the data following an initial extraction. Prevalence was calculated, median resistance (MR) and interquartile range (IQR) of resistance for each bacterium-antibiotic combination in each specific animal population (poultry, cattle…). Meta-analysis was not conducted because of the small number of articles available. All reports reporting ABR for an antibiotic were categories as no resistance (when resistance was <1%); very low resistance (1-24%); low resistance (25-49%); high resistance (50-74%) and very high resistance (75-100%) (Figure 1).

Table 1. Criteria for inclusion and exclusion.

Inclusion criteria
Studies reporting prevalence and molecular epidemiology of bacterial resistance in livestock animals
ABR in food animals and food products (meat, carcasses, egg, chicken, ready-to-eat meat/chicken, cheese, and sausage at supermarket)
ABR in food animals, exposed workers, and food products
Antimicrobial susceptibility testing by either disk diffusion or broth micro dilution, E-test
AST conducted using CLSI/EUCAST/CASFM/other relevant committee guidelines
Articles published in French and English.
Exclusion criteria
Data coming from outside West Africa French Speaking countries
Antimicrobial resistance in parasite, viruses, and fungi
Antimicrobial resistance in humans, wildlife and pets
Reports published in languages other than French and English

RESULTS

Number of studies per country and per year

A total of 28 studies from 6 countries were included in the review (Vounba et al., 2018, 2019**;** Cardinale et al., 2003; Bada-Alambedji et al., 2006; Fall-Niang et al., 2019; Fall et al., 2012; Shyaka et al., 2010; Stevens et al., 2006; Dione, 2009; Kadja et al, 2013; Mama et al., 2019; Sidibé et al., 2019; Coulibaly et al., 2010; Rene et al., 2014; Attien et al., 2013; Gblossi et al., 2012; Abdoulkarim et al., 2013, 2014; Caroline et al., 2019; Kagambèga et al., 2013; Somda et al., 2018; Deguenon et al., 2019; Boko et al., 2013; Ahouandjinou et al., 2016; Yao et al., 2018, 2017; Coulibaly et al., 2018; Guessennd et al., 2012. Three out of nine countries had no suitable report on antimicrobial resistance in animals as shown in Figure 2. The country in the considered region having the higher number of reports is Senegal followed by Ivory Coast. The majority of published articles are from the last decade as shown in Figure 3. The mean of published papers per year is less than 2 with 2019 being the year with a record number of published papers.

Situation of ABR in food producing animals in all countries

Animal population and bacteria of interest in studies

Investigations were mainly done in poultry (60.71%; 17/28) and cattle (35.71; 10/28) followed by Pigs (14.28%; 4/28) Sheep's (7.14%; 2/28), Goats (3.57%; 1/28) and Guinea fowl (3.57%; 1/28). Some studies investigated two or more species at the same time. The investigated bacteria were *Salmonella* spp. (50%; 14/28); *Staphylococcus* spp. (25%; 7/28); *Escherichia coli* (10.7; 3/28) and *Campylobacter* spp. (7.14%; 2/28). These bacteria were tested against 54 antibiotics belonging to 14 classes (Table 2). The most tested ATB were Tetracyclines (23 reports) followed by Gentamicin and association Sulfoxide-Trimethoprim (22 reports each). High to very high resistances were frequently reported for Tetracyclines (8 reports) followed by Sulfoxide-Trimethoprim, Streptomycin and Amoxicillin-clavulanic acid with 05 reports each (Table 3).

Samples tested and their origin

Studies included in this review investigated mainly in healthy animals (75%). Studies in diseased animals were performed in animals with mastitis (14.29%), Colibacillosis (7.14%) or Salmonellosis (7.14%). The majority of the studies were performed in samples collected in slaughterhouses or vendors in markets (60.71%; 17/28) and farms (46.42%; 13/28). Only one study was undertaken in samples from veterinary clinic and three studies investigated samples from both farm and slaughterhouse. Samples investigated were mainly carcasses (39.29% of the studies) and feces (39.29%). Two studies investigated carcass and feces at the same time. Milk was investigated

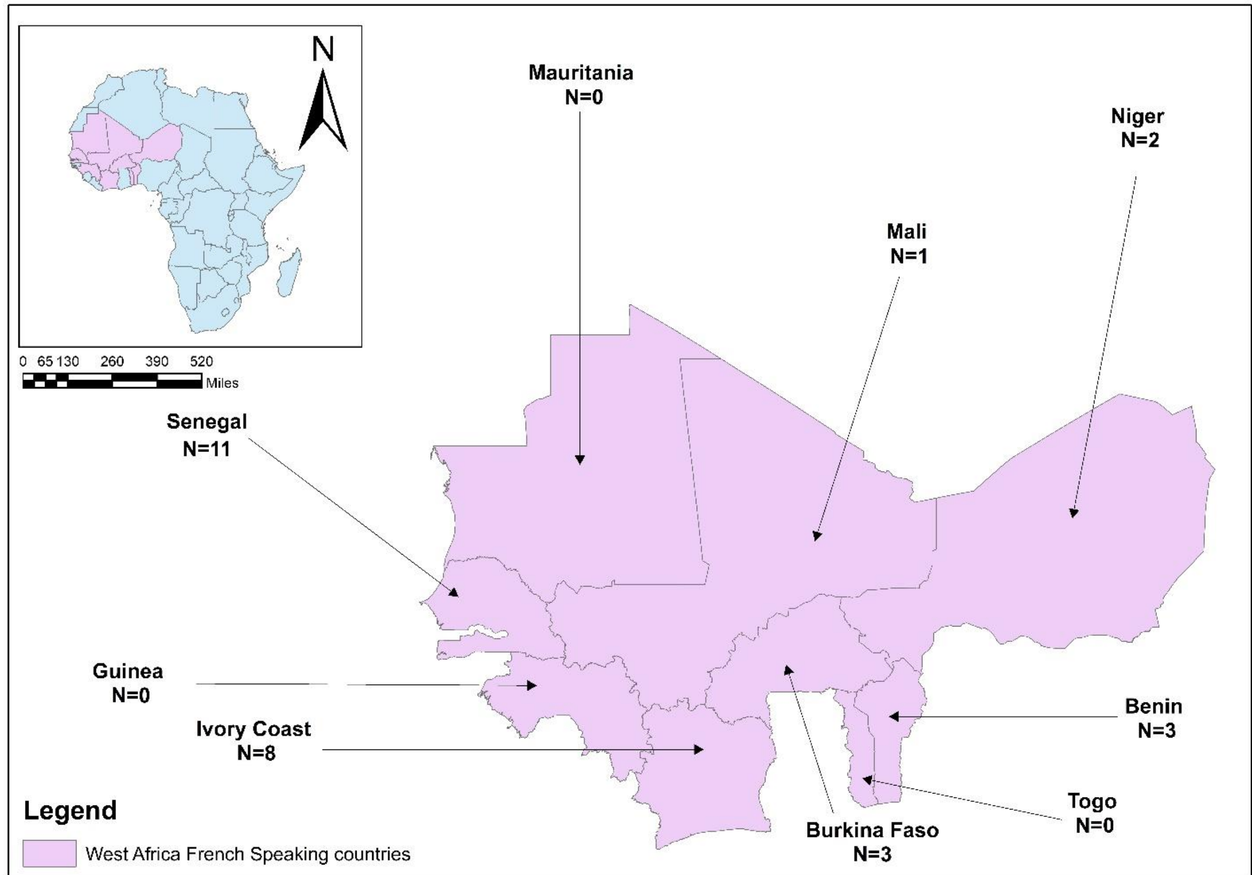

Figure 2. West Africa French speaking countries with number of included studies in each country.

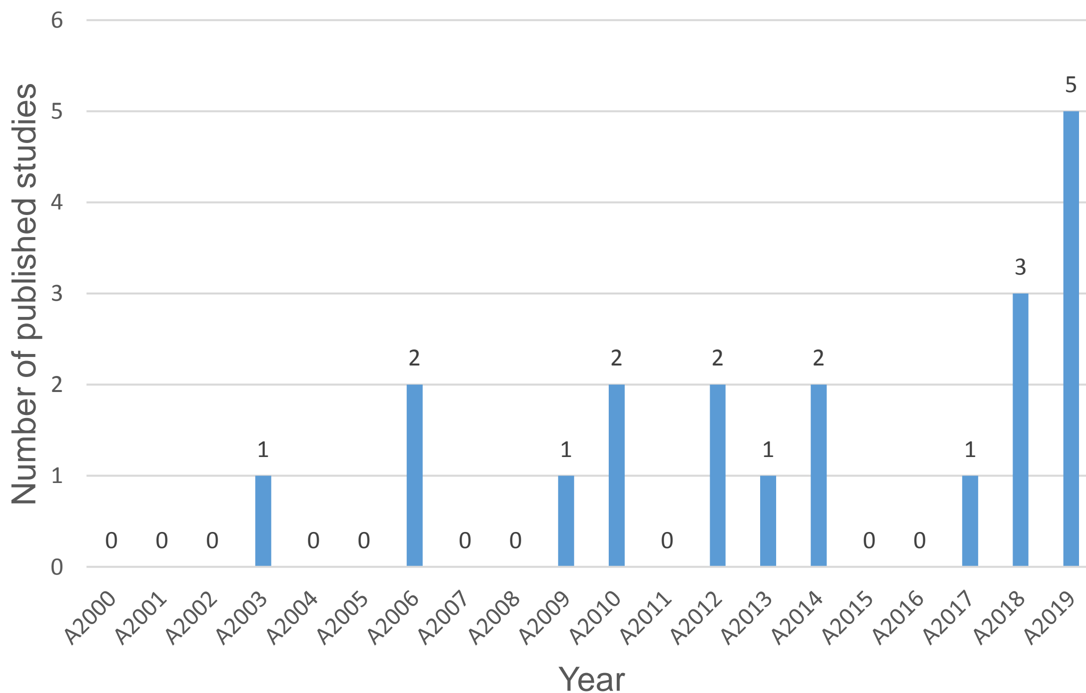

Figure 3. Number of publication over years.

Table 2. Antibiotics tested and their respective classes.

