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African Journal of Microbiology Research

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

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Table of Content

Vol. 13(28), pp. 520-530, October, 2019
DOI: 10.5897/AJMR2019.9145
Article Number: C7710EA62002
ISSN: 1996-0808

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African Journal of Microbiology Research

Full Length Research Paper

Qualitative assessment of *Uvaria chamae* (bush banana) crude extracts against wound isolated strains of *Pseudomonas aeruginosa* and *Proteus mirabilis* alongside resistance and plasmid profiles determination of the isolates

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Received 15 May, 2019; Accepted August 1, 2019

***Uvaria chamae* is a plant used traditionally in treatment of wounds and other ailments. This work was conducted on qualitative assessment of crude extracts of *U. chamae* against wound isolated strains of *Pseudomonas aeruginosa* and *Proteus mirabilis,* determining the resistance and plasmid profiles of the isolates. Standard techniques were employed in crude extraction of roots and leaves of the plant followed by phytochemical screening. The test isolates were obtained from University of Uyo Medical Centre and re-screened using standard microbiological techniques. Antibacterial activities of the extracts on the isolates were assessed using agar-well diffusion technique. Plasmids were analyzed using gel electrophoresis. *U. chamae* contains tannis, alkaloids and other bioactive components. Antibacterial activities showed that ethanolic roots extracts possessed greater inhibitory effects on test isolates than any other extract forms with zones of inhibitions (Z.I) that ranged from 18±2.0 to 21±1.2 mm and resistances with no zone of inhibitions (N.I) observed with *P. aeruginosa*-1, *P. mirabilis* -1 and *P. mirabilis-3* at 50 mg/ml concentration. Some test isolates were resistances to other extract forms and standard antibiotics. Some harbored plasmids ranged from 564 bps -23.13 kbp. Whereas resistance patterns of some isolates were not reverted, resistances of these bacteria to various antimicrobial agents are of public health implications.**

Key words: Antibacterial activities, *Uvaria chamae*, wound isolates, resistance, plasmids.

INTRODUCTION

Plants have been a source of medicine in the past centuries and today, plant-based formulation continues to play an important role in health care of greater number of people worldwide (Karachi, 2006). They are used in various complementary, traditional and alternate systems of treatment of human diseases (Sher, 2009). Scientists and the general public recognize the value of plants as a source of new or complimentary medicinal products. In some Africa countries, there has been an increasing concern to source for locally available drugs and

alternative medicine to treat ailments. This is due to the fact that many synthetic and routine drugs are inaccessible and increase loss of effectiveness and potency due to increased multi-drugs resistance organisms to these agents (WHO, 2004; Wang et al., 2010). Other factors are inability by many to afford conventional chemotherapeutic agents, pay hospital bills as well as economic recession with increased poverty in some African's countries including Nigeria. The aforementioned situations have rapidly increased the rate of scientific researches into the use, effects and constituents of medicinal plants especially in Nigeria (Ross et al., 2001). Hence many studies have been generated on the phytochemistry and medicinal potency of a number of plants commonly found in Nigeria. For instance, studies *by* Andy et al. (2008) on *Heinsia crinata*, Udoh et al. (2011) on *Centella asiatica*, Udoh et al. (2012) on *Lansiathera africana*, Udoh et al. (2017) and Udoh et al. (2018) who worked and reported on antimicrobial potencies of *Ocimum gratissimum* and *Uapaca Staudtii* plants respectively.

Uvaria chamae (P. Beauv) is one the plants commonly found Nigeria. It belongs to the family *Annonaceae* (Omale et al., 2013; Olumese et al., 2016) and is found growing naturally in the savannah and rain forest regions of Africa and tropical areas of the world. It is an evergreen plant that can grow to height of between 3.6 - 4.5 m (Olumese et al., 2016). *U. chamae* is commonly called "finger root" or "bush banana" in English. The plant is also known by the Efik people as "Nkarika Ikot", "Oko aja" or "Eruju" by the Yorubas, *"Kas Kaifi"* by the Hausas, IIgala people called it "Awuloko" or "Ayiloko" and "Akotompo" by the Fula-fainte of Ghana (Omale et al., 2013; Olumese et al., 2016).

U. chamae (P. Beauv) is used in trado-medical practices to cure various ailments as it is a plant with both medicinal and nutritional values. Every part of the plant has several local uses (Iwu, 1993; Okokon et al., 2006; Omale et al., 2013). Many researchers reported the use of this plant' parts in the local treatments. For instance, the root infusion and roots-bark have a widely spread reputation in native treatments. When pounded, it can be used in treatment of nose bleeding, deep wound treatment, sepsis, heart disease (bronchi, lungs), inflammation, blood in urine, pile, fever and, malaria (Adams and Moss, 1999; Etukudo, 2003; Okokon et al., 2006; Omajali et al., 2011). The stem ashes serve as salt substitution in food. The tender leaves are eaten as vegetables. The leaf juice is applied to wounds, sores ulcers, and cuts while the leaf in fusion prepared as lotion is used to treat injuries, swellings, ophthalmic and conjunctivitis (Iwu, 1993; Oluremi et al., 2010; Okwuosa et al., 2012). The fruits have aromatic flavor and is popularly used in beverage industry to add flavor to drinks and food. Moreover, the root yields yellow dye widely used in textile industries to dye fabrics and cosmetics (Igoli et al., 2005).

Wound infection is one of the clinical problems faced by physicians worldwide. Wound can be infected by a wide variety of microorganisms ranging from bacteria, fungus and parasites (Bowler et al., 2001; Yah et al., 2004). Among the bacteria that are frequently recovered from the wounds are *Pseudomonas aeruginosa* and *Proteus mirabilis* (Yah et al., 2004; Gus Gunzalez et al., 2006). According to Ejikeugwu et al. (2013), there are in fact some reports on the existence of multidrug resistance microorganisms and some extended spectrum activities among these clinically important bacterial pathogens, and according to Yah et al. (2007), is one of the ways bacteria confer resistance to the antimicrobials by acquisition of plasmids. Hence they develop resistance to available antibiotics on acquiring these important mechanisms. It is pertinent to search for alternative antimicrobial agents, as some plants prove to function in this aspect and also assess the resistance of these clinical isolates to the plant's crude extracts. *U. chamae* (P. Beauv) have been used locally to treat wound cases and is claimed to be very effective while *P. aeruginosa* and *P. mirabilis* are bacteria frequently isolated from the wounds which could sometimes develop resistance for routine antibiotics. Therefore, studies were conducted on qualitative assessment of *U. chamae* extracts against wound isolated strains of *P. aeruginosa* and *P. mirabilis* and the resistance and plasmid profiles of these bacterial isolates were also determined.

MATERIALS AND METHODS

Plant collection and identification

Fresh root and leaves of the plant were collected from Ikono Local Government Area of Akwa Ibom State, Nigeria. The plant part were identified as *U. chamae* (P. Beau) at the Department of Botany and Ecological Studies University of Uyo, Uyo, Akwa Ibom State, Nigeria by a Taxonomist and was taken to Pharmacognosy Laboratory, University of Uyo for crude extracts and phytochemical analysis. The study was conducted from June 2015 to October, 2015.

Source of test isolates

Six (6) isolates of *P. aeruginosa* and *P. mirabilis* bacteria isolated from wound samples were collected from University of Uyo Medical Centre Uyo, Akwa Ibom State, Nigera, re-cultured, and re-isolated using standard microbiological techniques. Re-characterizations and re-identification were done according to Holt et al. (1994) and

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Cheesbrough (2000). The isolated strains were labeled *P. aeruginosa* (*Pa*-1*), P. aeruginosa* (*Pa*-2)*, P. aeruginosa (Pa*-3) *P. mirabilis (Pm-1), P. mirabilis* (*Pm-2) and P. mirabilis* (*Pm*-3*)* respectively. They were maintained on Nutrient agar (Oxoid, USA) slants at 4°C prior to use.

Sample preparation: Extraction procedures and preparation extract concentration

The fresh root and leaves samples of *U. chamae* were prepared using Obi and Onuaha (2000), and Mukhtar and Tukur (2000) methods. The samples were shade-dried for one week (Andy et al., 2008; Ladpo et al*.,* 2010), thereafter the dried root and leaves were separately ground into fine powder using a mortar (Taura et al., 2014). The crude extracts of the root and leaves were extracted using standard procedures. The ethanolic extraction of *U. chamae* was carried out by soaking 500 g of the dried powdered root and leaves of *U. chamae* into separate sterile containers containing 1000 ml of 95% ethanol. The containers were covered and allowed to stand for 72 h under regular shaking condition at room temperature to allow maximum extraction of the bioactive components. The root and leaves extract were then filtered using Whatman filter paper (No. 1) and the filtrate were evaporated to dryness using a rotatory evaporator. The residue were retained as crude extracts in reagent bottles and maintained in the refrigerator until they were used. Similarly, for the aqueous extraction, 500 g of the dried powdered *U. chamae* leaves and root were separately weighed out and dispersed into separate sterile containers containng 1000 ml of water. The root and leaves solution were covered and shaken and were allowed to stand for about 72 h at ambient room temperature for maximum extraction, and then the infusion were filtered using Whatman filter paper (No. 1). The filtrates were evaporated to dryness using a rotatory evaporator. The residue were retained as crude extracts in reagent bottles and maintained in the refrigerator until they were used.

The concentration of the root and leaves extracts of *U. chamae* were prepared following the methods of Esinemore et al. (1998) and Akujobi et al. (2004) with some modifications. The crude extracts of 200, 150, 100 and 50 mg were respectively diluted with 1 ml of 20% dimethyl sulfoxide (DMSO) for ethanol and aqueous extracts respectively to obtain 200, 150, 100 and 50 mg/ml concentrations for the respective extracts. The reconstituted extract concentrations were stored at 15°C until required for use.

Phytochemical screening

Phytochemical screening tests were conducted on the respective root and leaves extracts using standard methods of Trease and Evans (1994) and Sofowora (2008), which were adopted and employed by Ekong and Udoh (2015a), and Udoh et al*.* (2017). The tests were; alkaloids test, saponin test, tannis test, test for cardiac glycosides, flavonoids determination and cyanogenic glycosides.

Antibacterial assay of *U. chamae extracts*

Ability of the *U. chamae* leaves and roots extracts to inhibit growth of the wound isolated strains of *P. aeruginosa* and *P. mirabilis* was determined using the agar-disc diffusion method (Ogbulie et al., 2004). Sterile filter paper discs of 6 mm in diameter were soaked in equal volumes of varying concentrations of extracts (50, 100, 150 and 200 mg/ml) and left for 2 h undisturbed. The 0.1 ml of 18 h peptone water culture of each of the test bacterial isolates was spread on the sterile Mueller-Hinton Agar (Difco Laboratories, Detroit, Mich) plates. The disc were picked with sterile forceps and placed at different areas on the surface of each plate inoculated with these isolates. Control experiments using routine antibiotic susceptibility test discs were also performed on the isolates by means of Kirby-Bauer disc diffusion method using the guidelines provided by Clinical Laboratory Standard Institute (CLSI, 2005). The antibiotics were: Ciprofloxacin, Tetracycline, Cephaloxin, Gentamicin, Amoxillin, and Nitrofurantoin. This was done by impregnating the disc on each of the culture plate. The sensitivity tests on both the crude extracts and standard antibiotics were performed in triplicates. The plates were incubated at 37°C for 24 h. Antimicrobial activity of the extracts on the test organisms was determined by measuring the zones of inhibition in Milliliter (mm) diameter of the respective disc. Clear zones of inhibition indicated the susceptibility of the organism to the extracts while absence of such zones showed no inhibitory effect of extracts on the test organism. The Z.I values > 15 mm = Sensitive, 12 – 15 mm = moderately sensitive and < 12 mm = Resistant.

Plasmid profiling using agar gel electrophoresis analysis

The plasmid profiling of the bacterial isolates was carried out using gel electrophoresis analysis. The method as described by Ehrenfeld and Clowell (1987), and Akinjogunla and Enabulele (2010), were adopted with slight modification to determine the plasmid profile of *P. aeruginosa* and *P. mirabilis* isolates. The test was also carried out to find out if the resistance pattern exhibited by the test isolates were plasmid mediated or not and to assess the molecular weight of the bacterial plasmids DNA that conferred resistance on these isolates to the crude extracts and the antibiotics (Lipps, 2008).

Plasmid curing

The curing of the resistant plasmids of the isolates was performed using sub-inhibitory concentration of 0.10 mg/ml of acridine orange as described by Sheikh et al. (2003), and Akortha and Filgons (2009) with sight modification. The purpose was to determine if the resistance of *P. aeruginosa* and *P. mirabilis* isolates to standard antibiotics and the crude extracts of *U. chamae* were plasmid or chromosomal mediated.

Statistical analysis

The values of zones of inhibitions (Z.I) recorded were calculated from the means of three measurements of zones of inhibitions on the triplicate cultures and their standard deviation of the mean was also calculated (Mean±SD).

RESULTS

The phytochemical analysis of both the ethanolic and aqueous crude extracts of *U. chamae* roots and leaves revealed that the plant contains some phytochemical constituents such as tannis, alkaloids, flavonoids, cardiac glycoside, and cyanogenic glycoside as bioactive components. These substances were found in varied concentrations depending on extract forms. Some components were abundantly present (+++) in ethanolic crude forms especially root extracts as compared to the crude leaves extracts in which some of these bioactive substances were found in trace (+) forms. For instance, in ethanolic root extract and ethanolic leaves extract, tannins, flavonoids and cardiac glycoside were abundantly

present (+++). However, it is noteworthy that in aqueous root extracts and aqueous leaves extracts these substances were found in trace (+) forms or in moderate amount (++) (Table 1).

The antimicrobial potency of ethanolc root extracts of *U. chamae* on test isolates was assessed. *P. aeruginosa -1* was resistant to ethanolic root extract with Z.I of 11±0.81 mm at 200 mg/ml, 10±0.48 mm at 150 mg/ml but N.I at 100 and 50 mg/ml respectively. Aqueous root extract of *U. chamae* had Z.I of 10±0.1 mm at 200 mg/ml, 9±0.62 mm at 150 mg/ml, and N.I was recorded for 100 and 50 mg/ml respectively for *P. aeruginosa -1. P. aeruginosa -2* had a wider Z.I of 21±1.2 mm from ethanolic root extract at 200 mg/ml, but for aqueous root extract, resistance was observed with *P. aeruginosa -2* as Z.I of 11±0.58 mm was recorded. *P. aeruginosa -3* was resistant to the ethanol root extract with Z.I ranging from 10±1.2 - 11±0.83 mm at 50 and 100 mg/ml but moderately sensitive at 200 mg/ml concentration with Z.I of 15±0.47 mm, while for aqueous root extract, resistance were recorded. Moreover, *P. mirabilis -1* was observed to be resistant to ethanolic root extract at 50 and 100 mg/ml but moderately sensitive at 150 mg/ml with Z.I of 15±2.0 and 16±1.75 mm at 200 mg/ml, while for aqueous root extract, Z.I ranged from N.I -13±0.57 mm. Outstandingly, for ethanol root extract, *P. mirabilis -2* progressively showed sensitivity with increased concentration of the extract. The Z.I recorded ranged from 11±1.15 - 20±0.67 mm at 50 – 200 mg/ml, respectively whereas for aqueous root extract, Z.I ranged from 8±0.6 -13±0.57 mm. *P. mirabilis -3* had resistance for ethanolic root extract at 50, 100 and 150 mg/ml but moderately sensitive at 200 mg/ml with Z.I of 15±1.67 mm respectively while for aqueous root extract, it was resistant as N.I and smaller Z.I were recorded. Assessing individual crude extract form at different concentrations, ethanolic roots extracts had greater inhibitory effects with zones of inhibitions (Z.I ranged from 18±2.0 mm -21±1.2 mm) on *P. aeruginosa* and *P. mirabilis* bacteria used in the study than aqueous root extracts (Z.I ranged from 8±0.1 - 15±2.0 mm) respectively (Table 2).

The antimicrobial potency of ethanolc leaves extracts on test isolates was also assessed. *P. aeruginosa -1* was resistant to ethanolic leaves extracts with N.I at 50 and 100 mg/ml, respectively but Z.I of 8±0.2 mm at 150 mg/ml and 9±1.0 mm at 200 mg/ml. Smaller Z.I values or N.I were also observed with *P. aeruginosa -1* isolate when subjected to crude aqueous leaves extracts. Inhibitory effect of ethanolc leaves extracts *on P. aeruginosa-2* increased with increased concentration Z.I of 8±0.6, 10±0.1, 12±1.2 and 13±0.57 mm at 50, 100, 150 and 200 mg/ml, respectively whereas for aqueous leaves extracts, *P. aeruginosa-2* was resistant to the extract form as N.I, 8±0.68 mm, 8±0.5 and 9±0.56 mm were recorded with used concentrations. *P. aeruginosa-3* had resistance against ethanolc leaves extracts as Z.I of 8±0.53 mm was recorded at 50 mg/ml, but at 100, 150 and 200 mg/ml and Z.I of 9±0.25, 9±0.047 and 10±0.5 mm respectively while for aqueous leaves extracts, *P.aeruginosa-3* was resistance as smaller Z.I were recorded. *P. mirabilis -1* was resistance to ethanolic and aqueous leaves extracts at all the concentrations used in the study as N.I and smaller Z.I were recorded. *P mirabilis -2*, was moderately sensitivity to ethanolic leaves extracts at 150 and 200 mg/ml with Z.I of 12±0.87 and 13±0.61 mm, respectively but resistance was observed with aqueous root extracts as *Pm*-2 had smaller Z.I values of 8±0.46 mm at 200 mg/ml. *P mirabilis -3* showed moderate sensitivity to ethanolic leaves extract at 200 mg/ml with Z.I of 13±0.52 mm but resistance at other concentrations used in the study as N.I while for aqueous leaves extracts *Pm-3* was resistance to the extract with N.I recorded at 50 and 100 mg/ml with smaller Z.I recorded at other concentrations. The potency of each form of leaves extract at different concentrations showed ethanolic leaves extracts had at least some inhibitory effects on test isolates (Z.I ranged from 7±1.0 - 13±0.61 mm) than the aqueous leaves extracts with zones of inhibitions (Z.I ranged from 7±0.2 - 11±0.2 mm) respectively (Table 3).

Some test isolates that exhibited multiple resistances to both the ethanolic and aqueous root and leaves extracts also showed resistances to some standard antibiotics used as control. *P. aeruginosa -1* was resistant to Cephaloxin, Nitrofuratoin and Amoxillin, moderately sensitive to Tetracycline and Gentamicin but highly sensitive to Ciprofloxacin with Z.I values of 18±0.76 mm. *P. aeruginosa-2* was highly sensitive to all the standard antibiotics with wider zone of Z.I of 25±0.67 mm recorded for Cephaloxin but *P. aeruginosa-3* was resistant to Cephaloxin, Gentamicin and Nitrofuratoin but highly sensitive to Cephaloxin with wider zones of Z.I of 31±0.88 mm. *P. mirabilis –1* formed resistance to Ciprofloxacin, Nitrofuratoin, Tetracycline and Amoxillin but high sensitivity was recorded with Gentamicin with wider zones of Z.I of 30±0.36 mm. *P. mirabilis-2* was resistant to all the standard antibiotics used in the study except Ciprofloxacin where it had the Z.I of 35±0.54 mm. *P. mirabilis–3* exhibited resistance to Ciprofloxacin, Cephaloxin, Gentamicin, Nitrofuratoin and Amoxillin but sensitive to Tetracycline with Z.I of 19±0.63 mm (Table 4).

The result of plasmid profiling of the strains of *P. aeruginosa and P. mirabilis* showed that some test isolates that exhibited multiple resistances to both the ethanolic and aqueous forms of both root and leaves extracts along with the control antibiotics used in the study was due to the fact that they harbored plasmids of different molecular weights ranging from 564 bps - 23.13 kbp. The multiple bands were clearly observed with *P. aeruginosa-1, P. aeruginosa* -3, *P. mirabilis* -1, and *P. mirabilis -3* with low molecular weight of 564 bp, and 2.03 kbp to a high molecular weight of 23.13 kbp (Figure 1).

Resistance patterns of some isolates were reverted after curing plasmids with acridine orange while others

Table 1. Phytochemical screening of crude extracts of *U. chamae.*

Some bioactive components of *U. chamae*	Ethanolic root extract	Aqueous root extract	Ethanolic leaves extract	Aqueous leaves extract
Tannins	+++	++	+++	++
Flavonoids	+++	++	+++	++
Saponins	-	-	-	-
Alkaloids	++	++	++	+
Cyanogenic Glycoside	++	++	+	+
Cardiac Glycoside	++	++	++	+

+ = Presents in trace amount, ++ = Presents in moderate amount, +++ = Presents in greater amount, - = Not present.

Table 2. Antibacterial activities of the crude root extracts of *U. chamae* on *Pseudomonas aeruginosa* and *Proteus mirabilis isolates.*

Test isolate	Concentration of the crude root extracts (mg/ml) and zones of inhibitions (mm)							
	Ethanolic root extract				Aqueous root extract			
	200	150	100	50	200	150	100	50
Pa-1	11±0.81	10±0.48	N.I	N.I	10±0.1	9±0.62	N.I	N.I
Pa-2	21±1.2	18±2.0	18±0.3	11±0.78	11±0.58	10±0.2	9±1.0	8±0.58
Pa-3	15±0.47	14±0.76	11±0.83	10±1.2	9±1.0	8±0.1	8±0.1	N.I
Pm-1	16±1.75	15±2.0	9±1.0	N.I	13±0.57	12±0.9	11±0.45	N.I
Pm-2	20±0.67	18±0.62	17±0.3	11±1.15	15±2.0	12±0.8	9±0.17	8±0.6
Pm-3	15±1.67	10±0.75	9±1.0	N.I	11±0.58	10±1.2	8±1.2	N.I

The values are means of three measurements of the zones of inhibitions on the triplicate cultures at the same concentration and standard deviation (means + standard deviation).

Table 3. Antibacterial activities of the crude leave extract of *U. chamae* on *Pseudomonas aeruginosa* and *Proteus mirabilis* isolates.

Test isolate	Concentration of the crude extracts (mg/ml) and zones of inhibitions (mm)							
	Ethanolic leaves extract				Aqueous leaves extract			
	200	150	100	50	200	150	100	50
Pa-1	9±1.0	8±0.2	N.I	N.I	8±0.46	8±0.26	N.I	N.I
Pa-2	13±0.57	12±1.2	10±0.1	8±0.6	9±0.56	8±0.5	8±0.68	N.I
Pa-3	10±0.5	9±0.047	9±0.25	8±0.53	10±1.0	10±1.0	7±0.2	N.I
Pm-1	11±0.7	8±0.58	7±1.0	N.I	11±0.2	11±0.2	N.I	N.I
Pm-2	13±0.61	12±0.87	11±0.87	10±0.5	8±0.46	9±0.1	8±0.52	N.I
Pm-3	13±0.52	9±0.23	9±1.2	N.I	9±0.73	8±1.15	N.I	N.I

The values are means of measurements of the different zones of inhibitions on the triplicate cultures and standard deviation (means + standard deviation). *Pa*-1= *Pseudomonas aeruginosa* -1, *Pa*-2 = *Pseudomonas aeruginosa* - 2, Pa-3 =*Pseudomonas aeruginosa* -3, *Pm*-1= *Proteus mirabilis* -1, *Pm*-2 = *Proteus mirabilis* - 2, *Pm*-3 = *Proteus mirabilis* -3. N.I. = No zone of inhibition.

still retained the plasmids. This was noted when some of the test organisms were found with no zone of inhibition after plasmid curing. Outstandingly was *P. aeruginosa* -1, where N.I and smaller Z.I in both the ethanolic and aqueous forms of the extracts at 50, 100, and 150 mg/ml concentration were recorded, whereas increased Z.I were observed from *P. aeruginosa* -2, *P. aeruginosa* -3, *P. mirabilis* -1, and *P. mirabilis* -2 at different extract forms, that might have possibly lost their plasmids after curing (Tables 5 and 6).

Furthermore, results obtained from antibiogram on test isolates after plasmid curing showed that the resistance patterns of some test isolates were reverted for standard antibiotics as some became sensitive while some isolates still harbored the plasmids. *P. aeruginosa* -1 with smaller Z.I was recorded for Cephaloxin and N.I was recorded against Amoxillin while increased Z.I was observed from *P. aeruginosa* -2 in nearly all the antibiotics with the wider

Table 4. Resistance pattern of *Pseudomonas aeruginosa,* and *Proteus mirabilis* species on standard antibiotics (Control).

Test isolate	Antibiotics used and zones of inhibition (mm)					
	Ciprofloxacin	Tetracycline	Cephaloxin	Gentamicin	Nitrofuratoin	Amoxillin
Pa-1	18±0.76	11±0.58	N.I.	12±0.48	8±0.58	N.I
Pa-2	20±0.48	22±1.2	25±0.67	18±0.34	20±1.0	22±1.2
Pa-3	16±1.75	25±1.0	31±0.88	N.I	N.I	15±0.48
Pm-1	N.I	8±0.58	20±0.68	30±0.36	N.I	10±0.58
Pm-2	35±0.54	11±0.47	N.I	12±0.48	N.I	11±0.84
Pm-3	N.I	19±0.63	10±0.5	11±0.47	15±2.0	14±0.47

The values are means of measurements of the different zones of inhibitions on the triplicate cultures and standard deviation (means + standard deviation). *Pa*-1= *Pseudomonas aeruginosa* -1, *Pa*-2 = *Pseudomonas aeruginosa* - 2, Pa-3 =*Pseudomonas aeruginosa* -3*, Pm*-1= *Proteus mirabilis* -1, *Pm*-2 = *Proteus mirabilis* - 2, *Pm*-3 = *Proteus mirabilis* -3. N.I. = No zone of inhibition.

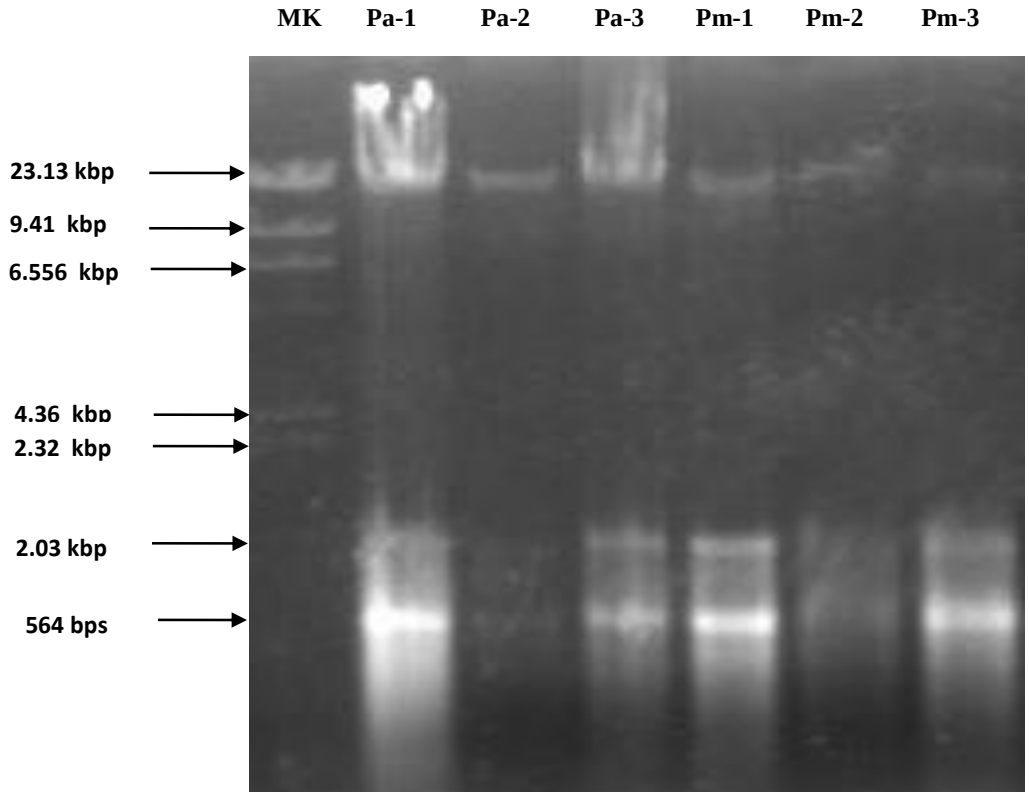

Figure 1. Plasmid profile of *Pseudomonas aeruginosa* and *Proteus mirabilis* isolates used in the study. MK - Molecular weight marker (Hind 111 digest), *Pa*-1= *Pseudomonas aeruginosa* -1, *Pa*-2 = *Pseudomonas aeruginosa* - 2, *Pa*-3 =*Pseudomonas aeruginosa* -3*, Pm*-1= *Proteus mirabilis* -1, *Pm*-2 = *Proteus mirabilis* - 2, *Pm*-3 = *Proteus mirabilis* -3. Molecular weight of plasmids ranged from 564 bps - 23.1 kbp.

Z.I of 35±1.2 mm recorded from Cephaloxin. The *P. aeruginosa* -3 and *P. mirabilis* -2 still had resistance against Nitrofuratoin with N.I, *P. mirabilis* -1 had smaller Z.I for Ciprofloxacin, Tetracycline, Nitrofuratoin and *P. mirabilis* -2 maintained resistance against Nitrofuratoin with N.I, and *P. mirabilis -3* isolates which initially had N.I were found with smaller Z.I and increased Z.I for Tetracycline (Table 7).

DISCUSSION

The results obtained from this study showed that the plants *U. chamae* possess some active substances as bioactive constituents*.* This result confirm reports of Omajali et al. (2011) and Olumese et al*.* (2016), who reported that the plant has several active ingredients. Moreover, Okokon et al. (2006) reported the antimalarial

Table 5. Antibacterial activities of the crude root extracts of *U. chamae* on *Pseudomonas aeruginosa* and *Proteus mirabilis* after plasmid curing.

Test isolate	Concentration of the crude leaves extracts (mg/ml) and zones of inhibitions (mm)							
	Ethanolic root extract				Aqueous root extract			
	200	150	100	50	200	150	100	50
Pa-1	12±0.49	10±0.52	N.I	N.I	11±0.2	9±0.2	N.I	N.I
Pa-2	22±1.5	17±0.21	18±0.3	15±0.1	12±0.31	10±0.6	10±0.5	11±0.1
Pa-3	20±0.9	21±1.25	15±0.75	15±0.2	12±0.31	12±0.4	9±1.0	9±0.5
Pv-1	18±0.55	16±0.16	13±0.31	13±0.5	15±0.75	14±0.2	12±1.0	10±0.6
Pv-2	20±1.16	16±0.21	12±0.56	12±0.6	13±0.52	13±0.8	12±0.3	N.I
Pv-3	15±0.16	14±0.16	12±0.80	13±1.1	10±1.1	10±0.6	N.I	N.I

The values are means of measurements of the different zones of inhibitions on the triplicate cultures and standard deviation (means + standard deviation). *Pa*-1= *Pseudomonas aeruginosa* -1, *Pa*-2 = *Pseudomonas aeruginosa* - 2, *Pa*-3 =*Pseudomonas aeruginosa* -3*, Pm*-1= *Proteus mirabilis* -1, *Pm*-2 = *Proteus mirabilis* - 2, *Pm*-3 = *Proteus mirabilis* -3. N.I. = No zone of inhibition.

Table 6. Antibacterial activities of the crude leaves extracts of *U. chamae* on *Pseudomonas aeruginosa* and *Proteus mirabilis* after plasmid curing.

Test isolate	Concentration of the crude leaves extracts (mg/ml) and zones of inhibitions (mm)							
	Ethanolic leaves extract				Aqueous leaves extract			
	200	150	100	50	200	150	100	50
Pa-1	11±0.5	11±1.2	N.I	N.I	8±0.02	N.I	N.I	N.I
Pa-2	15±0.2	13±1.2	12±0.2	11±0.5	11±0..1	8±0.48	9±0.2	N.I
Pa-3	18±1.2	12±0.2	13±0.6	12±0.8	10±0.8	9±0.67	9±1.1	9±0.8
Pm-1	15±0.3	14±0.5	12±0.3	10±0.2	12±1.2	10±0.2	9±1.0	9±0.4
Pm-2	17±0.3	13±0.54	13±0.5	13±0.5	13±0.3	12±0.8	11±0.1	N.I
Pm-3	20±1.2	16±0.7	14±0.9	12±0.6	10±0.45	10±1.1	8±0.2	N.I

The values are means of measurements of the different zones of inhibitions on the triplicate cultures and standard deviation (means + standard deviation). *P.a*-1= *Pseudomonas aeruginosa* -1, *P.a*-2 = *Pseudomonas aeruginosa* - 2, P.a-3 =*Pseudomonas aeruginosa* -3*, Pm* -1= *Proteus mirabilis* -1*, Pm* -2 = *Proteus mirabilis* - 2*, Pm*-3 = *Proteus mirabilis* -3. N.I. = No zone of inhibition.

Table 7. Resistance pattern of *Pseudomonas.aeruginosa* and *Proteus mirabilis* isolates on standard antibiotics (Control).