Class (Generation)	Antibiotic tested (Number of studies)
β-lactam+	Amoxicillin-Clavulanic Acid (14)
β-lactam(1)	Penicillin (4); Aztreonam (3); Cefalotin (7)
β-lactam(2)	Cefalexine (3); Cefoxitin (8); Cefuroxim(2) ; Methicillin(1); Oxacillin(3);
β-lactam(3)	Amoxicillin (7); Ampicillin(8); Cefixim(1); Cefotaxim(8); Ceftazidim (4); Ceftiofur(2); Ceftriaxone(8); Cephoperazone (1) ; Piperacillin (1)
β-lactam(4)	Ticarcilline (6) ; Cefepim (1)
β-lactam(NGC)	Imipenem(5); Mecilinam(1);
Quinolone(1)	Nalidixic Acid (11); Flumequine(3)
Quinolone(2)	Ciprofloxacine(14); Enrofloxacin(3); Norfloxacin(6); Ofloxacin(1); Pefloxacin(3)
Aminoglycoside	Amikacin(5); Gentamicin(19); Kanamicin(4); Neomycine(3); Sisomicin(1); Spectinomycine(2); Streptomycine(8); Tobramycine(4);
Phenicolé	Chloramphenicol (11)
Macrolide	Clindamycin(2); Erythromycin(5); Lyncomycin(1); Pristinamycin(1); Spiramycin(1)
Polypeptide	Colistin(5)
Tetracyclines	Doxycycline(5); Tetracyclin(18) ; Minocycline (3)
Organophosphate	Fosfomycine (2)
Furanes	Nitrofurane (3)
Ansamycin	Rifampicin (1)
Sulfonamides	Sulfoxides (7)
Sulfonamides +	Sulfoxide-Trimethoprim (19)
Diaminopyrimidine inhibitor.	Trimethoprim (5)
Glycopeptide	Vancomycin (1)

in 4 studies (14.28%) and only two studies (7%) investigated animal organs. Nasal and cloacal swabs were used as samples in one study each. Table 4 resumes the characteristics of included studies.

Resistance in poultry

Resistance was analyzed in *E. coli*, *Staphylococcus* spp. And *Salmonella spp.* subgroup. Resistance in *E. coli* strains from poultry was high to Tetracycline (MR: 97%; IQR [80.65- 98.5%]); Sulfoxide (MR: 81.4%; IQR [81.1-81.7%]); Sulfoxide-Trimethoprim (MR: 61%; IQR [46.57-68.85]) and Ampicillin (42.68%; IQR [40.58-60.43%]). Reported resistance in Salmonella strains from poultry was high to: Erythromycin (MR: 100%; IQR [99%-100%]; Amoxicillin-Clavulanic acid (47.76%; IQR [16.06-52.52%]); and Tetracyclines (46.04%; IQR [38.06-60.75]). Resistance of *Campylobacter* spp. to ciprofloxacin was 60% (IQR [55.25-65.75%])

Resistance in cattle

E. coli strains isolated from cattle's show very high resistances with resistance of 98.8% (IQR [98.2-99.4%]) for Tetracycline's; 97.65% (IQR [96.48-98.43%]) for Aztreonam and 96.45% (IQR [94.68-98.23]) for Ampicillin. Resistance of *Salmonella* spp. strains to Streptomycin was high, 58.58% (IQR [40.04-77.11%]), and resistance of *Staphylococcus* spp. was low in general for all antibiotics with resistance of 16.25% IQR [11.75-20.58%], 14.63% [13.82-31.32%], 10% [8.55-16%] respectively for Tetracycline's, Penicillin and Gentamicin.

Molecular investigations

Resistance and virulence genes in E. coli

Three studies investigated AMR and or virulence

Table 3. Antibiotics tested and the level of resistance reported.

ATB	Generation	No resistance (0%)	Very low resistance (1-24%)	Low resistance (25-49%)	High resistance (50-74%)	Very high resistance (75-100%)	Total reports*
Acid Nalidixic	1	5	5	4	0	1	15
Amoxicillin	3	1	3	1	1	1	7
Amoxicillin+Clavulinic Acid	4	3	4	2	4	1	14
Ampicillin	3	1	2	5	0	2	10
Amikacin	NGC*	1	3	0	0	1	5
Aztreonam	1	1	1	1	0	0	3
Cefalexin	2	2	0	1	0	0	3
Cefalotin	1	1	2	3	0	1	7
Cefixim	3	0	0	1	0	0	1
Cefotaxime	3	5	2	3	0	1	11
Cefoxitine	2	2	5	0	0	1	8
Ceftazidime	3	2	2	0	0	0	4
Ceftiofur	3	0	2	0	0	0	2
Ceftriaxone	3	0	5	0	0	3	8
Cefuroxime	2	0	1	0	1	0	2
Cephoperazone	3	0	0	1	0	0	1
Chloramphenicol	NGC	6	7	1	0	0	14
Ciprofloxacine	2	5	9	1	2	0	17
Clindamycin	NGC	0	2	0	0	0	2
Colistin	1	1	3	0	1	1	6
Doxycycline	NGC	0	4	1	0	0	5
Enrofloxacine	2	2	1	0	0	0	3
Erythromycine	NGC	1	3	0	0	4	8
Flumequine	1	0	2	0	1	0	3
Fosfomycine	1	1		0	0	0	2
Gentamicin	NGC	9	11	1	0	1	22
Imipenem	NGC	5	2	0	0	0	7
Kanamycin	NGC	0	3	0	1	0	4
Lincomycin	NGC	0	0	0	1	0	1
Mecillinam	NGC	1	2	0	0	0	3
Methicillin	2	0	1	0	0	0	1
Neomycin	NGC	1	2	0	0	0	3
Nitrofuran	NGC	0	1	1	1	0	3
Norfloxacin	2	3	2	1	0	0	6

Table 3. Contd.

Ofloxacin	2	0	1	0	0	0	1
Oxacillin	2	1	1	0	1	1	4
Pefloxacin	2	2	1	0	0	0	3
Penicillin	1	0	3	2	0	1	6
Pristinamycin	NGC	0	1	0	0	0	1
Rifampicin	NGC	0	1	0	0	0	1
Sisomicin	0	1		0	0	0	1
Spectinomycin	NGC	0	2	0	0	0	2
Spiramycine	NGC	0	0	0	0	0	0
Streptomycin	NGC	2	2	2	3	2	11
Sulfoxide	NGC	2	1	2	1	3	9
Sulfoxide-Trimethoprim	NGC	4	8	5	3	2	22
Tetracyclin	NGC	2	7	6	3	5	23
Ticarcillin	4	1	2	2	1	0	6
Tobramycine	NGC	2	2	0	0	0	4
Trimethoprim	NGC	2	2	1	0	2	7
Vancomycin	NGC	0	0	1	0	0	1

*Report: level of resistance reported for a combination of animal space and bacterium. *NGC: Non Generation Classification.

Table 4. Characteristics of included studies.

	Study Name	Study Population	Type of samples	Clinical Status	Setting	Organismes	Guidelines	AMR gene investigated	Virulence gene investigated	Study quality appraisal
	Bada-Alambedji et al. (2006)	Poultry	Carcass	Healthy	Vendors	*Salmonella* spp.	CASFM	NO	NO	H
	Cardinale et al. (2003)	Poultry	Carcass	Healthy	Abattoir/Vendor	*Compylobacter* spp.	CLSI	NO	NO	H
	Fall et al. (2012)	Pig	Carcass/Feces	Healthy	Farm/Abattoir	*Staphylococcus* spp.	NS	NO	NO	M
	Dione et al. (2009)	Poultry	Carcass/Feces	Healthy	Farm/Abattoirs	*Salmonella* spp.	CASFM	NO	NO	H
	Fall-Niang et al. (2019)	Poultry	Carcass	Healthy	Abattoir/Vendor	*Salmonella* spp.	CASFM	NO	NO	H
	M. KADJA et al (2013)	Goat/Sheep	Milk	Mastitis	Clinic	*Staphylococcus* spp	CASFM	NO	NO	M
Senegal	Mama et al. (2019)	Poultry	Nasal Sample	Healthy	Abattoir	*Staphylococcus* spp.	NS	NO	YES	M
		Cattle	Nasal Sample	Healthy	Abattoir	*Staphylococcus* spp.	NS	NO	YES	M
	Vounba et al. (2018)	Poultry	Feces	Healthy	Farm	*E.coli*	CLSI	YES	YES	H
	Vounba et al. (2019)	Poultry	Organs (liver, heart, intestines, and spleen)	Collibacillosis	Farm	*E.coli*	EUCAST	YES	YES	H
	Shyaka et al. (2010)	Cattle	Milk	Mastitis	Farm	*Staphylococcus* spp.	CASFM	NO	NO	M
	Stevens et al. (2006)	Cattle	Carcass	Healthy	Abattoir	*Salmonella* spp.	NS	NO	NO	M

Table 4. Contd.

Ivory Coast	Attien et al. (2013)	Beef, pig and Poultry	Carcass	Healthy	Vendors	*Staphylococcus* spp.	CASFM	NO	NO	M
	Coulibaly et al. (2010)	Poultry	Carcass	Healthy	Abattoir/Vendors	*Salmonella* spp.	NS	NO	NO	H
	Gblossi Bernadette et al. (2012)	Poultry	Organs/caeca	Healthy	Abattoir	*Camplylobacter* spp.	CLSI	NO	NO	H
	Rene et al. (2014)	Poultry	Carcass	Healthy	Abattoir/Vendors	*Salmonella* spp.	CASFM	NO	NO	H
	Yao et al. (2018)	Cattle	Feces	Healthy	farm	*E.coli*	CASFM	YES	NO	H
	Yao et al. (2017)	Cattle	Feces	Healthy	farm	*Salmonella* spp.	CASFM	NO	NO	H
	Coulibaly et al. (2018)	Cattle	Feces	Healthy	Abattoir	*E.coli/Salmonella* spp.	CASFM/EUCAST	NO	NO	H
	Guessennd et al. (2012)	Pig	Feces	Colibacillosis	farm	*E.coli*	CASFM	NO	NO	M
Mali	Sidibé et al. (2019)	Poultry	Cloacal Swab	Salmonellos diseas	Farm	*Salmonella* spp.	CASFM	NO	NO	H
Niger	Abdoulkarim et al. (2014)	Cattle	Milk	Mastitis	Farm	*Staphylococcus* spp.	CASFM	YES	NO	H
	Abdoulkarim et al. (2013)	Cattle	Milk	Mastitis	Farm	*Staphylococcus* spp.	CASFM	NO	YES	H
Burkina Faso	Kagambèga et al. (2013)	Cattle	Feces	Healthy	Abattoir	*Salmonella* spp.	NS	NO	NO	M
		Poultry	Feces	Healthy	Abattoir	*Salmonella* spp.	NS	NO	NO	M
		Pig	Feces	Healthy	Abattoir	*Salmonella* spp.	NS	NO	NO	M
	Caroline Bouda et al. (2019)	Poultry	Eggs	Healthy	Vendors	*Salmonella* spp.	EUCAST	NO	NO	H
		Poultry	Feces	Healthy	Farm	*Salmonella* spp.	EUCAST	NO	NO	H
	Somda et al. (2018)	Poultry	Carcass	Healthy	Abattoir/Vendors	*E.coli*	EUCAST	NO	YES	H
Benin	Boko et al. (2013)	Guinea fowl(pintades)	Feces	Mixte (Healthy/diseas)	farm	*Salmonella* spp.	CASFM	NO	YES	H
	Deguenon et al. (2019)	Poultry/sheep/pig	Feces	Healthy	farm	*Salmonella* spp.	NS	NO	YES	M
	Ahouandjinou et al. (2016)	Cattle	Carcass	Healthy	Abattoir	*Salmonella* spp.	CASFM	YES	NO	H

genes in *E. coli* strains. Somda et al. (2018) in BF investigated the presence of STEC, EPEC, ETEC, EIEC, and EAEC genes on grilled chicken meat samples by 16- plex PCR for the genes *uid*A, *pic*, *bfp*, *inv*E, *hly*A, *elt*, *ent*, *esc*V, *eae*A, *ipa*H, *agg*R, *stx*1, *stx*2, *est*Ia, *est*Ib, and *ast*. Only *st*Ia, *stx*2A, *inv*E, *ast*A, and *agg*R virulence genes were detected. Six diarrheagenic *E. coli* were detected as follows: EAEC and ETEC (in two samples each) and STEC and EIEC (in one sample each). No EPEC gene was detected.