Test isolate	Antibiotics used and zones of inhibitions (mm)					
	Ciprofloxacin	Tetracycline	Cephaloxin	Gentamicin	Nitrofuratoin	Amoxillin
Pa-1	19±0.6	11±1.2	9 ±0.53	12±0.48	8±0.58	N.I
Pa-2	28±0.4	22±1.2	35±1.2	21±0.52	20±1.0	24±0.21
Pa-3	17±0.52	28±0.	32±0.56	11±0 21	N.I	16±0.38
Pm-1	8±0.12	8±0.6	22±0.3	32±0.21	8±0-6	10±0.41
Pm-2	36±0.14	13±0.3	10±0.5	12±0.48	N.I	11±0.6
Pm-3	12±0.56	21±0.52	10±0.68	11±0.47	15±1.2	13 ±0.41

Pa-1= *Pseudomonas aeruginosa* -1, *Pa*-2 = *Pseudomonas aeruginosa* - 2, Pa-3 =*Pseudomonas aeruginosa* -3*, Pm*-1= *Proteus mirabilis* -1, *Pm*-2 = *Proteus mirabilis* - 2, *Pm*-3 = *Proteus mirabilis* -3. N.I. = No zone of inhibition.

activities of *U. chamae* while Omale et al. (2013) reported that the plants bioactive constituents have ability to neutralize snake venom in rats. Some researchers have discovered that antibacterial properties are usually associated with these bioactive constituents (Ameh, 2010; Udoh et al*.,* 2017). These substances have their different functions and are known to work using different mechanisms. For instance, alkaloids present in plant are known to function as anesthetic, spasmolytic and anti-cholinergic agent (Iroabuchi, 2008). According to Batisa

et al. (2004) and Burile et al. (2009), alkaloids have clinical importance and possess anticancer, antibacterial and anti-asthmatic activities. Tannins are known to inhibit the synthesis of cell proteins of bacteria by forming complexes that are irreversible with proline rich protein in bacteria. Tannins was reported to be responsible for the haemostatic activity where they arrest bleeding from damaged or injured vessels by precipitating protein to form vascular plugs (Okwu and Iroabuchi, 2004). Flavoniods which is also found in *U. chamae* in a very high concentration especially in ethanolic extracts is reported to function well in some biological aspects such as the protection against allergy, platelet aggregation, microbes invasion, ulcer, hepatoxin, viruses and tumors (Batisa et al., 2004; Burile et al., 2009). Nascimento et al. (2000) reported that flavonoid works well in human system in that it reduces the risk of estrogen-induced cancer by interfering with the enzymes that produce estrogen. Moreover, some researchers observed and reported that flavonoids are also found to form complexes with the extracellular soluble proteins leading to disruption of microbial cell membranes (Tsuchiya et al., 1996), while cardiac glycosides is useful in heart pumping (Godfraind, 1984; Ekong and Udoh, 2015b). The secondary metabolites or bioactive components present in this medicinal plant could be responsible for the therapeutic activity attributed to *U. chamae* (Monon et al., 2015). These natural substances are widely distributed in *U. chamae* and contribute greatly to their beneficial health effects. No wonder their natural antioxidant and antibacterial roles have attracted more interest in the plant' use in the prevention and treatment of inflammatory problem, cancer, and cardiovascular diseases (Vârban et al., 2009; Monon et al., 2015).

The results obtained in this study showed that the ethanolic and water extracts of *U. chamae* have some inhibitory effects on some wound isolated strains of *P. aeruginosa* and *P. mirabilis* as they inhibited the growth of some test isolates at varied concentrations. This indicates that the plant possess some active substances that can inhibit the growth of these microorganisms. These results support the traditional use by herbalists in the treatment of various ailments such as urinary tract infection, treatment of wounds just to mention but a few. It was also observed in the study that much of the active substances were found in the ethanolic extracts than the aqueous form and the lower inhibitory effects of the aqueous extracts could be that probably this ethanolic solvent extracted its active constituents more than the aqueous form and water was not potent enough to extract much of the bioactive substances from *U. chamae* leaves and roots. Thus, the results indicated that ethanol is a better extraction solvent for extraction of *U. chamae* plant active principles than the aqueous. This corroborates the reports of Obi and Onuoha (2000), and Ogueke et al. (2006). Other researchers like Ogbulie et al. (2007), Udoh et al. (2011), and Monon et al. (2015) also reported that ethanol is a better extraction solvent for most plants bioactive substances of medicinal importance. No wonder as observed in some areas in Nigeria, some trado-medical practitioners always employ or recommend the use of ethanol (local gin) for the maceration and extraction of plants for native treatments, with exception on where the patients request otherwise.

Moreover, the effectiveness of the antimicrobial potency was observed to be more active in the ethanolic root extracts than ethanolic leaves extracts of *U. chamae* This was evident when *P. aeruginosa -2* and strain of *P. mirabilis -2* had wider zones of inhibitions when they were subjected to ethanolic root extracts than to aqueous root extracts, thus showing that the pathogens were highly susceptible to the ethanolic root extracts than the other form. The results indicated that in general, the ethanolic root extracts had greater inhibitory effects on the isolates than the leaves extract forms. This agrees with Monon et al. (2015), who reported that the root extract of *U. chamae* has high antibacterial efficacy.

The present study also showed that some wound isolated strains of *P. aeruginosa* and *P. mirabilis* species used in the study were resistant to both the crude extracts of *U. chamae* and standard antibiotics but this does not portray that these antimicrobials have no antibacterial potency but rather, these strains of *P. aeruginosa* and *P. mirabilis* were able to acquire resistance genes that are commonly found on plasmids. In the study, the strains of *P. aeruginosa* and *P. mirabilis* species harbored resistant plasmids. Akinnibosun and Adeola (2015) also reported from their works some *P. aeruginosa* and *P. mirabilis* that acquired plasmids and formed resistance to multiple antibiotics used in their study.

The plasmids isolated from the test organisms varied widely with regard to size and molecular weight. Some isolates harbored low molecular weights, while some were high, and were even seen with multiple plasmids bands. The molecular weight of the plasmid varies depending on the size of the plasmids. Reports by Yah et al. (2007) in their work reported that the sizes of the plasmids from *Proteus* species varied. Norma et al. (2004) reported that most organisms acquire their antibiotic resistance genes through transposons, chromosomes or from other plasmids, and that it is necessary to elaborate and enhance ways of controlling these phenomena. Many other researchers such as Ruth et al. (2011), Mordi and Momoh (2009), and Ismaeil and Kadhim (2017), in their work reported that *Proteus* species developed a wide range of resistance to several antibiotics. Asad, and Amna (2004) likewise reported from their work plasmid-mediated multiple antibiotic resistance in *Proteus* isolates from their work. Enabulele et al. (2006), Yah et al. (2007) and Auwaerter (2008) reported that *Proteus* species vary in their susceptibility pattern due to transfer of plasmid resistant genes among the gram negative organisms. Murry et al. (1998), Mukhtar

and Tukur (2000) and Taura et al. (2014) likewise observed and ascertained that *P. aeruginosa* is inherently resistant to many antibiotics and can have the possibility of mutating to even more resistant strain during treatment. Moreover, according to Piddock (2006), these genes conferring antibiotic resistance are commonly found on elements known as integrons and transposons, which facilitate movement between different replicons such as between the bacterial chromosome and a plasmid. Moreover, Nikaido (2009) reported that R-plasmids found in bacteria often contain several resistance genes and these genes are steadily maintained in the host strains of bacteria in a stable manner and are transferred efficiently to other neighboring drug-susceptible bacterial cells which later become resistant to multiple antimicrobial agents. Remarkably, many such genes are often present on a single R- plasmid, so that multidrug resistance can be transferred to a susceptible bacterium in a single conjugation event. Hence, it is wise that treatment of infections caused by these isolates should be guided by adequate laboratory sensitivity test result.

It was further observed in this study that after plasmid curing, some isolates were susceptible to the crude extract and the routine antibiotics with increased zones of inhibition. This corroborates with report by Akinnibosun and Adeola (2015), who in their work recorded increased zones of inhibition after plasmid curing of the test isolates. However, in the study, some test isolates were still resistant to both crude extracts of *U. chamae* and standard antibiotics. This probably may be that some of them harbored resistant genes that could not be eliminated by acridine orange and possibly, the resistance observed in this work could be both plasmid and chromosomal mediated. This agrees with Bush et al. (1995), who stated that, the production of plasmid or chromosomal encoded β-lactamase enzyme is the most common mechanism of resistance in Gram-negative bacteria causing clinical significant infections. Piddocks (2006) affirmed that genes encoding efflux pumps resistance can be found on the chromosome and these genes can chromosomally encode multidrug resistance (MDR) efflux pumps. The reason why the intrinsic mechanism for multidrug resistance could be chromosomal is that it probably may be hard to remove by acridine orange as observed in this work.

Conclusion

The fact that crude extracts of both ethanolic and aqueous forms of *U. chamae* showed some antimicrobial activities against most of the test organisms is a major breakthrough in appreciating the medicinal potential of this plant especially in the management of wound infection. *U. chamae* could be exploited for the isolation of active principles for drug formulation with proper dose given for treatments of various infectious diseases caused by these test isolates. However, the resistance and plasmid profiles of these wound isolated strains of *P. aeruginosa* and *P. mirabilis* used in the study for both the crude extracts of *U. chamae* and standard antibiotics is of a great concern as the isolates exhibited multidrug resistance patterns that are mediated by both plasmids and chromosomes. Therefore, assessment and caution should be given during prescription and administration of any antimicrobial agents for proper and effective treatment of cuts, wounds and other ailments caused by these organisms.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors are grateful to Mr Etefia who brought the plant; other staff of Pharmacognosy Laboratory, University of Uyo, Akwa Ibom State; staff of Microbiology Laboratory, University of Uyo, Akwa Ibom State, Nigeria; along with staff of Biotechnology and Biochemistry Units, Nigerian Institute for Medical Research (NIMR), Yaba, Lagos State, Nigeria where extraction of plasmids was carried out for their assistance and cooperation.

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Vol. 13(28), pp. 531-537, October, 2019
DOI: 10.5897/AJMR2018.8941
Article Number: 6C35CAC62004
ISSN: 1996-0808

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African Journal of Microbiology Research

Full Length Research Paper

Effect of abattoir wastes on stream quality in the Bolgatanga municipality, Ghana-west Africa

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Received 17 July, 2018; Accepted 12 November, 2018

This research was undertaken to determine abattoir wastes effect on the stream quality along Kollaa stream in Sawaba area of Bolgatanga municipality. The study also determined the microbiological quality and some physicochemical parameters of the stream that takes delivery of untreated waste from the abattoir and the extent of pollution through discharge of waste from abattoir sites into stream water. Three water samples were collected from (effluent discharge point, upstream and downstream) in the stream using sterile containers. Biological oxygen demand (BOD) was determined by calculating the difference in the initial and final dissolved oxygen. Dissolved oxygen (DO) was determined using dissolved oxygen probe and meter. Total dissolved solids were determined using HM Digital TDS meter. Fecal and total coliform coliforms counts were investigated by using multiple tube fermentation methods. Salmonella and total bacterial count and were determined using standard plate count method and pour plate method. The study results revealed that the stream contained high coliform counts attributable to the release of abattoir waste into the stream. Water samples from effluent discharge point had the highest BOD value of 60.5 mg/L, DO had 1.1 mg/L and TDS had 80 mg/L. Effluent discharge also had fecal coliform count of 80 mpn/100 ml, total coliform count of 500 mpn/100 ml and total bacteria count of 30 cfu/ml x 10^5. Upstream sample had BOD value of 2.1 mg/L, DO had 0.9 mg/L, TDS had 74 mg/L, fecal coliform count was 20 mpn/100 ml, total coliform count was 80 mpn/100 ml and total bacteria count was 10 cfu/ml × 10^5. Downstream sample also had BOD value of 2.1 mg/L, DO was 0.6mg/L, TDS was 70 mg/L, fecal coliform counts was 41 mpn/100 ml, total coliform was 70 mpn/100 ml and total bacteria counts was 20 cfu/ml × 10^5. All samples had no Salmonella count. Presence of the bacterial in the stream made it unwholesome for drinking purposes.

Key words: Abattoir, pathogens, stream quality, effluent discharge, physical qualities, microbiological quality, upstream, downstream.

INTRODUCTION

Abattoir is a designated area where animals are killed for their meat. An Abattoir is a registered and an approved premise for hygienic killing and inspection of animals meats, possessing and storage or preservation of meat for human consumption (Nouri et al., 2008). The processing activities of cow result in essential meat

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provisions. It is a vital protein source and beneficial by-product production such as skin, leather and bones. This sometimes cause pollution of the environment and other health threatening hazards to humans and. (Nouri et al., 2008) defines meat hygiene as a system of principle designed to ensure the safety of meat product and that they are wholesome and processed under hygienic condition that makes them worthy of human consumption. When waste from the slaughter house are not well treated and released into running water, it has very high chance of contaminating surface water with enteric pathogens and other nutrients. In practices of these natures, it becomes a major pollutant of the country towns and cities. The abattoir wastes that consist of liquid, solid and fats could be a source of high organic matter. The solid component of the wastes is made up of hairs, condensed meat, bones, aborted fetus and undigested ingest. The liquid part in the other vein comprise of blood, dissolved solids, urine, guts contents and water, while fat waste primarily is made up of fat. Water resources pollution often brings about the destruction of primary producers. This in turn causes diminishing effect of fish stock consequently leading to the decrease in tertiary diet (Aina and Adedipe, 1991).

Waste from killed Animals is mostly contaminated by microbes living normally or entering it from the environments such as those resulting from processing operations (UNESCO, 2006). Abattoir can serve as a valuable source of nutrients for crops when decomposed. The same material poses greater threat to water quality. Animal waste may also contain heavy metals traces, organic solid, salts, trace, viruses, bacteria, other microbes and sediments. Abattoir discharge composition is complex and could be extremely harmful. Its adverse effects on the environment could reduce oxygen content of stream; hence depriving aquatic life of oxygen. Unhealthy discharge of animal waste can also result in the transmission of zoonotic diseases onto humans subjects by contact with animal feces (Raymond, 1977). Untreated Abattoir discharge in streams brings about high utilization of phosphorous, oxygen and nitrogen (Weobong and Adinyira, 2011). For instance, animal blood discharged into stream could exhaust the Dissolved (DO) of the water body. Improper discharge of paunch manure may increase oxygen requirements on the receiving environment or produce huge population of decomposers (microorganisms) which may be pathogenic. Furthermore, improper disposal of animal feces may cause oxygen-depletion in the receiving environment. It could also lead to nutrient-over enrichment of the receiving system and increase accumulation rate of toxins in biological systems (Nwachukwu et al., 2011). Unhygienic discharge of abattoir waste could also bring about transmission of pathogenic microbes onto humans that may cause outbreaks of water borne diseases such as cholera, typhoid, diarrhea, fever, and wool sorter diseases.

Escherichia coli infection source was revealed to have come from raw beef contaminated in the slaughterhouse with feces containing bacterium (Bello and Odeyemi, 2009). Discharge from abattoir is responsible for the contamination of underground and surface waters bodies, quality of air as well as reduction in quality of health of residents within the catchment areas of abattoir (Katarzyna et al., 2009; Odoemelan and Ajunwa, 2008). This study therefore investigates some of the physical and bacteriological characteristics of discharged abattoir effluents into surrounding stream as it affects humans lives who use the stream water domestically. The outcome will help create public consciousness on the state and health implications of abattoir discharge on the water bodies.

MATERIALS AND METHODS

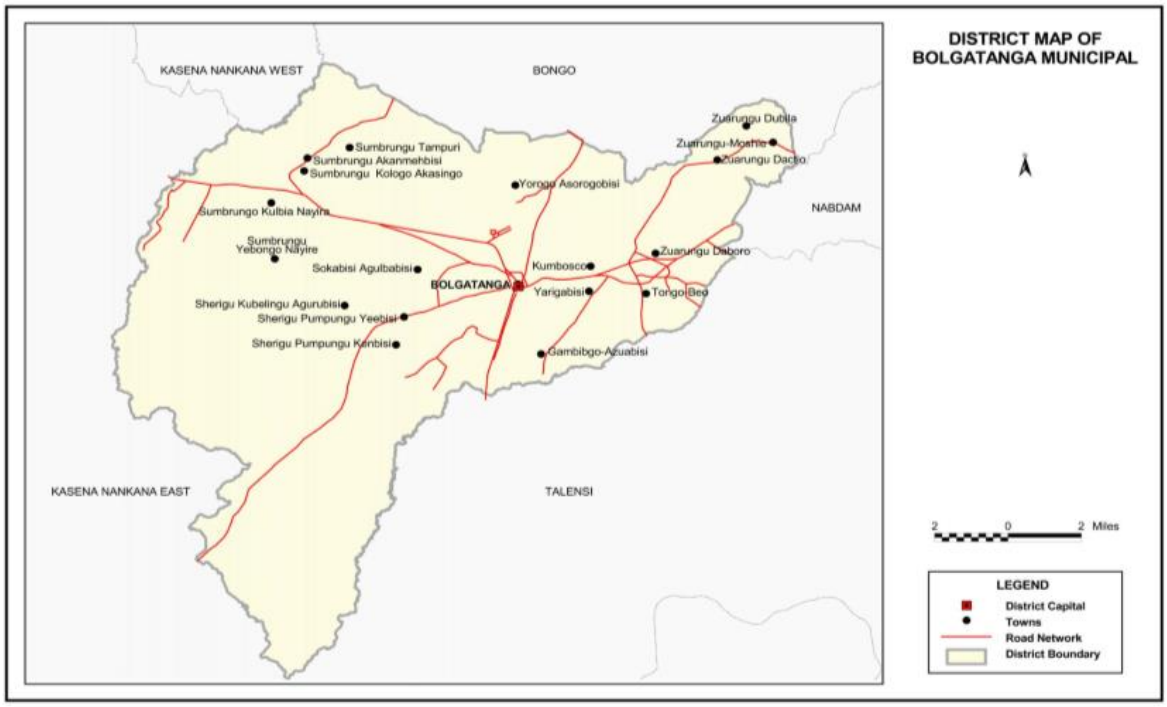
Study area

The study was conducted at the Bolgatanga Abattoir in Bolgatanga Municipality, Upper East Region of Ghana from January to April, 2015. The abattoir is located at Sawaba area in the municipal. The samples were collected along Kuulaa stream where most of the abattoir wastes are discharged. The wastes from the slaughtering and the dressing grounds in the abattoir are washed into open drainages untreated and are carried into a nearby stream. Bolgatanga is the Regional capital of the Upper East city in the Northeastern part of Ghana. The Municipality forms part of the 13 Districts and Municipals in the Region. The region is the largest urban area among the districts in the Region. Bolgatanga is bounded with Bongo District to the North, Nabdam District to the east, Talensi District to the south and Kassena Nankana Municipal to the west. Bolgatanga is the administrative capital of the municipality. (Figures 1 to 3) The climate is tropical one and characterized by a single season rainfall regime (May to October) and a longer dry season with that stretches October to April without rainfall. Temperatures can rise to maximum of 40°C in March/April and a minimum 12°C for November/December. The vegetation of the municipality is that of the savanna type with notable trees such as baobab, and acacia trees. The low vegetation is destroyed by fire during the dry season or dried by the sun. The inhabitants of the district belong predominantly to different peoples of Northern extradition of Ghana. Bolgatanga is cosmopolitan town. There are different peoples in the north, but members of the major ethnic groups include the Akan, Ewe, and Ga peoples. Majority of the population in the 1990s lived, in spite of the urban structure of the district, from agriculture, 19% commercial, 12% industry, mainly handicrafts, and just 7.4% were employed in public services. There are some jobs in the mining and construction and in the form of some metal-working companies, repair shops, painting companies etc.; but these represent a very small minority. The Bolgatanga Municipal is the home of the Upper East Regional capital. The Municipality forms part of the 13 Districts and Municipals in the Region. This, of course, makes it the largest urban centre in the Region. The population of the municipality according to 2010 population and housing census stands at 131,550 with 62,783 males and 68,767 females (Ghana Statistical Service, 2014).

Sampling design

Sampling was carried out three times at three strategic points of the stream along the stream using random grab sampling. Samples

Figure 1. Ghana map.



Source: Ghana Statistical Service GIS

Figure 2. Bolgatanga municipal map.

Figure 3. Maps indicating the Abattoir and Kuula stream in Bolgatanga. Yellow spot = abattoir; blue spot =school; red lines = contours; light blue = effluent discharge**;** white dotted lines = dagmew; deep blue = kuula stream; green triangle = bridge.

were taken in duplicate to enhance the dependability of data. Samples collection point was the mid-width of the stream. 1.0 litre sterile plastic container cleaned with 10% nitric acid and rinsed thrice with distilled water was used for sample collections. Three one-litre samples were collected at each of the three sampling points designated Up Stream (US), Effluent Discharge (ED) and Down Stream (DS).

Sample analysis

The standard methods adopted in investigating physicochemical characteristics of water and wastewater samples and bacteriological determination were in consistence with the American Public Health Association series of Standard Methods of Examination of Water and Effluent (APHA, 2005).

Collection and analysis of water samples

To enhance aseptic conditions in the laboratory before analysis, all the equipment and apparatus used were sterilized using hot air oven and autoclave. All media used were prepared aseptically according to manufacturer instructions and were subjected to moist heat sterilization at 121°C for 15 min using the autoclave. Samples of water were collected into sterilized 2.5 ml dark bottles that contain dissolved oxygen and transported in a dark environment to the Danida Laboratory of UDS for analysis (Table 1). The bottles were aseptically used to collect water samples from three points along the stream where the abattoir waste is drained analysis. The control and the first sample were collected from about 100 m upstream away from the abattoir. This is used to determine the different in the stream water quality after effluent discharged. The second sample was taken at the discharge point of the abattoir effluent into the stream. The remaining point was at point down the stream (downstream) 100 m away and sampling was conducted at about 11:30 am so as to allow the effluent enough time to reach the stream from the abattoir. Sample containers were concealed in ice chest with ice packs imbedded and transported to the laboratory. Samples of water were picked fortnightly within a four-month period (January- April, dry season) (Table 2). There was a total of eight collections (twice a month) from each sampling point for the effluent and downstream and influent.

Bacteriological and physicochemical analysis

The methods used for the analysis were standard methods for examination of bacteriological and physical characteristics of wastewater as laid down by the American Public Health Association

Table 1. Description of sampling points.

Sampling point	Description
Point 1- Upstream	About 100 m to the point of introduction of abattoir waste
Point 2 - Midstream	Point of effluent discharge
Point 3- Downstream	100 m away where the effluent mixes with the receiving water body.

Table 2. Periods of the samples collection.

Month	1st week	2nd week	3rd week	4th week
January	x	x		
February		x	x	
March		x	x	
April			x	X

Table 3. Mean physicochemical characteristics of samples.

Sample	Parameter		
	DO mg/L	BOD mg/L	TDS mg/L
Upstream (US)	0.9 ±1.41	2.1± 2.84	74 ± 1.41
Downstream (DS)	0.6 ± 1.41	2.4 ± 2.84	70 ± 1.41
Effluent Discharged (ED)	1.1 ± 1.41	60.5 ± 4.24	80 ± 2.84
EPA Ghana/ GSB standard for drinking water influent	4 - 15	0.5	1000
EPA Ghana standard for effluent	>50	<50	>75

US= Upstream, DS= downstream, ed= effluent discharge. Source: Environmental Protection Agency, Ghana.

series of standard methods of examination of water and effluent (APHA, 2005). Physical characteristics such as dissolved oxygen (DO), biochemical oxygen demand (BOD5), total dissolved solids (TDS) were used to examine the quality of water and pollution effects of waste from abattoir. Initial dissolved oxygen (DO) content was done using a modified azide method. Biochemical oxygen demand (BOD5) was calculated by taking the numerical difference between the initial DO and final DO (APHA, 2005). Total bacteria count was determined using Standard plate count method and pour plate method. Total and fecal coliform loads were determined using Multiple tube fermentation/ Most probable number method. Both single and double strength Lactose broth were used for the analysis. Fecal coliform and Total coliform bacteria were incubated for 24 h at 44 ± 1°C and 36 ± 1°C respectively.

RESULTS

From Table 3, dissolved oxygen in effluent discharge was 1.1 mg/L, upstream recorded 0.9 mg/l and downstream was 0.6 mg/L. Biochemical oxygen demand in the effluent discharge was 60.5 mg/L, downstream had 2.4 mg/L and upstream had 2.1 mg/L. Total dissolved solids in effluent discharge had 80 mg/L, upstream had 74 mg/L and downstream had 70 mg/L. From Table 4, the fecal coliform in downstream water sample was 41mpn/100ml, effluent discharge had 80 mpn/100 ml and upstream water sample had 20 mpn/100 ml. The total coliform count in effluent discharge was 500mpn/100ml, upstream water sample had 80 mpn/100 ml and downstream water sample had 70 mpn/100 ml. Total bacterial count in effluent discharge was 30cfu/ml x 10^5, upstream had 10 mpn/100 ml and downstream had 20 mpn/100 ml. *Salmonella* count was not detected in all the samples collected. There is no significant difference ($P<0.05$) between the discharged effluents water and other stream water sample because all samples were polluted.

DISCUSSION

The result as presented in (Table 1) showed that the concentration of dissolved oxygen (DO) is lower downstream with (0.6 mg/L) followed by upstream (0.9 mg/L) and higher at the discharge point with (1.1 mg/L). Animal blood is known to possess high oxygen demand. Blood from beef cattle had a BOD which accounted for the high dissolved oxygen at the discharge point. The implication of this fact is that discharge of waste containing blood of animal onto streams could critically reduce the dissolved oxygen (DO) of the aquatic environment.

Table 4. Bacteriological quality of samples.

Sample	Parameter			
	Fecal coliform Bacteria (MPN/100 ml)	Total coliform bacteria (MPN/100 ml)	Total bacterial counts × 10^5cfu/ml	*Salmonella* counts × 10^2 cfu/ml
Upstream (US)	20 ± 2.82	80 ±4.82	10 ± 2.12	0 ± 0.00
Downstream (DS)	41 ± 4.82	70 ± 4.82	20 ± 2.12	0 ± 0.00
Effluent Discharged (ED)	80 ± 4.82	500 ± 0.00	30 ± 2.82	0 ±0.00
EPA Ghana/ GSB standard for drinking water influent	0	<10	0	0
EPA Ghana standard for effluent	<10	<400	<10	<10

US= Upstream, DS= downstream, ED= effluent discharge. Source: Environmental Protection Agency, Ghana.

Similarly, the levels of BOD differs across the stream, with the discharge source obtaining the highest value of 60.5mg/l, while the upstream had the least value of 2.1 mg/L. BOD deals with the quantity of oxygen needed for aerobic decomposition of organic materials. It shows the amount of oxygen required for microbes to breakdown organic matter (Chukwu, 2008). The high BOD value recorded at the point of discharge is an indicative that there are higher amounts of biodegradable materials from the abattoir. The observed high BOD in effluent discharge can be linked to excessive organic matter load arising from waste meat, blood, skins, salts, and rumen ingest discharge. This scenario is not unique to the Bolgatanta Abattoir (Bush, 2000). BOD is a very vital indicator for water quality parameter and is used as a vehicle for water quality assessment. However, higher BOD is dependent on higher organic matter content in the discharged abattoir waste.

This high BOD obtained at the discharge point perhaps correlates with the high DO levels observe at this point. The contents BOD obtained in this study is higher than the stipulated value of potable drinking water. The high BOD obtained for this study could be attributed to the release of huge amounts of abattoir effluents into the stream. The high BOD observed in this research is in agreement with the value as revealed by Omole and Longe (2008), Akan et al. (2010). High BOD in some stream could be attributed to the discharge of large quantities of abattoir effluents. Furthermore, the level of total dissolved solid (TDS) ranged from 70 to 80 mg/L, with discharge point having 80 mg/l; while the downstream had 70 mg/L (Table 3). The values of TDS upstream and downstream are within EPA standard of drinking water. According to Ewa et al. (2011), total or fecal coliform bacteria are usually present in water polluted by waste animals and humans subjects. The result in (Table 4) showed that the discharge point has the highest amount of total coliform of 500mpn/ml, followed by the downstream with value of 70mpn/ml. The high content at the point of discharge was apparent, as it is the point of organic waste entry into the river from the intestinal contents of the slaughtered animals. High counts in the effluent is a powerful indicator of excessive pollution and therefore not hygienic to be disposed in the environment. The high counts could be because of the excreta from the intestines of the cattle, goat and cows, which is washed to the effluent. The presence of fecal coliform in the effluent show recent fecal pollution – this reveal that greater risk of pathogen presence (Figueras, 2000; Cadmus et al., 1999; Coker et al., 2001). The reduction in TDS, Fecal coliform bacteria, Total coliform bacteria and total bacterial in downstream may be attributed to the self-purification capacity of effluent through the stream course. However, high value at the discharge point is linked to the discharge of many materials of solid wastes from the slaughtered animals as well as lack of sedimentation facility to separate the solid wastes from the liquid wastes before discharge. It could also be attributed to the fact that lesser water is used for washing animal carcass in this section of stream (Osibanjo and Adie, 2007; Akan et al., 2010). Microbiological analysis was positive due to the presence of coliform, apparently due to fecal contamination from the abattoir wastewater that seeped into surrounding stream water. The result of bacterial analysis of stream water sample reveals that the bacterial count of water from the Kuula stream in Bolgatanga exceeds EPA standard of drinking water in Ghana. EPA guideline requires that water intended for drinking should not contain any pathogen or micro-organisms indicative of fecal contamination. Thus, the stream has trace of fecal coliform or total coliform. The presence of bacterial count mean that the stream is receiving organic pollutants not only from the abattoir effluents, but also from other source of organic pollution like agricultural and household wastes. Reduction in bacterial counts downstream may be due to activities of other antagonistic organisms that utilized them. There was no *Salmonella* detected in the samples collected. More so, foul odor of the cow feces deposited and other activities going on around the abattoir place caused air pollution and had negative impact on the health of the community people living

around the place.

Conclusion

The study showed that physical characteristics of the stream water samples are within tolerable limits; microbiological in the other vein exceeds the limits. Solid and liquid wastes generated at the abattoir pollute the stream water. Physical and microbiological qualities of the effluent exceeded the EPA-Ghana standards. The discharge of abattoir waste into streams in Bolgatanga had negative impacts on the microbiological quality of streams, especially at mid-stream for humans' consumption. This may have adverse effect on the health of consumers drinking at the mid-stream and downstream. However, it is recommended that the sanitation in the local meat processing industries should be closely monitored. There should be adequate waste treatment before abattoir wastes are discharged and waste management practices must be ensured to reduce waste disposal. Environmental regulatory bodies should embrace re-use and recycling of waste in order to protect the water resources from negative impacts of abattoir wastes.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Vol. 13(28), pp. 538-543, October, 2019
DOI: 10.5897/AJMR2019.9154
Article Number: 6263BD462018
ISSN: 1996-0808

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African Journal of Microbiology Research

Full Length Research Paper

Prevalence and antibiotic susceptibility patterns of extended spectrum beta-Lactamase-producing *Klebsiella oxytoca* isolated from urine samples of patients visiting private laboratories in Abakaliki Metropolis

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Received 11 June, 2019; Accepted 30 July, 2019

Studies have shown that *Klebsiella oxytoca* is a major cause of infections in humans. This study was designed to determine the prevalence and antibiotic resistance pattern of extended spectrum beta-lactamase (ESBL)-producing *K. oxytoca* from urine samples of patients visiting private laboratories in Abakaliki, Ebonyi State. A total of 150 mid-steam urine samples of patients visiting three main private laboratories in Abakaliki, Ebonyi State were analyzed for the presence of *K. oxytoca* using standard bacteriological identification methods. Out of the 150 urine samples cultured, seven (7) were phenotypically identified as *K. oxytoca*. Isolated *K. oxytoca* were screened for ESBL production by antibiotic susceptibility test using second and third generation cephalosporins, and double disc synergy test. Susceptibility of ESBL-positive *K. oxytoca* to various classes of antibiotics was done on Mueller-Hinton agar (Oxoid, England) by Kirby Bauer disk diffusion technique. Results showed that 3 (42.8%) out of the seven (7) *K. oxytoca* isolates were ESBL-producers. All the ESBL-producers were completely resistant (100%) to ofloxacin, gentamicin, cefoxitin, sulphamethoxazole, ciprofloxacin, nitrofurantoin and ertapenem. The average multiple antibiotic resistance index (MARI) of the *K. oxytoca* isolates was one (1) and this explains their high multi-drug resistance trait. This study revealed that ESBL-producing *K. oxytoca* isolates exhibited complete resistance to all antibiotics tested against them. The multi-drug resistant traits expressed by these *K. oxytoca* isolates in our study area could lead to grave public health consequences if not curtailed.

Key words: *Klebsiella oxytoca*, extended spectrum beta-lactamase (ESBL), antibiotics, multi-drug resistance, urine.

INTRODUCTION

K. oxytoca is a gram-negative, rod-shaped, lactose-fermenting, non-motile, aerobic rod-shaped bacterium. Since it was isolated in the late nineteenth century, it has become a known human pathogen. Infections caused by

K. oxytoca could lead to a variety of diseases such as urinary tract infections (UTIs), liver abscess, pneumonia, meningitis, blood stream infection, infection of the heart and valves, and prostate infection (Paterson and Bonomo, 2005). ESBL is a rapidly evolving group of β-lactamases which have the ability to hydrolyze third-generation cephalosporins and aztreonam but are inhibited by clavulanic acid (Philippon et al., 1989). ESBL-producing *Klebsiella* spp. have been established since the 1980s as a major cause of nosocomial infections. Interestingly, in the late 1990s, some community-acquired pathogens which commonly cause UTIs and diarrhea were also found to be ESBL-producers (Paterson and Bonomo, 2005). ESBLs are usually encoded by genes located on large plasmids, and these plasmids sometimes carry genes for resistance to other antimicrobial agents including trimethoprim, tetracycline, sulphonamides, aminoglycosides, and chloramphenicol (Paterson, 2000). Some recent studies have shown fluoroquinolone resistance mediated by co-transfer of the *qnr* determinant on ESBL-producing plasmids (Mammeri et al., 2005). As a result, multidrug resistance trait is now a frequent characteristic of ESBL-producing enterobacteria. Thus, ESBL-producing bacteria pose a major problem in clinical therapeutics. Throughout bacterial history, resistance to β-lactamases has become a useful trait ever since the clinical application of β-lactam antibiotics in the treatment of bacterial infections. These drugs exhibit a Darwinian selection; thus killing susceptible bacteria and allowing the resistant strains to survive. Resistance to β-lactamase may be intrinsic to a particular species, as observed in enterococci, which have intrinsically insensitive penicillin binding proteins (PBPs). Alternately, it may be acquired via spontaneous mutation or DNA transfer. The frequent causes of resistance in Gram-positive cocci such as pneumococci and methicillin-resistant *Staphylococcus aureus* (MRSA) are alterations in the normal PBPs or the acquisition of additional β-lactam–insensitive PBPs. This study focuses on the isolation, phenotypic characterization, and determination of the antibiotic resistance patterns of *K. oxytoca* in urine samples of patients visiting private laboratories in Abakaliki metropolis.

MATERIALS AND METHODS

Study area

This research was conducted in three main private laboratories namely; Best diagnostics, comprehensive medical and research laboratory (Lab A), New life medical laboratory (Lab B), and City of david medical laboratory (Lab C), all in Abakaliki metropolis, the state capital of Ebonyi State. Ebonyi State is located in the South Eastern part of Nigeria. It shares boundary with Enugu, Cross River, Abia, and Benue states. It is between longitude 7°30' and latitude 60°45' E.

Sample collection

A total of 150 mid-stream urine samples (50 urine samples from each of the three laboratories) were collected using sterile universal containers which have been labeled with the patients' information. The patients were given instructions on how to collect the urine samples. The collected urine samples were then immediately transported to the Laboratory unit of Applied Microbiology Department, Ebonyi State University, Abakaliki for bacteriological analysis.

Isolation and phenotypic identification of the isolates

The urine samples were aseptically streaked on MacConkey agar, cysteine lactose electrolyte deficient (CLED) agar, and incubated at 37°C for 24 h. After incubation, the plates were observed for typical *Klebsiella* growth on MacConkey agar (red or pink colonies) and CLED (yellow to whitish blue mucoid colonies). These suspected *K. oxytoca* isolates were further characterized using standard microbiology techniques such as Gram-staining, catalase test, motility test, and other biochemical tests which include indole test, citrate utilization test, oxidase test, methyl red test, Voges-Proskauer test, and sugar fermentation test (Cheesbrough, 2004).

Screening of bacterial isolates for ESBL production

Screening the *K. oxytoca* isolates for ESBL production was done by observing their sensitivities to 2nd and 3rd generation cephalosporins; such as ceftazidime (30 g), cefotaxime (30 g), cefepime (30 g), and aztreonam (3). These antibiotics were aseptically placed at a distance of 30 mm apart on Mueller-Hinton agar (Oxoid, UK) plate that was previously inoculated with standardized inocula of the test bacterium using a sterile swab stick in order to get a confluent growth. The plates were allowed to stand for about 30 min for pre-diffusion of the antibiotics and after which was incubated for 18 to 24 h at 37°C. After the incubation time, the zones of inhibition were measured in millimeter using a metre rule and results were interpreted according to clinical and laboratory standard institute (CLSI) chart. ESBL production was suspected if any of the test bacteria showed reduced susceptibility or is resistant to any of the antibiotics used for the screening studies according to the CLSI guidelines (CLSI, 2014).

Phenotypic screening/confirmation of ESBL production by isolated *K. oxytoca* using double disk synergy test (DDST)

The *K. oxytoca* isolates that showed reduced susceptibility to any of the 2nd and 3rd generation cephalosporins were phenotypically confirmed for ESBL production using the double disc synergy test (Iroha et al., 2009). DDST was performed as a standard disc diffusion assay on Mueller-Hinton (MH) agar (Oxoid, UK) plates in

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Table 1. Prevalence of *Klebsiella oxytoca* in the three laboratories.

Source of urine sample	Number of urine sample collected	Number of *Klebsiella* species isolated (%)	Number of *K. oxytoca* isolated (%)
Lab A	50	12 (24.0)	4(33.3)
Lab B	50	9 (18.0)	2(22.2)
Lab C	50	7 (14.0)	1(14.2)

Lab A: Best Diagnostics, Comprehensive Medical and Research Laboratory, Lab B: New Life Medical Laboratory, Lab C: City of David Medical Laboratories.

line with CLSI criteria (CLSI, 2014). Sterile swab sticks were dipped into bacterial suspension(s) standardized to 0.5 McFarland turbidity standards, and was inoculated on MH agar plates. Antibiotic disc of amoxicillin/clavulanic acid (20/10 µg) was placed at the center of the MH agar plate and antibiotic discs containing cefotaxime (30 µg) and ceftazidime (30 µg) each was placed at a distance of 15 mm (center to enter) from the central disc, amoxicillin/clavulanic acid (20/10 µg) and the plates was incubated at 37°C for 18 to 24 h. ESBL production was suspected phenotypically when the zones of inhibition of the cephalosporins (cefotaxime 30 µg and ceftazidime 30 µg) increased in the presence of amoxicillin/clavulanic acid disk (20/10 µg). A≥ 5mm increase in the inhibition zone diameter for either of the cephalosporins (cefotaxime and ceftazidime) tested in combination with amoxycillin-clavulanic acid versus its zone when tested alone confirmed ESBL production phenotypically (Iroha et al., 2009).

Antibiotics susceptibility test

Antibiotic susceptibility test was carried out on all the *K*. *oxytoca* isolates using Kirby Bauer disc diffusion method. The standardized test organisms were aseptically swabbed on Mueller-Hinton agar plates using sterile swab sticks and each of the antibiotic discs (Oxoid, UK) was aseptically placed on the swabbed MH agar plates using sterile forceps. The MH plates were allowed for 15 minutes on the work bench for pre-diffusion of the antibiotics. The plates were then incubated at 37°C for 18 to 24 h. The inhibition zone diameter (IZD) produced by the test antibiotics on the test isolates were measured in millimeter (mm) using a ruler and interpreted according to the standard breakpoints of the Clinical Laboratory Standard Institute (CLSI) in order to classify them as resistant (R), intermediate (I), or susceptible (S) (CLSI, 2014).

Multiple antibiotic resistance (mar) index calculation

The *K. oxytoca* isolates were tested against seven different antibiotics to determine the prevalence of multiple antibiotic resistance traits among isolates. Multiple antibiotic resistance (MAR) index was calculated as a/b; where 'a' denotes the number of antibiotics to which the isolates showed resistance, and 'b' denotes the total number of antibiotics tested against the isolates.

RESULTS

In our study, 50 urine samples were each collected from three different private laboratories (Best Diagnostics, Comprehensive Medical and Research Laboratory (Lab A), New Life Medical Laboratory (Lab B), City of David Medical Laboratories (Lab C)), and later analyzed. Out of a total of 150 urine samples analyzed, seven (Lab A = 4, Lab B = 2, Lab C = 1) were positive for *K*. *oxytoca* (Table 1). Results showed that 3 (42.8 %) out of the seven (7) *K. oxytoca* isolates were phenotypically identified as ESBL-producers using disc diffusion technique (Tables 2 and 3).

DISCUSSION

The widespread application of beta-lactam antibiotics in most healthcare institutions today and communities unarguably established problems which have led to increased mortality, morbidity and cost of health care (Blomberg et al., 2005). Understanding the antibiotic resistance profiles of urinary tract bacteria is very pertinent not only in helping clinicians in the prescription of appropriate antibiotics but also for evidence-based recommendations especially in empirical antibiotic treatment of UTI (Blomberg et al., 2005). Results showed that 3 (42.8%) out of the seven (7) *K. oxytoca* isolates were phenotypically identified as ESBL-producers. This is a serious cause of concern as many clinicians revert to fluoroquinolones for the treatment of infections caused by Gram-negative pathogens (Paterson, 2007). In this study, all the ESBL-producers were found to be completely resistant (100%) to ofloxacin, gentamicin, cefoxitin, sulphamethoxazole, ciprofloxacin, nitrofurantoin, and ertapenem (Tables 4 and 5). The observed resistance (100%) to ciprofloxacin by ESBL-producing *K*. *oxytoca* isolates in our study does not completely agree with the study conducted by Akujobi and Ewuru (2010) who reported 37.6% resistance to ciprofloxacin among ESBL-producers. Also, our findings are in great consonance with that of Sasirekha et al. (2010) who reported a high resistance frequency value of 68% by Enterobacteriaceae to ciprofloxacin. Interestingly, aminoglycosides have been recorded to have good antibacterial activity against gram-negative organisms of clinical importance. This study revealed that all the *K*. *oxytoca* isolates were completely resistance to gentamicin. Our study contradicts the

Table 2. Preliminary screening for ESBL production in *K. oxytoca* isolated from urine sample of patients visiting the three different laboratories.

Antibiotics	Total number of isolate	Susceptible (%)	Resistance (%)
Cefepime	7	0	100
Aztreonam	7	0	100
Cefotaxime	7	0	100
Ceftazidime	7	0	100

Table 3. Prevalence of ESBL-Producing *K. oxytoca* in urine samples collected from patients visiting the three different laboratories.

Sample source	Number of *Klebsiella oxytoca* isolated (%)	Number of isolated *Klebsiella oxytoca* positive for ESBL production (%)
Lab A	4(57.1)	2(50)
Lab B	2(28.6)	1(50)
Lab C	1(14.3)	0(00)
Total	7(100)	3(100)

Lab A: Best Diagnostics, Comprehensive Medical and Research Laboratory; Lab B: New Life Medical Laboratory; Lab C: City of David Medical Laboratories, ESBL: Extended spectrum beta-lactamase.

Table 4. Antibiotic susceptibility patterns of ESBL-Producing *K. oxytoca* isolated from laboratory A.

S/N	No. of isolate	Antibiotics	Resistance (%)	Susceptible (%)
1	2	Ofloxacin	2(100)	0 (0)
2	2	Gentamicin	2 (100)	0(0)
3	2	Cefoxitin	2 (100)	0 (0)
4	2	Sulphamethoxazole	2 (100)	0 (0)
5	2	Ciprofloxacin	2 (100)	0 (0)
6	2	Nitrofurantoin	2(100)	0 (0)
7	2	Ertapenem	2(100)	0 (0)

findings of Sasirekha et al. (2010) who reported that 41.8% of the *Klebsiella* isolates in their study were resistant to gentamicin. This study is in concord with that of Al-Zarouni et al. (2008), who reported that over 90% of ESBL isolates in their study exhibited resistance to cephalosporin and aztreonam. Interestingly, Haque and Salam (2010), Sasirekha et al. (2010), and Ullah et al. (2009) reported antibiotic resistance frequencies of 59% and 55.5% to gentamicin for similar isolates in India and Bangladesh. These differences might possibly be due to the selective pressure on aminoglycosides as a result of increased use of gentamicin in various regions (Miller and Sabatelli, 1997). Chowdhury et al. (1994) reported in Bangladesh that 65 to 92% of Enterobacteriaceae isolated from urine samples were mostly resistant to frequently used antibiotics such as tetracycline, ampicillin, and co-trimoxazole. Recent findings indicated that bacteria harbouring multiple antibiotic resistance genes have suddenly become increasingly prevalent (Perez et al., 2007). In our study, all the *K. oxytoca* isolates were completely resistant (100%) to ofloxacin. The result of this study is in total agreement with other studies by Sasirekha et al. (2010) and Ullah et al. (2009), where resistance frequencies to antibiotics were reported to be 75 to 98% among *Enterobacteriaceae*. Also, third generation cephalosporins have been used for treatment of Gram-negative bacterial infections (Samaha-Kfoury and Araj, 2003). In this present study, all the *K. oxytoca* isolates were completely resistance to cefoxitin. Our study is also in concord with the work done by Sasirekha et al. (2010) in India where 85% of *Klebsiella* isolates were resistant to cefoxitin. In this study, the ESBL-

Table 5. Antibiotic susceptibility patterns of ESBL-producing *K. oxytoca* isolated from laboratory B.

S/N	No. isolate	Antibiotics	Resistance (%)	Susceptibility (%)
1	1	Ofloxacin	1 (100)	0 (0)
2	1	Gentamicin	1 (100)	0(0)
3	1	Cefoxitin	1 (100)	0 (0)
4	1	Sulphamethoxazole	1 (100)	0 (0)
5	1	Ciprofloxacin	1 (100)	0 (0)
6	1	Nitrofurantoin	1 (100)	0 (0)
7	1	Ertapenem	1 (100)	0 (0)

Table 6. MARI value of ESBL producing *K. oxytoca* from laboratory A.

S/N	Organism	Mari value
1	*K. oxytoca*	1
2	*K. oxytoca*	1
Total		2

Table 7. MARI value of ESBL-producing *K. oxytoca* from laboratory B.

S/N	Organism	Mari value
1	*K. oxytoca*	1
Total		1

producing isolates exhibited high resistance profiles. This agrees with the report of Okesola and Fowotade (2012) in Western Nigeria, where 70% *Klebsiella* isolates among the *Enterobactericeae* isolates were recorded among outpatients. The high rate of antibiotic resistance obtained from this study might possibly be attributed to poor antibiotic policy, irrational use of third generation cephalosprins (Shobha et al., 2007), and the emergence of antibiotic-resistant bacteria in hospitals. However, it has been reported that *Kebsiella* spp. were more resistant than *E. coli* which may be due to the fact that *Klebsiella* spp. harbour some virulence factors such as polysaccharide capsules, production of endotoxin, and carbapenemases, which make it more resistant than *E. coli* (Lin et al., 2011). Thomson (2001), in his findings, reported more resistance to cefotaxime and ceftazidime. This could probably signify overuse and irrational use of third-generation cephalosprins. In the present study, 3 (42.8%) out of the seven (7) *K*. *oxytoca* isolates were ESBL-producers. This study is similar to the findings of Livrelli et al. (1996) and Lal et al. (2007) who reported *Klebsiella* spp. as the leading bacteria that produce ESBLs. The high prevalence of ESBLs in *Klebsiella* spp. is of serious concern as infections caused by this bacterium is very common and antibiotic resistance exhibited by this bacterium may be due to the presence of the capsule that gives extra level of protection to the cells, presence of multi-drug resistance efflux pump, and their ability to acquire and disseminate resistance plasmids (Chaudhary and Aggarwal, 2004; Yusha'u et al., 2010). Also, the average MARI value of the ESBL-producing *K*. *oxytoca* isolates was one (1) (Tables 6 and 7). This result shows that the organisms expressed high resistance frequencies (100%).

Conclusion

In West Africa, especially Nigeria, β-lactam antibiotics are the most commonly prescribed drugs against gram-negative aerobic bacilli infections according to literature. The selective pressure exerted by the overuse of these β-lactam antibiotics especially in the treatment of some life-threatening infections, most likely result in strains developing ESBL enzymes. Results obtained from our study showed that the *K*. *oxytoca* isolates obtained from urine samples of patients were multi-drug resistant as they were resistant to at least two different classes of antibiotics. Our study also revealed the presence of ESBL-producing *K*. *oxytoca* strains in the urine samples of patients. High frequency of resistance exhibited by these *K*. *oxytoca* isolates against second and third generation cephalosporins indicates that very soon, these antibiotics may no longer be effective in the treatment of infections caused by these *Enterobacteria* (*K*. *oxytoca*) isolated from urine samples of patieints in Abakaliki. This is a serious public health problem and if not properly addressed, could metastasize into serious consequences. Although this study showed low prevalence of ESBL-producing *K. oxytoca* isolates from urine samples of patients, however, it is paramount to properly monitor antibiotic resistance in *Klebsiella* spp. so as to avert serious public health problem. Thus, proper antibiotics screening should be incorporated before antibiotics administration in clinical practice in order to

detect bacteria that harbour multi-drug resistance traits and curtail the abuse of antibiotics.

CONFLICT OF INTEREST

The authors have not declared conflict of interests.

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Vol. 13(28), pp. 544-551, October, 2019
DOI: 10.5897/AJMR2019.9155
Article Number: 7D752ED62076
ISSN: 1996-0808

http://www.academicjournals.org/AJMR

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Review

Probiotics *Lactobacillus* strains: A promising alternative therapy against to biofilm-forming enteropathogenic bacteria?

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Received 12 June, 2019; Accepted 24 September, 2019.

Biofilms formation stands out in context of persistent intestinal infections caused by Enterobacteriaceae, which are associated with a high resistance to antimicrobial agents' and phagocytosis by host defense cells. Hence, understanding the mechanisms involved in this process becomes major for the development of new preventive and therapeutic strategies. Lactic acid bacteria, including species of the genus *Lactobacillus*, have been associated with the prevention or dispersion of biofilms formed by pathogenic microorganisms. This effect is often associated with the antimicrobial substances production, among them organic acids, bacteriocins, hydrogen peroxide and biorsurfactants. However, the antibiofilm action of *Lactobacillus* seems to be strain-specific and may not be demonstrated by strains of the same genus. Thus, diet supplementation with beneficial microorganisms represents a possible strategy for prevention and treatment of intestinal infectious diseases, such as persistent or acute diarrhea caused by enteropathogenic bacteria. However, *in vitro* and *in vivo* further studies are needed to clarify the efficacy of different probiotic candidates, including commercially available products.

Keyword: Enterobacteria, biofilm, *lactobacillus*, antimicrobials.

INTRODUCTION

The term biofilm describes a lifestyle characterized by microbial adhesion with production of extracellular polymer substances, constituting a gelatinous network that protects the cells and its associated with numerous cases of infections in human beings (Schiebel et al., 2017). In a liquid environment the primary event of biofilm formation, mainly in Gram-negative bacteria such as *Escherichia coli* and *Salmonella,* is related to the flagellar apparatus, which provide an initial approach between the bacterial cell and the surface (Misselwitz et al., 2012; Guttenplan and Kearns, 2013). Afterwards, three sequential steps, initial microbial adhesion, attachment either by exopolysaccharide production and cell surface structures, and colonization by growth of attached organisms, guarantee their survival in complex environments (Tolker-Nielsen, 2015).

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The biofilm formation is considered as an essential factor in the pathogenesis of various enteropathogenic bacteria such as enteroaggregative *E. coli* (EAEC), which has been frequently implicated with several kinds of diarrhea (Meng et al., 2011; Dallman et al., 2012; Lääveri et al., 2014; Bamidele et al., 2019). Thus, diseases related with the ability of bacteria to form a structured microbial community are generally difficult to treat due to intrinsic biofilm resistance against antimicrobial agents (Haney et al., 2018; Bjarnsholt et al., 2013). In the attempt to explore new forms that may contribute to the prevention and treatment of infectious intestinal diseases, probiotics are standing out in merit of their protective effect against bacterial pathogens (Ramos et al., 2012; Sikorska and Smoragiewicz, 2013; Osama et al., 2017). Lactic acid bacteria (LAB), including *Lactobacillus* species, have been associated with the prevention or dispersion of biofilms formed by enteropathogenic microorganisms (Kaur et al., 2018; Miquel et al., 2016). Therefore, this review aims to describe the biotechnological application of *Lactobacillus* strains as probiotic agents and their role to counteract with enteropathogenic microbial biofilms, which may represent a promising alternative therapy.

Infectious intestinal diseases and enteropathogens

There is a complex beneficial relationship in intestine environment between host and gut microbiota that seeks a homeostatic equilibrium in which both are favored (Paixão and Castro, 2016). Thus, the interruption of this synchrony may result in the prevalent growth of pathogenic microorganisms, causing tissue inflammation and immunological regulation failures (Iacob et al., 2019). The ingestion of contaminated food or water are among the various causes of intestinal dysbiosis (Gabliardi et al., 2018). Food-borne outbreaks caused by several Enterobacteriaceae have been reported in Brazil, with emphasis on classical microorganisms such as *E. coli* and *Salmonella* sp. (Feltes et al., 2017).

Although many microorganisms cause foodborne infections, the severity of the disease, clinical manifestations, and duration of symptoms differ significantly (AL-Mamun et al., 2018). For instance, *Vibrio cholerae* and some strains of *E. coli* are capable to induce secretory diarrhea by exotoxins production (Choi et al., 2016; Chau et al., 2016; Chen et al., 2019). It triggers the intracellular cAMP and cGMP levels and result in the activation of the cyclic transmembrane conductance regulator nucleotide (CFTR) of Cl^- channels, increasing fluid secretion into intestinal lumen (Thiagarajah et al., 2015). Furthermore, the high production of cAMP, cGMP and Ca^{2+} by host cells induced through enterotoxins inhibits the sodium-hydrogen antiporter (NHE3), which is the main exchanger channel responsible for maintaining of cellular sodium balance (Beltrán et al., 2015; Hodges and Gill, 2010).

On the other hand, bacteria such as *Campylobacter jejuni*, *Shigella* spp. and enteric *Salmonella* invade epithelial cells and trigger a massive neutrophilic infiltrate into the mucosa, followed by cellular transmigration into intestinal lumen (Navaneethan and Giannella, 2008). Thus, a response is established with recruitment of immune cells and release of cytokines, which characterizes inflammatory diarrhea (Hodges and Gill, 2010). It should be noted that enteropathogenic bacteria also alter the expression of transport proteins, as well, influencing the absorption of Na^+ and Cl^- (Marchelletta et al., 2013).

Intestinal microbiota may act as a very competitive environment, since it can host over 2000 bacterial species (Novik and Savich, 2019). However, high nutrients availability combined with a constant microorganisms' influx makes the gastrointestinal environment ideal for development of sessile communities by enteropathogens, called biofilms, fact closely related to chronic intestinal infections (Rosenvinge et al., 2016). Biofilm formation represents an ability developed by the most microorganisms, which facilitates colonization on new surfaces with increased tolerance to environmental stresses (Miquel et al., 2016). Thus, the morphology, cellular density, as well as their physiological state are associated to wide range of antibiotic resistance (Singh et al., 2017).

The development of the biofilm starts from a response of the planktonic cells to environmental signals in order to raise survival chances, altering the expression of hundreds of genes (Oliveira et al., 2015). In short, this process begins by the cell attachment to (a)biotic surface, reversibly adhered. Then, irreversibly attachment happens after cell proliferation with microcolonies formation, and polymer matrix production. Finally, maturation is achieved, followed by detachment or dispersion of mature biofilm parts, which may determine the appearance of cellular clusters on new colonization sites (Tolker-Nielsen, 2015).

External bacterial structures play an important role in biofilms formation by enteropathogens, which may include fimbriae, flagella and capsules (Rabin et al., 2015). The first one stands out for facilitating bacterial aggregation and adhesion on several substrates, being very common in Gram-negative organisms. These cellular interactions are also triggered by the presence of specific components encoded by plasmids, such as adhesins and curli, potent inducers of the inflammatory response in the host (Wolska et al., 2016).

Genetic mechanism directly influences the ability to form cell agglomerates and, consequently, the pathogenesis of each bacterial species (Wolska et al., 2016). Schiebel et al. (2017) related the expression of *fimH* and *agn43* genes in pathogenic *E. coli* strains with the ability to form biofilms by encoding type 1 fimbriae and Ag43 antigen. It is known that adhesins are essential for initial cell adhesion on the surface and Ag43 is

capable of promoting auto cell-to-cell aggregation (Schiebel et al., 2017). Nascimento et al. (2014) also associate the participation of type 1 fimbriae with strong biofilm production by enteropathogenic *E. coli* strains (EPEC). Also in EPEC isolates, bundle-forming pilus (BFP) and the EspA filament are involved in the formation of microcolonies on epithelial cells and abiotic surfaces (Saldaña et al., 2009 and Moreira et al., 2006).

Probiotics microorganisms

Probiotics are living microorganisms that, when administrated in adequate amounts, confer a health benefit on the host, and can be found in foods or as dietary supplements and medications (WHO, 2002; Hill et al., 2014). The most commonly used are those belonging to *Lactobacillus* and *Bifidobacterium* genus, which are commensals bacteria that live in or on human bodies (Chew et al., 2015; Novik and Savich, 2019). Currently, the main claim for use of probiotic bacteria are: helping healthy microbiota maintenance, reducing the numbers or colonization of pathogenic bacteria, promoting the digestion of lactose by intolerant individuals, relieving constipation and increasing the absorption of vitamins and minerals (Novik and Savich, 2019). In addition, due to evidence of antimicrobial effect against several pathogens, the interest on the metabolic performance of these organisms has increased in recent years (Abdelhamid et al., 2018; Do Carmo et al., 2018; Osama et al., 2017).

Probiotics strains can affect pathogenic microorganisms through different mechanisms, such as enhancing the intestinal barrier function, increasing mucin production and modulating the immune system activity (Miquel et al., 2016; Hu et al., 2017; Vieco-Saiz et al., 2019). Other factors such as metabolites production, nutrients competition and suppression of toxin production are also involved in probiotic action (Markowiak and Slizewska, 2017). All of those effects can be triggered by metabolites, cell wall components, DNA fragments, as well as the adhesion of probiotic cells to host epithelium (Oelschlaeger, 2010).

Recent *in vitro* studies concluded that the use of LABs, specially *Lactobacillus* species, are related to positive results against enteropathogens (Turková et al., 2013; Ruiz et al., 2017; Prabhurajeshwar and Chandrakanth, 2017; Kaur et al., 2018). However, when it comes to clinical trials, the effects against bacterial infections are related to regular consumption of food sources, such as yogurts and curds, as well as supplementation (Varavallo et al., 2008; Halder and Mandal, 2016; Prabhurajeshwar and Chandrakanth, 2017).

Other effect associated to probiotics strains is the inhibition of virulence-related gene expression, such as toxins production (Rätsep et al., 2017). Thus, the efficacy of certain strains for the treatment of diarrheal diseases is probably associated with their ability to protect the host against toxins action, including those produced by cyanobacteria and fungi (Oelschlaeger, 2010). Carey et al. (2008) reported that 15 different *Lactobacillus* strains were able to inhibit the expression of shiga-toxin production by EHEC O157:H7, from the production of organic acids in sub-bactericidal concentrations with consequent pH reduction. Rätsep et al. (2017), testing the combination of xylitol with *Lactobacillus plantarum* detected the suppression of spores' germination and outgrowth into vegetative toxin producing cells of *C. difficile,* which reduces the colonization of gut with the pathogen.

Enterobacteria and *Lactobacillus* Interactions

Lactobacillus genus corresponds to an important group of microorganisms related to ferment dairy products, as starters or as secondary microbiota, as well as food preservation (Ruiz et al., 2017). Several species of this group have been accepted with GRAS (*Generally Recognized as Safe*) status, which identifies a microorganism or microbial derivatives as safe for use in food industry (Cui et al., 2017; Gabliardi et al., 2018). *Lactobacillus* is frequently found in environments with low molecular oxygen tension, such as intestinal and urinary tract of humans, sharing their habitat with several types of potentially pathogenic microorganisms, among them pathogenic enterobacteria (WGO, 2017; Ruiz et al., 2017). Thus, these microorganisms have antagonistic properties against to pathogenic bacteria through metabolites production that render a hostile environment, such as organic acids, hydrogen peroxide, biosurfactants and bacteriocins (Fijan, 2014; Davoodabadi et al., 2015; Yeganeh et al., 2017; Abdelhamid et al., 2018; Fernandes, 2019; Vieco-Saiz et al., 2019).

Although lactic and/or acetic acids are considered to have low acidity, it is noteworthy the bactericidal effect against numerous pathogens, especially under conditions with nutrient limitation (Fijan, 2014). In these conditions, acids in the non-dissociated form penetrate the cytoplasm, where they dissociate and decrease the intracellular pH, interfering on cellular metabolic processes (Hughes and Webber, 2017). In addition, these acids increase the permeability of the outer membrane of Gram-negative organisms, compromising their integrity, what may potentiate the action of other antimicrobial substances such as bacteriocins (Gálvez et al., 2010). Hydrogen peroxide produced by many strains of *Lactobacillus* is also capable of inducing stresses in the outer membrane of some bacteria, such as uropathogenic *E. coli* (UPEC) which affects the structure of fimbriae and prevent their cell adhesion ability (Costa et al., 2012).

In addition, Halder and Mandal (2016) have shown that *Lactobacillus* from different species, individually, have

demonstrated excellent *in vitro* inhibition of enterobacteria growth, such as *E. coli* and *K. pneumoniae*. Also, when tested different strains combination from the same genus, they showed a synergistic effect against *E. coli* (Halder and Mandal, 2016). The use of isolated species, as well as blends containing different strains combined have been shown to be useful in the treatment of gastrointestinal diseases *in vivo* (Vuotto et al., 2014). Nevertheless, bactericidal capacity does not necessarily predict an antibiofilm action (Kaur et al., 2018). Considering the increasing ability of pathogens to generate persistent infections related to biofilms formation, probiotics administration may be able to modulate and prevent the proliferation of invasive microorganisms *in vivo* (Vuotto et al., 2014).

Antibiofilm strategies

The trend in health promotion through natural means leads to interest in non-chemical antibiotic agents, including microbial products, capable of reducing bacterial biomass (Challinor and Bode, 2015; Miquel et al., 2016). Two mechanisms are verified to be able of modulating the formation of these communities: destabilization of mature biofilms irreversibly attached or the inhibition of bacterial surface attachment (Miquel et al., 2016). In this perspective, *in vitro* and *in vivo* studies shown that probiotics are useful to modify the composition of the exopolymeric matrix, affecting the primary cell adhesion and/or colonization by exclusion/competition, or even trigger a cellular dispersion from biofilm (Gutiérrez et al., 2016).

Recent researches highlight the antibiofilm feature of *Lactobacillus* genus. Osama et al. (2017) demonstrated antimicrobial and antibiofilm action of *Lactobacillus rhamnosus* and *Lactobacillus gasseri* strains against *Pseudomonas aeruginosa*, *E. coli* and *Staphylococcus aureus*, three pathogens commonly involved in persistent infections associated with biofilm formation. As also, Abdelhamid et al. (2018) identified effective results in the biofilm eradication from multidrug resistant (MDR) *E. coli* isolates through bioactive compounds acting as antimicrobials. Fernandes (2019) demonstrated that cell free supernatant produced by standard and commercial probiotic strains (*L. acidpohillus* LA14, *L. acidophillus* ATCC 4356 and *L. rhamnosus* ATCC 9595) exerted strong bactericidal and antibiofilm action against MDR *E. coli* isolated from fish fillet samples.

However, the results obtained are attributed to several mechanisms of action which requires further investigations. For instance, an effective strategy to avoid the first step of biofilm formation it's through to biosurfactants use, that impairs microbial adhesion modifying the physicochemical cell surface properties (Gómez et al., 2016; Sharman and Saharan, 2016; Kaur et al., 2018). The antibiofilm activity is also related to the production of bacteriocins, which are antimicrobial peptides produced by certain bacteria, that act suppressing biofilm formation and have a high applicability as food bioconservatives, since they have a broad spectrum against many food spoilage microorganisms, among them *E. coli* (Mathur et al., 2017; Novik and Savich, 2019).

The most of bacteriocins secreted by *Lactobacillus* belong to class II, heat stable, whose effect is related to the membrane destabilization with pores formation, plasma content extravasation and consequent cell death (Paixão and Castro, 2016). It is already recognized that bacteriocins action is potentiated under acidic conditions, which highlights the importance of organic acids secreted by various probiotic strains (Gálvez et al., 2010). The assembly of these antimicrobial peptides is controlled according to population density and communication between the cells through *Quorum Sensing* (QS), a process that bacteria use to coordinate gene expression and allow the production of virulence factors (Lixa et al., 2015).

QS control has become one of the purposes in the development of new strategies for the treatment of bacterial biofilm infections (Wu et al., 2015). Through QS, bacteria tend to produce low molecular weight chemical signals called autoinducers (AIs) that, when diffusing into the medium might be internalized to induce the differential genes expression that alter cellular metabolism (Lixa et al., 2015). Several enterobacteria are recognized for producing and responding to these AIs, so they can express virulence factors, succeed in colonization, and consequently to establish intestinal infections. Therefore, the use of probiotics emerges also due to produce small biologically active molecules capable of interfering on bacterial pathogens QS (Li et al., 2011; Liu et al., 2016). Some elucidations about the main mechanisms of action by *Lactobacillus* strains are able to exert an antibiofilm action are summarized in Table 1.

Although numerous *in vitro* evidences about antimicrobial activity of *Lactobacillus* strains, relevant actions of these probiotic preparation have been also observed by classical *in vivo* infectious models, as bacterium- and/or rotavirus-infected animals (Nakazato et al., 2011; Quigley et al., 2019; Jiang et al., 2017; Vlasova et al., 2013; Zhang et al., 2013). Normally, the probiotics effects have been related to normalization of intestinal microbial communities, competitive exclusion of pathogens associated with gut epithelia, bacteriocin production, production of short-chain fatty acids and modulation the activity of the immune system (Plaza-Diaz et al., 2019). Therefore, investigations performed by *in vivo* models are strongly recommended to help in the clarification of the mechanisms of action by each probiotic candidate.

In human beings, probiotic *Lactobacillus* strains can survive after oral administration and efficiently colonize

Table 1. *In vitro* evaluation of the mechanisms of action involved with antibiofilm feature of *Lactobacillus* genus against enterobacteria.

Strains	Source	Mechanisms of action
L. rhamnosus EMC 1105	Standard	Production of organic acids and inhibitory effect on proteolytic activities of *P. aeruginosa*, *E. coli* and *S. aureus* (Osama et al., 2017).
L. gasseri EMC 1930		
L. plantarum ATCC 1363 *L. acidophilus* ATCC 314 *L. casei* ATCC 25598	Homemade fermented milk	Lactobacilli supernatant had antimicrobial activity against the biofilm produced by ciprofloxacin-resistant uropathogenic *E. coli* strains in pasteurized milk, referred to lactic acid production. It was reported an important anti-adhesive effect, as well (Yeganeh et al., 2017).
L plantarum KSBT 56	Fermented milk product	*Lactobacillus* inhibited the growth, invasion and the biofilm formation of *Salmonella enteritidis* due to the production of organic acids and down regulation of virulence related genes (Das et al., 2013).
L. sakei and *L. curvatus*	Salami	Reduce *Listeria monocytogenes*, *Salmonella* and *E. coli* O157:H7 biofilm formation. This effect was attributed to biosurfactant and bacteriocin production, as well as mechanisms of pathogens exclusion through their trapping (killing of cells embedded in biofilms) (Gómez et al., 2016).
L. helveticus	Goatcheese	
L. casei	Ripened cheese	
L. jensenii ATCC 25258	Standard	Anti-adhesive and antibiofilm abilities mediated by biosurfactant production against multidrug resistant *Acinetobacter baumannii, E. coli and S. Aureus* (Sambanthamoorthy et al., 2014).
L. rhamnosus ATCC 7469		
L. hevelticus	Yak milk cheese	Antimicrobial and antiadhesive properties by the biosurfactant against various pathogenic and nonpathogenic microorganisms (Sharman and Saharan, 2016).
Lactobacillus strains	Vaginal samples and dairy products	Reduction of surface hydrophobicity and suppression of motility affected *E. coli* phenotypic characteristics important in the contacts with the substratum during the early stages of biofilm settlement. The results also suggested the peptides or protein factors also contributed to antibiofilm effect (Vacheva et al., 2012).
L. acidophilus La-5	Dairy products	Secretion of low molecular weight molecules that binds the autoinducers (AI-2 or AI-3) that altered the QS system in *E. coli* O157:H7, decreasing attachment to tissue culture cells (Mendellin-Peña and Griffiths, 2009).
L. plantarum CIRM653	Food	Production of strain-specific derived bioactive molecules cause destabilization of *Klebsiella pneumoniae* (multi-resistant) of pre-formed biofilm architecture, induced by transcriptional modifications of biofilm-related genes (Lagrafeuille et al., 2018).

different parts of gastrointestinal tract, but the maintenance of the strain into gut environment seems to depends on intake frequency of probiotic preparation (Saxelin et al., 2010; Balgir et al., 2013; Arioli et al., 2018; Taverniti et al., 2019). That may explain when some of these probiotic strains come to randomized clinical trials, the results are occasionally inconclusive, and these intriguing outcomes put on doubt the clinical value of this treatment method (Chau et al., 2018; Ten Bruggencate et al., 2015; Hegar et al., 2015; Piescik-Lech et al., 2013). However, it must be considered that the anti-infectious action of *Lactobacillus* is not equally effective for all disease prevention or treatment indication. Several factors are involved on efficacy of probiotic strains, among them adequate doses for appropriate periods, besides diseases type and mechanisms of action of strains (Liu et al., 2019; Islam, 2016; Floch et al., 2015). In other words, the correct choose of probiotic must be strain-specific, and should not be generalized even among strains into the same species (Sniffen et al., 2018;

McFarland et al., 2018; Liu et al., 2018).