In Senegal, Vounba et al. (2018) investigated Antimicrobial Resistance (AMR) genes in *E. coli* isolated from diseased chicken. Many AMR genes were detected, including variants of $bla_{CTX\text{-}M}$ encoding resistance to third-generation cephalosporins. Most fluoroquinolone-nonsusceptible isolates were carriers of mutations in *gyrA (Ser83Leu, Asp87Asn, and/or Asp87Tyr)* and/or *parC (Ser80Ile)* genes. *A total of 84.5%* isolates exhibited at least one of the virulence markers of Avian Pathogenic *Escherichia Coli* (APEC), among which 39.7% were defined as potential virulent APEC. The same author investigated *E. coli* from healthy chicken in Senegal (Vounba et al., 2019) and reported the presence of AMR genes. According to this report, 95% of tested farms harbored isolates carrying mutations in *gyrA* (*Ser83*Ile and *Asp*87Asn) and *par*C (*Ser*80Ile). 3GC resistance was mediated by $bla_{CMY\text{-}2}$ or $bla_{CTX\text{-}}$M genes, $bla_{CTX\text{-}M}$ bein Stevens g of genotypes $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}8}$ and the genotype $bla_{CTX\text{-}M\text{-}15}$. The most prevalent AMR genes were those encoding resistance against tetracycline (tet genes) and trimethoprim-sulfamethoxazole (*dfr*A genes). Genes encoding

resistance against streptomycin (*aadA*1) or ciprofloxacine (*qnr*B) were also detected.

Resistance and virulence genes in Salmonella strains

Boko et al. (2013) screened all confirmed Salmonella isolates in Guinea fowl by polymerase chain reaction (PCR) for the presence of several virulence-associated genes located on Salmonella Pathogenicity Islands (SPI) 1 to 5: *prg*H, *inv*A, *sit*C, *spa*I, *inv*E (SPI-I); *spi*C, ssaU, *ttr*B (SPI-2); *mgc*T, *mis*L (*SPI*-3); *orf*L (*SPI*-4); *pip*D (SPI-5). All isolates belonging to five serotypes tested positive by PCR for most of the target genes (Deguenon et al., 2019). The genes of virulence that were targeted for amplification by PCR were *inv*A, spvR, *spv*C, *fimA* and *stn*. All Salmonella isolates were positive for the presence of *inv*A genes, *fim*A and *stn*. The *spv*C gene was present in 10% and *spv*R gene in 20% of the isolates.

Resistance and virulence genes in Staphylococcus strains

Mama et al., 2019 in Senegal tested six S. aureus isolates of cow origin. All the isolates showed resistance for penicillin (with *bla*Z gene) and two of them to tetracycline (with ***tet* (**K) gene). All the isolates hosted hemolysin-encoding genes. The following genotypes were observed for non *S. aureus* strains: tetracycline [*tet*(K), *tet*(L)], trimethoprim/sulfamethoxazole (SXT) (*dfr*G, *dfrK*), penicillin (***bla***Z), erythromycin [*msr*(A)/ *msr*(B)].

In Niger, Abdoulkarim et al., 2014 tested all the isolates resistant to Amoxicillin in his study for the presence of *bla*Z gene (coding for β-lactamase) by polymerase chain reaction (PCR). 90% of the isolates tested positive for the blaZ gene suggesting that the blaZ gene encodes the production of β-lactamase by most penicillin-resistant S. aureus of this study. Previously, the same author (Abdoulkarim et al., 2013) virulotyped *S. aureus* strains by PCR for the presence of genes coding for Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMM) to colonize the host epithelia and extracellular matrix components (*clf*A, *clfB*, fnbA, cna, ebpS and *sdr*C). This was done for capsular and other surface antigens conferring resistance to phagocytosis (*cap*5H, *cap*8H, *spa* and *ica*A); exfoliative toxins active on extracellular matrix components (*et*A, *et*B and *et*D); haemolysins and leukocidins counteracting the cells of the host's innate immune response (*hla, hlb, hld, hlgAC, lukD, lukM, lukF-PV* and *luk*S-PV); and for enterotoxins causing diarrhoea by action on the host enterocytes (*sea, seb, sec, sed, seg, seh, sei, sej and sen*). All or most of the *S. aureus* isolates gave amplified fragments with most for the genes coding for surface antigens: *clfA* (91%), *clf*B (100%), *fnbA* (100%), *cna* (100%), *ebpS* (100%), sdrC (87%), *cap*5H (83%), *spa* (96%), *ica*A (96%); and with several for the toxin-encoding genes: *et*D (74%), *hla* (96%), *hlb* (100%), *hld* (96%), *hlgA*C (74%), *luk*D (96%), *luk*M (96%). Conversely, a minority of isolates tested positive for the following genes: *cap*8H (4%), *lukF-PV* (17%), *lukS*-PV (9%); only one isolate (4%) tested positive for 2 of the 9 enterotoxin-encoding genes (*sej* and *seg*);

Situation of ABR per country

Senegal

Eleven studies from Senegal are included in this qualitative review. 39 different antibiotics were tested in these studies namely : Methicillin ; Penicillin; Ampicillin; Amoxicillin; Amoxicillin+ Clavulanic Acid; Nalidixic Acid; Cefotaxime; Ceftazidime; Ticarcilline; Imipenem; Cefoxitine; Cephalexin; Cephoperazone; Cefalotin; Cefoxitin; Ceftiofur; Ceftriaxone; Flumequine; Gentamicin; Spectinomycine; Streptomycin; Spiramycine; Kanamycin; Amikacin; Pefloxacin; Norfloxacin; Ciprofloxacin; Neomycin; Erythromycin; Tetracycline; Doxycycline; Chloramphenicol; Nitrofurane; Sulfoxide; Sulfoxide-Trimethoprim; Tobramycin; Trimethoprim; Colistin; Rifampicin; Tetracyclines were the most frequently tested antibiotics in the studies (90.9%) followed by the association Sulfoxide-Trimethoprim (81.81) and Gentamicin tested in 72.72% of the studies.

Most of the studies in Senegal were performed in poultry (63.63%; 7/11). Four studies out of the 7 in poultry was performed in carcass sampled in slaughterhouses or vendors and 2 studies performed on feces sampled in farms, one (1/7) on animal's organs and one on nasal samples. Salmonella (3/7), *E. coli* (2/7), Campylobacter (1/7) and *Staphylococcus* spp. (1/7) were the studied bacteria in poultry. The ABR in Campylobacter was 75% to Ciprofloxacin; in *Staphylococcus* spp., resistance was 83.33% for Tetracycline and 50% for Sulfoxide-Trimethoprim.

In *Salmonella* spp. the resistance was mainly observed to Tetracyclines (66%; [56.34-70.5]), Trimethoprim (59.1%; [50.55%-67.65%); sulfoxides (56.55%; [48.83-64.28%]) Streptomycin (50.86%, IQR: [39.33-62.44]) and association Sulfoxide-Trimethoprim (47% [43.5-63.5]). The highest resistance in *E. coli* was observed for the same antibiotics: Tetracycline (98.50%; [97.75-99.25%]), Sulfoxide (81.04% [81.1-81.7%]) Sulfoxide-Trimethoprim (68.85%; [64.93-72.78]) and Ampicillin (58.15%; [48,23%-68,08%]). Two studies focus on cattle (2/11; 18.18%) and bacteria of interest was *Staphylococcus* spp. (milk samples) for one study and *Salmonella* spp. (carcass samples) for the other study. Only one study on ABR was performed in pigs (Carcass and feces samples) and one on small ruminants (Sheep and goat with milk as sample matrix). *Staphylococcus* spp. as bacteria of interest was

investigated in these two studies.

Ivory coast

Twenty eight different antibiotics were tested in a total of 8 studies included in this qualitative review: Pristinamycin; Oxacillin; Ofloxacin; Amoxicillin; Amoxicillin+Clavulinic Acid; Nalidixic Acid; Cefotaxime; Cefuroxime; Imipenem; Cefalotin; Ceftriaxone; Gentamicin; Kanamycin; Rifampicin; Vancomycin; Lyncomycine; pefloxacin; Ciprofloxacin; Erythromycin; Tetracycline; Doxycycline; Chloramphenicol; Sulfoxide-Trimethoprim; Sixomicin; Tobramycin; Piperacillin; Cefepime; Minocycline. The association Amoxicillin-Acid Clavulanic was tested in all the studies followed by Association Sulfoxide-Trimethoprim tested in seven of the eight studies.

Studies were mainly conducted in poultry (4/8) and cattle (3/8) and Salmonella was the bacteria of interest in four studies followed by *E. coli* in three studies. Globally, the highest prevalence of resistance in Salmonella in poultry was observed with Amoxicillin (50.47% IQR [49.55-51.37]); Amoxicillin-Acid Clavulanic (MR=47.76% [44.50-50,01]) and Cefalotin (MR=42.99%; IQR[41.77%-44.22%]). In cattle, Salmonella was most frequently resistant to Tetracycline's (MR=42.99%; IQR [41.77%-44.22%]). One of the three studies in poultry investigated resistance of *Campylobacter* spp. to antibiotics and reported resistances to these bacteria. Indeed, interesting resistances were reported to: Nalidixic Acid (78%) Ciprofloxacin (50%) ; Erythromycin (13,5%) and Amoxicillin + Clavulanic Acid (11.8%) (Gblossi et al., 2012).