Meanwhile, it is worthy to highlight that those desirable effects trough probiotic administration encourage and indicate *Lactobacillus* species as promising therapy strategies against enteropathogens (Szajewska et al., 2016; Bustos and Chamorro, 2018). In the end, the real impact of probiotic administration on the microbial ecology of the gastrointestinal tract and on animal health is far from being understood (Ten Bruggencate et al., 2014; Suez et al., 2018).

Conclusion

In conclusion, the biotechnological application of *Lactobacillus* strains as probiotics presents effective results to control microbial biofilms formed by enteropathogenic bacteria, representing a promising alternative for medicine use. The inhibitory effect seems to be strain specific and is referred to metabolic compounds, such as organic acids, hydrogen peroxide, bacteriocins, biosurfactants and QS inhibitors. Although, *in vitro* activity does not always correspond to *in vivo* results, which shows that further clinical trials are needed to predict select real beneficial strains.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGMENT

The authors show gratitude to the institutions University Center INTA and Federal University of Ceara for support during the course of this research.

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Vol. 13(28), pp. 552-561, October, 2019
DOI: 10.5897/AJMR2019.9176
Article Number: F06363362078
ISSN: 1996-0808

http://www.academicjournals.org/AJMR

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African Journal of Microbiology Research

Full Length Research Paper

Prevalence of HEV markers among healthy and patients with hepatitis B and C in Upper Egypt

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Received 22 July, 2019; Accepted 5 September, 2019.

Hepatitis E virus (HEV) infection has a worldwide distribution and represents an important cause of acute hepatitis. This study aimed to investigate the frequency of HEV infection, and risk factors associated with this infection in healthy individual and patients with hepatitis B and C in Upper Egypt. Samples were collected from different governorates (Luxor, Assuit, Aswan and Sohag governorates) in Upper Egypt. Serum samples from all subjects were tested for hepatitis B surface antigens, hepatitis C virus antibodies and hepatitis E virus antibody using enzyme linked immunosorbent assay (ELISA). Anti-HEV positive samples were tested for HEV RNA using reverse transcriptase polymerase chain reaction (RT-PCR) method. Between March 2017 to October 2018, 358 participants (8.66%) were seropositive anti-hepatitis C virus, (4.75%) Hepatitis B surface antigens positive, 1.2% were seropositive for both HBsAg and anti- HCV and 85.47% subjects were negative both for HBsAg and anti-HCV. The overall seroprevalence of anti-HEV in this study was 9.22%. Seroprevalence of anti-HEV increased significantly with age. Anti-HEV IgM and IgG positive samples were tested for HEV RNA. All anti-HEV antibodies samples were negative for Hepatitis E virus RNA by reverse transcriptase polymerase chain reaction (RT-PCR) method. The highest seroprevalence of Anti-hepatitis E was detected in 15 females (11.45%) compared to males (18, 7.93%). Pregnant women showed highest seroprevalence of anti-HEV compared to non-pregnant women. Prevalence of anti-HEV was higher among community residing in rural versus urban areas (11.02 vs. 5.74% respectively). There was high prevalence of anti-HEV antibodies in individual users of the river as a principal source of bathing water versus individuals bathing at home. HEV may be responsible for sporadic self-limited cases of acute hepatitis in Upper Egypt.

Key words: Pregnant, HBsAg, Hepatitis E virus (HEV) RNA, Upper Egypt.

INTRODUCTION

Infection with hepatitis B virus (HBV) and hepatitis C virus (HCV) is a greater global health problem (Flichman et al., 2014). It is estimated that around 350 million people are chronically infected with HBV and around 200 million people are infected with HCV worldwide (Zaheer et al., 2014). HEV super infection in patients with chronic HBV or HCV infections and autoimmune hepatitis has been assisted with clinical out comes in several geographical

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settings (Pischke et al., 2014).

The hepatitis E virus (HEV) is an etiological agent for acute hepatitis in endemic areas (Meng, 2010). HEV was first detected in 1983 (Balayan et al., 1983; Sreenivasan et al., 1984). The causative agent is a single-stranded, non-envelope, positive-sense RNA virus, which is about 27 to 34 nm in diameter. HEV is classified as the only member of the genus Hepevirus in the Hepeviridae family (Meng, 2010). It has a 7.2 kb gene with three open pen reading frames (ORFs): ORF1 encodes a non-structural protein; ORF2 encodes a capsid protein, and ORF3 encodes a cytoskeleton-associated phosphoprotein (Zafrullah et al., 1997; Tyagi et al., 2001).

Virus particles are relatively stable in the environment and have recovered from waste samples (Clemente-Caseres et al., 2003). Of the more than 20 million infections that are estimated to occur globally each year, ~ 70,000 infections result in death. This great grandeur of death occurs in resource-poor countries in Asia, Africa and Latin America (WHO, 2016).

Five different human pathogen HEV-genotypes (HEV-1 to HEV-4 and HEV-7) have been distinguished (Smith et al., 2016). HEV1 and 2 are only human pathogens, endemic in Asia and Africa and transmitted through the fecal-oral route, often through contaminated water in areas with better sanitation. They can cause large waterborne outbreaks with mortality rates ranging from 0.2 to 4%, and up to 25% in pregnant women (WHO, 2010; Kamar et al., 2014). HEV3 is endemic in America, Europe and Japan, and HEV4 in China, Taiwan and Japan. HEV3 and HEV4 generally cause acute self-limited sporadic infections (Renou et al., 2014). In addition, HEV-7 was reported in one person in the United Arab Emirates and was associated with several camel products (Lee et al., 2016).

Antibodies respond to HEV infection with specific IgM which often cannot be detected at the beginning of infection and increases early in the fourth week; this increase cannot be detected after 3 months. IgG reaches its peak immediately at the end of the fourth week after infection and can last for years (Dalton et al., 2008).

In Egypt, the prevalence of HEV in humans is higher than in other countries (Fix et al., 2000). Hepatitis E virus is likely endemic in Egypt and appears to be a frequent infection. Characteristics of HEV genotypes circulating in Egypt show the dominance of genotype 1 HEV associated with other North African isolates circulating in patients with acute symptoms in Egypt (Blackard et al., 2009).

High prevalence rates were recruited in the rural areas of Cairo and the Nile Delta (Saad et al., 2007). Several studies were conducted in Nile Delta for the prevalence of anti-HEV (Stoszek et al., 2006), anti-HEV IgG (Gad et al., 2011) and HEV RNA (Zaki and Othman, 2011).

Transmission can occur through contaminated drinking water (HEV-1 and HEV-2) or contaminated food (HEV-3 and HEV-4) (Hakim et al., 2017). Hepatitis E virus infections are mainly transmitted by means of contaminated water, but may also be transmitted via food or blood transfusions, or vertically from mother to fetus (Aggarwal and Jameel, 2011). The disposal of human waste in rivers which are also used as a source for drinking, washing and cooking is found to be most significantly associated with an increased prevalence of antibodies to HEV (Corwin *et al.,* 1999).

There is currently very little data on HEV infection among populations in Upper Egypt. This study aimed to investigate the occurrence of HEV infection and risk factors associated with this infection in healthy individuals and patients with hepatitis B and C in different governorates in Upper Egypt.

MATERIALS AND METHODS

Blood samples collection

Between March, 2017 and October, 2018 blood samples were collected from healthy and patients with hepatitis B and C from different governorate (Luxor, Assuit, Aswan and Sohag governorates) in Upper Egypt. Demographic data including age, sex, residence, contact with animals, blood transfusion, sanitary conditions and source of drinking water were collected using a questionnaire.

Blood samples were taken from each subject with cubital vein punctures. The site was thoroughly cleaned using 70% isopropyl alcohol in water and 1% iodine for 1 min and left to dry. Precautionary measures were taken to avoid site contamination. Five milliliters of blood were collected using sterile needles and distributed to clean plastic. Blood samples were centrifuged at 4000 rpm for 10 min, and the stored serum was stored in duplicate at -70°C for HBsAg, anti-HCV, anti-HEV and HEV RNA.

Serological detection

Serum markers for HCV infection

Anti-HCV was studied using third-generation ELISA tests (Ortho Diagnostics, Raritan, NJ, USA; and Abbott Diagnostics, North Chicago, IL, USA).The results were read using EL x 800 universal micro-plate reader, (Biotek Instruments Inc.). All positive samples were retested using the same method (double ELISA).

Serum markers for HBV infection

The HBsAg markers were serologically assessed using a third generation enzyme immunoassay (Murex HBsAg Version 3, Abbott-Murex, South Africa). Results were read using EL x 800 universal microplate reader, (Biotek Instruments Inc.). All positive samples were retested using the same method (double ELISA).

Serum markers for HEV infection

IgG and IgM were detected using third generation Enzyme Immunoassay (EIA) according to the manufacturer's instructions (DIA.PRO, Milano, Italy). The Cut-off was calculated by addition of 0.350 with mean optical density value of the Negative Control (NC) and samples were considered as positive when ratio of the test

Table 1. The oligonucleotides that used for the amplification of HEV RNA.

Sequence	Location	Direction	Position in the genome of HEV*
′*5*-AAT TAT GCC CAG TAC CGG GTT G-3′	External	Forward	5708-5687
′*5*-CCC TTA TCC TGC TGA GCA TTC TC-3′	External	Reverse	6414-6395
5′-GTT ATG CTT TGC ATA CAT GGC T-3′	Internal	Forward	5993-5972
5′- AGC CGA CGA AAT CAA TTC TGT C-3′	Internal	Reverse	6319-6298

*Numbering of the nucleotide positions given by the strain HEV Burma (number in the database GenBank M73218).

result of sample (od 450 nm) and the cut-off value was above 1 (or ≤1), according to the manufacturer's instruction. Results were read using EL x 800 universal microplate reader, (Biotek Instruments Inc.). All positive samples were retested in duplicate with the same EIA assay to confirm the initial results.

Molecular detection of HEV by RT-PCR

RNA extraction

Detection of serum HEV RNA by nested RT-PCR was performed using a QIAGEN One-Step RT-PCR kit according to the manufacturer's instructions. The primers were adopted after Huang et al. (2002). Briefly, a reaction tube contained 50 µL of the reaction solutions, including 10 µL of the 5 × QIAGEN One-Step RT-PCR buffer, 2 µL of the dNTP mix (containing 10 mM of each dNTP), 10 µL of the 5 × Q-Solution, 2 µL of the external forward and reverse primer (100 pM μL^{-1}) (Table 1), 2 µL of the QIAGEN One-Step RT-PCR enzyme mix, 1 µL of the RNase Out RNA inhibitor (10 U µL−1 ; Gibco BRL, Gaithersburg, MD), 10 µL of the template RNA, and 11 µL of the RNase free water. The thermal cycling conditions included one step of reverse transcription for 30 min at 50°C and an initial PCR activation step for 15 min at 95°C. This was followed by 40 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 50°C, and extension for 1 min 15 s at 72°C, and a final incubation for 10 min at 72°C. A nested PCR was conducted with the following components: 3 µL of the RT-PCR product, 5 µL of the 10×PCR buffer, 5 µL of $MgCl_2$ (25 mg mL^{-1}), 4 µL of the dNTP mix (10 mM of each dNTP), 1 µL of the internal forward and reverse primer (100 pm μL^{-1}) (Table 1), 0.5 µL of Takara Ex Taq polymerase (5 U μL^{-1}), and 30.5 µL of the double-distilled H_2O. The thermal cycling conditions for the nested PCR included 5 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 45°C, and extension for 1 min 15 s at 72°C. This was followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 53°C, and extension for 1 min 15 s at 72°C, and a final incubation for 7 min at 72°C. Sterile distilled water was used as a negative control. The positive control was the strain of human HEV. Positive and negative controls were included in each run with specific molecular weight markers.

Electrophoresis agarose gel detection

The PCR-HEV product amplified was detected by 1.5% agarose gel electrophoresis, stained with etidium bromide and observed under an ultraviolet light reaction. The expected product of universal nesting RT-PCR is 348 bp.

Statistical analysis

Data were analyzed using the SPSS version 16. Qualitative variables are described as numbers and percentages. Chi square or Fisher's exact test is used for comparison between groups; as appropriate. Odds ratios and their 95% confidence intervals were calculated. A p value ≤ 0.05 was considered statistically significant.

RESULTS

A total of 358 subjects from different regions in Upper Egypt were included in this study. From 358 participants, 31 (8.66%) were seropositive anti-hepatitis C virus, 17 (4.75%) Hepatitis B surface antigens positive, 4 (1.2%) were seropositive for both HBsAg and anti-HCV and 306 (85.47%) participants were negative for both HBsAg and anti-HCV. The highest seroprevalence of anti-HEV was detected in patients infected with HBV 5/17(29.41%), followed by patients positive for HCV 4/31 (12.90%), while samples negative for HBsAg and anti-HCV had the lowest seroprevalence of 24/306 (7.84%) with high statistically significant (T test =0.000**) (Table 2).

Of the 33 Anti-hepatitis E virus positive, 2 (6.06%) were positive for anti-HEV IgM, 26 (78.78%) were positive for anti-HEV IgG and 5 (15.15%) were positive for anti-HEV IgM and IgG. Seroprevalence of anti-HEV increased significantly with age; from 4.25% in participants below 30 years of age, 11.69% in 31-40 years of age, 15.30% in 41-50 years of age and a slight decline of 13.16% over those of 51-60 years of age, 3.12% in participants above 60 years of age. This age related prevalence of anti-HEV antibodies are high statistically significant (p =0.000**) (Table 3 and Figure 1).

Out of the 358 population tested for anti-HEV, 227 (63.40%) were males whereas 131 (36.60%) were females. The highest seroprevalence of anti-hepatitis E was detected in females (15; 11.45%) compared to males (18; 7.93%). This gender related prevalence of anti-HEV antibodies are non-statistically significant (p =0.63 n.s) (Table 4 and Figure 2).

The overall seroprevalence of anti-HEV in our study was 9.22%. The highest seroprevalence was found in Assuit governorates (15.38%), compared to 8.57 and 6.30 % in the Sohag and Luxor governorates respectively, while the lowest seroprevalence was found in Aswan governorates (5.26%). This results are non-statistically significant (p =0.68 n.s.) (Table 5 and Figure 3).

Table 2. Seroprevalence of HEV infection in patients with HBV, HCV infection and in healthy population.

Parameter	Total no. of participants	Anti-HEV antibodies		T test significant
		Positive, n (%)	Negative, n (%)	
Anti-HCV	31	4 (12.90%)	27 (87.10%)	**0.000
HBsAg	17	5 (29.41%)	12 (70.59%)	**0.000
Positive for anti-HCV and HBsAg	4	0 (0%)	4 (100%)	**0.000
Negative for anti-HCV and HBsAg	306	24 (7.84%)	282 (92.16%)	**0.000
Total	358 (100%)	33 (9.22%)	325 (90.78%)	**0.000

**, high statistically significant.

Table 3. Seroprevalence of IgG and IgM anti-HEV antibodies in different age groups.

Age (years)	Total no. of participants	Seropositive of Anti-HEV markers				Significant	
		Anti-HEV IgM	Anti-HEV IgG	Anti-HEV IgG and IgM	Total	X^2	P-value
>30	94	1 (1.06%)	3 (3.19%)	0	4 (4.25%)		
31- 40	77	0	7 (9.09%)	2 (2.60%)	9 (11.69%)		
41-50	85	1 (1.18%)	10 (11.76%)	2 (2.36%)	13 (15.30%)		
51- 60	38	0	4 (10.53%)	1 (2.63%)	5 (13.16%)	0.000**	36.16
60<	64	0	2 (3.12%)	0	2 (3.12%)		
Total	358	2 (0.56%)	26 (7.26%)	5 (1.40%)	33 (9.22%)		

**, High statistically significant.

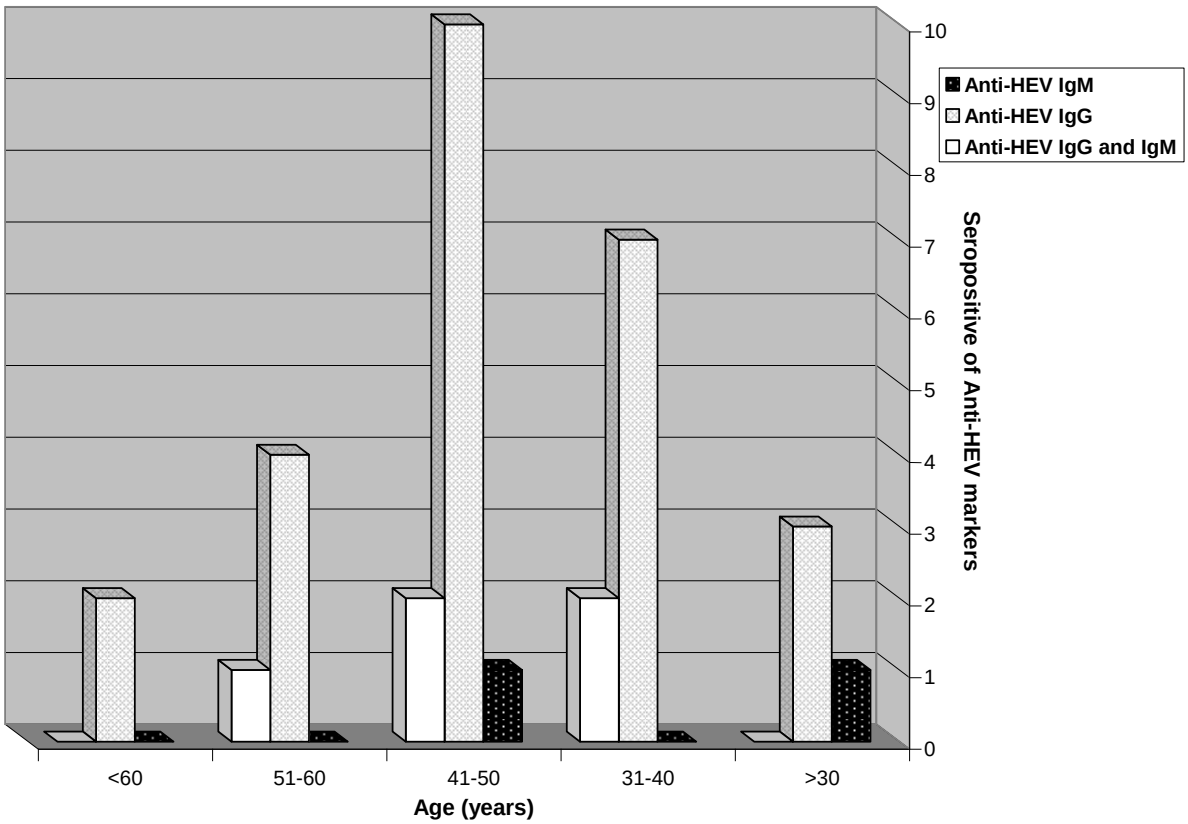

Figure 1. Seroprevalence of IgG and IgM anti-HEV antibodies in different age groups.

Table 4. Seroprevalence of Anti-HEV related to gender.

Gender	Total no. of participants	Seropositive of Anti-HEV markers				Significant	
		Anti-HEV IgM	Anti-HEV IgG	Anti-HEV IgG and IgM	Total	X^2	P-value
Male	227	0	15 (6.61%)	3 (1.32%)	18 (7.93%)	2.56	0.63 n.s.
Female	131	2 (1.53%)	11(8.39%)	2 (1.53%)	15 (11.45%)		
Total	358	2 (0.56%)	26 (7.26%)	5 (1.40%)	33 (9.22%)		

n.s., non-statistically significant.

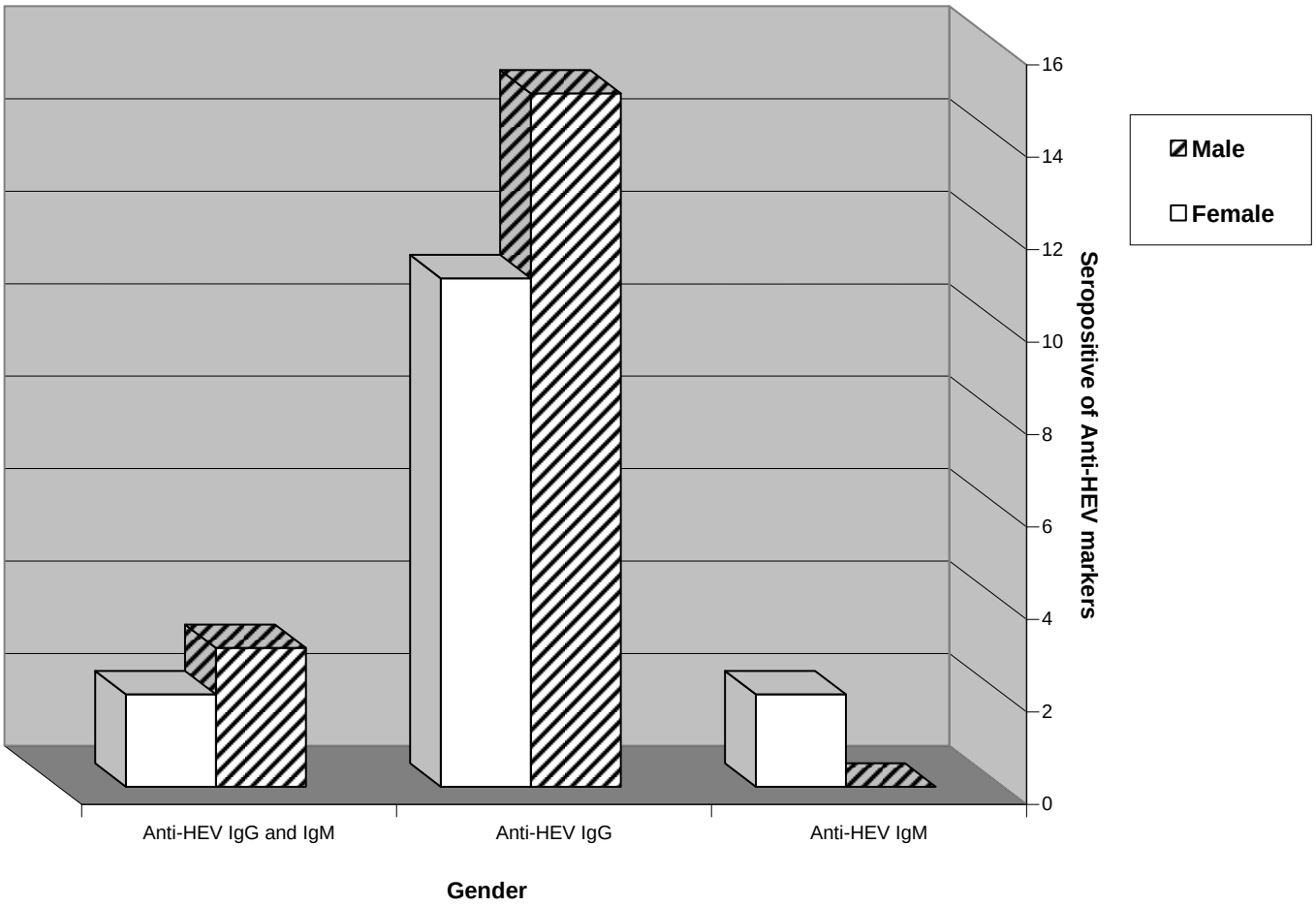

Figure 2. Seroprevalence of Anti-HEV related to gender.

Table 5. Seroprevalence of anti-HEV at different regions in upper Egypt.

Region	Total no. of participants	Anti- HEV		Significant	
		Positive, n (%)	Negative, n (%)	X^2	P-value
Aswan	57	3 (5.26%)	54 (94.74%)	7.12	0.068 n.s
Luxor	127	8 (6.30%)	119 (93.70%)		
Sohag	70	6 (8.57%)	64 (91.43%)		
Assuit	104	16 (15.38%)	88 (84.62%)		
Total, n (%)	358 (100%)	33 (9.22%)	325 (90.78%)		

n.s., non-statistically significant.

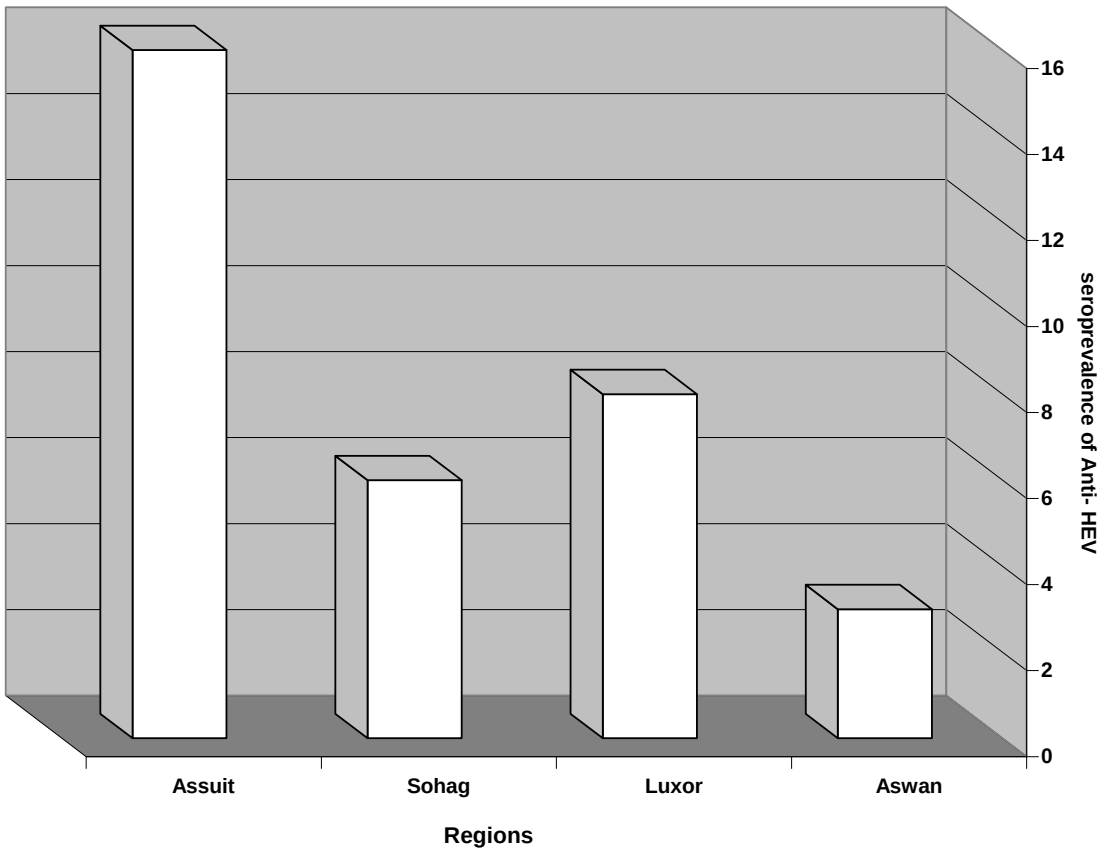

Figure 3. Seroprevalence of anti-HEV at different regions in Upper Egypt.

Many healthcare exposures are associated with anti-HEV, including residence area, contact with animals, source of drinking water, habit of bathing in the river, sanitary conditions, pregnant women and blood transfusion. The strongest of these associations is for pregnant compared to non-pregnant women; our results showed that 5 of 27 (18.52 %) of pregnant women were seropositive for anti-HEV versus 10 of 104 (9.62 %) for non-pregnant women, with none statistically significant (P = 0.85). There was a significant association between presence anti-HEV antibodies and residence area. Prevalence of anti-HEV was higher among community residing in rural versus urban areas (11.02 % vs. 5.74 % respectively) (Table 6).

Source of drinking water as a risk factor was associated with anti-HEV seroprevalence with a non-significant difference (P=0.59), the results revealed that subjects who use underground as drinking water source had the highest prevalence with 12.76%, followed by those who use tap water (9.33%) and least for those who use filtered water with 7.63%. There was high prevalence of anti-HEV antibodies in individual uses of the river as a principal source of bathing water verus individuals bathing at home (10.24 verus 8.66% respectively) (Table 6).

Although non-statically significant between presence of anti-HEV antibodies and contact of participants with animals (P= 0.86), out 152 that gave history of contact with animals, 15 (9.87%) were seropositive for anti-HEV antibodies compared to non with contact with animals (18/206; 8.74%) (Table 6).

The risk of infection with HEV through blood transfusion was assessed, and the results revealed a non-significant difference between those that have and those that have not and association of anti-HEV seroprevalence with blood transfusion (P= 0.848). Subjects with a history of blood transfusion accounted for a low seropositivity than those with no history of transfusion with 7.69% versus 9.48%, respectively (Table 6).

The type of toilet system used was associated with HEV seroprevalence. Results indicated that those who use pit for defecation accounted for the highest anti-HEV seroprevalence compared as those who use sewer (11.63 % verus 5.59 % respectively), with non-statically significant difference (p=0.08) (Table 6).

DISCUSSION

Egypt has the highest prevalence of IgG HEV in the

Table 6. Risk factors associated with HEV infection.

Risk factor	Total no. of participants	Anti- HEV		Significant	
		Positive, n (%)	Negative, n (%)	X^2	P-value
Residence area					
Urban	122	7 (5.74%)	155 (94.26%)	4.81	0.028*
Rural	236	26 (11.02%)	210 (88.98%)		
Contact with animals					
Yes	152	15 (9.87%)	137 (90.13%)	0.033	0.86 n.s
No	206	18 (8.74%)	188 (91.26 %)		
Bathing in the Nile river					
Yes	127	13 (10.24%)	114(89.76%)	0.092	0.76 n.s
No	231	20 (8.66%)	211(91.34%)		
Sanitary conditions					
Sewer	143	8(5.59%)	135(94.41%)	3.05	0.08 n.s
Pit	215	25(11.63%)	190(88.37%)		
For women (n=131)					
Pregnant	27	5 (18.52%)	22(81.48%)	0.037	0.85 n.s
Non-pregnant	104	10 (9.62%)	94(90.38%)		
Blood transfusion					
Yes	52	4 (7.69%)	49 (92.31%)	0.037	0.848 n.s
No	306	29 (9.48%)	277(90.52%)		

* - statistically significant; n.s. - non-statistically significant

world, which reaches 80-90% (Stoszek et al., 2006). However, most infections are unclear, although fecal-oral transmission is the main suspect. Other reports confirm that HEV can be transmitted through blood transfusions (Gotanda et al., 2007). It is not clear how this transmission is important in areas that are highly endemic like Egypt.

Abd-Al Aziz et al. (1999) investigated the prevalence of HEV antibodies among apparently healthy Egyptians with different age groups and reported that HEV was endemic in Egypt, especially in rural areas.

In this present study, we examined 358 stored sera among healthy and patients with Hepatitis B and C in Upper Egypt for markers of HEV infection using anti-HEV IgM and IgG ELISA for detection of serological markers and RT-PCR for detection of HEV RNA. Anti-HEV antibodies were detected in 33 out of 358 (9.22%) of the all individuals. Reports from Egypt about the seroprevalence of HEV in blood donors indicate that prevalence ranges from 0.45 up to 28.57% (Tadesse et al., 2013; Ibrahim et al., 2011).

This study also searched the seroprevalence of HEV antibodies among HBsAg and HCV antibodies. Out of 358 were 31 (8.66%) seropositive anti-HCV, 17 (4.75%) HBsAg positive, 4 (1.2%) were seropositive for both HBsAg and anti-HCV and 306 (85.47%) subjects were negative for both HBsAg and anti-HCV. The highest seroprevalence of anti-HEV was detected in patients infected with HBV 5/17(29.41%), followed by patients infected with HCV 4/31 (12.90%) compared to individual without anti-HCV and HBsAg 24/306 (7.84%) with high statistically significance (T test =0.000**). In agreement with other findings (Atiq et al., 2009), our results indicate that the HEV seroprevalence was higher in patients with HBV-related liver diseases compared to healthy individuals, suggesting patients with HBV-related liver diseases might have a higher risk for HEV infection (Nghiem et al., 2015).

The overall seroprevalence of anti-HEV in our study was 33 (9.22%), 2 (6.06%) were positive of anti-HEV IgM, 26 (78.78%) were positive for anti-HEV IgG and 5 (15.15%) were positive for anti-HEV IgM and IgG. The highest seroprevalence was found in Assuit governorates 15.38%, compared to 8.57% and 6.30% in the Sohag and Luxor governorates respectively, while the lowest seroprevalence was found in Aswan governorates (5.26%), which is more than figures obtained from blood donors in Germany (5.94%) (Vollmer et al., 2012), Spain (2.8%) (Mateos et al., 1999), Ghana (4.6% anti-HEV IgG and 5.9% anti-HEV IgM) (Meldal et al., 2013), but it is lower than what was reported in previous Egypt study which was 45.2% (43/95) in blood donors and 39.6% (38/96) in hemodialysis patients (AbdelHady et al., 1998).

In the current study, seroprevalence of anti-HEV increased significantly with age; from 4.25% in patients

below 30 years of age, 11.69% in 31-40 years of age, 15.30% in 41-50 years of age and a slight decline of 13.16% over those of 51-60 years of age, 3.12% in subjects above 60 years of age. Similar findings from seropositivity related to increasing age were also reported in other studies among people living in HEV endemic (Cheng et al., 2012) and non-endemic regions (Bernal et al., 1996).

Although no statistically significant difference was observed with regards to sex, pregnancy were investigated in the present study as potential risk factors for HEV infection. The highest seroprevalence of anti-HEV was detected in females compared to males (11.45 and 7.93% respectively), while 18.52 % of pregnant women were seropositive for anti-HEV versus 9.62% for non-pregnant women. Agreement with reports conducted in Egypt recorded that males have 26.8% seropositivity while the female seropositivity was 50.8% (El-Tras et al., 2013). Other research conducted in Egypt reported 67.7 and 84% of anti-HEV prevalence among healthy adults and pregnant women respectively (Stoszek et al., 2006).