Another study investigated the resistance of *Staphylococcus* spp. in three species (beef pork and chicken) but did not report resistance per specie (Attien et al., 2013). According to this study, resistance to staphylococcus in food producing animals in Ivory Coast was 100% to erythromycin, 62% to Amoxicillin-acid-clavulanic and 58% to Oxacillin. To finish, Guessennd et al. (2012) investigated *E.coli* resistance to antibiotics in pigs and reported notable resistance of 76.7, 66.7 and 56.7% respectively for Tetracycline, Cefotaxime and Sulfoxide-Trimethoprim.

Benin

Three studies were identified from Benin and antibiotic susceptibility was performed against 24 antibiotics namely: Oxacillin; Ampicillin; Amoxicillin; Amoxicillin-Clavulanic; Nalidixic Acid; Cefotaxime; Cefuroxime; Imipenem; Cefoxitine; Cefalotin; Ceftriaxone; Flumequine; Gentamycin; Amikacin; Ciprofloxacin; Neomycin; Tetracycline; Chloramphenicol; fosfomycine; Sulfoxide; Sulfoxide-Trimethoprim; Trimethoprim; Colistin; gentamicin.

Studies in Benin were done on poultry/sheep/pigs for one study, guinea fowl for another study and cattle. Two studies were performed in feces samples and one on carcass from cattle. *Salmonella* spp. strains were investigated in the three studies. Salmonella isolates from poultry/sheep/pigs were resistant to Amoxicillin (100%), Amoxicillin-Acid clavulanic (100%) Cefotaxime (100%) Cefoxitin (100%) Cefalotin (100%) Gentamycin (100%) Amikacin (100%) Trimethoprim (100%); Ceftriaxone (85%). Those from Guinea flow were resistant to Oxacillin (100%), Sulfoxide (100%) and Colistin (100%). In cattle, High resistance was reported from Ampicillin (87.77), Ceftriaxone (88.49), Sulfoxide-Trimethoprim.

Burkina Faso

In BF, three published studies reported ABR in food producing animals. The three studies tested a total of 21 antibiotics: Ampicillin; Amoxicillin-Clavulanic acid; Nalidixic Acid; Aztreonam; Cefotaxime; Mecilinam; Ticarcilline; Imipenem; Cephalexin; Ceftriaxone; Gentamycin; Streptomycin; Ciprofloxacin; Norfloxacin; Erythromycin; Tetracycline; Chloramphenicol; Sulfoxide; Sulfoxide-Trimethoprim; Trimethoprim; Colistin.

Common antibiotics in the studies were: Imipenem; Gentamycin; Streptomycin; Ciprofloxacin; Tetracycline and Chloramphenicol. Disc diffusion method was used in all studies. Salmonella and *E. coli* were the bacteria studied. Salmonella resistance was reported in two studies and *E. coli* in One study. Resistance of *E. coli* to Tetracycline in chicken meat was 64.3%. Salmonella from food producing animals in BF was resistant to Erythromycin (MR=100%); Amoxicillin-Clavulanic (MR=55.85%; [54.23-57.48%]); Ticarcillin (MR=49%; [45.78-53.13%]) and Tetracycline (MR=42.1%; IQR [34.05-43.75%]).

Mali-Niger

Three published studies were found for these countries. In Mali, the only published paper reported resistance to salmonella in poultry while in Niger the two included studies reported resistance of *Staphylococcus* spp. in Cattle milk. The three studies used disc diffusion method and followed the CASFM guidelines. Resistance of salmonella in Mali was high for Erythromycin (98%); Colistin (94%); Streptomycin (90%); Kanamycin (67%); Flumequine (65%) and Tetracycline (59%). In Niger, moderate to low resistance of *S. aureus* was observed to penicillin (30.5% [21.75-39.25]); Gentamycin (16% [13-19]); and Tetracycline (10.5% [9.25-11.75]).

DISCUSSION

As a threat to a century of gains made since the discovery

of antibiotics and the contribution of these drugs to improvements in animal and human health and wellbeing, antibiotic resistance has become a global concern. Sub-Saharan Africa has a high incidence of invasive bacterial infections in humans and this condition increase demand for both preventive and therapeutic antimicrobials. However, antibiotics are widely used in domestic and commercial animal husbandry and this can contribute to the annihilation of the therapeutic arsenal used in human medicine and on which the continent's and the world relies to overcome these infections. Indeed, the development of resistances in animals can threat human health through dissemination of resistance bacteria or resistance genes from animal to human through food chain or direct contact.

Being in human health or veterinary medicine, sub-Saharan Africa has the least antimicrobial surveillance strategies of all world regions. Only six (15%) of the 41 WHO Africa region member states carry out surveillance for bacterial antimicrobial resistance in human health (Williams et al., 2018). In animal health, surveillance of antibacterial resistance in animals is almost nonexistent in Africa countries. Then, the lack of consistency in the measurement and reporting of antibiotic susceptibility data in animals makes it difficult to know the situation in food producing animals in different countries. To address this issue, scientists are making efforts in research to yield data that can draw attention of decision makers to set up surveillance network. Unfortunately, these efforts although useful are often rare and scattered that they do not allow the visibility of the magnitude of the situation. That is why a synthesis of the scientific data generated here and there over a long period is often useful to give visibility of the situation and draw attention. This summary of the situation of antibiotic resistance in food producing animal in West Africa French-speaking countries from 2000 to 2019 (20 years) trailed this objective.

In a recent systematic review and meta-analysis, some published papers tried to report the situation of ABR in food producing animals in all African countries (Founou et al., 2018). However, it failed sometime to include some interesting studies from French countries in west Africa may be because some are published in French or are not index in high databases as PubMed or simply did not met authors inclusion criteria. The present review has the advantage of zooming on the situation of antibiotic resistance in French-speaking countries, where interesting scientific work is often not very visible because of language limitations. Indeed, this study proved that antibiotic-resistant is globally under investigation in West Africa French speaking countries with only 28 studies included from 6 countries out of the 9 countries in the study area. This is similar to the situation previously reported by Founou et al. (2018) who reported data from 12 countries out of 54 in Africa. However, unlike this study, which includes only one study from Senegal, many data were reported from Senegal and data from other French speaking countries, giving the real situation in this geographic area.

Our systematic review has demonstrated widespread prevalence of antibiotic resistance in food producing animals in West Africa French speaking countries. Poultry and cattle were the most studied species with *Salmonella* spp. and *Staphylococcus* spp. as bacteria's of interest in most investigations. The fact that poultry is the specie most concerned by studies is not surprising and is rather interesting because poultry farming is the sector that uses antibiotics the most, often in modern farms constituted by semi-intensive or intensive systems unlike other livestock sectors which for the most part still extensive (Schneider et al., 2010). Salmonella as bacteria of interest in studies would be linked to the role of salmonellosis in food poisoning and to the interest of this bacterium in several monitoring programs (Jajere, 2019).

Many studies investigated ABR on samples from healthy animals and this is a good indicator according to WHO advisory group on integrative surveillance who estimated that samples from both healthy animals and sick animals are useful for surveillance but samples from healthy animals should be the primary focus for surveillance because such samples can provide an unbiased measure of antimicrobial resistance in animals source of human food supply (WHO, 2017).

Studied bacteria were Salmonella, *Staphylococcus* spp., *E. coli* and *Campylobacter* spp. . These bacteria are important bacteria commonly investigated and monitored for resistance in many monitoring systems. According to WHO advisory group worldwide, Salmonella is the first priority for inclusion in a program of integrated surveillance of antimicrobial resistance in foodborne bacteria. Campylobacter spp. is also an important foodborne pathogen and should be included in program of integrated surveillance of antimicrobial resistance in foodborne bacteria. Because Escherichia coli are common and some strain variants may cause disease, E. coli is used as a sentinel organism for antimicrobial resistance. *E. coli* also serve as reservoirs of resistance genes that can be transferred to human pathogens transiting the intestinal tract; as such, it provides information on the flow of Gram-negative resistance trend in the food chain (WHO, 2017).

The majority of studies included used the disk diffusion method and CASFM guidelines. This reduces the impact of the variation in AMR methodology on the pooled estimation. Resistance was found to be high in *E. coli* strains from poultry to Tetracycline (MR: 97%; IQR [80.65-98.5%]); Sulfoxide (MR: 81.4%; IQR [81.1-81.7%]); Sulfoxide-Trimethoprim (MR: 61%; IQR [46.57-68.85]) and Ampicillin (42.68%; IQR [40.58-60.43%]). Similar high rates of resistance in *E.coli* have been reported for tetracycline (10.6- 95%), ampicillin (6.02- 95.7%) and trimethoprim/sulfamethoxazole (4.49-80%) in a previous

review (Alonso et al., 2016).

In Salmonella strains, high resistance were found to: Erythromycin (MR: 100%; IQR [99-100%]; Amoxicillin-Clavulanic (47.76%; IQR [16.06-52.52%]); and tetracycline (46.04%; IQR [38.06-60.75]). Full resistance to Erythromycin (100%) is not surprising as Salmonella is known to have natural resistance to this antibiotic (Braoudaki and Hilton, 2005). The findings are comparable with previous review in the African region by Founou et al. (2018) who identified similar resistance of Salmonella. Indeed, these authors' reported a high level of resistance of Salmonella (80.9% [95% CIs; 54-93.8%) in a Meta-analysis of all reported resistance to all antibiotics. This findings highlights serious concerns relating to the use of these antibiotics in animals and corroborate similar findings in Cameroun where Moctar et al. (2019) reported high levels of resistance of *E. coli* and Salmonella spp. to all classes of antibiotics tested who are usually antibiotics of critical importance for humans and animals according to WHO and OIE classifications. Very few publications associated molecular investigation of resistance to phenotypic resistance. This is because the most important in these countries remain the inventory of the situation of antibiotic resistance before any thorough investigation.

Given the findings of this review, harmonization efforts are urgently needed in West Africa French speaking countries. Standardizing bacteria of interest, antibiotic to be tested (to avoid testing bacteria against antibiotics for which they have a natural resistance such as salmonella against erythromycin which has been reported a lot in studies when this is not really of interest to public health), AMR methods, interpretation guidelines, report format and prioritizing animal population of interest, could allow better comparability of results and situation from all countries. Also in this sense, technical and financial support of laboratories in these countries is a necessity to support the monitoring of the situation in human and food producing animals in a one health approach as recommended by WHO (2017) so as not to undermine the efforts undertaken around the world to combat resistances to antibiotics. The limitations of the current review include the exclusion of English language countries as Nigeria and Ghana biasing this view of the situation in the wall West Africa Sub region. For example, only in Nigeria, with similar inclusion and exclusion criteria, the review of (Oloso et al., 2018) reported 77 studies from animal sector. This shows that the inclusion of the situation in English-speaking countries would provide a better view of the situation in West Africa. A further limitation is combining AMR results from different species across different countries to have median resistance for a bacterium. However, given the observed trends, it is believed that the resolution of the obtained data was sufficient to show general trend of AMR in livestock in French Speaking countries. Moreover, since no monitoring system exist, this review can draw attention of sub regional institutions as ECOWAS to further support the establishment of One Health monitoring networks for the wall countries as it is done in many part of the word especially in Europe with EUCAST monitoring network (Silley et al., 2011).