Hepatitis E is an important hygienic infectious problem worldwide with a high incidence in developing countries, mainly in Asia and in Africa (Dalton et al., 2008). Our results showed a significant association between presence of anti-HEV antibodies and residence area. Prevalence of anti-HEV was higher among community residing in rural versus urban areas (11.02 vs. 5.74% respectively). This is consistent with the findings in Egypt (Stoszek et al., 2006), in eastern China (Dong et al., 2007), and in Bangladesh (Labrique et al., 2009), which showed that prevalence was significantly higher in persons living in rural neighborhood than those in urban settings.

Contaminated water or water supplies are an important source of HEV infection especially after outbreaks in humans (Buti et al., 2006). The wells are most likely not polluted due to the large number of toilets for water. Lack of drinking water treatment and low sanitation standards have been involved in a greater outbreak in developing countries where contamination of drinking water with animal or human feces is common (Ashbolt, 2004). This study revealed that subjects who use underground drinking water source had the highest prevalence with 12.76%, followed by those who use tap water (9.33%) and least for those who use filtered water with 7.63%. This is consistent with the findings in other studies (Galiana et al., 2008; Eker et al., 2009).

Antibodies to HEV have been detected in various animal species, suggesting that exposure to pets and other animals can play a role in the cycle of HEV transmission (Okamoto et al., 2004). In agreement with other studies (Stoszek et al., 2006; Eker et al., 2009; Kuniholm et al., 2009), the current study found that HEV IgG and IgM seropositivity were positively associated with having pets and other animals in the household 15/152 (9.87%) compared to non with contact with animals (18/206; 8.74) of the study population.

The present study showed no strong evidence that HEV is transmitted through the transfusion of blood or blood products which is consistent with finding that was reported in Turkey with no significant association (Arankalle and Chobe, 2000; Eker et al., 2009).

The type of toilet system used was associated with HEV seroprevalence. Results indicated that those who use pit for defecation accounted for the highest anti-HEV seroprevalence compared to those who use sewer (11.63 verus 5.59 % respectively), with non-statically significant difference ($p=0.08$). This is consistent with the findings of Eker et al. (2009) who reported high rates of seropositivity among people with individual incompatibility to the hygiene rules, and denotes that personal hygiene is an important factor for the prevention of HEV infection. The highest prevalence of HEV infection occurs in regions where low standards of sanitation promote the transmission of the virus (CHP, 2011).

Our failure to detect HEV RNA by RT-PCR may be explained by the characteristic low level viremia in HEV infection in Egypt (Blackard et al., 2009). It is believed that it is most likely due to low levels of viremia, because 33 individual samples were tested for positive pools and all were negative. This low level viremia may also decrease the potential risk of transmission of HEV by blood transfusion in Egypt.

Conclusion

Seroprevalence of HEV-antibody among healthy people and patients with Hepatitis B and C infection in Upper Egypt was high, so that a careful surveillance in the general population is required and further appropriate investigations are needed to identify the exact mode of transmission and risk groups in Egypt.

The seroprevalence of anti-HEV antibody in pregnant women is high in Egypt especially in rural areas. With chronic HBV coinfection, a marked increase in anti-HEV antibody was seen. The need for HEV vaccination for those at risk, especially pregnant ladies, should be considered.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The author acknowledges Microbiologists Ahmed Ibrahim and Ahmed Khalifa, and the Ministry of Health, Luxor and Assiut Governorate for their assistance during sample collection.

ABBREVIATIONS

ELISA, Enzyme-linked immunosorbent assay; **HBV,**

Hepatitis B virus; **HCV,** Hepatitis C virus; **HEV,** Hepatitis E virus; **HBsAg,** Hepatitis B surface antigen; **IgG,** Immunoglobulin G; **ORFs,** open reading frames; **RT-PCR,** Reverse transcriptase polymerase chain reaction.

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Vol. 13(28), pp. 562-567, October, 2019
DOI: 10.5897/AJMR2019.9160
Article Number: CB8CF7D62080
ISSN: 1996-0808

http://www.academicjournals.org/AJMR

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African Journal of Microbiology Research

Full Length Research Paper

Diketopiperazine alkaloids produced by the endophytic fungus *Penicillium citrinum* and evaluation of their antileishmanial activity

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Received 26 June, 2019; Accepted 26 August, 2019

Chromatographic fractionation of the antileishmanial extract obtained from fermentation of the endophytic fungus *Penicillium citrinum*, isolated from leaves of *Ageratum myriadenia*, yielded three diketopiperazine alkaloids; *cyclo*(L-Pro-L-Leu) (1), *cyclo*-(L-Pro-L-Phe) (2) and tryprostatin B (3). The structures of these compounds were established on the basis of spectroscopic methods and comparison with the literature. Compounds 1 and 2 were active against both amastigote-*like* forms of *Leishmania* (*Leishmania*) *amazonensis* and intracelular amastigotes of *L.* (*Leishmania*) *infantum* with approximately 50% of parasite growth inhibition at 100 µM. None of the compounds were considered toxic against human leukemia monocyte cell line (THP-1) at 100 µM. It is the first report about isolation of these diketopiperazines from *P. citrinum* and their antileishmanial potential against *L.* (*L.*) *infantum*.

Key words: Microfungi, natural products, *in vitro*, *Leishmania*, amphotericin B, cytotoxic activity.

INTRODUCTION

Penicillium is cosmopolitan genera and comprises pathogens, saprobes, opportunists or endophytes taxa, which are able to produce a large number of secondary metabolites being reported 1338 exometabolites (Frisvad, 2015). *Penicillium citrinum* is assigned to section Citrina with a worldwide distribution, occurring commonly in soils; they are known as producers of the mycotoxins citrinin and citreoviridin (Houbraken et al., 2011).

Penicitrinine A has been claimed as antitumor agents in patents (Zheng et al., 2015; Ying et al., 2015). Other secondary metabolites isolated from these species such as penicillanthranin A and chrysophanol, which are anthraquinone-citrinin derivatives, have exhibited moderate antibacterial activity against *Staphylococcus*

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aureus, both with MIC values of 16 μg/ml (Khamthong et al., 2012).

In previous study, it was shown that the crude extract from the fungus *Penicillium* citrinum UFMGCB 579, isolated from leaves of bioactive plant species *Ageratum myriadenia*, had antileishmanial activity (Rosa et al., 2010) in the assays using amastigotes-*like* forms of *Leishmania* (*Leishmania*) *amazonensis*. Chemical investigation of the ethyl acetate extracts from the broth and mycelia of *P. citrinum* UFMGCB 579 led to the isolation of three metabolites. In this study, the occurrence of three diketopiperazine alkaloids in addition to the evaluation of their antileishmanial activity is reported.

MATERIALS AND METHODS

General experimental procedures

Thin-layer chromatography (TLC) analyses were conducted on silica gel G-60/F_{254} (0.25 mm, Merck) and the spots were visualized under visible light after heating the plate sprayed with a mixture (1:1) of ethanolic solutions of vanillin (1% w/v) and sulfuric acid (10% v/v). Gel Permeation Chromatography (GPC) was carried out by using two coupled glass columns (Büchi column n° 17980) filled with Sephadex LH-20 TM (GE Healthcare, U.S.A.) gel. Semi-preparative HPLC purifications were carried out by a Shimadzu chromatographic system (Shimadzu, Kyoto, Japan), equipped with a LC6AD pump and a dual wavelength detector (SPD10A). The mass spectra were acquired in a maXis ETD high-resolution ESI-QTOF mass spectrometer (Bruker Daltonics, Bremen, Germany). The optical rotations were determined on a Modular Circular Polarimeter MCP 300 (Anton Paar, Ashland, Virginia, USA). 1D and 2D nuclear magnetic resonance (NMR) experiments were run on a Bruker Avance 400 MHz spectrometer (Bruker Biospin, Rheinstetten, Germany).

Fungal isolation

The endophytic fungus *P. citrinum* was isolated from the plant *Ageratum myriadenia* (DC) R.M. King & H. Rob (Asteraceae) that was deposited in the herbarium of the "Departamento de Botânica da Universidade Federal de Minas Gerais" under the code BHCB 5816. A fungal sample was deposited at "Coleção de Microrganismos e Células da Universidade Federal de Minas Gerais" under the code UFMGCB 579 and at GenBank by accession number FJ466725 (Rosa et al., 2010).

Fermentation and extraction

The fungus was cultured in a bioreactor containing 10 L of MEC medium (2% malt extract, 0.1% peptone, 1.5% glucose) for fourteen days at 28°C and 150 rpm. Both filtrate and biomass were extracted with ethyl acetate (EtOAc). The organic solvent was removed using a rotary evaporator to afforded 3.3 g of EtOAc extract.

Isolation of secondary metabolites from the endophytic fungi

All extract was subjected to GPC using EtOH as mobile phase at flow rate of 120 ml/h to produce 16 fractions after TLC analyses. Fraction 6 (210 mg) was purified on semi-preparative HPLC, using a Shim-pack® C18 column (5 μm, 250 x 20 mm i.d.), detection at λ 220 and 254 nm, flow rate of 7 ml/min and eluted with mixture of MeOH:H_2O (MeOH, 10–100% in 60 min and 100% for 10 min) to obtain **1** (12 mg). Fraction 8 (220 mg) was also purified as same conditions and yielded compounds **2** (5 mg) and **3** (2.8 mg), respectively.

Cyclo(L-Pro-L-Leu) ((3S,8aS)-3-(2-methylpropyl)-2,3,6,7,8,8a-hexahydropyrrolo[1,2-a]pyrazine-1,4-dione)(**1**) is a colorless amorphous solid (8.0 mg); $[\alpha]_D^{25}$ –117.0 (*c* 0.40, MeOH). The molecular formula is $C_{11}H_{18}N_2O_2$, as determined by HR-ESI-MS (*m/z* 211.1419, $[M+H]^+$; calc. for $C_{11}H_{19}N_2O_2$: 211.1441). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 0.96 (d, *J*= 6.58 Hz, H-12b), 1.0 (d, *J*= 6.58 Hz, H-12a), 1.76 (m, H-11), 2.02 and 1.90 (m, H-4b and a), 2.35 and 2.13 (m, H-5b and a), 2.07 and 1.53 (m, H-10b and a), 3.57 (m, H-3), 4.02 (dd, *J*=9.38, 3.47 Hz, H-9), 4.12 (t, *J*=8.14 Hz, H-6), 6.07 (br s, NH).^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 21.2 (CH_3, C-12b), 22.8 (CH_2, C-4), 23.3 (CH_3, C-12a), 24.7 (CH, C-11), 28.1 (CH_2, C-5), 38.7 (CH_2, C-10), 45.5 (CH_2, C-3), 53.4 (CH, C-9), 59.0 (CH, C-6), 166.2 (C=O, C-1), 170.3 (C=O, C-7).

Cyclo-(L-Pro-L-Phe) ((3S,8aS)-3-benzyl-2,3,6,7,8,8a-hexahydropyrrolo[1,2-a]pyrazine-1,4-dione) (**2**) is a colorless amorphous solid (5.0 mg); $[\alpha]_D^{25}$ –71.5 (*c* 0.25, MeOH). The molecular formula is $C_{14}H_{16}N_2O_2$, as determined by HR-ESI-MS (*m/z* 245.1255, $[M+H]^+$; calc. for $C_{14}H_{17}N_2O_2$: 245.1285).^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 1.80 (m, H-4), 2.09 and 1.24 (m, H-5), 3.16 (d, *J*=5.01 Hz, H-10), 3.36 and 3.54 (m, H-3), 4.06 (ddd, *J*= 10.75, 6.44, 1.67 Hz, H-6), 4.43 (tt, *J*= 5.01, 5.01, 0.99 Hz, H-9), 7.23 (br m., H-2'/6' and H-4'), 7.27 (br m, H-3'/5').^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 21.4 (CH_2, C-4), 28.0 (CH_2, C-5), 36.8 (CH_2, C-10), 44.6 (CH_2, C-3), 56.3 (CH, C-9), 58.7 (CH, C-6), 126.7 (CH, C-4'), 129.6 (CH, C-2'/-6'), 128.1 (CH, C-3'/-5'), 136.0 (C, C-1'), 165.5 (C=O, C-1), 169.5 (C=O, C-7).

Tryprostatin B ((3S,8aS)-3-[[2-(3-methylbut-2-enyl)-1H-indol-3-yl]methyl]-2,3,6,7,8,8a-hexahydropyrrolo[1,2-a]pyrazine-1,4-dione) (**3**) is a pale yellow amorphous solid (2.8 mg); $[\alpha]_D^{25}$ –29.5 (*c* 0.14, MeOH). The molecular formula was $C_{21}H_{25}N_3O_2$, as determined by HR-ESI-MS (*m/z* 352.2019, $[M+H]^+$; calc. for $C_{21}H_{26}N_3O_2$: 352.2020). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 1.65 and 1.38 (m, H-14), 1.75 (br s, H-21), 1.76 (br s, H-22), 1.95 and 0.93 (m, H-13), 3.20 (dd, J= 14.80, 6.90 Hz H-8b), 3.24 (dd, *J*= 11.3, 3.9 Hz, H-15b), 3.34 (d *J*= 4.60 Hz, overlapped, H-8a), 3.49 (m, H-15a) 3.50 (m, H-18), 3.97 (ddd, *J*=10.87, 6.51, 1.44 Hz, H-12), 4.37 (ddd, *J*=6.80, 4.6, 1.8 Hz, H-9), 5.35 (tt, *J*= 7.21, 7.21, 1.34 Hz, H-19), 6.96 (td, *J*= 7.52, 7.52, 1.15 Hz, H-5), 7.02 (td, *J*= 7.52, 7.52, 1.15 Hz, H-6), 7.27 (d, *J*= 7.94 Hz, H-7), 7.46 (d, *J*= 7.81 Hz, H-4), 8.52 (s, NH-1). ^{13}C NMR (101 MHz, CD_3OD-d_4) δ ppm 18.0 (CH_3, C-22), 22.5 (CH_2, C-14), 25.9 (CH_3, C-21), 26.2 (CH_2, C-18), 28.6 (CH_2, C-8), 29.0 (CH_2, C-13), 46.0 (CH_2, C-15), 57.2 (CH, C-9), 60.1 (CH, C-12), 104.5 (C, C-3), 111.7 (CH, C-7), 119.1 (CH, C-4), 119.8 (CH, C-5), 121.9 (CH, C-6), 122.1 (CH, C-19), 129.5 (C, C-3a and C-2), 134.7 (C, C-20), 137.3 (C, C-7a), 167.5 (C, C-17), 170.4 (C, C-11).

Assays of anti-leishmanial activity

Stationary phase promastigotes of *L.* (*Leishmania*) *amazonensis* (strain IFLA/BR/67/PH-8) were stimulated to differentiate into amastigote-*like* forms by rising the incubation temperature to 32°C and lowering the pH of the medium to 6.0. The parasites were seeded in 96-well plates using 90 μl of parasite suspension at 16 × 10^8 parasites per milliliter, followed by 10 μl of the sample-tests to give a final concentration of 100 μM. Amphotericin B at 0.5 μM was used as positive control. The plates were incubated at 32°C for 72 h and the number of parasites was estimated using the methyl thiazolyl tetrazolium (MTT). The results were calculated from the absorbance measurements using the percentage of parasite death

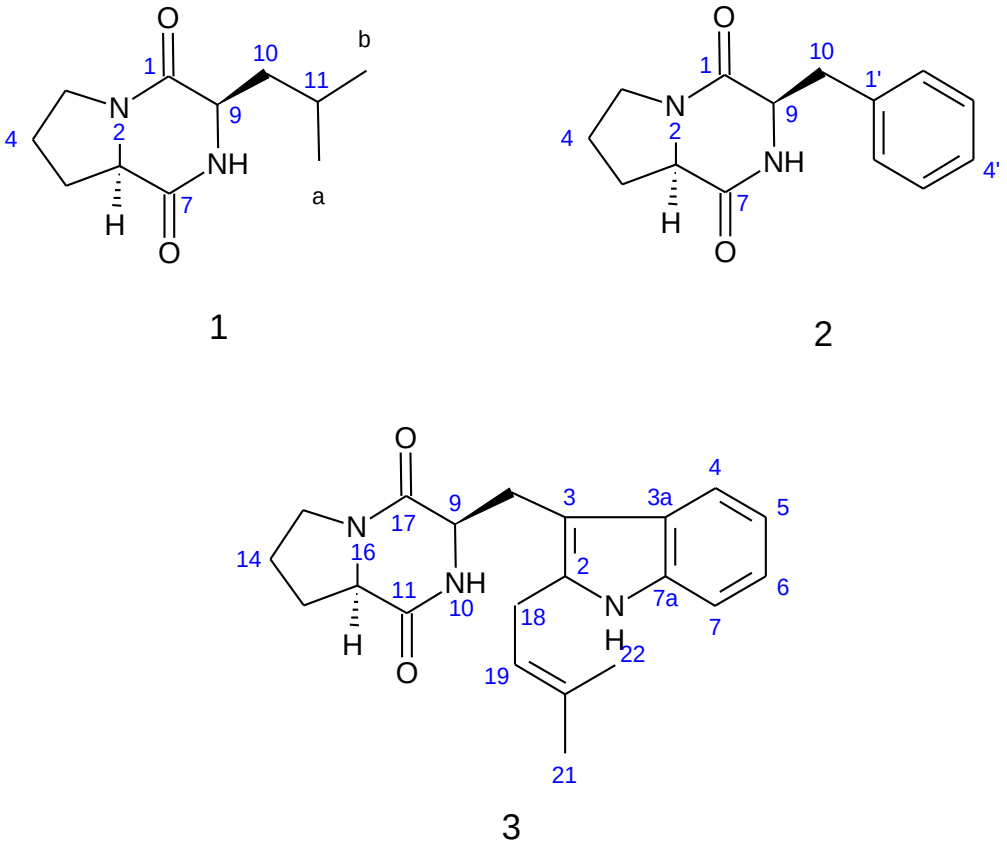

Figure 1. Structure of *cyclo*-(L-Pro-L-Leu) (**1**), *cyclo*-(L-Pro-L-Phe) (**2**) and tryprostatin B (**3**) isolated from *Penicillium citrinum.*

in relation to the controls without drug (Teixeira et al., 2002).

THP-1, maintained in RPMI 1640 medium supplemented with 10 % FBS cells and differentiated in the presence of 20 ng/ml phorbol myristate acetate (PMA) for 72 h at 37°C, were infected at a parasite/macrophage ratio of 10:1 for 3 h with *L.* (*Leishmania*) *infantum* (strain MHOM/MA/67/ITMAP-263) promastigotes expressing the firefly luciferase as reporter gene. Non-internalized parasites were removed by five washes with HEPES/NaCl buffer (20 mM HEPES, 0.15 M NaCl, 10 mM glucose, pH 7.2). The infected cells were then treated with 100 µM of the sample-tests and of amphotericin B at 0.5 µM. After 72 h, RPMI was aspirated and the luciferase activity was assessed by adding 20 µl of reconstituted One-Glo™ Luciferase Assay System solution as enzyme substrate (Promega, Madison, WI, USA). Luciferase activity was measured in a luminometer SpectraMax M5 (Molecular Devices, Sunnyvale, CA, USA) using 1 s integration/well (Roy et al., 2000).

Cell viability assays

Non-infected THP-1 macrophages were used as signal background while non-treated infected THP-1 cells were used as control for growth comparison (Garcia et al., 2013). THP-1 macrophages were seeded in 96-well plates at a density of 2×10^5 cells per well. After 72 h treatment with compounds at a final concentration of 100 µM, the cell death was estimated using the MTT. The results were calculated from the absorbance measurements using the percentage of cell death in relation to the controls of untreated cells.

Statistical analysis

Statistical analyses were performed using Graph Pad Prism software 5.03 (GraphPad Software, Inc., San Diego, CA, USA). Differences were assessed by analysis of variance (ANOVA). Differences were considered statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

In an ongoing survey of the bioactive potential of microorganisms present in the Brazilian ecosystems, the EtOAc extract of an endophytic fungus *P. citrinum* UFMGCB 579 was able to inhibit the growth of amastigotes-*like* forms of *L. (L.) amazonensis* by 86% at 20 µg/ml and showed an IC_{50} value of 4.6 µg/ml (Rosa et al., 2010). The crude EtOAc extract was subjected to preparative GPC by medium pressure liquid chromatography to afford a series of fractions. HPLC purification yielded the known compounds *cyclo*-(L-Pro-D-Leu) (**1**), *cyclo*-(L-Pro-L-Phe) (Campbell et al., 2009) (**2**) and tryprostatin B (**3**) (Cui et al., 1996) (Figure 1).

These compounds were elucidated by comparing their spectral data (MS, and 1D and 2D NMR data) and specific rotation values with those in the literature values.

The *cyclo*-(L-Pro-Leu) (**1**) was reported to be isolated

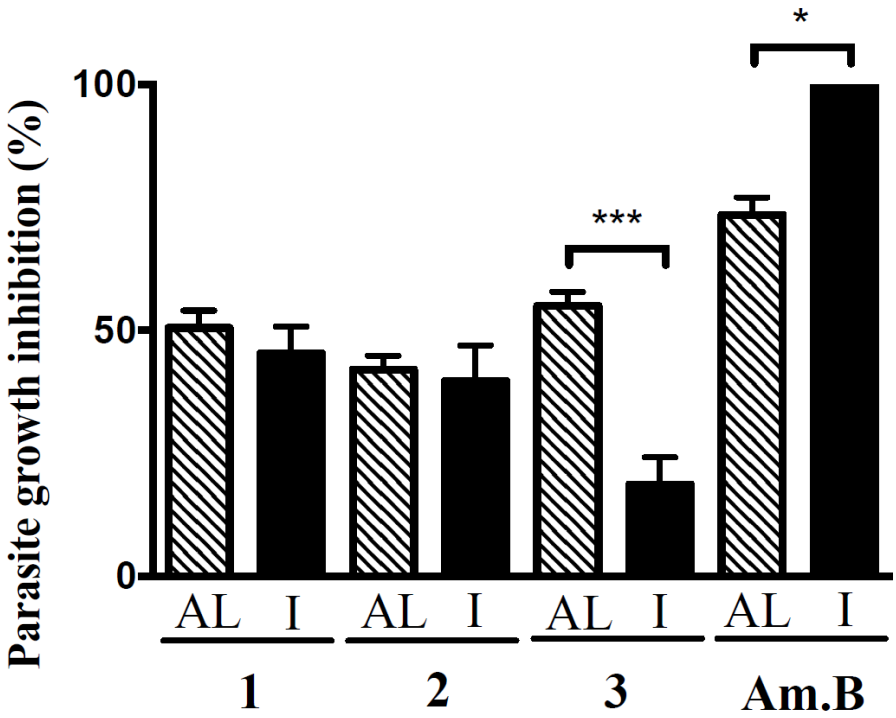

Figure 2. Antileishmanial effects of compounds **1**, **2** and **3** at 100 µM and of amphotericin B (Am.B) at 0.5 µM. Compounds were assayed against intracelular amastigotes (I) of *Leishmania infantum* and amastigote *like*-forms (AL) of *Leishmania amazonensis*. The results showed here are representative of at least 3 experiments in triplicates. Compounds **1** and **2** showed similar activities against intracelular amastigotes and amastigote-*like* forms of the parasite. Compound **3** was more active against amastigote-*like* forms. Statistical differences were calculated using One- way ANOVA with multiple comparisons: * $p < 0.05$; *** $p < 0.0001$.

from the EtOAc extract of the culture broth of the marine fungus *Penicillium chrysogenum*, which was obtained from the North China Sea (Wang et al., 2014) and it was obtained together with *cyclo*-(L-Pro-L-Phe) (**2**) from a neomycin resistant mutant of the marine-derived fungus *Penicillium purpurogenum* G59 (Wang et al., 2016).

Compounds **1** and **2** have antibacterial activity against gram positive and gram negative bacteria (Kumar et al., 2012; Mangamuri et al., 2016) and tryprostatin B (**3**) is a microtubule inhibitor on the cell cycle progression in the M phase of mouse tsFT210 cells (Cui et al., 1996). Compound **3** has also immunosuppressive activity against mouse splenic lymphocytes stimulated with lipopolysaccharide (IC_{50} 3 µg/ml) (Fujimoto et al., 2000) and cytotoxic activity against human leukemia cancer cell line K562 (IC_{50} 21.1 µM) (Wollinsky et al., 2012).

Compounds **1** and **2** were active against both intracellular amastigotes and amastigote-like forms of *Leishmania* parasites, showing approximately 50% of parasite growth inhibition at 100 µM (Figure 2). Compound **3** was more active in the amastigote-*like* model than in the intracellular amastigote form of the parasite ($p < 0.001$), showing a reduction of parasite growth of 55 and 19%, respectively. In addition, treatment with amphotericin B reduced more intracellular amastigote form of the parasite than amastigote-*like* form ($p < 0.05$). This could be explained by the different susceptibility of different species of *Leishmania* or by an activation of the compound by the macrophage. The variation of susceptibility to drugs in different species and strains of *Leishmania* is described in clinical isolates and reference strains, reinforcing the difficulty to find a single drug that can treat all forms of leishmaniasis (Fernández et al., 2012). The intracellular amastigote model is the best to represent human infection and it is important in the drug discovery process because it is the only *in vitro* model that allows the discovery of pro-drugs that need to be metabolized by macrophages in order to become active, such as antimony, and of drugs that activates the immune response of the macrophages in order to eliminate the parasites (Siqueira-Neto et al., 2010).

None of the compounds (**1-3**) were toxic for THP-1 cells when they were tested up to 100 µM ($p < 0.0001$, Figure 3). They reduced the viability of cells less than 10% at 100 µM (**1** = 1%; **2** = 0%; **3** = 8%). Amphotericin B, although more active than the compounds against the parasites, is also more toxic, showing 95% of cell death at the same concentration.

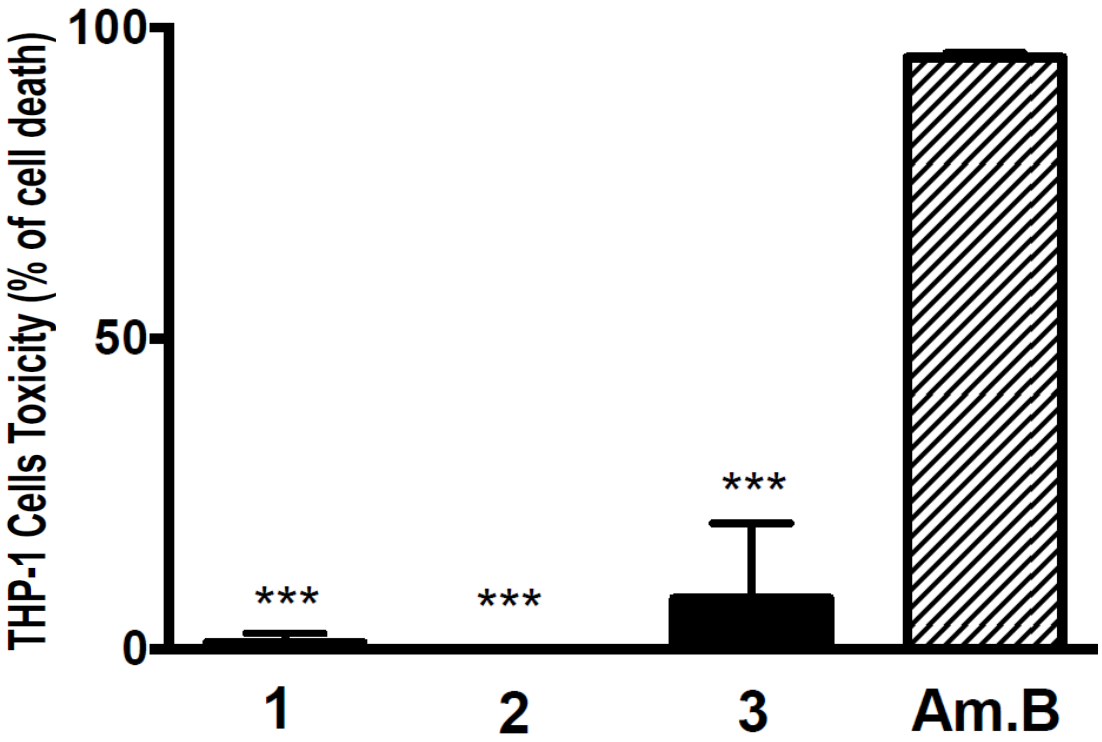

Figure 3. Cytotoxic effects of compounds (**1**-**3**) and, amphotericin B at 100 µM against human monocyte-derived THP-1 macrophages. The results showed here are representative of at least 3 experiments in triplicates. None of the compounds (**1**-**3**) were considered toxic. Statistical differences were calculated using One- way ANOVA with multiple comparisons: *** $p < 0.0001$.

Conclusion

This study demonstrated that the endophytic fungus *P. citrinum* is a source of the diketopiperazine alkaloids. To the researchers' knowledge, this is the first report on the occurrence of tryprostatin B in *Penicillium* species and of the diketopiperazines (**1-2**) in the *P. citrinum.* The biological evaluation of these compounds showed that they are not toxic for THP-1 although they have shown weak antiparasitic potential against two *Leishmania* species in comparison with amphotericin B.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors are grateful to the FAPEMIG (APQ-00193-08), CAPES and to the Program of Technological Development in Tools for Health (PDTIS-FIOCRUZ), RPT13A platform for the use of its facilities. They are especially grateful to Rubens Lima do Monte Neto for helping with bioassays and Silvia Reni Bortolin Uliana for providing the *Leishmania* strain.

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Vol. 13(28), pp. 568-579, October, 2019
DOI: 10.5897/AJMR2019.9165
Article Number: 2BFBD0762128
ISSN: 1996-0808

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Impact of vessels on sediment transport and diversity in Lake Taihu, China

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Received 5 July, 2019; Accepted 7 October, 2019.

In shallow lakes, waves from different sources play a major role in sediment transport and the release of associated nutrients. This study was conducted to analyze the impacts of waves generated by wind or vessels, on nutrient concentrations and microbial diversity in China's third largest lake, Taihu. The study area is fixed at 100 m length of sailing vessel and 10 m width from where water samples were collected, near the Taihu Laboratory for Lake Ecosystem Research (TLLER) Station, at Meiliang Bay. The average flow velocity changed during passages of the vessel from -0.1 to + 0.1 m/s with high-speed vessel compering by the time before and after passages for one hour. The significant wave height ranged from 0.002 to 0.698 m. Turbidity ranged from 29.5 to 65.5 NTU. The concentration of Chl-a (μgL^{-1}) and nutrients total nitrogen (TN), total phosphorus (TP), ammonium (NH_4^+) and phosphate (PO_4) were resuspended in the three depths of the water column, as a response to the turbulence caused by waves-waves collision and rivers waves. Microbial amount concentration in the two layers changed over time. It increased in the surface from 5.03 to 8.3 ng μl^{-1} and decreased in the bottom from 5.03 μl^{-1} to 2.93 ng μl^{-1} over a 45 min period. This study points to the importance of sediment resuspension as a factor influencing nutrient availability and microbial community structure, and as such it plays an important role in the eutrophication dynamics of Lake Taihu.

Key words: Destructive interference, wave, vessels, hydrodynamics, nutrients resuspension, microbial diversity.

INTRODUCTION

Water quality is affected by natural and anthropogenic factors. Natural factors include drainage density, lake interchange coefficient, temperature and precipitation within the watershed, lake morphology, water depth, wind and waves. The anthropogenic factors include industrial effluent, agricultural runoff, and effluent from wastewater treatment facilities (Epele et al., 2018; Wang et al., 2009; Xian et al., 2007; Zhou et al., 1996). Algal blooms are indicative of accelerating eutrophication and resultant of poor water quality (Li et al., 2017; Qin et al., 2010; Stumpf et al., 2012). In Lake Taihu, China, poor water quality has become a serious threat to lake, reservoir and other freshwater ecological and economic status and sustainability (Li et al., 2017; Qin et al., 2010). Sediments

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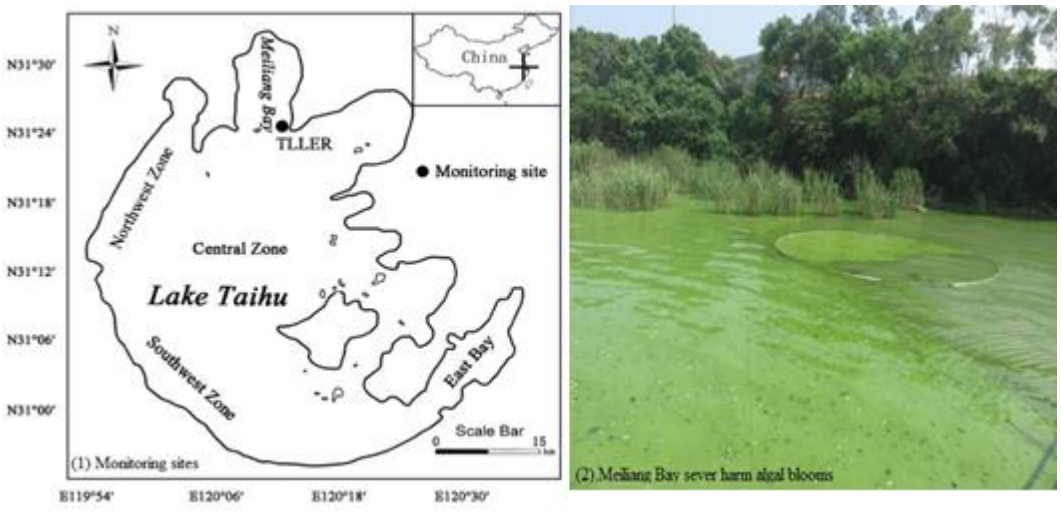

Figure 1. Map shows the sites of field of experiments.

are a major internal source of nutrients in shallow lakes like Taihu, where sediment resuspension resulting from currents, waves, dredging, and ship-induced waves can be a source of nutrient enrichment (Gabel et al., 2012; Garrad and Hey, 1987; Maynord, 2005; Qin et al., 2007; Schneider et al., 2007). Microorganisms play a major role in the degradation of organic matter, and the cycling of nutrient elements in freshwater lake sediments (Junier et al., 2007). The lake microbial community is influenced by fluctuating environmental factors, including conductivity, nutrients and organic matter. An increase of organic matter content in the sediment promotes bacterial activity and can influence diversity of prokaryotic populations (Edlund et al., 2006; Fry et al., 2006; Muylaert et al., 2002; Nelson et al., 2007). Excessive nutrient inputs can increase the growth rate of some microorganisms while also affecting microbial habitats and diversity (Weisse, 1991). Studies have shown that the vertical variation of general microbial groups is related to sediment dynamics (Koizumi et al., 2004; Wilms et al., 2006). It is important to know how the partial and temporal distribution of microorganisms responds to environmental drivers accordingly therefore this study: 1) examined impacts of waves generated by wind and vessel on nutrient loads in each layer of water column, 2) measured microbial abundance in water layers, and 3) examined the relationships between microbial abundance and sediment resuspension.

MATERIALS AND METHODS

Study area

Lake Taihu is a large freshwater lake located in the lower Yangtze River Delta between 30° 56′– 31° 33′ N and 119° 53′–120° 36′ E. It has a surface area of 2338 km^2 and a mean depth of 1.9 m. Meiliang Bay is a semi-enclosed bay with a surface area of 129.3 km^2, 1.9 m average depth and it is located in the northern part of Lake Taihu (Gao et al., 2017; Li et al., 2017; Liu et al., 2014). Meiliang Bay is extremely eutrophic, as indicated by cyanobacterial blooms during summer leading to severe water quality problem (Gao et al., 2017; Li et al., 2017). Meiliang Bay has average annual wind speed that varies from 3.5 to 5.0 m/s, with dominant summer winds from the southeast and dominant winter winds from the northwest (Gao et al., 2017; Li et al., 2017; Wu et al., 2013) (Figure 1).

Field observations and instrumentation

Field observations were made on 26 May 2016 in Meiliang Bay Taihu Laboratory for Lake Ecosystem Research, Chinese Academy of Sciences (TLLER). Wind speeds and wind direction, were monitored by a PH-Ⅱ handheld weather station and PHWD wind direction transducer; the frequency was set at 5 min. The wind monitoring instruments were positioned at 10 m above the water surface. The data collection interval was 5 min. The data were calibrated with the automatic wind speed and direction recorder (10 m above the water surface, 10 min monitoring interval) located at the TLLER. Wave parameters, including wave height, wave energy, and wave period were measured by Red Blue Reader (RBR) duo T.D wave situated at 50 cm below the water surface. The acoustic Doppler velocimeter (ADV) is the most important instrument to determine and obtain a good understanding for suspended sediment transport. In our field study, we used Acoustic Doppler Velocimeter (ADV) 100 Hz and optical backscatter sensor (OBS-A3) instruments, which have capability to measure the field velocities and suspended sediment concentration (SSC) in the bed layer of Lake Taihu. During the field observation, the vessel moved from one point to another and returned to the beginning several times over a time frame of 60 min. The goal was to understand the precision of effect wave induced by wind (vessel). Water samples were collected during the experiment. The sampling site was located in Meiliang Bay (31°25′7.41″N, 120°12′46.90″E (Figure 1).

Sample collection techniques

Water samples were collected near the TLLER (311240N, 1201130E) in Meiliang Bay on 26 of May 2016. Samples were

collected at different depths; 5 cm above the bottom layer, which is considered descriptive of the water column because of the shallow, middle layer (1 m above the bottom) and near surface 1.8 m above the bottom. One type of sterile bottles (20 samples) was dispensed for DNA extraction and the other 40 samples were collected for water quality from the same site--Meiliang bay and in-lake monitoring stations. Water quality samples were transported to the laboratory for filtration to obtain suspended particulate matter (SPM) content by filtering only 250 mL of water through cellulose acetate membranes (0.45 µm), followed by analyses directly including total phosphorus (TP); total nitrogen (TN); sediments solid; chlorophyll-a (Chl-a) phosphate (PO_4) and ammonium (NH_4^+). These nutrient measurements were according to the Chinese standard methodology for Lake Eutrophication Surveys (Jin and Tu, 1990). This technique is similar to the American standard methods (APHA, 1998), for those parameters (James et al., 2009) while the DNA samples were filtered by using 0.2 µm polycarbonate membrane for DNA extraction in Meiliang Bay. Then the total DNA was removed using the procedure illustrated by Tang et al. (2009) and Zhou et al. (1996). Finally, DNA was measured using 454 pyrosequencing of 16S rRNA genes by using polymerase chain reaction (PCR). The first time sample was collected at zero time, the second time samples was after passages of vessel after 10 min, the third time samples was 20 min, the fourth time samples was 45 min. The vessel moved for 1 h continuously from one point to another point, 100 m distances far away from site of collection the samples.

Data analysis

Acoustic method measures high-frequency suspended sediment concentration (SSC). To obtain high-frequency SSC, we combined Acoustic Doppler Velocimeter (ADV Ocean, SonTek Inc.) and Optical Backscatter Sensor (OBS) turbidity meter, and bottle samples. The first step was to use bottle samples to calibrate with OBS turbidity, and then SSCs was measured by OBS (one datum every 3 min) arithmetic mean value of 3-min echo intensity (EI) data measured by ADV (which frequency was 10 data per second) and obtained logarithmic relationship between EI and SSC. Finally, high-frequency simultaneous SSC and water vertical flow velocity were obtained by ADV according to Gao et al. (2017) and Voulgaris and Meyers (2004). Wind speed and direction were measured at the meteorological station every 5 min at 5 m above water surface. The wind speed was scaled using the logarithmic profile law Equation 1:

$$U_{10} = U_{measured} \frac{\ln(\frac{10}{z_0})}{\ln(\frac{z}{z_0})} \quad (1)$$

Where, z is the height of the instrument with respect to the water surface level, and z0 is a sea-surface roughness length set equal to 10.3 m (Donelan et al., 1993).

Various simplified wave methods were applied in shallow lake based on characteristic wave methodology. Wave (ζ) record was calculated by using RBR duo T.D simple method. The wavelength was calculated according to the observed wave parameters given in Equation 2:

$$L_s = (\frac{gT_s^2}{2\pi}) \tanh(\frac{2\pi h}{L_s}) \quad (2)$$

Where, Ls is the significant wavelength, Ts is the significant wave period, and h is the depth of observation points. Nutrient concentrations are shown as mean, minimum and maximum values at each layer.

RESULTS

Wave characteristics

On 26 May, 2016, the significant wave height ranged from minimum value of 0.002 to maximum value of 0.612 m; the wave energy increased after time experiment from 0.03 to 0.09 NdBar$^{\wedge 2}$ m$^{\wedge -3}$ and maximum wave height ranged from 0.005 to 0.023 m; the wind speed ranged from 0.5 to 2.5 (m/s) (Figures 2 and 3).

Frequency distribution of wind directions

Major wind directions can have impact on the upper and lower velocities of flow area study and inverse wave flow during strong wind speed. For this determination, wind directions were separated into 14 sides North; East South East; North North East; East; South South East; North East; South West; South; West South West; West North West; West; North West; East North East and North North West. The flow directions were also converted into the same 14 directions to compare them with wind directions (Figure 4).

Wind directions data are presented as dominate north direction with frequencies N (0.166); ESE (0.05); NNE (0.05) E (0.1); SSE (0.0166); NE (0.016); SW (0.066); S (0.016); WSW (0.016); WNW (0.1); W (0.016); NW (0.066); ENE (0.016); NNW (0.083) (Figure 4a). The blue color demonstrates the ranges of wind directions while the white represents the degree of the opposite flow directions which were considered before the experiments. It can be perceived from the frequency statistics that the quadrant directed to the north side creates the highest percent (16.66%) of total frequencies, followed by NNW (8.33%), WNW (10%) and East (10%) of total wind directions (Figure 4b). Thus, it is important to consider them as prevailing wind directions for impact assessments on the flow before vessel passage. Each flow direction was deliberated opposite if it was 90° more than the direction of that precise wind as shown in Figure 4.

Average flow velocities distributions

Table 1 shows that there was a significant strong correlation determination by positive value of P (value < 0.5). The average flow velocities changed during passages of vessel compared to the time before passages of vessel; during the time passages of vessel, the curve shows that the flow average velocities had value that ranged from -0.1 to + 0.1 m/s with high-speed vessel as sweep and ejection events. The varying curve of the flow velocities looks similar and has kelvin wave shape especially after 16 min averages seconds recording measured by Acoustic Doppler velocity (ADV) near distance fixed. The average flow velocities curve

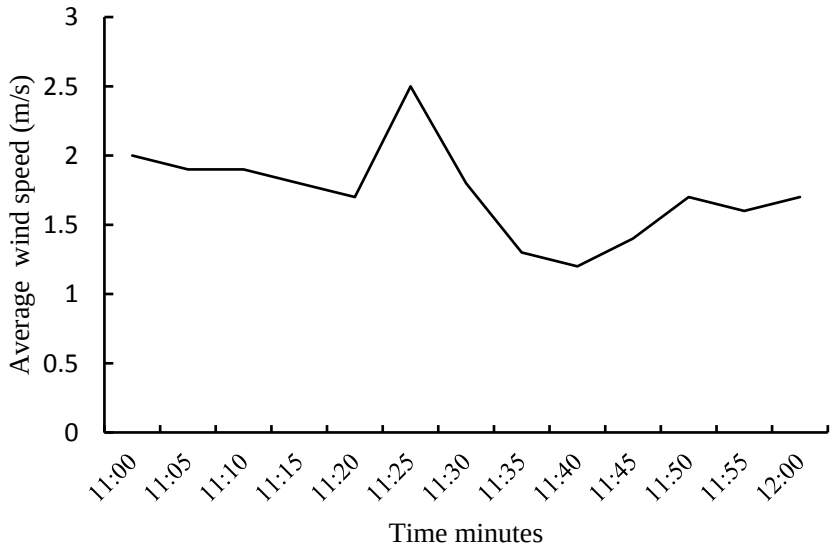

Figure 2. Average wind speed (m/s) during passages vessel.

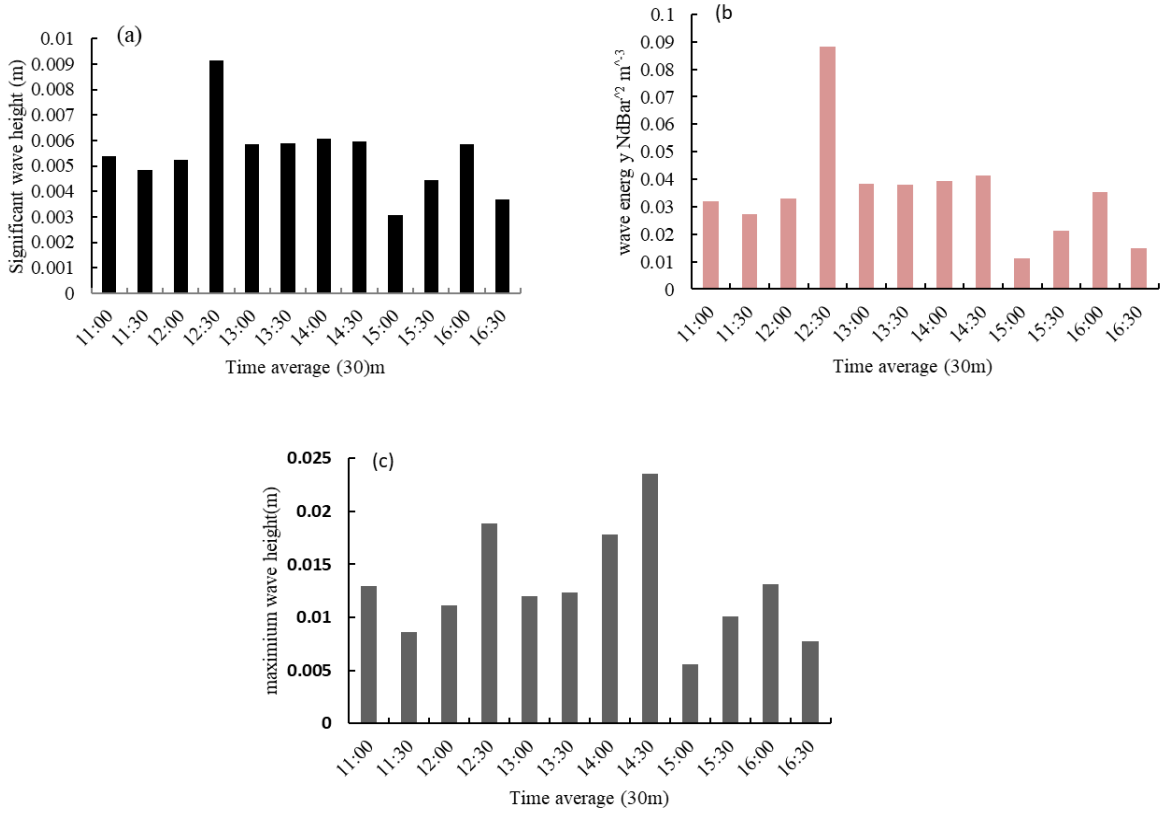

Figure 3. Wave features during vessel passages (a) significant wave height (m) (b) total wave energy NdBar^2 m^-3 (c) maximum wave height (m).

gives a clear event of ejection and sweep processes (Figure 5). While, the statistical data of the average velocities during passages and after 5 h passages vessel show that there is no significant correlation determination by P (value > 0.5) with values ranging from minimum value of -0.11 to maximum value of 0.06 (Figure 6; Table 2).

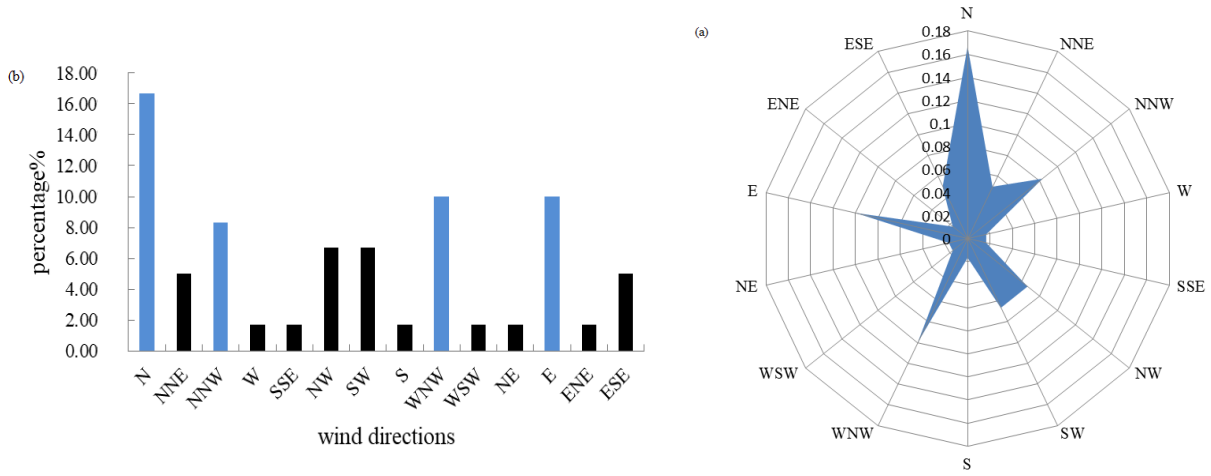

Figure 4. Chart diagrams showing (a) the method approved to estimate flow directions with dominant wind north directions (b) the percent of wind direction.

Table 1. Shows the statistical significance of the average flow velocity cm/s during moving vessel and before moving vessel.

ANOVA					
Source of Variation	**SS**	**Df**	**MS**	**P-value***	**F crit**
Between Groups	0.0147	1	0.015	0.0500	3.922
Within Groups	0.214	116	0.002		
Total	0.228	117			

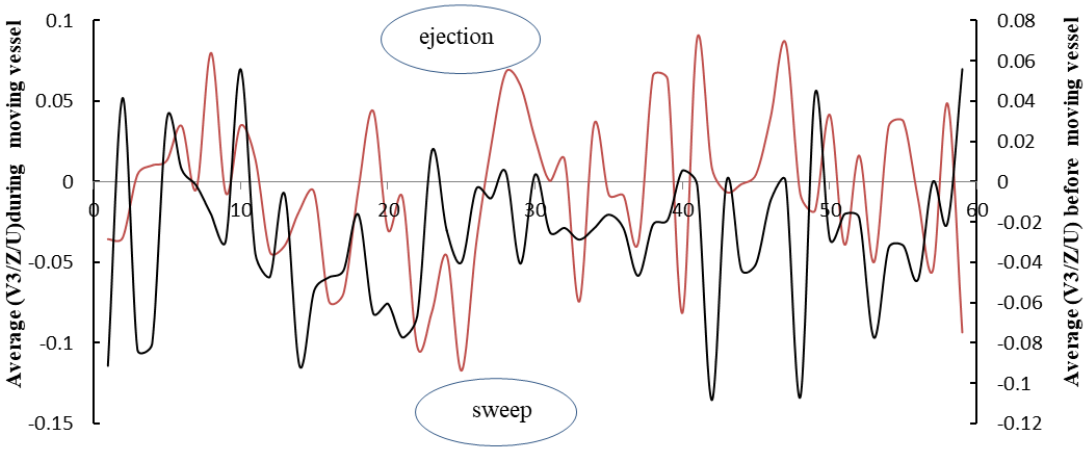

Figure 5. Average flow velocities (V3/Z/U) (cm/s) for 60 minutes before moving vessel and 60 minutes during moving vessel.

Turbidity and SSC

Nutrients are released from sediment resuspension processes, which encouraged algal growth in the open shallow lakes. The result shows positive relationship between SSC and turbidity (R^2=0.538; P<0.5) during passages of vessel (Figure 7).

Wind speed ranged from a minimum of 1.5 to maximum of 2.5 ms^{-1}. Water temperature and pH measurements averaged as 14.3°C and 9.17. Correspondingly, turbidity

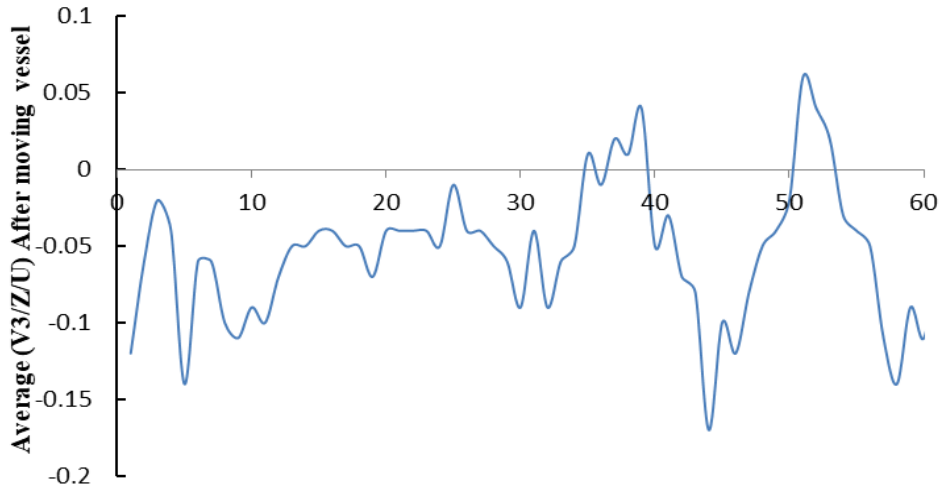

Figure 6. Average flow velocities (V3/Z/U) cm/s for 60 minutes after 5 h moving vessel.

Table 2. Shows the statistical significance of the average flow velocities cm/s during moving and after moving vessel.

Source of Variation	SS	df	MS	F	P-value*	F crit
Between Groups	0.071	1	0.071	32.478	9.300	3.922
Within Groups	0.251	116	0.002			
Total	0.322	117				

*There is no significant correlation P (value > 0.05).

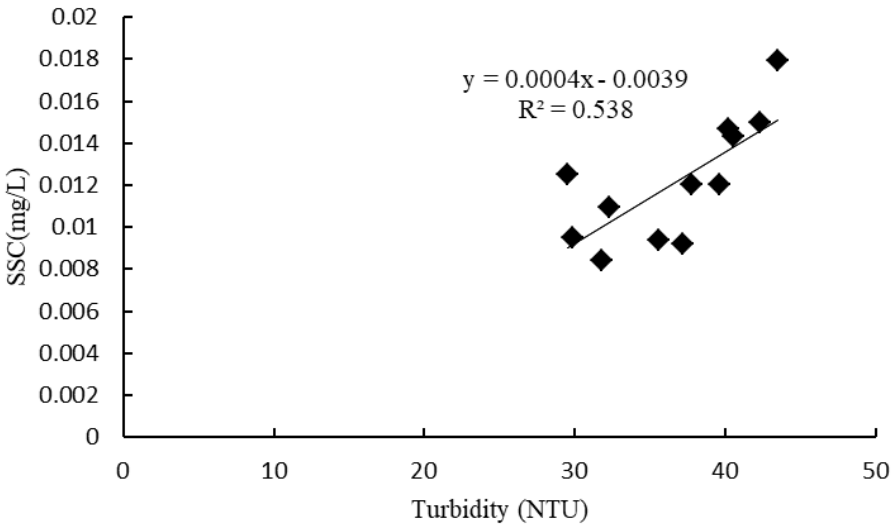

Figure 7. The relationship between SSC (mg/L) & Turbidity (NTU).

ranged from 29.5 NTU at 11:00 to a maximum of 50.00 NTU at 12:00 (Figure 8a). Concentrations of TP increased from a minimum of 0.5 mg^{-1} at 11: 00, to a maximum of 0.14 at 14:00. The data showed a positive correlation between wind speed and turbidity R^2= (0. 95, P<0.05) and Figure 8b shows correlation between wind speed and total phosphorus (TP) concentrations [R^2 = 0.7698, P (value < 0.5)].

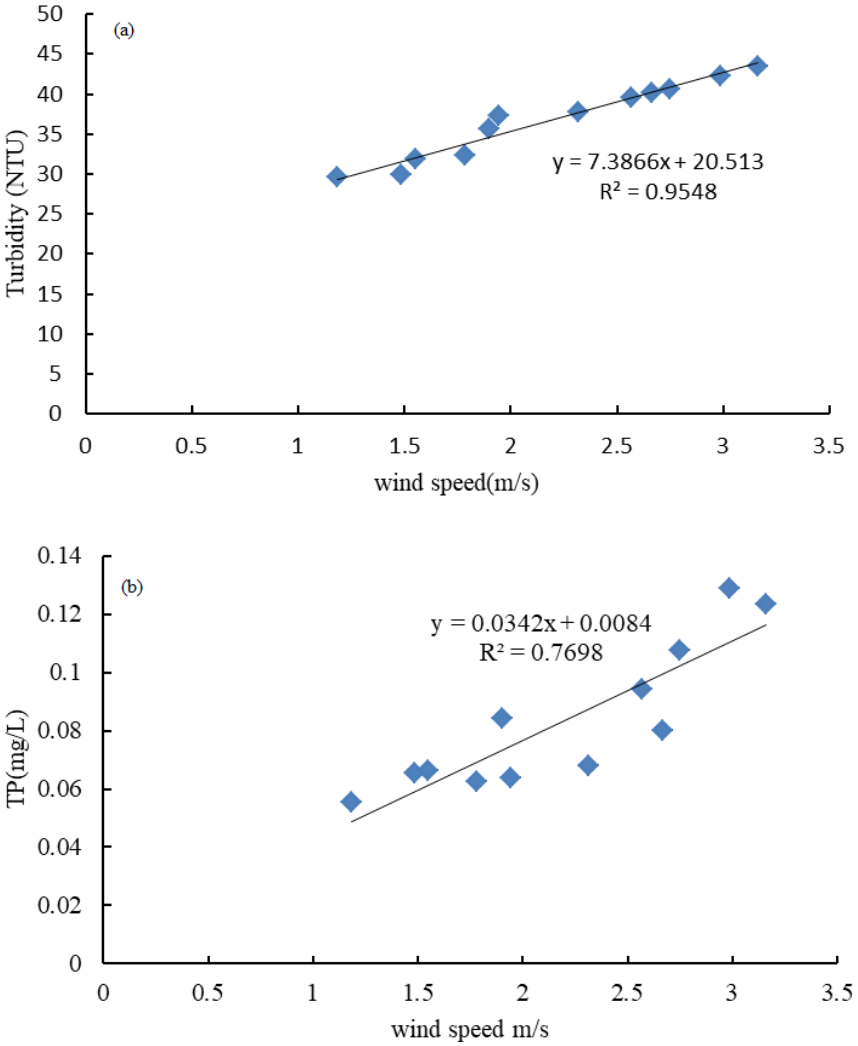

Figure 8. Shows relationship between (a) the turbidity (NTU) and wind speed (m/s) and (b) total phosphorus (TP) (mg/L) and wind speed (m/s).

Nutrients loads

Figure 9 shows the average of chlorophyll-a (μgL^{-1}) and nutrient concentrations during the experiment at three depths; bottom, middle and surface layers. The results demonstrated that the concentration of Chl-a (μgL^{-1}) decreased at bottom from 21.5 to 12.5 μgL^{-1}, middle layer (45.1-22.9 μgL^{-1}) and surface layer (50.2-34.4 μgL^{-1}). TN increased at each layer; bottom (1.8-2.7 mgL^{-1}), middle layer (1.7-2.5 mgL^{-1}) and surface layer (1.7 -2.1 mgL^{-1}), while TP increased in the middle layer (0.09-0.12 mgL^{-1}) and modestly decreased in the bottom (0.8-0.09 mgL^{-1}), and surface layer (0.09-0.11 mgL^{-1}). NH_4 increased in the bottom (0.14-0.19 mgL^{-1}), middle (0.13-0.18 mgL^{-1}) and surface (0.14-0.18 mgL^{-1}). PO_4 decreased in the bottom layer (0.02-0.03 mgL^{-1}), middle layer (0.01-0.02 mgL^{-1}) and surface layer (0.01- 0.06 mgL^{-1}). SS also increased in each layers (7.93-11.67, 7.8-9.07, 10.93-11.4 mgL^{-1}).

Wave-effect and diversity

Lake Taihu has suffered from severe eutrophication and harmful algal blooms for several decades (Abdul et al., 2017; Li et al., 2017; Paerl and Huisman, 2008). There are multiple sources of pollution, including urban runoff, industrial sewage, and agricultural runoff, and Meiliang Bay is the main drinking water source of Wuxi City (Epele et al., 2018; Li et al., 2017; Zheng et al., 2015). This Bay is the most pollution part and growing different microbial diversity and harmful algal blooms in the Lake. Table 3 shows changes in the distribution of microbial diversity in the two layers during the experiment, with the abundance concentration of total DNA in the bottom layer at 5.03 $ng/\mu l^{-1}$ and 8.3$ng/\mu l^{-1}$ in the surface layer. Ten minutes after start of the experiment the concentrations in surface and bottom layers decreased to 3.52 and 5.50 $ng/\mu l^{-1}$ respectively.

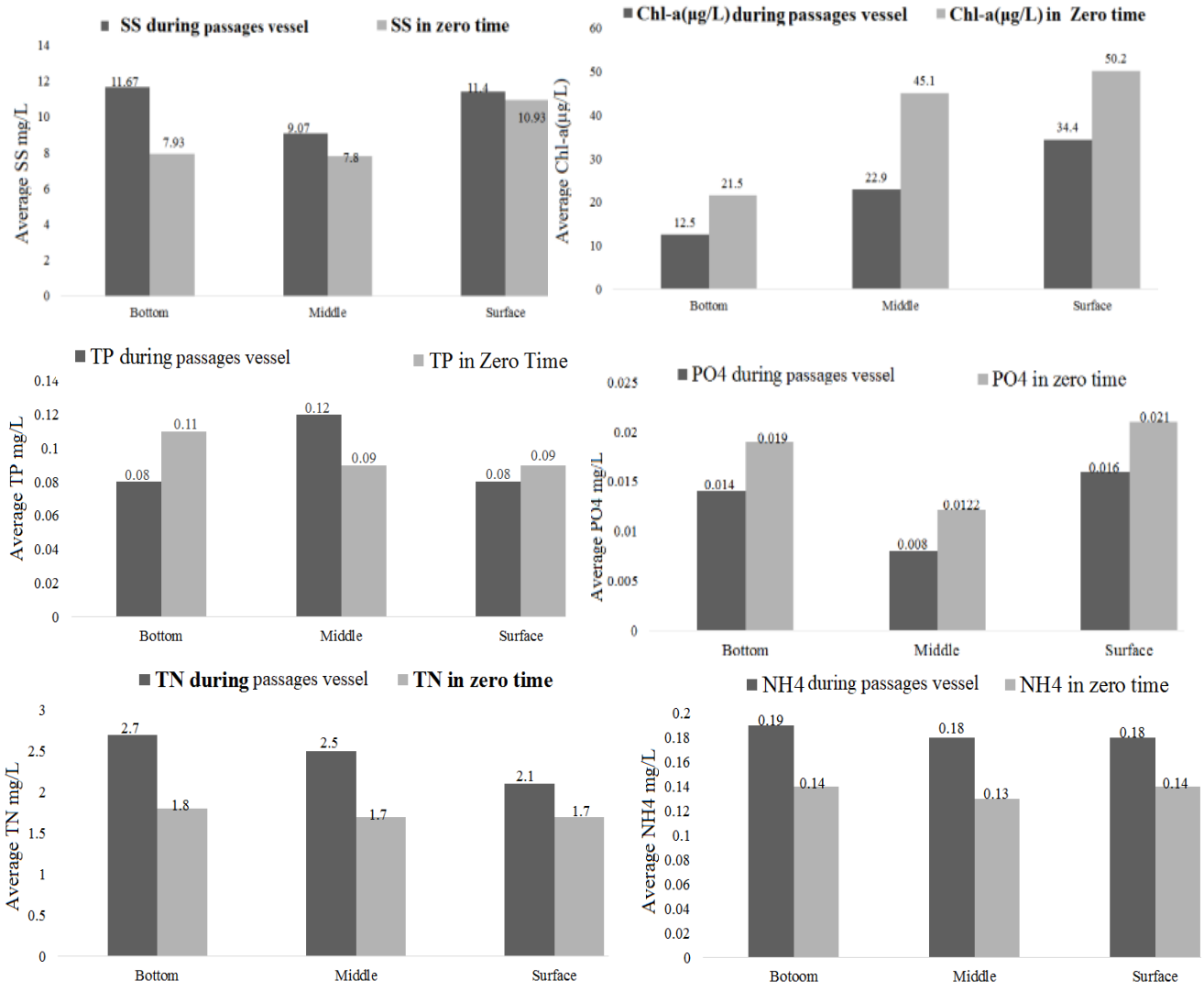

Figure 9. Sediment suspension during time experiments.

Table 3. Showed the concentration of microbial community in two layers.

Time during the experiment	Site	Con.(ng/µl)
Con. 1: in normal state	Bottom	5.03
	Surface	8.3
Con. 2: after 10 min	Bottom	3.52
	Surface	5.50
Con. 3: after 20 min	Bottom	3.30
	Surface	7.08
Con. 4: after 45 min	Bottom	2.93
	Surface	9.92
Con. 5: after experiment 2 h	Bottom	3.28
	Surface	15.5

After 20 min, the concentration increased slightly at the surface, to 7. 08 ng/µl^{-1}, and decreased at the bottom to 3.30 ng/µl^{-1}. The concentration at the bottom decreased to 2.93 and increased at the surface to 9.92 ng/µl^{-1} after 45 min passages vessel. Two hours after the experiment, the concentration increased at the bottom to 3.28 ng/µl^{-1} and increased to 15.5 ng/µl^{-1} at the surface.

DISCUSSION

Wave characteristics and hydrodynamic disturbances

The waves in Lake Taihu are produced by surface strong wind disturbance, and their characteristics essentially depend on wind speed, wind fetch, water depth and size of the vessel. In large shallow lakes, open water area waves are generated when the wind blows across the lake surface (Huang and Liu, 2009); wind energy was absorbed by the surface waves and turbulence, and then degenerated by bottom friction (Green and Coco, 2014; Li et al., 2017). The strong wind can cause algal blooms and other microbial communities to be completely mixed throughout the water column. In addition, the main factor inducing the distributions of wind speed and wave parameters is wind direction (You et al., 2007).

The direction of wind during experiment varied considerably, but dominated from the north. Other study analyzed the directions in Lake Taihu and found the predominant winds were southeast, during summer and the dominant wind speed range was 1.7-6.0 m/s (You et al., 2007). Li et al. (2017) confirmed that the wave heights created by southeast winds were considerably more than wave heights produced by northwest winds during the two periods (5/25/2014 and 5/27/2014), even with similar wind speeds. This is mostly due to varying wind fetch lengths and wind energies. The association between wind speed and wave height at the site showed that the wind speed and wave height were closely related. The waves generated by vessels have raised concerns about their effects on the water body, such as bank erosion, and damage to aquatic plants (Erfurt-Cooper 2009; Gabel et al., 2017). Also, Gabel et al. (2012) demonstrated that ship-induced waves can affect the physical characteristics of lake and river shorelines. Pettibone et al. (1996) found that the waves from a ship cause resuspension of bacteria-colonized bed sediments. The resuspension events occur frequently, internal nutrient loading and high concentrations of suspended particulate matter which can negatively impact in the water quality (Hartmann and Ronneberger, 1996; Li et al., 2017).

In the present study, when the vessel moved forward in the length area of 100 m and 10 m width from site collected the data, and returned to the starting point, it generated progressive waves. The progressive waves were created by the forward movement and the waves created by dominant north wind from the opposite direction collided. The analyses of wind and wave characteristics at steady time and during the experiment showed little to no significant change. This is possibly because both waves have the same amplitude and energy and caused phenomena called destructive interference. The significant wave height ranged from minimum value of 0.002 to maximum value of 0.612 m, the wave energy increased during time experiment from 0.03 to 0.09 NdBar$^{\wedge 2}$ m$^{\wedge -3}$ and the wind speed ranged from 0.5 to 2.1(m/s).

This findings show the likelihood of wave-wave to collision caused by the waves from the vessel and waves from north winds in the opposite direction. However, the presence of Southeast and Southwest winds caused reverse waves after hitting the solid stage of the site monitoring the Lakeshore and led to exerting an equal downward force on the end of the string. This new force creates a wave pulse that propagates from right to left with the same speed and amplitude as incident wave, but with opposite polarity (upside down) of strong reflectional waves according to Newton's third law (Brown, 1989; Svendsen and Madsen, 1981). The same effort and strength that led to impact on the water surface of the study area lead to generate small waves downwards causing resuspension of sediments and increased the microbial biomass at the time of experiment. Also, the dominant winds from the north directed towards the lakeshore in Meiliang bay caused the same inverse waves, which could be the most important reason for sediment resuspension in this bay (Figures 5 and 6).