Conclusion

Tackling the public health threat posed by antimicrobial resistance requires effective antimicrobial resistance surveillance programs. The essential need for robust antimicrobial resistance surveillance systems is emphasized in the Global Action Plan on Antimicrobial Resistance (WHO, 2017). Based on this study, antibiotic resistance is high in food producing animals in West Africa French speaking countries. It is necessary to design a carefully planned, multi-sectoral, surveillance plan, which can be used for research and monitoring of resistances in all countries and sectors in a one health approach. The relevant ministries and governments should enforce registration and monitoring of veterinary drugs use, promote good practices in antimicrobial use by trained professionals.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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In-vitro assessment of antibacterial activity of crude methanolic and aqueous extracts of *Mitracarpus villosus*

Sunday Ocholi Samson, Emmanuel Edegbo and Cornelius Arome Omatola*

Department of Microbiology, Faculty of Natural Sciences, Kogi State University, Anyigba, Kogi State, Nigeria.

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Globally, there is an increasing report of bacterial resistance to currently available antibiotics. The urgent need for newer therapeutic modalities was the incentive for the current evaluation of bactericidal and inhibitory effects of methanolic and aqueous extracts of *Mitracarpus villosus* on selected antibiotic resistant Gram-positive and Gram-negative bacteria. Ten-fold serial dilution was used to test the effects of the extracts. The percentage yield of extractable components was 22% for methanol and 8.3% for water. The minimum inhibitory concentration (MIC) of methanolic extract was 0.001 mg/mL against *Streptococcus pyogenes, Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa,* and *Salmonella* Typhi showing resistance to antibiotics and 0.01 mg/mL for *Staphylococcus aureus*, *Bacillus subtilis,* and *Klebsiella pneumoniae* while the minimum bactericidal concentration (MBC) was 0.1 mg/mL for all the isolates except *S. aureus* and *E. coli* with MBC of 0.01 mg/mL. Similarly, the MIC of aqueous extract was 0.01 mg/mL for *S. pyogenes, S. aureus, B. subtilis,* and *K. pneumoniae*; 0.1 mg/mL against *S. faecalis* and *E. coli;* 0.001 mg/mL against *P. aeruginosa* and *S. typhi*. The MBC of the aqueous extract at 0.1 mg/mL was active against *S. pyogenes*, *S. faecalis*, *P. aeruginosa, K. pneumonia,* and *S. typhi*; 1.0 mg/mL was active against *S. aureus*, *B. subtilis,* and *E. coli*. Findings from the present study indicate that the extracts of *M. villosus* have the potential to be used for treatment and cure of infections caused by selected bacteria. The methanolic extracts show more potent activity. Further study on a larger scale at varied concentrations including a test for potential toxicity in cultured cells are needed to depict their safety profiles before recommendation for inclusion in the antibacterial armamentarium.

Key words: Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), *M. villosus*, bioactive compounds.

INTRODUCTION

A large number of compounds of therapeutic values are traditionally produced from plants that possess antimicrobial activity (Khameneh et al., 2019). Successful defense mechanisms developed by these plants are responsible for the scarcity of infective diseases in those plants (Bolla et al., 2011). A report of Sivananthan (2013)

*Corresponding author. E-mail: omatola.ca@ksu.edu.ng. Tel: 08061259317.

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indicates the potential inhibitory effects of some bioactive compounds on the life processes of disease-causing bacteria, either alone or in combination with other agents. Several studies have explored the potential of medicinal plant administration for the treatment of bacterial related infections (Irobi and Daramola, 1994; Panda, 2014; Martins and Nunez, 2015). However, most of these published reports are from countries where traditional medicines for curative treatment of diverse diseases are frequently adopted owing to economic or practical reasons. Kokoska et al. (2019) and Shedoeva et al. (2019) mention the antimicrobial activity of *Aloe vera* that has been used for the local treatment of wounds, burns, and infections since ancient times from several *in vitro, in vivo,* and clinical studies. One major drawback is the concern of safety. A recent study highlighted the toxicity property of some of the diverse plants frequently used in traditional medicine (Mabona and Van Vuuren, 2013). While this needs to be addressed, it is not an exaggeration to say that the health benefits accrued to their use have been enormous.

The plant *Mitracarpus villosus* is abundantly distributed on farmlands as a weed across the Southern and Northern parts of Nigeria (Onyiloyi et al., 2005), and as also in Senegal, Mali, Liberia, and Gambia (Abere et al., 2007). Previous phytochemical studies and antimicrobial sensitivity tests of the leaf of *M. villosus* extract reported the potential antibacterial activity of the plants on a few organisms (Irobi and Daramola, 1994; Gbala and Anibijuwon, 2018). Most traditional medicines in West Africa use *M. villosus* as a remedy for toothaches, headaches (Dalziel, 1992), amenorrhea, leprosy, hepatic diseases, skin diseases (Kerharo and Adam, 1974), venereal disease (Medical Online News, 2019), neurological disorders (John-Africa et al., 2014), dyspepsia, and dysentery (Fabri et al., 2012). Antibacterial effects of the crude ethanolic extract have also been reported for *Bacillus subtilis, Streptococcus pyogenes, Escherichia coli,* and *Staphylococcus aureus* (Okoye and Okafor, 2013).

Information regarding the *M. villosus* (Swartz) DC plant commonly used for local treatment of wound infections is scarce. Traditional plants of known medicinal values are a rich source of active biological compounds with high antibacterial properties (Karygianni et al., 2014). The presence of compounds of low molecular weight, phytoalexins, in medicinal plants including the *M. villosus* plant has been shown to possess antibacterial activity (Hemaiswarya et al., 2008). Bioactive compounds including flavonoids, polyphenols, glycosteroids, and terpenoids have been reported in *M. villosus* (Hemaiswarya et al., 2008). Daiane and Cecilia (2015) and Ekpendu et al. (2001) reported a reasonable amount of triterpenes, terpenoids, and glycosides, in association with *M. villosus* known to exert various medicinal properties.

Nigeria has an abundant flora, and many species are believed to possess curative activities. Nevertheless, many of these claims have limited scientific validation. The current study may validate the antibacterial activities of *M. villosus* especially against multidrug-resistant bacteria including *Pseudomonas aeruginosa, S. aureus,* and *E. coli*, frequently implicated in delayed healing and infections.

Considering the increasing resistance to currently available antibiotics, the need to develop novel and more potent antibacterial agents to combat the global challenge of antimicrobial resistance is of high priority. Antibiotic resistance remains a serious threat to an effective prevention and treatment of an ever-increasing range of infections caused by bacteria (WHO, 2020). Antibiotic resistance contributes to increase in the cost of health care due to longer duration of illness, additional tests, use of more expensive drugs and more intensive care. Factors such as accumulation of mutations in bacterial genome due to drug selection pressure or natural mutations, misuse and overuse of antimicrobials are associated with increase prevalence of antibiotic resistance (WHO, 2020). The problem of multi-drug resistance is compounded by the widespread indiscriminate use of antibiotics that over time has enhanced bacterial pathogenic potential leading to default in treatment of infections caused by them. This study sought to determine the susceptibility of selected antibiotic resistant Gram-positive and Gram-negative bacteria commonly implicated in diseases in the study area to crude methanolic and aqueous extracts of *M. villosus*. Extract of *M. villosus* is frequently used in different communities in Africa for treating various diseases (Neuwinger, 2000) without knowing the active ingredients and pharmacological basis of their action. Identifying the bioactive compounds and its refinement as well as optimization may set the pace for future studies on the toxicity and safety profiles of the use of the plant extract.

MATERIALS AND METHODS

Sample collection and preparation

The whole plant of *M. villosus* (Swartz) DC was collected from the Botanical Garden of Kogi State University, Anyigba, Nigeria (Figure 1). The plant was identified and authenticated in the Department of Plant Science and Biotechnology of the same university. The sample (whole plant of *M. villosus)* was washed and dried at room temperature in the laboratory for two weeks. Using mortar and pestle, the sample was pulverized to powdery form and placed in a polythene bag until ready for extraction. The maceration method with Soxhlet (solvent) and aqueous (water) were used to extract the bioactive agents.

Test organisms

Eight bacteria were used: *S. pyogenes, S. aureus, Enterococcus faecalis, B. subtilis, E. coli, P. aeruginosa, Klebsiella pneumoniae,*

Figure 1. Field image of *Mitracarpus villosus* (Swartz) DC plant.

and *Salmonella* species. The bacteria are clinical isolates with resistance to cotrimoxazole, ampicillin, tetracycline, cefotaxime, amoxicillin clavulanic acid, ciprofloxacin, ceftazidime, cefoxitin, and gentamicin in the study area. They were obtained from the stock culture of the Microbiology Laboratory, Kogi State University, Anyigba.

Aqueous extraction was carried out as described by Irobi and Daramola (1994). Briefly, about 120 g of the powdered plant sample of *M. villosus* was weighed and dispensed in 250 mL of distilled water in a clean and sterile beaker. The resulting solution was boiled for 20 min on a Bunsen burner and then cooled. The extract was filtered and the filtrate was collected in a sterile and clean conical flask.

For Soxhlet extraction, about 120 g of pulverized plant sample was subjected to Soxhlet extraction as described by Akinyemi et al. (2000) using 800 mL of extraction solvent (methanol) at 80°C for 6 h for complete extraction. This was performed in the chemistry laboratory of Kogi State University, Anyigba. 120 g of the powdered plant sample was weighed, packaged in a serviette paper, tied with a thread, and placed inside the thimble. The latter was loaded into the main chamber of the Soxhlet extractor. The solvent for extraction was placed in a distillation flask which was then connected to the heating element. The Soxhlet extractor was placed atop the flask followed by a reflux condenser and the cycles of extraction began. After 6 h of several cycles, the compound was concentrated in the distillation flask and evaporated to dryness on a rotary evaporator at 78°C for 60 min. The soluble component was retained while the non-soluble portion of the extracted solid still in the thimble was discarded. The same procedure was repeated until the desired material was completely extracted from the plant.

Phytochemical screening of extracts

Both water and methanol extracts were screened for the presence of tannins, saponins, alkaloids, glycosides, flavonoids, carbohydrates, terpenes, and anthraquinones by adopting the methods described by Trease and Evans (2009) (Table 1).

Test for carbohydrates

One gram of the extract (water or methanol) was transferred to the test tube and 5 mL of an equal mixture of Fehling's solution A and B was added and boiled in a water bath, the formation of a brick-red precipitate is suggestive of the presence of reducing sugar.