Dynamic nutrients loads

The increased turbidity and the positive correlation between wind speed, turbidity R^2= (0. 97, P<0.05) may be due to the impact of waves generated by wind (vessel). The wind speed during experiment did not exceed the critical point 3.5 m/s that gave key factor for the resuspension to occur in the water body during weak wind speed and nutrients released. This may consider the cause of eutrophication phenomena and make algal bloom grew in the lake maybe by waves generated by vessels. The mixing of sediments resuspension into the water column does lead to nutrient release, the recurrence and period of resuspension events, as well as relation bioavailability of nutrients (TP, TN, NH_4, PO_4); therefore, factor in determining the magnitude of water quality impacts from waves.

The slope of relationship (TP=3.98x+55.4, R^2 = 0.7698, P (value < 0.5) (Figure 9) during May 26, 201, had weak wind occurrence, suggesting its potential to provide accurate prediction of the size of change in TP due to sediment resuspension in Meiliang Bay. The enrichment of water column by TP from the sediments could promote nitrogen limitation if a significant proportion of mobilized TP is bioavailable. Furthermore, a

large flux of bioavailable phosphorus could maintain eutrophic conditions, in spite of large reductions in external phosphorus loading. On the other hand, if resuspended nutrients continue to exist largely as particle-bound and inaccessible to the biota, the effect on water quality could be comparatively small. Our assessments of resuspension are similar to other shallow eutrophic lake which were determined by Anthony and Downing (2003). Our results in Taihu Lake therefore show that resuspension of the bottom area may be influenced by waves. Frequent wave influence may be by strong wind, vessels, and disturbance of sediments. Efforts should be taken to research more about waves induced by wind, vessels to minimize influence of them, distinguish their impact exactly from the separate wave, which can further minimize the resuspension possibility in the shallow lake.

Many organisms were found in the sediments in higher concentrations than in the water column (Burton et al., 1987). Some microorganisms such as, Sediment-bound enteric bacteria may become resuspended by a verity of perturbations including dredging, storm events, boats and ships traffic (Garrad and Hey, 1987; Pettibone et al., 1996; Yousef et al., 1980). The concentration of some indicator microorganisms increase of 40-50 times in waters below dredging operation as compared to upstream waters (Grimes, 1980; Pettibone et al., 1996). The results indicated (Table 1) that there was a slight decrease of the microbial abundance concentration in the bottom layer especially during time passages of vessel 45 min and increased in the surface layer. This result may be because momentum wave shear stress and wave-wave collided the waves generated by vessel or SE, SW wind. This collide induced turbulence which increased the dissolved solids, soluble reactive phosphorus, turbidity and resuspending the microbial community associated with bottom sediments in the water bodies. Sediments may be resuspended as flocculated particles and sediment gathers can be a dominant form of sediment transport in freshwater. Generation of a wave makes resuspension in the layers (Perkins et al., 2014; Van Donsel and Geldreich, 1971).

In addition, slight increase of microbial abundance in surface layer also provides the suspension process in the lake. Moving the vessel generated waves, resuspending bacteria-colonized bed sediments. The microbes in different layers may also move by motility or a momentum change because the average velocity hydrodynamics change during the experiment. At the beginning of experiment, microbes move from the bed to water column, leading to increased microbial abundance in the others layers Table 1; and at the bottom layer, the wave's energy transmission to sediments particles attached. This leads to a different transport dynamic of sediments thus changing their mode of transport and deposition processes. The vessels had an impact on the physical characteristic of lake and river shorelines (Gabel et al., 2012; Garrad and Hey, 1987). Vessels induced waves and currents are effects on sediment resuspension and shoreline erosion and it began decades ago (Gabel et al., 2017; Garrad and Hey, 1987; Mason and Bryant, 1975). As a result of flow velocities changed and nutrients concentrations during passages of vessel induced waves and currents. This led to increase effect of shear stresses and sediments particles suspended fallowing by nutrients released which is the reason for supplying algal bloom for growth in a huge quantity. Increased wave energy, turbidity and changing the velocities during the time passages boat are similar with the results of (Gabel et al., 2012; Gao et al., 2017; Garrad and Hey, 1987) (Figures 5, 6). This led to alterations of the bottom morphology, sediment grain sizes and bank collapses (Gabel et al., 2017; Garrad and Hey, 1987; Nanson et al., 1994).

The vessel generated waves and the shear stress from those waves motivated the bottom sediment partials and liberation nutrients and maybe this shear stress is momentum mechanisms according to the turbulent burst that happened, ejections and sweeps during passages of vessel. The results are given a start point for more concentration of the mechanisms happened during momentous events on the bottom layer. In Lake Taihu there were a lot of vessels traveling in the lake and maybe the amount eroded materials depends on the characteristics of the vessels and its waves such as, travelling speed, distance to shore Lake, significant wave height, wave length. Also the characteristics of the location water depths are different in the lake, steepness of shoreline, sediment characteristics, grain size and cohesively, therefore leading to different erosion or suspension rates in the lake. May be in Meiliang bay the lakeshore was eroded from different waves generated by vessels and north winds.

Nutrient concentrations during the experiment at three depths

The average Chl-a μgL^{-1} and nutrient concentrations during the experiment at the three depths showed changes, with the concentration of Chl-a μgL^{-1} decreasing at the bottom. Wind-induced waves, especially strong winds, participate in 95% of sediment resuspension and nutrient release and are important for understanding how hydrodynamics affect eutrophication. The water depth at sites of data collection was only a few meters because Lake Taihu is a shallow lake, hence causing long-period waves move to the bottom layer and infiltrate the sediment water boundary that may bring about sediment resuspension and nutrient liberation (Jin and Tu, 1990; Li et al., 2017). Our results appear similar to Carper and Bachmann (1984) which showed that the resuspension of sediments in the lake by waves action and moments shear stress resulted in increased turbidity, thereby circling sediment nutrients back into the water column.

Conclusion

Field observations, analysis of the water column characteristics, effects of vessels and wind-generated waves on SS and microbial abundance were determined in this study. Examinations of nutrient loads in water layers suggest that both waves generated by wind or vessels can be responsible, by increasing resuspension and turbidity in the water column. In the lake, the variances of induced wave such as wind, boats and ships passages may differentially impact the concentration of suspended solid. Our results indicate that waves have a significant impact on suspended sediment concentration. Management strategies in Lake Taihu have thus far concentrated on the mitigation of algal blooms by nutrient load reductions. Future studies should focus on field observations of vessel waves shear stress, their effects on sediment resuspension and nutrient cycling, and their roles in eutrophication and algal bloom the changes in the hydrodynamic of the shallow Lake.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The research was supported by National key research and development plan (2016YFC0401703), and Chinese National Science Foundation (5177090079, 51579071, 51379061, 41323001, 51539003); National Science Funds for Creative Research Groups of China (No. 51421006); the program of Dual Innovative Talents Plan and Innovative Research Team in Jiangsu Province; the Priority Academic Program Development of Jiangsu Higher Education Institutions, and the Fundamental Research Funds for the Central Universities

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Vol. 13(28), pp. 580-608, October, 2019
DOI: 10.5897/AJMR2019.9206
Article Number: E8A2C8662130
ISSN: 1996-0808

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Characterization of bacterial pathogens associated with milk microbiota in Egypt

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Received 31 August, 2019; Accepted 7 October, 2019.

Milk is a substantial source of nutrients needed by all humans across lifespan development. Given its nutritional composition, milk is considered a vehicle for various microbes including beneficial and pathogenic bacteria. In this study, 270 milk samples comprising raw cow and buffalo milk and pasteurized milk with different shelf-life durations were tested along with pasteurized organic milk for the presence of *Staphylococcus aureus and Escherichia coli.* Collectively, 21 *E. coli* and 14 *S. aureus* isolates were cultivated and identified from total milk samples. All *E. coli* and *S. aureus* isolates exhibited resistance to erythromycin and penicillin, respectively. Serogroups O26, O128, and O111 were the most frequently identified amongst *E. coli* isolates, whereas staphylococcal enterotoxins (SEs) were inconsistently produced across *S. aureus* isolates. The molecular profile showed clustering of 6 isolates of *E. coli* by harboring *stx1, stx2, eaeA* genes, and 5 isolates of *S. aureus* by *mecA* gene. Findings revealed the bacteriological quality of popularly consumed milk in Egypt, including raw and pasteurized milk with preference to pasteurized organic milk and 7-day shelf life (7DSL) pasteurized milk. However, raw milk and 3MSL pasteurized milk were the major sources of *E. coli* and *S. aureus*, posing a serious public health issue.

Key words: Raw milk, pasteurization, *Staphylococcus aureus and Escherichia coli,* shelf-life.

INTRODUCTION

Milk and dairy products are substantial sources of macro- and micronutrients needed by humans that make them prone to contamination with microbial pathogens. Simultaneously, milk nutrients support the growth of specific beneficial microbes (e.g. lactobacilli and bifidobacteria) that promote human health and fitness

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(Fernandes, 2009). Though the ingestion of contaminated milk either raw or pasteurized is the major cause of serious food-poisoning outbreaks, potentially result from microbial toxins production (Dhanashekar et al., 2012). Contaminated milk may harbor harmful microbes that lead to either milk spoilage (e.g. *Pseudomonas* and thermoduric microbes such as *Clostridium* and *Bacillus*) or the emergence of public health issues (e.g. *Listeria, Salmonella, E. coli,* and *S. aureus*) (Bennett et al., 2013; Quigley et al., 2013b). Milk is sterile at secretion in udder but is contaminated with extraneous microbes before leaving the animal udder (Elgadi et al., 2008). In developing countries especially rural areas, raw milk is directly used for either consumption or local dairy production (FAO, 2011; Zeinhom and Abdel-Latef, 2014). Raw milk has a short shelf life that could be extended by heating. However, in the dairy industry, the shelf life of pasteurized milk is greatly influenced by the microbiological quality of the used raw milk (Murphy et al., 2016). In general, spoilage of commercialized milk and dairy products is attributable to various contamination sources including; pre-pasteurization psychrotophic growth, the degradable activity of heat-resistant microbial enzymes, and contamination after pasteurization process which is the most probable source (Sarkar, 2015). Gram-negative rods are the major psychrotrophic bacteria inhabiting raw milk (e.g., Enterobacteriaceae family including coliform bacteria that encompasses 5 to 33% of milk psychrotrophic bacteria) and proliferate during storage with the production of thermoresistant degradative enzymes (De Oliveira et al., 2015; Barbano et al., 2006; Mallet et al., 2012; Lewis and Gilmour, 1987). In addition, some Gram-positive bacteria contaminate raw milk with less frequent existence compared to Gram-negative psychrotrophs such as *Staphylococcus* species (Vithanage et al., 2016).

In general, pasteurization and ultra-high temperature (UHT) sterilization are the most commonly used techniques in the dairy industry for proper preservation and prolonged usability periods (Rais et al., 2013). Pasteurization meant to destroy common pathogens inhabiting raw milk microbiotas, especially those responsible for milk spoilage and influencing the shelf-life duration. Furthermore, pasteurization inactivates microbial enzymes that catalyze the breakage of milk macro-nutrients (e.g., lipids and proteins) and result in spoilage and invalidity of dairy products for consumption (Sarkar, 2015). In UHT treatment, heating is applied in the range of 135 to 150°C for up to 4 s for safe commercial dairy products combined with prolongation of the milk shelf-life duration (up to 12 months) (Vranješ et al., 2015).Though, aseptic packaging is crucial in both techniques that assure safety and extended usability of final dairy products (Deeth, 2017).

There is a considerable number of published studies that have been conducted on the prevalence of *E. coli* and *S. aureus* in milk (Kandil et al., 2018; Vahedi et al., 2013). Milk and dairy products are one of the major causes of the transmission of pathogenic *E. coli* strains into the human (Ombarak et al., 2019; Momtaz et al., 2012). With the advent of the high throughput sequencing technology, *Escherichia coli* was reported as a dominant inhabitant of the healthy human gut microbiome (Desmarchelier and Fegan, 2016). However, some *E. coli* strains exhibited virulence traits that enabled them to infect different body organs and cause illness (Awadallah et al., 2016; Zeinhom and Abdel-Latef, 2014). Noteworthy, diarrheagenic *E. coli* strains increasingly become the leading cause of pediatric diarrhea. The most important diarrheagenic *E. coli* that threaten human health worldwide are enteropathogenic *E. coli* (EPEC) (the etiological agent of watery diarrhea in infants), enterohemorrhagic *E. coli* (EHEC) (leads to hemorrhagic colitis and hemolytic-uremic syndrome), entero-aggregative *E. coli* (EAEC) (causes persistent diarrhea), and enterotoxigenic *E. coli* (ETEC) (known to cause traveler's diarrhea) (Nataro and Kaper, 1998). The pathogenicity of diarrheagenic *E. coli* is attributed to possessing genetically encoded virulence traits. For instance, enterohemorrhagic *E. coli* (EHEC) causes illness through the expression of intimin outer membrane protein encoded by *eae* gene and required for tissue colonization along with the production of Shiga toxins (ST) (e.g., *Stx1, Stx2* or *Stx2* variants) (Kaper et al., 2004). However, Enteropathogenic *E. coli* (EPEC) lacks *ST* genes, but exhibits its pathogenicity through the formation of A/E lesions on the intestinal cells, and is identified as *eae*-harboring diarrheagenic *E. coli* (Aidar-Ugrinovich et al., 2007).

S. aureus is a facultative anaerobic Gram-positive coccus and one of the world top pathogens that causes food-poisoning (Tirado and Schmidt, 2001; Hennekinne et al., 2012). Globally, enterotoxigenic *S. aureus* is implicated in udder infection of dairy cows combined with improper handling and poor storage conditions that result in frequent contamination of milk and dairy products. *S. aureus* produces several toxins including classical staphylococcal enterotoxins (SE) (SEA to SEE), in addition to other new types (SEG to SElU2) (Argudín et al., 2010). *S. aureus* could be inactivated by pasteurization however, thermostable SEs were found to retain their biological activity after the thermal treatment (Asao et al., 2003). Furthermore, more recent evidence suggests that SEA is the leading cause of staphylococcal food poisoning worldwide (Argudín et al., 2010). In order to verify the prevalence of genes encoding SE in *S. aureus* isolated from milk and dairy products, the phenotypic/serotypic assays of SE production should be conducted (Morandi et al., 2007). Of the classical techniques used for SE serotyping analysis, the gel-

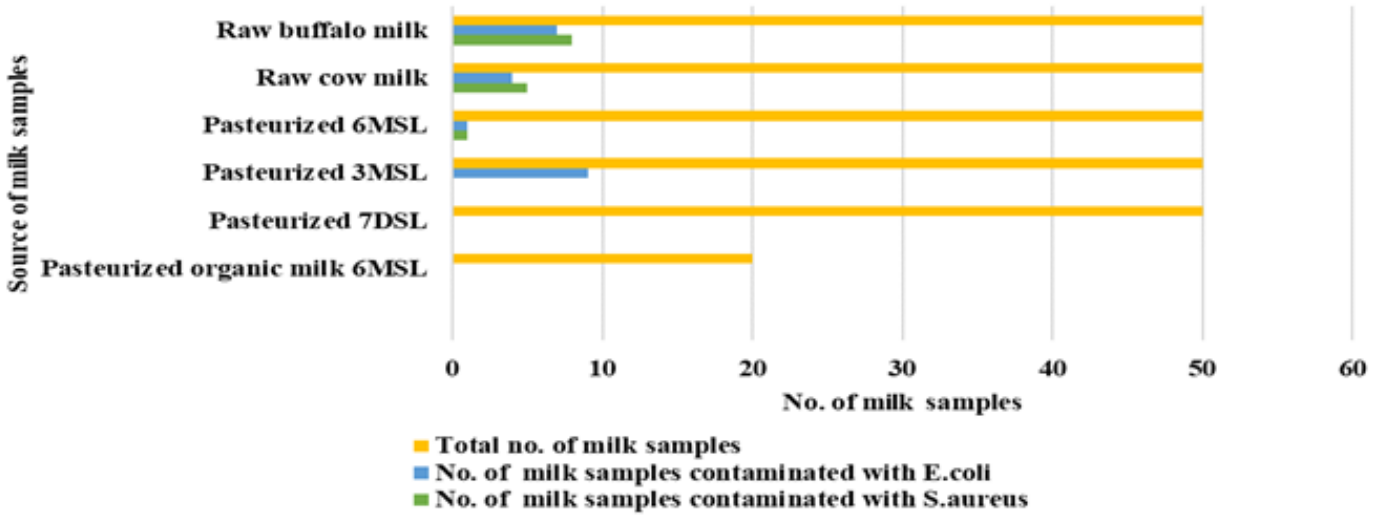

Figure 1. Prevalence of isolated and identified *E. coli* and *S. aureus* contaminants across milk samples collected from different sources. 3MSL: 3-month shelf life; 6MSL: 6-month shelf life and 7DSL: 7-day shelf life.

diffusion test, agglutination test, and reverse passive latex agglutination (RPLA) test kits (Wu et al., 2016). When compared to molecular techniques, the serological tests have limited sensitivity and specificity for SEs detection and cannot be used for total quantification of SE (Wu et al., 2016).

So far, culture-dependent methods are still used as a routine protocol for the microbial assessment of raw and pasteurized milk. However, the detection of bacterial species that exist at subdominant levels is needed since the conventional laboratory methods are not enough to support the *in vitro* growth of milk-associated microbiota (Quigley et al., 2013a). Nowadays, culture-based foodborne pathogen detection methods have been developed to reduce the inspection time and improve product quality. One of the most informative and cost-effective molecular-based detection techniques is the multiplex PCR, which enables the screening of multiple target genes within a single reaction (Postollec et al., 2011).

In developing countries, consumption of raw milk is not prohibited and the advanced pasteurization techniques are still neither regulated nor implemented. Given the nutritional importance of milk and its widespread consumption particularly, among women and children, the study aimed to investigate the bacteriological quality of popularly consumed milk in the Delta area, Egypt for the presence of *E. coli* and *S. aureus* as major milk contaminants. The identified isolates were subjected to further testing for their potential pathogenicity through serotypic characterization and molecular profiling along with their antibiotic susceptibility profile.

MATERIALS AND METHODS

Samples collection

Two hundred and seventy milk samples (10 ml each) were randomly collected (from January to June 2017) from local grocery stores and farmer vendors in El-Beheira governorate that represents Delta area in Egypt as street vendors are coming from different villages of neighbor Delta governorates. The milk samples included 100 samples of raw milk (50 samples of cow milk and 50 samples of buffalo milk), and 170 samples of pasteurized milk (50 samples of 6-month shelf life (6MSL), 50 samples of 3-month shelf life (3MSL), 50 samples of 7-day shelf life (7DSL) and 20 samples of pasteurized organic milk (6MSL)) (Figure 1 and Table S1A). All milk samples were collected in an icebox and brought to the laboratory to assess them for the presence of *E. coli* and *S. aureus* contaminants.

Isolation and identification of *E. coli*

Under aseptic conditions, 1 ml of each milk sample was drawn, homogenized with 10 ml of nutrient broth and incubated overnight at 37°C. Next day, 100 µl of the cultivated broth were streaked on MacConkey agar plate and incubated overnight for selection of enteric Gram-negative (Gm-ve) bacteria. Every lactose-fermenting (LF) colony was picked up using sterile toothpicks and streaked on Eosin methylene blue (EMB) agar plate, then incubated overnight at 37°C for further purification. Colonies exhibited blue-black color with a metallic green sheen were isolated and examined under a light microscope for gram stain. *E. coli* candidates were biochemically confirmed using indole, methyl Red, Voges Proskauer, citrate, triple sugar iron, and urease tests (Table S1B) according to Kreig and Holt (1984) and Miller (1992).

Isolation and identification of *S. aureus*

For isolation of *S. aureus*, 100 µl of overnight cultivated milk

samples were streaked on Mannitol salt agar (MSA) plate and incubated overnight for bacterial growth. A yellow colony grown on a red/pink (MSA) medium was picked up and then streaked on a Baired parker (BP) agar plate for further purification. Every unique single colony was gram stained and visualized under the light microscope. The identification of *S. aureus* isolates was confirmed by performing a specific scheme of biochemical tests including coagulase, oxidase and DNase tests (Table S1C) according to MacFaddin (2000) and Lachica et al. (1971). 50% glycerol stocks of all identified bacterial isolates under this study were prepared and stored at -80°C freezer for further experiments.

Antibiotic susceptibility testing

The susceptibility of *E. coli* and *S. aureus* isolates to antibiotics were tested using the agar disk diffusion method. 11 antibiotics including ampicillin 10 µg (AML), amoxicillin/clavulanic 30 µg (AMC), imipenem 10 µg (IPM), cefipime 30 µg (CPM), cefotaxime 30 µg (CTX), gentamicin 10 µg (CN), azithromycin 15 µg (AZM), chloramphenicol 30 µg (C), tetracycline 30 µg (TE), sulphamethoxazole/trimethoprim 1.25/23.75 (SXT) and ciprofloxacin 5 µg (CIP) were used for the screening of *E. coli* isolates. With respect to testing *S. aureus* isolates, 9 antibiotics including penicillin 10U (P), cefoxitin 30 µg (CX), vancomycin 30 µg (VA), gentamicin 10 µg (CN), erythromycin 15 µg (E), chloramphenicol 30 µg (C), tetracycline 30 µg (TE), sulphamethoxazole/trimethoprim 1.25/23.75 (SXT) and ciprofloxacin 5 µg (CIP) were used. Following 16 to 18 h of aerobic incubation at 37°C, the plates were examined for bacterial growth and the diameter of inhibition zones surrounding antibiotic disks were scored in millimeter (mm). The zone diameters were interpreted as resistant (R), intermediate (I) or susceptible (S) according to (CLSI, 2017).

Serotyping of *E. coli* isolates

Serotyping of *E. coli* isolates was performed using rapid diagnostic *E. coli* antisera sets (Denka Seiken Co, Japan) for lab diagnosis of Enteropathogenic serotypes according to the manufacturer's instructions. All antisera were obtained and absorbed with the corresponding cross-reacting antigens to remove the non-specific agglutinins.

Staphylococcal enterotoxins (SE) production test using SET-RPLA assay

S. aureus isolates were tested for enterotoxin production (SEA to SED) using SET-RPLA assay (SET-RPLA; Denka Seiken Co. Ltd., Tokyo, Japan) (Park and Szabo, 1986). The serotypic assay was performed according to the manufacturer's instruction

Genomic DNA purification

DNA was purified from *E. coli* and *S. aureus* isolates along with used reference strains using a genomic DNA purification QIAamp kit (Qiagen, Germany) according to the manufacturer's recommendations. The used reference strains for *E. coli* were: *E. coli* O157:H7 Sakai (positive for *stx1, stx2, eaeA,* and *hylA* genes*)* and *E. coli* K12 DH5α (a non-pathogenic negative control strain). Whereas enterotoxigenic *S. aureus* strains ATCC 13565 (positive for *sea* gene), ATCC 14458 (positive for *seb* gene), ATCC 19095 (positive for *sec* gene), ATCC 23235 (positive for *sed* gene), 95-S-739 (positive for *mecA* gene) were used as positive controls for *S. aureus* molecular profiling, and *S. xylosus* ATCC 29971 was used as a negative control.

Molecular shiga toxin profiling and *eaeA* gene in *E. coli* isolates

The multiplexed-PCR technique was used for molecular profiling of *E. coli* isolates through amplification of shiga toxin-encoding genes; *stx1*, *stx2* along with intimin-encoding gene (*eaeA*). The PCR reaction was performed using primers listed in (Table 1) in a Thermal Cycler (Master Cycler, Eppendorf, Hamburg, Germany). Approximately 50 ng of bacterial DNA was added to 12.5 µl DreamTaq Green PCR Master Mix (2X) (Thermo), 0.5 µl (5 pmol) of each primer and the final volume was adjusted to 25 µl by adding sterile ultrapure water. The amplification conditions started by initial denaturation for 3 min at 95°C followed by 35 cycles of 95°C for 20 s, 58°C for 40 s, and 72°C for 90 s. The final cycle was followed by 72°C final extension for 5 min. The amplified DNA fragments were separated by 1.5% of agarose gel electrophoresis (Applichem, Germany, GmbH) in 1x TBE buffer and captured as well as visualized on a UV transilluminator. A 100 bp plus DNA Ladder (Qiagen, Germany, GmbH) was used to determine each amplicon size and strains; *E. coli* O157:H7 Sakai and *E. coli* K12 DH5-α were used as a positive and negative control, respectively.

Molecular enterotoxin profiling and *mecA* gene in *S. aureus*

The genotypic profile of *S. aureus* isolates was generated based on the presence of *sea*, *seb*, *sec* and *sed* SE-encoding genes using multiplexed PCR along with conventional PCR for *mecA* gene amplification. PCR conditions used in *E. coli* molecular profiling were adapted by changing the annealing temperature to 50°C for 1 min and 56°C for 30 s for multiplexed and conventional PCR, respectively. *S. aureus* strains ATCC 13565, ATCC 14458, ATCC 19095, ATCC 23235 and 95-S-739 were used as positive controls for *sea, seb, sec, sed* and *mecA* genes*,* respectively and *S. xylosus* ATCC 29971 was used as a negative control. Sequences of the used primers are listed in (Table 2).

RESULTS

Prevalence of *E. coli* and *S. aureus* contaminants across milk samples

In the current study, a total of 21 (7.8%) *E. coli* isolates were identified in particular, from raw and pasteurized 3MSL milk samples (Figure 1 and Table S1B). At the other side, raw and pasteurized 6MSL milk samples were the main sources of *S. aureus* isolates (14 isolates, accounting for 5.2% of the total milk samples) (Table S1C). Interestingly, pasteurized 7DSL and organic 6MSL samples exhibited negative bacterial growth (Figure 1).

Antibiotic susceptibility testing

Findings revealed the resistance of all *E. coli* isolates to

Table 1. Primers used for molecular profiling of *E. coli* isolates.

Primer	Oligonucleotide sequence (5' → 3')	Product size (bp)	References
stx1 (F)	5′ACACTGGATGATCTCAGTGG′3	614	Olowe et al. (2014)
stx1 (R)	5′ CTGAATCCCCCTCCATTATG ′3		
stx2 (F)	5′CCATGACAACGGACAGCAGTT′3	779	
stx2 (R)	5′CCTGTCAACTGAGCAGCACTTTG′3		
eaeA (F)	5′ GTGGCGAATACTGGCGAGACT ′3	890	Kargar and Homayoon (2015)
eaeA (R)	5′ CCCCATTCTTTTTCACCGTCG ′3		

Table 2. Primers used for molecular profiling of *S. aureus* isolates.

Primer	Oligonucleotide sequence (5' → 3')	Product size (bp)	Reference
sea (F)	5′ TTGGAAACGGTTAAAACGAA′3	120	Rall et al. (2008)
sea (R)	5′ GAACCTTCCCATCAAAAACA ′3		
seb (F)	5′ TCGCATCAAACTGACAAACG ′3	478	
seb (R)	5′ GCGGTACTCTATAAGTGCC ′3		
sec (F)	5′ GACATAAAAGCTAGGAATTT ′3	257	
sec (R)	5′ AAATCGGATTAACATTATCC ′3		
sed (F)	5′ CTAGTTTGGTAATATCTCCT ′3	317	
sed (R)	5′ TAATGCTATATCTTATAGGG ′3		
mecA (F)	5′ TAGAAATGACTGAAC GTCCG ′3	533	
mecA (R)	5′ TTGCGATCA ATGTTACCGTAG ′3		

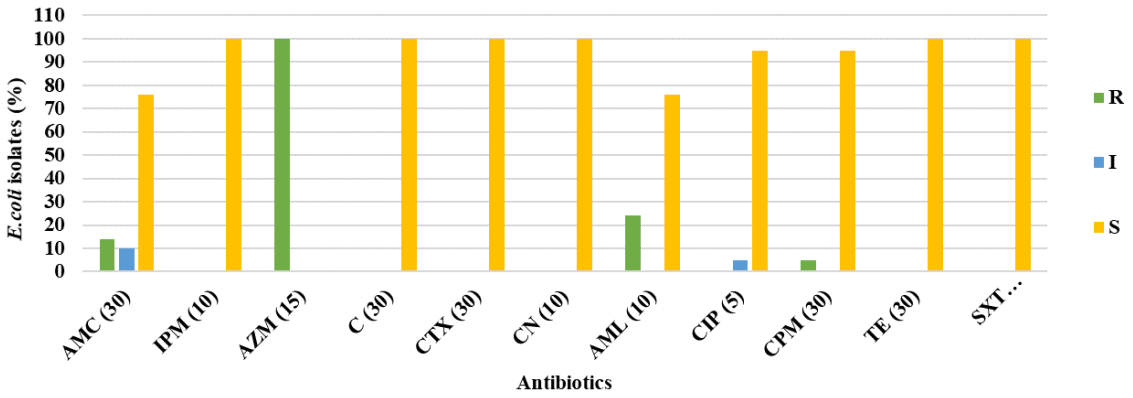

Figure 2. Antibiotic susceptibility patterns of *E. coli* isolates. R: Resistant; I: Intermediate; S: sensitive.

erythromycin (100%) whereas 24 and 14% of the total *E. coli* isolates exhibited resistance to amoxicillin and amoxicillin/clavulanic acid, respectively. Of note, all *E. coli* isolates were susceptible to imipenem, chloramphenicol, gentamicin, cefotaxime, tetracycline, and sulfamethoxazole (Figure 2). Similarly, all *S. aureus* isolates showed resistance to penicillin followed by far behind cefoxitin (50%) and sulfamethoxazole (29%). Meanwhile, vancomycin and ciprofloxacin inhibited the growth of all *S. aureus* isolates (Figure 3).

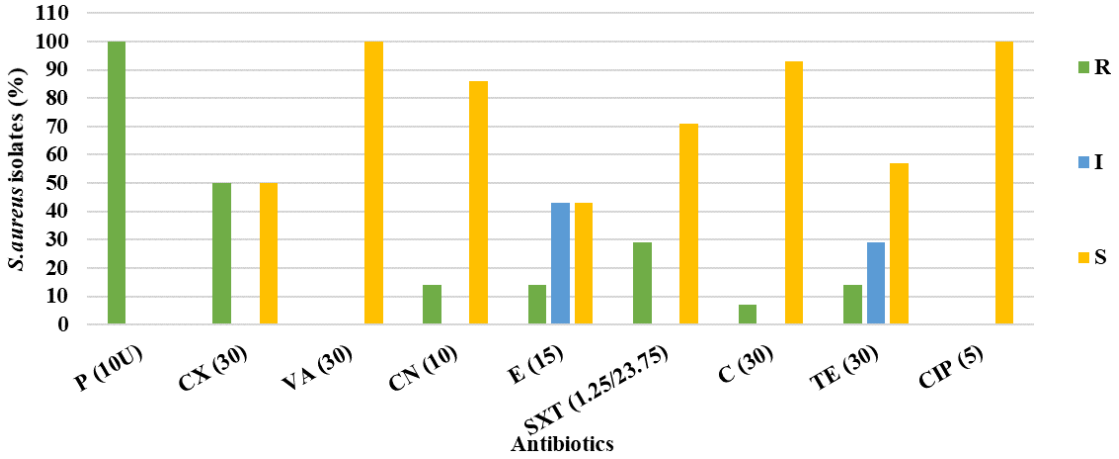

Figure 3. Antibiotic susceptibility patterns of *S. aureus* isolates. R: Resistant; I: Intermediate; S: sensitive.

Serotyping of *E. coli* and *S. aureus* isolates

The serological typing of *E. coli* isolates showed that EHEC was the most dominant pathotype accounting for 62% (13 out of 21 isolates), followed by far behind ETEC (19%, 4 isolates), EPEC (14%, 3 isolates), and EIEC (5%, 1 isolate) (Table 3). Interestingly, O26, O128, and O111 were the most prevalent serogroups identified in 29, 19 and 14% of the isolates, respectively. With respect to staphylococcal enterotoxin production, RPLA assay showed that 3 out of 14 isolates (21.4%) produced different SE listed in (Table 4).

Molecular profiling of *E. coli* and *S. aureus* isolates

The molecular profiling of *E. coli* isolates showed positive results for the presence of *stx1, stx2, eaeA* genes accounting for 90.5% (19 out of 21) of total *E. coli* isolates, and spanning different sources of milk samples (Table). However, *stx1*, *stx2*, *eaeA* genes were amplified altogether in 31.6% (6 out of 19) of *E. coli* isolates. Of note, these 6 isolates were purified from raw milk and 3MSL pasteurized milk (Figure 4 (A and B) and Table 3). Interestingly, 35.7% (5 out of 14) of *S. aureus* isolates exhibited positive PCR products for *mecA* gene, exclusively collected from raw milk (Figure 4) (C and D) and (Table 4). Only 3 *S. aureus* isolates showed positive results for the tested SE-encoding genes with an exception for *sed* gene (Table 3).

DISCUSSION

Bacterial contamination of milk may originate from diverse sources mainly; infected udders and unhygienic practices during the milking process. Of the major bacterial contaminants of milk; *E. coli* and *S. aureus* that are responsible for serious food-poisoning outbreaks worldwide (Vahedi et al., 2013). In the current study, 270 milk samples including raw and pasteurized milk of different shelf life durations (Figure 1) were tested for the presence of *E. coli* and *S. aureus* contaminants. Interestingly, 11% (11 out of 100) of raw milk samples were the source of approximately half of the identified *E. coli* isolates (11 out of 21 *E. coli* isolates). This percentage was significantly lower than previously published reports from Iran and Egypt, where *E. coli* was identified from 42% (Vahedi et al., 2013), 33% (Hassan et al., 2015) and 60% (Kandil et al.,2018) of tested milk samples. 36.4% (4 out of 11 isolates) of identified *E. coli* isolates from raw milk originated from 8% (4 out of 50 samples) of raw cow milk (Figure 1). Similarly, cultivated raw buffalo milk samples resulted in the isolation of 14% (7 out of 50) of *E. coli* isolates which is a lower rate compared to previously published studies (Ranjbar et al., 2018). These findings indicated a relatively good bacteriological quality of raw milk in El-Beheira area when compared to previous studies (Bali et al., 2013; Garedew et al., 2012; Disassa et al., 2017; Reta et al., 2016). With regard to pasteurized milk, 5.9 % of tested samples resulted in the isolation of 10 *E. coli* isolates (9 out of 50 samples (18%) from 3MSL, and 1 out of 50 samples (2%) from 6MSL milk samples). Contrarily, in other published work (Kandil et al., 2018; Hassan et al., 2015; Garedew et al., 2012), none of the pasteurized/sterile milk samples was reported for in vitro bacterial growth of *E. coli.*

The incidence of *S. aureus* in milk is increasingly ubiquitous as a result of the widespread of various

Table 3. Summary of the serological identification and molecular profiling along with the antibiotic resistance patterns of *E. coli* isolates.