Test for cardiac glycosides

One gram of the extract was dissolved in 1 ml of glacial acetic acid containing traces of Ferric chloride solution and the resulting mixture was transferred into a dry test tube and 1 ml of concentrated sulphuric acid was added down the side of the test tube to form a lower layer at the bottom. The formation of a purple-brown ring is suggestive of cardiac glycoside's presence (Bhatt, 2019).

Test for saponins

To 1 g of the extract, 10 mL of distilled water was added, shaken vigorously for 30 s, and the tube allowed to stands in an upright position. The tube was observed for 30 min for honeycomb froth that persisted for 10-15 min, indicative of saponins.

Test for steroids and triterpenes

One gram of the extract and equal volume of acetic acid anhydride

Table 1. Phytochemical screening for methanolic and aqueous extracts of *Mitracarpus villosus*.

Phytochemical compound	Methanolic extract	Aqueous extract
Glycosides	+	-
Anthraquinones	-	-
Terpenoids	+	-
Steroids	+	+
Alkaloids	-	-
Saponins	+	+
Tannins	+	-
Flavonoids	+	+

Present (+); Absent (-).

were mixed gently in a test tube after which 1 mL of concentrated sulphuric acid was added down the side of the test tube to form a layer. Color changes were observed immediately and over 1 h. Immediate formation of blue color at the upper layer and a reddish color indicated the presence of steroids and triterpenes, respectively.

Test for flavonoids

To 1 g of extract, a few drops of ferric chloride solution were added and the formation of a green precipitate indicated the presence of phenols.

Test for tannins

Three to 5 drops of ferric chloride solution were added to a portion of 1 g of the extract, and the resulting dark green precipitate indicated the presence of tannins.

Test for alkaloids

A few drops of Wagner's reagent were added to a portion of 1 g extract and the formation of a whitish precipitate is suggestive of alkaloids presence.

Test for free anthraquinones

Five milliliter of chloroform was added to a portion of 1 g extract in a dry test tube and then shaken to mix properly. The resulting solution was filtered to obtain filtrate, mixed with an equal volume of 10% ammonia solution. The formation of bright pink color in the aqueous (upper) layer is suggestive of free anthraquinones.

Determination of percentage yield of plant extract

The following is the equation for plant extract (Terblanche et al., 2017):

$$\%\ Extraction\ yield = \frac{\text{Weight of the dry extract after solvents have been removed}}{\text{Weight of the dry plant before extraction}} \times 100$$

$$= \frac{X_1 - X_2}{X_3} \times 100$$

where X_1 = weight of powdered leaves, X_2 = weight of chaff after extraction, X_3 = amount (g) of finely grounded plant material used.

Methanol extract = 120 - 93.6 = 26.4 g

$$\%\ yield = \frac{26.4}{120} x100 = 22\%$$

Aqueous Extract = 120 – 110.6 = 9.4 g

$$\%\ yield = \frac{9.4}{120} x100 = 8.3\%$$

Preliminary screening of extract concentrations for antibacterial activity by agar well diffusion method

One milliliter of each test organism at 10^8 CFU/mL was used to flood Mueller-Hinton agar plates (prepared separately for methanolic and aqueous extracts) at aseptic conditions and allowed to solidify. On each of the solidified agar plates, six wells were done with a sterile cork-borer (diameter of 6 mm). The prepared plant extracts, 100% extract concentration, diluted concentrations (1, 0.1, 0.01 and 0.001 mg/mL) and distilled water as control, were loaded into each of the wells separately. Following the incubation at 37°C for 24 h, the presence of visible zones of inhibition around the wells shows the extract can inhibit the test organism at that concentration. The wells filled with distilled water show no visible zone of inhibition (NCCLS, 2006).

Evaluation of antibacterial activity by macrodilution technique

Following the National Committee for Clinical Laboratory Standards (2006), suspensions of micro-organisms was prepared in sterile normal saline and adjusted to 0.5 MacFarland standard (1.5×10^8 Cfu/mL), minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined (Jones et al., 2002; Wikler, 2006). Mueller-Hinton broth was added (1 mL) to each tube. Then, 100% concentration of the whole plant extract was added to the first well and the other four concentrations of the extract (1, 0.1, 0.01 and 0.001 mg/mL, respectively) were prepared by serial macrodilution technique. Bacterial suspensions (1.5×10^8 CFU/mL), 1 mL each was added to each tube, and mixed. In each case of the extract (aqueous or methanolic), a medium in the tube containing bacterial suspension and another containing extracts were used as positive and negative controls, respectively. The tubes were incubated for 24 h at 37°C and the cloudiness was examined via unaided eyes. Tubes without turbidity were considered as MIC and subcultured on a Mueller-Hinton broth, incubated for 24 h at 37°C. The MBC was determined according to the methods described by Irobi and Daramola (1994) with little adjustments. The

Table 2. Results of preliminary screening of antibacterial effect of crude methanolic and aqueous extracts of *M. villosus* against selected Gram-positive and Gram-negative bacteria.

Test organism	Methanolic extract	Aqueous extract	Control
Gram-positive bacteria			
Streptococcus pyogenes	-	-	+
Staphylococcus aureus	-	-	+
Streptococcus faecalis	-	-	+
Bacillus subtilis	-	-	+
Gram-negative bacteria			
Escherichia coli	-	-	+
Pseudomonas aeruginosa	-	-	+
Klebsiella pneumonia	-	-	+
Salmonella Typhi	-	-	+

Growth (+); No growth (-).

Table 3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of crude methanolic and aqueous extracts of *M. villosus* against selected Gram-positive and Gram-negative bacteria.

Gram-positive bacterial isolate	Methanolic extract (mg/mL)		Aqueous extract (mg/mL)	
	MIC	MBC	MIC	MBC
Streptococcus pyogenes	0.001	0.1	0.01	0.1
Staphylococcus aureus	0.01	0.01	0.01	1.0
Streptococcus faecalis	0.001	0.1	0.1	0.1
Bacillus subtilis	0.01	0.1	0.01	1.0
Gram-negative bacterial isolate				
Escherichia coli	0.001	0.01	0.1	1.0
Pseudomonas aeruginosa	0.001	0.1	0.001	0.1
Klebsiella pneumonia	0.01	0.1	0.01	0.1
Salmonella Typhi	0.001	0.1	0.001	0.1

lowest concentration at which the extract did not permit any growth of a particular microorganism was considered MBC. This experiment was repeated three consecutive times for each methanolic and aqueous extracts.

RESULTS AND DISCUSSION

The percentage yield of crude aqueous extract and methanol extract was 9.4 g (corresponding to 8.3%) and 26.4 g (corresponding to 22%), respectively. Table 1 depicts the results of the phytochemical screening. In the methanolic extract, anthraquinones and alkaloids tested negative while glycosides, terpenoids, steroids, saponin, tannin, and flavonoids tested positive. In the aqueous extract, glycosides, anthraquinones, terpenoids, alkaloids, and tannins tested negative while flavonoids, steroids, and saponins tested positive. The results of the antibacterial effects of the crude extract varied at different concentrations against both Gram-positive and Gram-negative bacteria are shown in Table 2. Results showed that none of the selected organism could grow in the presence of either the methanolic or water crude extract preparations. The Minimum Inhibitory Concentrations (MICs) and the Minimum Bactericidal Concentrations (MBCs) of the crude extracts against the selected bacteria are depicted in Table 3. The MIC of methanolic extract at 0.001 mg/mL was active against *S. pyogenes, S. faecalis*, *E. coli*, *P. aeruginosa,* and *S.* Typhi except three isolates. The MBC of the methanolic extract was 0.1 mg/mL for all the isolates except *S. aureus* and *E. coli* which was at 0.01 mg/mL. Similarly, the MIC of aqueous extract at 0.01 mg/mL was active against *S. pyogenes, S. aureus, B. subtilis,* and *K. pneumonia* while the MBC was at 0.1 mg/mL against all the isolates except *S. aureus*, *B. subtilis,* and *E. coli*.

Our findings showed that both extracts (methanolic and aqueous) have strong inhibitory effects on the growth of the test organisms. The results of the phytochemical

study in extracts of *M. villosus* revealed the presence of bioactive agents such as tannins, flavonoids, saponins, glycosides, terpenoids, and steroids, all of which exhibited antibacterial activity. Our finding supports previous reports by Ubani et al. (2012) and Gbala and Anibijuwon (2018) which indicated that tannins, flavonoids, saponins, glycosides, terpenoids, and steroids are capable of bacterial growth inhibition. However, variations in active substance detection have been documented. Ouadja et al. (2018) identified phenolic and alkaloids in a different species (*Mitracarpus scaber)* of the same plant which were not identified in the current study, suggesting that variability in bioactive compounds extraction or bio-responsiveness in plant estrogen levels may affect their detection. Ubani et al. (2012), also attributed differences in bioactive substance detection to variation in geographical location as a result of soil mineral concentration.

Results of MIC and MBCs fractions of the methanolic and aqueous extracts shown in Tables 2 and 3 revealed the promising antibacterial activity of the extracts against species of Gram-positive and Gram-negative bacteria such as *S. pyogenes, S. aureus, S. faecalis, B. subtilis*, *E. coli*, *P. aeruginosa*, *K. pneumoniae, S. typhi,* etc. This finding suggests that, isolation of the active compounds is needed to enable further development of this plant as a source for anti-bacterial substances and the potential pharmacognostic properties of the extract when developed, could surmounts the global treatment challenges associated with antibiotics resistant pathogens frequently encountered in clinical practice.

Findings from the present study showed that the methanolic extract of *M. villosus* inhibited the selected test organisms better than the aqueous extract, as can be seen from the lower MIC and MBC. This is supported by the higher amounts of bioactive compounds obtained in Table 1 that are known to inhibit and/or kill the bacteria at lower concentrations compared to aqueous (water) extract. Previous reports of Abdelrahim et al. (2017) also showed that the methanol extract of *Mitracarpus* has a higher inhibitory effect on the growth of *B. subtilis, S. aureus, E. coli,* and *P. aeruginosa* compared to the aqueous extract.

The fact that crude extracts of this medicinal herb inhibit some Gram-positive and Gram-negative bacteria indicates the presence of potent antibacterial activities which can be developed (Zahra et al., 2020). Although both the aqueous and methanolic extracts of *M. villosus* produced inhibitory action against the bacteria, the latter was more active against the bacteria. The need for lower concentrations of the methanol extracts for antibacterial activity compared to the aqueous extracts could be attributed to the efficacy of solvents for extraction of active compounds and also suggests that methanol is a better solvent for isolation of antibacterial principles.

In conclusion, methanolic and aqueous extracts of *M. villosus* have been shown to possess antibacterial activity on all selected test organisms in this study. The inhibitory effect demonstrated by this extract against test isolates even at their lower concentrations depicts the ethnopharmacological benefit. This observation supports other scientific articles that reported that extract of the plant has potential therapeutic values against *S. pyogenes, S. faecalis*, *E. coli*, *P. aeruginosa, S.* Typhi, *S. aureus*, *B. subtilis,* and *K. pneumonia* resistant isolates commonly associated with various ailments as claimed by traditional medicine healers. Despite the inhibitory effects of extracts of *M. villosus* on selected clinical isolates that have shown relative resistance to the conventional antibiotics including cotrimoxazole, ampicillin, tetracycline, cefotaxime, amoxicillin clavulanic acid, and ciprofloxacin in the study area (Mofolorunso et al., 2020), further study on individual isolated compounds and their potential for toxicity and safety profiles are needed.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Comparison of disk-diffusion and E-test methods for *in-vitro* susceptibility of *Streptococcus pneumoniae* isolates to oxacillin and ceftriaxone

Walekhwa Michael[1*], Margaret Muturi[2], Tonui Josephat[1], Suge Titus[3] and Kabera Beatrice[4]

[1]Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, Kabarak University, Kenya.
[2]Department of Medical Laboratory Sciences, Faculty of Medicine, Kenyatta University, Kenya.
[3]Department of Pharmacology and Pharmacognosy, Faculty of Pharmacy, Kabarak University, Kenya.
[4]Department of Pathology and Laboratory Medicine, Getrudes Children's Hospital, Kenya.

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Susceptibility of *Streptococcus pneumoniae* (pneumococci) to oxacillin and ceftriaxone was compared using disk diffusion and E-test methods. A total of 206 children attending Gertrude's Children's Hospital (GCH) were recruited. Sterile Copan Flocked Swabs were used to obtain nasopharyngeal swabs. Samples were inoculated on gentamicin blood agar and initial identification was done on the basis of colony morphology. Optochin test was performed to definitively identify the isolates as pneumococci. Antibiotic testing was done using disk diffusion and E-test methods on Muller Hinton agar enriched with 5% defibrinated sheep blood. A total of 42 (20%) isolates were recovered from the samples. Based on disk diffusion method, 74 and 40% of the isolates were resistant to oxacillin and ceftriaxone while; on the basis of E-test, 45 and 14% of the isolates were resistant to oxacillin and ceftriaxone, respectively. About 40% (*n*=17) of isolates that had zone diameters ≥ 20 mm which is considered susceptible to oxacillin by disk diffusion, had MICs ≤0.06 µg/ml correspondingly susceptible by E-test. Most isolates deemed susceptible to (≤0.5 µg/ml) ceftriaxone by E-test (72%, *n*=30) also exhibited susceptibility (≥27mm) to the antimicrobial agent by disk diffusion. E-test presents more sensitive results compared to disk diffusion. Isolates that exhibit resistance to penicillin and ceftriaxone by disk diffusion should be confirmed by E-test before being reported as resistant.

Key words: E-test, disk diffusion, antibiotic resistance, ceftriaxone and oxacillin.

INTRODUCTION

Pneumococcus (*Streptococcus pneumoniae*) kills over 1 million children across the world every year (Köksal et al*.,* 2017). Remarkably, more than 50% of these fatalities occur in Africa (Pagan, 2011). To reverse this trend, the World Health Organization (WHO) recommends two major interventions: inclusion of higher valence conjugate vaccines in national immunization programs and judicious use of antibiotic agents (WHO, 2011). Kenya introduced the 10 valent pneumococcal conjugate vaccine (PCV-10) in her national immunization program (KEPI) in 2011 (Ojal et al*.,* 2019). The uptake of the vaccine overtime surpassed the recommended threshold and cases of

pneumococcal infections due to the vaccine serotypes reduced remarkably (Hammitt et al., 2014). Ironically, pneumococcal disease has continuously remained one of the leading etiologies of child morbidity and mortality in Kenya (Heath et al., 2018). As a result, appropriate use of antibiotics seems to be the only other promising alternative (Esposito and Principi, 2013).

Information on susceptibility and resistance profiles to relevant antibiotics is paramount if their effective use is to be achieved (Reeve et al., 2015). This can only be made possible by use of reliable, accurate, precise and affordable laboratory methods. Often, the choice of the method used is determined by its reproducibility, accuracy, cost, practicality among others (OIE., 2012). Antibiotics in the class of macrolides are used to treat pneumococcal infections but mostly among persons above the age of five years. For pediatrics, the recommended agents are mainly beta-lactams like ampicillin (Gamache, 2019). For children aged between 2 days to 5 years, cephalosporins like cefotaxime or ceftriaxone can be administered as alternative drug therapy and as a single therapy (Gamache, 2019). While this antibiotic plan has been effective among pediatrics over-time, cases of penicillin resistance have been on the rise lately (Daniel, 2020). Unfortunately, studies have reported that most penicillin resistant pneumococci have also exhibited high resistance to fallback alternatives like cephalosporins (Kim et al., 2016). Considering the fact that ceftriaxone (cephalosporin) is one of the agents used in cases where penicillins have failed, it is important to understand its *invitro* effectiveness against pneumococci.

Laboratory assays such as broth dilution, agar dilution, disk diffusion and molecular based methods are used to profile susceptibility of bacteria to several antibiotic agents (OIE., 2012). The broth dilution method determines the lowest concentration of an antibiotic agent that hinders growth of bacteria (Clinical and Laboratory Standards Institute (CLSI), 2008). The test is done using varying antimicrobial concentrations against optimal bacterial inocula. The point between the lowest minimum inhibitory concentration (MIC) and the next point is recorded. As such, broth dilution method may not always signify total values and may therefore be prone to errors. The technique is not flexible to the changing surveillance needs because dilution panels are often predesigned and available commercially (Yakoob, 2014). Therefore, utilization of reference organisms for quality checks is fundamental to ensure reproducibility and accuracy of results. Procurement of test panels and relevant equipment for broth dilution assays may be costly and therefore the test may not be viable for some laboratories especially in the developing world (Turnidge and John, 2015).

In agar dilution method, gradually changing serial dilutions of the antibiotic agent is integrated in a preferred culture medium. Bacterial inoculum is thereafter applied on the surface of the media plate (Wiegand et al., 2008). Except for organisms that exhibit swarming, this method represents the most reliable and reproducible results (Griffin et al., 2000). However, the technique may be labor intensive and expensive especially if not automated. The ability to accurately determine endpoints and purity of the inocula makes it partially inappropriate. The disk diffusion (Kirby Bauer) method is relatively affordable and effective (Shields et al., 2018). According to Hudzicki (2016), the method measures capacity of antimicrobials to inhibit growth of bacteria under optimal conditions. The radius of the zone around an antibiotic disk in which the bacterium has not grown is directly proportional to the efficacy of the antibiotic agent against that bacterium. While the manual measurement of the inhibition zone may present a platform for inaccurate results, automated methods of measurement are now available (EUCAST, 2015). The technique is easy to perform, provides relatively reproducible results and is generally less costly.

E-test is a gradient based technique that utilizes both dilution and diffusion capacities of the antimicrobial agent of interest (Romney and Hindler, 2015). The point at which the lower part of the growth intersects with the ellipse strip represents the minimum inhibition concentration (MIC) value (Bremmer et al., 2017). The test provides a substitute method to detecting even low levels of resistance for a range of bacteria (Amy, 2016). However, it may be slightly expensive given the high cost of purchasing commercially available gradient strips. Molecular based methods like polymerase chain reaction (PCR) and gene expert detect the presence of resistant genes (Dunne et al., 2017). The techniques are highly rapid and obviously less labor intensive. Unfortunately, the level of expertise, quality of reagents and equipment required make molecular based methods unpopular for economically emerging regions.

Against this backdrop, most laboratories in the developing world routinely use disk diffusion and e-test methods for antimicrobial susceptibility testing (AST). Therefore, continuous surveillance and quality checks to establish reproducibility and reliability of results obtained using these methods may be fundamental to provision of effective clinical management. This study compared

*Corresponding author. E-mail: walekhwam@gmail.com. Tel: +254-705-290520.

ORCID: 0000-0002-9038-6597.

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sensitivity levels of the disk diffusion and e-test methods using oxacillin and ceftriaxone antibiotic agents against Streptococcus pneumoniae isolates.

MATERIALS AND METHODS

Subject recruitment

Samples were collected between February 2017 and February 2018 from children below the age of five years seeking care at Gertrude's Childrens hospital (GCH). The participants were clinically diagnosed of a variant of pneumococcal disease by the resident clinician. Biological mothers or legal guardians gave voluntary written assent permitting the recruitment of their children to the study. Any child who had a known history of an immunosuppressive condition and those who had used any antibiotic agent two weeks prior to collection of the sample were excluded.

Research design and sampling technique

The study used cross-sectional descriptive design. Study participants were studied within a specified period of time and findings inferred to the entire population of interest. Purposive sampling method was used to recruit subjects because the researchers had pre-determined rationale that prospective participants required to satisfy before being recruited.

Ethical considerations

Ethics approval for the study was given by Kenyatta University Ethics Review Committee (KU/ERC/APPROVAL/VOL. 1 (12)). The research permit was given by the National Commission of Science Technology and Innovation (NACOSTI/P/17/65428/15801). The study was further approved by the Gertrude's Childrens Hospital Research Committee to be conducted at the outpatient clinic of GCH (GCH/ERB/VOL.MMXVII/121). A written informed assent was given by parents or legal guardians to prospective subjects before recruitment.

Sample size determination

To determine the minimum sample size, the formula for Fisher et al. (1991) was used, with a prevalence rate of 17% (Githii et al*.,* 2013).

$$n = \frac{z^2 \hat{p}(1-\hat{p})}{m^2}$$

Where:

n= Desired minimal sample size (where population is ≥10,000);
z= Standard normal deviation = 1.96;
p= Prevalence rate;
m= the desired degree of accuracy @ 95% confidence level= 0.05 and;
$n=1.96^2 \times 0.17(1-0.17) / 0.05^2=217$

Sample size (*n*) = 217

Since the target population is ≤10000, the value of n was further adjusted as follows:

nf= *n*/1+ {*n*/N}

Where:

nf= Desired minimum sample size (where population is ≤10,000)
n= Calculated sample size N= Total population nf= 217÷ [1+ (217/5,000)] *nf*= 207 subjects

Sample collection and processing

Nasopharyngeal swabs were collected by use of Copan Flocked Swabs (FLOQSwabs®) according to (CDC, 2015). Collected samples were immediately suspended in Amies Transport Media and kept in a cool box with ice until transportation to laboratory within three hours of collection. At the laboratory, samples were immediately streaked on 5% sheep blood agar (BA) containing 5.5 Pg/ml of gentamicin (GBA) and incubated in anaerobic conditions at 37°C for 24-48 h. Gentamicin was added to prevent growth of bacteria other than *Streptococcus pneumoniae*. Initial identification of the pneumococcus was on the basis of colony morphology as follows: α-hemolysis, draughtsman's appearance and a mixture of either large or small gray-mucoid colonies. Initially identified isolates were further cultured on blood agar in anaerobic conditions at favorable conditions. Optochin test was done on each sample that had initially demonstrated features akin to pneumococcus for definitive identification. Fresh cultures were thereafter obtained from the BA plates, inoculated in brain heart infusion agar (BHI) enriched with 5% defibrinated sheep blood and stored at -80°C.

Disk diffusion (Kirby-Bauer) method

The stored isolates were thawed for a period of 30-45 min at room temperature in a sterile room. Colony suspension equivalent to 0.5 McFarland standard was prepared and inoculated evenly on Mueller Hinton agar enriched with 5% sheep blood. A sterile antibiotic disk dispenser (ADD) was used to dispense ceftriaxone (10 μg) and oxacillin (1 μg) impregnated disks onto a 100 mm petri dish. The preparations were incubated in anaerobic conditions at 37°C for 24-48 h. A linear caliber was used to measure the diameter of the zone of inhibition including the diameter of the disk which was 6 mm. The results were interpreted based on Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2017). *S. pneumoniae* ATCC 49619 strain was used as part of quality control (QC).

E-test method

The stored isolates were thawed and fresh bacterial colony suspension equivalent to 0.5 McFarland standard was prepared, which was lawned evenly on Mueller Hinton agar enriched with 5% sheep blood. A sterile forceps was then used to place E-test strips (bioMe'rieux) containing gradually increasing concentrations of oxacillin and cetriaxone antibiotics on the opposite sides of each plate. The point on the epsilometer strip where the antibiotic agent no longer inhibited growth of the test organism was considered as the minimum inhibitory concentration (MIC).

Data analysis

Data were entered and analysed using SPSS version 22. Descriptive statistics and bivariate analysis were done to compare sensitivity levels of the pneumococci to oxacillin and ceftriaxone using disk diffusion and E-test methods. The analysis was done at 95% CI.

Table 1. Susceptibility of the pneumococci to oxacillin and ceftriaxone using disk diffusion method.

Name of antibiotic	Number of isolates (*n=42*)	Percent
Oxacillin (1 µg, ≥20 mm)		
Susceptible	8	19
Intermediate susceptible	3	7
Resistant	31	74
Ceftriaxone (10 µg, ≥27 mm)		
Susceptible	23	55
Intermediate susceptible	2	5
Resistant	17	40

Clearance zone classifications for oxacillin (1 µg) were: <15 mm: resistant, >16 mm<19 mm: intermediate susceptible and ≥20 mm: susceptible. Clearance zone classifications for ceftriaxone (10 µg) were: <24 mm: resistant, ≥25 mm≤26 mm: intermediate susceptible and ≥27 mm: susceptible.

RESULTS

Sensitivity of pneumococcus to oxacillin and ceftriaxone by disk diffusion method

A total of 42 pneumococci isolates were recovered from the samples and thereafter subjected to the study antibiotic agents using both E-test and disk diffusion methods. Based on disk diffusion findings, 74% (*n*=31) and 40% (*n*=17) of the isolates were resistant to oxacillin and ceftriaxone respectively. On the other hand, 55% (*n*=23) of the isolates were susceptible to ceftriaxone while 19% (*n*=8) were susceptible to oxacillin by disk diffusion as shown in Table 1.

E-test MICs (µg/ml) for pneumococci to oxacillin and cetriaxone

The minimum inhibitory concentration (MIC) results obtained from E-test showed varied sensitivity patterns for the pneumococcal isolates. More isolates exhibited resistance to oxacillin (45%, *n*=19) while 14% (*n*=6) were resistant to ceftriaxone. Isolates susceptible to oxacillin and ceftriaxone were 41% (*n*=17) and 72% (*n*=30) respectively as shown in Table 2. The distribution of MICs of the isolates on oxacillin compared to the respective zone diameters showed at ≤17 mm, 45% were resistant at ≥2.0 MIC. Further, 10% (*n*=4) and 5% (*n*=2) were intermediately susceptible at 0.12-1 and ≤ 0.06 MICs respectively. Interestingly, at ≥20 mm cut off, only 40% of the isolates were susceptible at ≤ 0.06 MIC as shown in Table 3. The minimum inhibitory concentration (MIC) for ceftriaxone compared with disk diffusion results showed that at ≤24 mm cut-off point for disk diffusion, only 14% (*n*=6) of the isolates were resistant at ≥2.0 MIC. Further, at ≥27 mm 72% (*n*=30) of the isolates were susceptible to oxacillin at ≤ 0.5 MIC. Finally, at 25-26 mm point, 12% (*n*=5) and 2% (*n*=1) were intermediately susceptible to the antibiotic at 1 and ≤ 0.5 MIC respectively as shown in Table 4.

DISCUSSION

Understanding sensitivity levels of disk diffusion and e-test methods of antimicrobial resistance is a vital step in the clinical management infections. This study compares sensitivity of the two methods in determining antibiotic susceptibility of clinically important *S. pneumoniae* isolates. Based on disk diffusion, 74% (*n*=31) and 40% (*n*=17) of the isolates were classified as resistant to oxacillin and ceftriaxone respectively while 55% (*n*=23) and 19% (*n*=8) were considered susceptible to ceftriaxone and oxacillin respectively (Table 1). The disk diffusion results indicate that more isolates were sensitive to ceftriaxone compared to oxacillin. However, it is imperative to note that ceftriaxone was tested at a higher concentration (10 µg) compared to oxacillin (1µg) and it is a broad spectrum cephalosporin with extended half-life. It may therefore not be unreasonable if one attributed these results to the reasons stated above. Further, the results demonstrate almost clear-cut coherence with that of Waqas (2019), which reported efficacy of 84.7% for ceftriaxone and 71.4% for penicillin.

The results obtained on e-test for both antimicrobial agents show that 45% (*n*=19) and 14% (*n*=6) of the isolates were resistant to oxacillin and ceftriaxone, respectively; while 40% (*n*=17) and 72% (*n*=30) were susceptible to oxacillin and ceftriaxone, respectively (Table 2). The pattern of e-test susceptibility results was consistent with disk diffusion results based on the number of isolates that were sensitive or resistant to the antimicrobial agents tested. Overall, ceftriaxone showed better efficacy compared to oxacillin in both methods. Previous studies have reported discrepancies in the results from e-test and disk diffusion methods (Erfani et al*.,* 2011). Notable is that the number of isolates resistant to both antibiotic agents was lower when using e-test method. This is a crucial comparator indicator of the two methods which qualifies e-test as being relatively superior to disk diffusion. Manoharan et al. (2003) reported that e-test presented minor to no errors when

Table 2. E-test minimum inhibitory concentrations (MICs) (µg/ml) for pneumococci (*n*=42) to oxacillin and ceftriaxone.

Antibiotic agent	Resistant	Intermediate	Susceptible
Oxacillin	19 (45%)	6 (14%)	17 (41%)
Ceftriaxone	6 (14%)	6 (14%)	30 (72%)

Interpretive MICs for oxacillin were**:** ≥2.0 µg/ml: resistant, 0.12-1.0 µg/ml: Intermediate and ≤0.06 µg/ml: Susceptible. MICs for ceftriaxone were: ≥2.0 µg/ml: resistant, 1: intermediate and ≤0.5 µg/ml: susceptible. QC strain ATCC 49619.

Table 3. Distribution of pneumococci (*n*=42) strains by MICs (µg/ml) of oxacillin and clearance zone diameter around a 1 µg oxacillin disk.

Zone of inhibition (mm) 1 µg oxacillin)	Number of isolates inhibited by oxacillin MIC (µg/ml)		
	≤ 0.06	0.12-1	≥2.0
≤17	0	0	19 (45%)
18-19	2 (5%)	4 (10%)	0
≥20	17 (40%)	0	0

Table 4. Distribution of pneumococci (*n*=42) by MICs (µg/ml) of ceftriaxone and respective clearance zone diameter around a 10 µg ceftriaxone disk.

Zone of inhibition (10 µg ceftriaxone disk mm)	Number of isolates inhibited by ceftriaxone MIC (µg/ml)		
	≤ 0.5	1	≥2.0
≤24	0	0	6 (14%)
25-26	1 (2%)	5 (12%)	0
≥27	30 (72%)	0	0

used to assay for AMR by *Haemophilus Influenzae* to chloramphenicol and ampicillin agents, respectively. This additional evidence enhances possibility of concluding that e-test method may be a little more sensitive than disk diffusion A comparison of the distribution of MICs with the corresponding zone diameters for the test antimicrobial agents revealed that 40% (n=17) of isolates with > 20 mm zone diameter for oxacillin had a corresponding MIC ≤ 0.06 µg/ml while 45% (n=19) had MIC≥2.0 µg/ml. On the contrary, 72% (n=30) isolates subjected to ceftriaxone had a corresponding MIC ≤ 0.5 µg/ml with fewer isolates (14%, n=6) having MIC ≥2.0 µg/ml. The findings demonstrate a higher efficacy of ceftriaxone against the pneumococcal isolates with possible resistance to Oxacillin exhibited by majority of the isolates. According to the CLSI, MIC (e-test) testing should be performed on all *Streptococcus pneumoniae* isolates showing oxacillin zones ≤ 19 mm before reporting as resistant (Horna et al*.,* 2016). E-test and disk diffusion methods are relatively easy to perform; present reproducible and reliable results and do not require high technical expertise. Unfortunately, although fast and consistent, procurement of e-test strips is relatively costly and may not constitute a viable option for routine assaying of AMR especially in low-income countries.

Conclusion

E-test method presents more sensitive results as compared to disk diffusion in terms of quantitative classification of the level of resistance. However, both methods are non-laborious, reliable techniques which can be adopted in resource limited settings.

RECOMMENDATION

Pneumococci isolates that exhibit resistance to ceftriaxone by disk diffusion method may be confirmed by e-test method before being reported as resistant.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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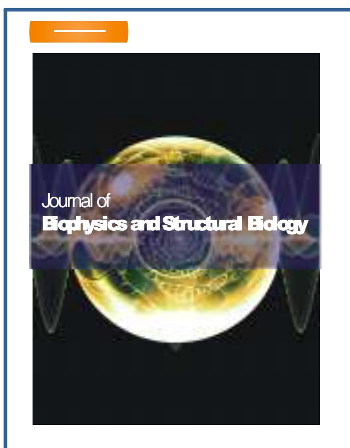

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