Sample source	Sample code	Serotyping characterization	Serodiagnosis	Molecular profiling	Antibiotic failed to inhibit bacterial growth
Raw buffalo milk (n = 7)	EB15	EIEC	O124	-	E, AMC, AML
	EB16	EHEC	O121:H7	*stx2*	E
	EB23	EHEC	O111:H2	*stx1,eaeA*	E, AML, CPM
	EB25	EHEC	O26:H11	*stx1,stx2*	E
	EB26	EHEC	O26:H11	*stx1,stx2,eaeA*	E
	EB24	EPEC	O146:H21	*stx2*	E
	EB39	EPEC	O15:H2	*stx2*	E, AML
Raw cow milk (n = 4)	EC27	EHEC	O121:H7	*stx2*	E
	EC29	EHEC	O111:H2	*stx1,stx2,eaeA*	E
	EC30	ETEC	O128:H2	*stx1*	E
	EC32	ETEC	O128:H2	*stx1*	E, AML
Pasteurized 3MSL milk (n = 9)	ET2	EHEC	O91:H21	*stx1, stx2*	E
	ET4	ETEC	O128:H2	*stx1*	E
	ET5	EPEC	O119:H6	-	E
	ET6	EHEC	O26:H11	*stx1,stx2,eaeA*	E
	ET7	EHEC	O111:H2	*stx1,stx2,eaeA*	E
	ET33	EHEC	O26:H11	*stx1,stx2,eaeA*	E
	ET35	EHEC	O91:H21	*stx1, stx2*	E, AMC, AML
	ET37	EHEC	O26:H11	*stx2,eaeA*	E
	ET38	EHEC	O26:H11	*stx1,stx2,eaeA*	E
Pasteurized 6MSL milk	ES41	ETEC	O128:H2	*stx1*	E

EHEC: Enterohaemorrhagic *E. coli*, ETEC: Enerotoxigenic *E. coli*; EPEC: Enteropathogenic *E. coli*; EIEC: Enteroinvasive *E. coli.*

Table 4. Summary of the serological identification and molecular profiling along with the antibiotic resistance patterns of *S. aureus* isolates.

Sample source	Sample code	Serotyping characterization	Molecular profiling	Antibiotic failed to inhibit bacterial growth
Raw cow milk	SC93	SEC	*sec*	P, CX, SXT, C
	SC118	SEA	*sea, mecA*	P, E, SXT, TE
	SC95	-	-	P, CX, SXT, TE
	SC75	-	-	P, CN
	SC55	-	-	P
Raw buffalo milk	SB57	SEA, SEB	*sea, seb, mecA*	P, CX
	SB119	-	-	P, CX, SXT
	SB61	-	-	P, CX, CN
	SB81	-	*mecA*	P, CX
	SB113	-	*mecA*	P, CX
	SB48	-	-	P
	SB67	-	*mecA*	P
	SB68	-	-	P
Pasteurized 6 MSL milk	SS94	-	-	P, E

SEA: Staphylococcal enterotoxin A; SEB: Staphylococcal enterotoxin B; SEC: Staphylococcal enterotoxin C.

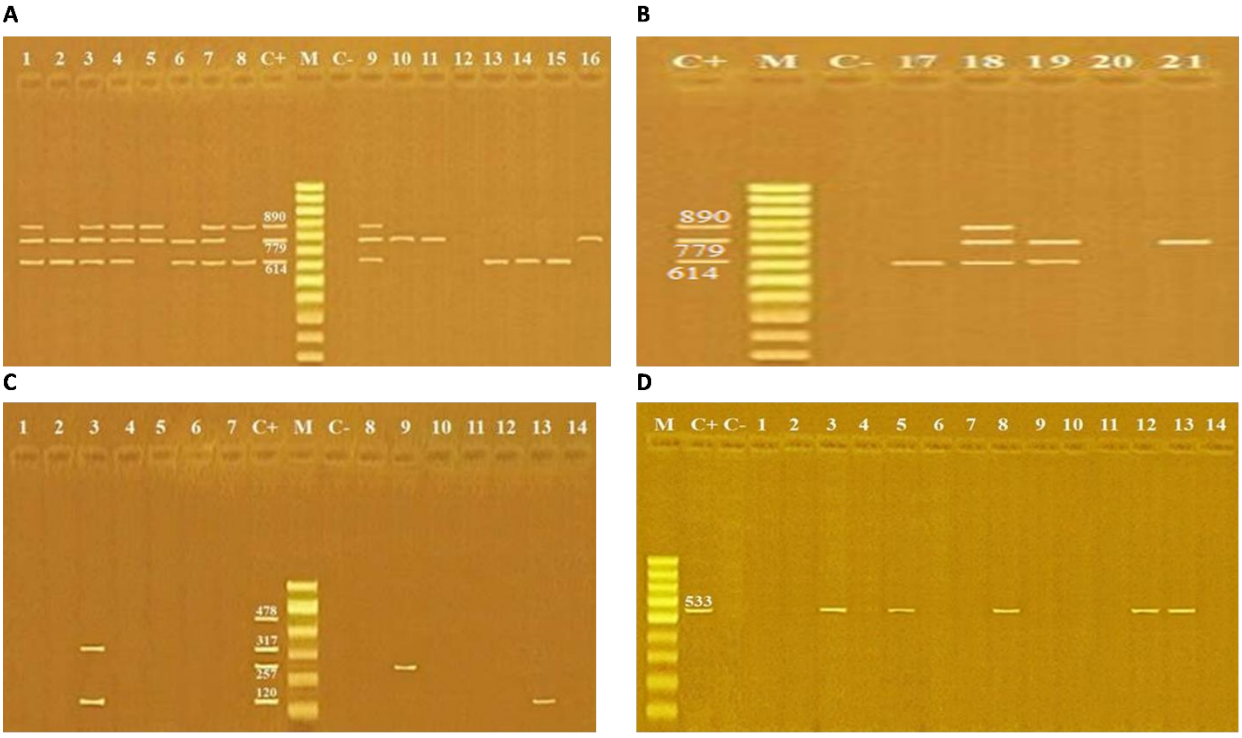

Figure 4. Molecular profiles of bacterial contaminants associated with tested milk samples: panel A and B show the multiplexed PCR profile of identified *E. coli* isolates for the presence of *stx1*(614 bp), *stx2* (779 bp) and *eaeA* (890 bp) genes, panel C shows the multiplexed PCR profile of identified *S. aureus* isolates for the presence of *sea* (120 bp), *seb* (478 bp), *sec* (257 bp) and *sed* (317 bp) genes, and panel D shows the PCR profile of identified *S. aureus* isolates for the presence of *mecA* gene (533 bp).

pathogenicity factors including; toxin-mediated virulence, invasiveness, and antibiotic resistance (Kadariya et al., 2014). 14 *S. aureus* isolates from all tested milk samples were biochemically identified. Approximately, 93% (13 out of 14 isolates) of *S. aureus* isolates were cultivated from raw milk (100 samples) accounting for 13% of the tested samples (Figure 1). The results came in accordance with those obtained by Zeinhom et al. (2015) and Mansour et al. (2017) that reported 12 and 16.3% of tested raw milk samples were contaminated with *S. aureus*, respectively. However, moderate and high contamination levels were also reported worldwide indicating the crucial importance of livestock health combined with the hygienic practices of milking on the safety of the dairy industry. For instance, a study from Egypt recorded the highest contamination incidence rates of raw milk with *S. aureus* accounting for 80% of the tested samples (Kandil et al., 2018). Interestingly, 10% (5 out of 50 samples) of the raw cow milk samples were contaminated with *S. aureus* that is comparatively lower than a previous report (24.2%) from Reta et al. (2016) in Ethiopia. However, only 0.6% (1 out of 170 samples) of pasteurized milk (6MSL milk) was contaminated with *S. aureus*. This result is consistent with a report published by Kandil et al. (2018) where *S. aureus* had zero existence in pasteurized milk samples in Egypt. In contrast, a higher contamination rate (14.92%) had been reported in Algeria (Matallah et al., 2019).

Globally, the unsupervised use of antimicrobial agents in the treatment of animal and human infections have been contributed to the emergence of antimicrobial resistance (Van Boeckel et al., 2015). The antimicrobial resistance mainly originates from the transfer of resistance genes across microbes enabling them to survive in the presence of antimicrobial agents that eventually resulted in failure of antibiotic therapeutic protocols (Blair et al., 2015). Furthermore, the overuse of antibiotics in animal husbandry as growth promoters could be a potential source of bacterial resistance through dissemination of resistant microbes from intestinal microbiotas of livestock that contaminate the surrounding environment and enhance the transmission of resistant genes to autochthonous bacteria (resident microbes) of the surface water systems (McEwen and

Collignon, 2018). In this study, all *E. coli* isolates exhibited susceptibility to tetracycline, ciprofloxacin, sulfamethoxazole and chloramphenicol (except for one isolate that was resistant to ciprofloxacin) (Figure 2) which disagreed with reports published by Nobili et al. (2016), Schroeder et al. (2002), Mora et al. (2005), Abebe et al. (2014) and Ranjbar et al. (2018). However, the results reported by Tadesse et al. (2018) were relatively similar to our study where the *in vitro* growth *E. coli* was restrained by gentamicin, ciprofloxacin, and tetracycline. Of note, erythromycin inhibited the growth of all *E. coli* isolates, whereas Tadesse et al. (2018) reported a considerably moderate percentage (60%) of erythromycin resistance. Interestingly, only 14% of *E. coli* isolates were resistant to amoxicillin-clavulanic acid, while Nobili et al. (2016) reported a significantly higher percentage (100%). Furthermore, all *E. coli* isolates exhibited sensitivity to tested sulfa-drug antibiotic that disagreed with reports from Tadesse et al. (2018) and Nobili et al. (2016) where the susceptibility levels were 40 and 50%, respectively. Regarding the antibiotic resistance patterns of *S. aureus*, the isolates exhibited resistant to penicillin, cefoxitin, sulfamethoxazole, tetracycline, gentamicin, and erythromycin (Figure 3) which concurred with the findings published by Hoque et al. (2018) and Reta et al. (2016). Interestingly, 29% of *S. aureus* isolates showed resistance to sulphamethoxazole-trimethoprim that completely agreed with Hoque et al. (2018), and spiking high when compared to those reported by Reta et al. (2016) and Umaru et al. (2013). Despite previous studies, Umaru et al. (2013) and Reta et al. (2016) reported variable sensitivity rates (44.3 and 6.9%, respectively) of *S. aureus* isolates to vancomycin, findings showed absolute susceptibility of all tested isolates to it. Similarly, all *S. aureus* isolates were susceptible to ciprofloxacin that disagreed with findings reported by Hoque et al. (2018) and Zeinhom et al. (2015).

Enterohemorrhagic *Escherichia coli* (EHEC) strains comprise a subgroup of Shiga-toxin (ST)-producing *E. coli* (STEC) and are the most frequently implicated in severe clinical illness worldwide (Vendramin et al., 2014). In this study, we found that 62% of *E. coli* isolates were serologically identified as EHEC (Table 3), known to cause outbreaks of bloody diarrhea. This percentage is higher than Vanitha et al. (2018), Vendramin et al. (2014), Momtaz et al. (2012) and Ranjbar et al. (2018). Interestingly, the molecular profiling showed that 90% (19 out of 21 isolates) of *E. coli* isolates were positive for *stx* genes, whereas 42.8% (9 out of 21 isolates) of them were positive for both *stx1* and *stx2* genes (Figure 4 and Table 3). However, these results were higher than that reported in previous studies (Tabaran et al., 2017; Nobili et al., 2016; Neher et al., 2015; Virpari et al., 2013) (Figure 4 and Table 3). Furthermore, 38% (8 out of 21 isolates) of *E. coli* isolates harbored *eaeA* gene and serotypically characterized as EHEC including O26 and O111 serogroup (Figure 4 and Table 3). These results were congruent with previously published studies (Momtaz et al., 2012; and Vanitha et al., 2018) where 33.33 and 36% of identified *E. coli* isolates were positive for *eaeA* gene, respectively. Contrarily, in a study conducted by Nobili et al. (2016), all STEC isolates exhibited negative results for *eaeA* gene.

S. aureus isolates are able to produce enterotoxins posing a public health threat. This means that the detection of SE in milk is very crucial for the bacteriological assessment of milk and dairy products (Wu et al., 2016). In the current study, the molecular detection of SE-encoding genes was greatly helpful for proper characterization of SE-producing *S. aureus*. In general, multiplex PCR detection could infer the presence of genes but does not consider their expression. Therefore, RPLA technique is needed to emphasize the SE production (van Belkum, 2003). Here, SET-RPLA assay showed that 21.4% (3 out of 14 isolates) of *S. aureus* isolates produced classic enterotoxins (SEA, SEC, SED) (Figure 4 and Table 4), which is in line with results reported by Fagundes et al. (2010). Interestingly, the molecular profiling of *S. aureus* isolates for SE-encoding genes confirmed the results of SET-RPLA technique (Figure 4 and Table 4) and agreed with previously published reports (Mansour et al., 2017). In contrast, in a study performed by Rall et al. (2008), a higher prevalence rate of *S. aureus* was reported, whereas 68.4% of the *S. aureus* isolates were positive for one or more enterotoxins-encoding-genes. Of note, Arcuri et al. (2010) detected SE genes in 13.6% of mastitic cow milk and 41.7% of a bulk milk tank. In general, methicillin-resistant *S. aureus* (MRSA) strains have the ability to express multiple antibiotic resistance genes that pose a global threat to animal and human health (Shah et al., 2019). In this study, *mecA* gene was detected in approximately 36% of the total *S. aureus* isolates that indicated the potential emergence of MRSA outbreaks from consumption of contaminated raw milk in particular, in traditional societies (Figure 4 and Table 4). Noteworthy, similar percentages (22.2 and 20%) of MRSA detection in milk were reported by Umaru et al. (2013) and Hoque et al. (2018), respectively.

Conclusion

To conclude, findings revealed that raw and 3MSL pasteurized milk are most prone to be contaminated by the pathogenic *E. coli* and *S. aureus* isolates, that poses serious health issues upon direct consumption of milk from these sources. Noteworthy, pasteurized organic milk and 7DSL milk were found to be of the highest

bacteriological quality when tested for the presence of *E. coli* and *S. aureus*. Eventually, our findings implicitly highlighted the importance of constituting strict regulations with regard to milk handling in local farms and dairy plants to minimize the chance of milk contamination and the transmission of bacterial pathogens along with their antimicrobial resistance from dairy animals to humans.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGMENT

The authors would like to thank the Department of Microbiology, Faculty of Veterinary Medicine, Damanhour University for their kind cooperation and contribution to this study. This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

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Table S1A. Primary microbiological testing of milk samples for the presence of *E.coli and S.aureus*

Sample code	Sample source	Pasteurization technique	*E.coli*		*S.aureus*	
			Macconkey	EMB	Mannitol salt	BP
B1	Buffalo milk	Raw (Non pasteurized)	LF	+	+	-
B2	Buffalo milk	Raw (Non pasteurized)	LF	-	+	+
B3	Buffalo milk	Raw (Non pasteurized)	LF	-	+	+
B4	Buffalo milk	Raw (Non pasteurized)	LNF	-	+	+
B5	Buffalo milk	Raw (Non pasteurized)	LNF	-	+	+
B6	Buffalo milk	Raw (Non pasteurized)	LNF	-	+	-
B7	Buffalo milk	Raw (Non pasteurized)	LF	+	+	-
B8	Buffalo milk	Raw (Non pasteurized)	LF	+	+	-
B9	Buffalo milk	Raw (Non pasteurized)	LF	-	+	+
B10	Buffalo milk	Raw (Non pasteurized)	LF	+	+	-
B11	Buffalo milk	Raw (Non pasteurized)	LF	+	+	-
B12	Buffalo milk	Raw (Non pasteurized)	-	-	+	+
B13	Buffalo milk	Raw (Non pasteurized)	LF	+	+	+
B14	Buffalo milk	Raw (Non pasteurized)	LF	+	+	+
B15	Buffalo milk	Raw (Non pasteurized)	LF	-	+	+
B16	Buffalo milk	Raw (Non pasteurized)	LF	-	+	+
B17	Buffalo milk	Raw (Non pasteurized)	LF	-	+	+
B18	Buffalo milk	Raw (Non pasteurized)	LF	-	+	+
B19	Buffalo milk	Raw (Non pasteurized)	LF	-	-	ND
B20	Buffalo milk	Raw (Non pasteurized)	LF	+	+	+
B21	Buffalo milk	Raw (Non pasteurized)	-	-	-	ND
B22	Buffalo milk	Raw (Non pasteurized)	LF	+	+	-
B23	Buffalo milk	Raw (Non pasteurized)	LNF	-	NA	-
B24	Buffalo milk	Raw (Non pasteurized)	LF	+	+	+
B25	Buffalo milk	Raw (Non pasteurized)	LF	-	NG	ND
B26	Buffalo milk	Raw (Non pasteurized)	LF	-	+	+
B27	Buffalo milk	Raw (Non pasteurized)	LF	-	NA	-
B28	Buffalo milk	Raw (Non pasteurized)	LF	-	+	-
B29	Buffalo milk	Raw (Non pasteurized)	LF	-	+	-
B30	Buffalo milk	Raw (Non pasteurized)	LF	-	NA	-
B31	Buffalo milk	Raw (Non pasteurized)	LF	+	+	-
B32	Buffalo milk	Raw (Non pasteurized)	LF	-	NG	ND

Table S1A. contd.

B33	Buffalo milk	Raw (Non pasteurized)	LF	-	-	-
B34	Buffalo milk	Raw (Non pasteurized)	LF	-	+	+
B35	Buffalo milk	Raw (Non pasteurized)	LF	-	-	-
B36	Buffalo milk	Raw (Non pasteurized)	LF	-	+	+
B37	Buffalo milk	Raw (Non pasteurized)	LF	-	+	+
B38	Buffalo milk	Raw (Non pasteurized)	LF	-	+	-
B39	Buffalo milk	Raw (Non pasteurized)	LF	-	NA	-
B40	Buffalo milk	Raw (Non pasteurized)	LF	-	-	-
B41	Buffalo milk	Raw (Non pasteurized)	LF	+	+	+
B42	Buffalo milk	Raw (Non pasteurized)	LF	+	+	+
B43	Buffalo milk	Raw (Non pasteurized)	LNF	-	-	-
B44	Buffalo milk	Raw (Non pasteurized)	LNF	-	NA	-
B45	Buffalo milk	Raw (Non pasteurized)	LNF	-	-	-
B46	Buffalo milk	Raw (Non pasteurized)	LNF	-	+	+
B47	Buffalo milk	Raw (Non pasteurized)	LF	+	+	+
B48	Buffalo milk	Raw (Non pasteurized)	LF	+	-	-
B49	Buffalo milk	Raw (Non pasteurized)	LF	-	NA	-
B50	Buffalo milk	Raw (Non pasteurized)	LF	-	-	-
C1	Cow milk	Raw (Non pasteurized)	LNF	-	+	-
C2	Cow milk	Raw (Non pasteurized)	LNF	-	NA	-
C3	Cow milk	Raw (Non pasteurized)	LNF	-	-	-
C4	Cow milk	Raw (Non pasteurized)	-	-	+	-
C5	Cow milk	Raw (Non pasteurized)	LNF	-	-	-
C6	Cow milk	Raw (Non pasteurized)	LNF	-	NA	-
C7	Cow milk	Raw (Non pasteurized)	-	-	+	+
C8	Cow milk	Raw (Non pasteurized)	LNF	-	+	+
C9	Cow milk	Raw (Non pasteurized)	-	-	+	-
C10	Cow milk	Raw (Non pasteurized)	LF	+	+	-
C11	Cow milk	Raw (Non pasteurized)	LF	-	+	-
C12	Cow milk	Raw (Non pasteurized)	-	-	NA	-
C13	Cow milk	Raw (Non pasteurized)	LNF	-	+	+
C14	Cow milk	Raw (Non pasteurized)	LF	+	+	-
C15	Cow milk	Raw (Non pasteurized)	LF	+	+	-
C16	Cow milk	Raw (Non pasteurized)	LNF	-	+	-

Table S1A. contd.

C17	Cow milk	Raw (Non pasteurized)	LF	+	+	-
C18	Cow milk	Raw (Non pasteurized)	LNF	-	+	-
C19	Cow milk	Raw (Non pasteurized)	LNF	-	+	-
C20	Cow milk	Raw (Non pasteurized)	-	-	+	-
C21	Cow milk	Raw (Non pasteurized)	LF	+	+	-
C22	Cow milk	Raw (Non pasteurized)	LNF	-	+	+
C23	Cow milk	Raw (Non pasteurized)	LF	+	+	-
C24	Cow milk	Raw (Non pasteurized)	LNF	-	+	+
C25	Cow milk	Raw (Non pasteurized)	-	-	+	-
C26	Cow milk	Raw (Non pasteurized)	-	-	+	-
C27	Cow milk	Raw (Non pasteurized)	LNF	-	+	-
C28	Cow milk	Raw (Non pasteurized)	LNF	-	+	-
C29	Cow milk	Raw (Non pasteurized)	-	-	+	-
C30	Cow milk	Raw (Non pasteurized)	-	-	NA	-
C31	Cow milk	Raw (Non pasteurized)	LNF	-	NG	ND
C32	Cow milk	Raw (Non pasteurized)	LNF	-	NG	ND
C33	Cow milk	Raw (Non pasteurized)	LNF	-	NA	-
C34	Cow milk	Raw (Non pasteurized)	LNF	-	NG	ND
C35	Cow milk	Raw (Non pasteurized)	LNF	-	NA	-
C36	Cow milk	Raw (Non pasteurized)	LNF	-	+	+
C37	Cow milk	Raw (Non pasteurized)	LNF	-	+	+
C38	Cow milk	Raw (Non pasteurized)	-	-	+	+
C39	Cow milk	Raw (Non pasteurized)	-	-	+	+
C40	Cow milk	Raw (Non pasteurized)	LNF	-	+	+
C41	Cow milk	Raw (Non pasteurized)	LF	+	+	+
C42	Cow milk	Raw (Non pasteurized)	LNF	-	+	-
C43	Cow milk	Raw (Non pasteurized)	-	-	+	-
C44	Cow milk	Raw (Non pasteurized)	-	-	+	-
C45	Cow milk	Raw (Non pasteurized)	LNF	-	+	-
C46	Cow milk	Raw (Non pasteurized)	LF	+	+	-
C47	Cow milk	Raw (Non pasteurized)	LF	+	+	+
C48	Cow milk	Raw (Non pasteurized)	LNF	-	+	+
C49	Cow milk	Raw (Non pasteurized)	LNF	-	+	+
C50	Cow milk	Raw (Non pasteurized)	LNF	-	+	+

Table S1A. contd.

S1	Pasteurized 6MSL	UHT	LF	-	NA	-
S2	Pasteurized 6MSL	UHT	LF	-	NA	-
S3	Pasteurized 6MSL	UHT	LF	-	+	-
S4	Pasteurized 6MSL	UHT	LF	-	+	-
S5	Pasteurized 6MSL	UHT	LF	-	+	-
S6	Pasteurized 6MSL	UHT	LF	-	+	-
S7	Pasteurized 6MSL	UHT	NG	ND	+	-
S8	Pasteurized 6MSL	UHT	NG	ND	NG	ND
S9	Pasteurized 6MSL	UHT	NG	ND	NA	-
S10	Pasteurized 6MSL	UHT	NG	ND	+	-
S11	Pasteurized 6MSL	UHT	LF	-	NG	ND
S12	Pasteurized 6MSL	UHT	LF	+	+	-
S13	Pasteurized 6MSL	UHT	LF	+	+	-
S14	Pasteurized 6MSL	UHT	NG	ND	NG	ND
S15	Pasteurized 6MSL	UHT	NG	ND	+	+
S16	Pasteurized 6MSL	UHT	LF	-	+	+
S17	Pasteurized 6MSL	UHT	LF	-	NG	ND
S18	Pasteurized 6MSL	UHT	NG	ND	NG	ND
S19	Pasteurized 6MSL	UHT	NG	ND	NG	ND
S20	Pasteurized 6MSL	UHT	NG	ND	NG	ND
S21	Pasteurized 6MSL	UHT	NG	ND	NG	ND
S22	Pasteurized 6MSL	UHT	NG	ND	NG	ND
S23	Pasteurized 6MSL	UHT	NG	ND	NG	ND
S24	Pasteurized 6MSL	UHT	LNF	-	NG	ND
S25	Pasteurized 6MSL	UHT	NG	ND	NG	ND
S26	Pasteurized 6MSL	UHT	NG	ND	NG	ND
S27	Pasteurized 6MSL	UHT	NG	ND	NG	ND
S28	Pasteurized 6MSL	UHT	NG	ND	NG	ND
S29	Pasteurized 6MSL	UHT	LNF	-	NG	ND
S30	Pasteurized 6MSL	UHT	NG	ND	NG	ND
S31	Pasteurized 6MSL	UHT	NG	ND	NG	ND
S32	Pasteurized 6MSL	UHT	NG	ND	NG	ND
S33	Pasteurized 6MSL	UHT	NG	ND	NG	ND
S34	Pasteurized 6MSL	UHT	NG	ND	NG	ND

Table S1A. contd.

S35	Pasteurized 6MSL	UHT	NG	ND	NG	ND
S36	Pasteurized 6MSL	UHT	NG	ND	NG	ND
S37	Pasteurized 6MSL	UHT	NG	ND	NG	ND
S38	Pasteurized 6MSL	UHT	NG	ND	NG	ND
S39	Pasteurized 6MSL	UHT	NG	ND	NG	ND
S40	Pasteurized 6MSL	UHT	LNF	-	NG	ND
S41	Pasteurized 6MSL	UHT	NG	ND	NG	ND
S42	Pasteurized 6MSL	UHT	NG	ND	NG	ND
S43	Pasteurized 6MSL	UHT	NG	ND	NG	ND
S44	Pasteurized 6MSL	UHT	NG	ND	NG	ND
S45	Pasteurized 6MSL	UHT	NG	ND	NG	ND
S46	Pasteurized 6MSL	UHT	NG	ND	NG	ND
S47	Pasteurized 6MSL	UHT	NG	ND	NG	ND
S48	Pasteurized 6MSL	UHT	NG	ND	NG	ND
S49	Pasteurized 6MSL	UHT	NG	ND	NG	ND
S50	Pasteurized 6MSL	UHT	NG	ND	NG	ND
T1	Pasteurized 3MSL	Sterilized	LF	+	NG	ND
T2	Pasteurized 3MSL	Sterilized	LNF	-	NG	ND
T3	Pasteurized 3MSL	Sterilized	LF	+	+	+
T4	Pasteurized 3MSL	Sterilized	NG	ND	+	+
T5	Pasteurized 3MSL	Sterilized	LF	+	NA	-
T6	Pasteurized 3MSL	Sterilized	LF	+	NG	ND
T7	Pasteurized 3MSL	Sterilized	LF	+	NA	-
T8	Pasteurized 3MSL	Sterilized	NG	ND	+	-
T9	Pasteurized 3MSL	Sterilized	NG	ND	+	-
T10	Pasteurized 3MSL	Sterilized	NG	ND	+	-
T11	Pasteurized 3MSL	Sterilized	LF	+	+	-
T12	Pasteurized 3MSL	Sterilized	LNF	-	NA	-
T13	Pasteurized 3MSL	Sterilized	NG	ND	+	-
T14	Pasteurized 3MSL	Sterilized	NG	ND	+	-
T15	Pasteurized 3MSL	Sterilized	NG	ND	NG	ND
T16	Pasteurized 3MSL	Sterilized	NG	ND	+	-
T17	Pasteurized 3MSL	Sterilized	NG	ND	+	-
T18	Pasteurized 3MSL	Sterilized	LF	+	+	+

Table S1A. contd.

T19	Pasteurized 3MSL	Sterilized	LF	+	NA	-
T20	Pasteurized 3MSL	Sterilized	LF	+	NG	ND
T21	Pasteurized 3MSL	Sterilized	LF	+	+	-
T22	Pasteurized 3MSL	Sterilized	LF	+	NG	ND
T23	Pasteurized 3MSL	Sterilized	LF	+	NG	ND
T24	Pasteurized 3MSL	Sterilized	NG	ND	NG	ND
T25	Pasteurized 3MSL	Sterilized	NG	ND	+	-
T26	Pasteurized 3MSL	Sterilized	NG	ND	NG	ND
T27	Pasteurized 3MSL	Sterilized	NG	ND	NG	ND
T28	Pasteurized 3MSL	Sterilized	NG	ND	NG	ND
T29	Pasteurized 3MSL	Sterilized	LF	+	NA	-
T30	Pasteurized 3MSL	Sterilized	NG	ND	NA	-
T31	Pasteurized 3MSL	Sterilized	NG	ND	NA	-
T32	Pasteurized 3MSL	Sterilized	NG	ND	NG	ND
T33	Pasteurized 3MSL	Sterilized	NG	ND	NA	-
T34	Pasteurized 3MSL	Sterilized	NG	ND	NG	ND
T35	Pasteurized 3MSL	Sterilized	NG	ND	NG	ND
T36	Pasteurized 3MSL	Sterilized	NG	ND	NG	ND
T37	Pasteurized 3MSL	Sterilized	NG	ND	NG	ND
T38	Pasteurized 3MSL	Sterilized	NG	ND	NG	ND
T39	Pasteurized 3MSL	Sterilized	NG	ND	NA	-
T40	Pasteurized 3MSL	Sterilized	NG	ND	NG	ND
T41	Pasteurized 3MSL	Sterilized	LNF	-	NG	ND
T42	Pasteurized 3MSL	Sterilized	LNF	-	NG	ND
T43	Pasteurized 3MSL	Sterilized	NG	ND	NG	ND
T44	Pasteurized 3MSL	Sterilized	NG	ND	NA	-
T45	Pasteurized 3MSL	Sterilized	LNF	-	NA	-
T46	Pasteurized 3MSL	Sterilized	NG	ND	NG	ND
T47	Pasteurized 3MSL	Sterilized	LNF	-	NG	ND
T48	Pasteurized 3MSL	Sterilized	NG	ND	NG	ND
T49	Pasteurized 3MSL	Sterilized	LNF	-	NG	ND
T50	Pasteurized 3MSL	Sterilized	LNF	-	NG	ND
D1	Pasteurized 7DSL	ultra processed	NG	ND	+	-
D2	Pasteurized 7DSL	ultra processed	NG	ND	NA	-

Table S1A. contd.

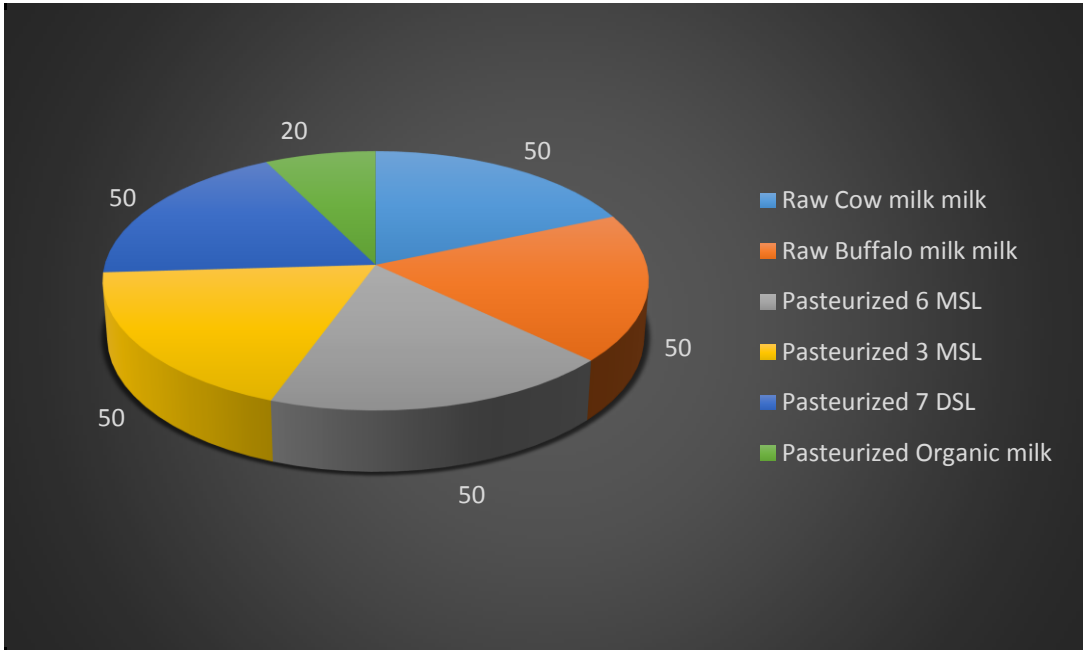
D3	Pasteurized 7DSL	ultra processed	NG	ND	NA	-
D4	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND
D5	Pasteurized 7DSL	ultra processed	NG	ND	NA	-
D6	Pasteurized 7DSL	ultra processed	NG	ND	NA	-
D7	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND
D8	Pasteurized 7DSL	ultra processed	NG	ND	NA	-
D9	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND
D10	Pasteurized 7DSL	ultra processed	LNF	-	NG	ND
D11	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND
D12	Pasteurized 7DSL	ultra processed	LNF	-	NG	ND
D13	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND
D14	Pasteurized 7DSL	ultra processed	LNF	-	NG	ND
D15	Pasteurized 7DSL	ultra processed	LNF	-	NG	ND
D16	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND
D17	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND
D18	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND
D19	Pasteurized 7DSL	ultra processed	NG	ND	NA	-
D20	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND
D21	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND
D22	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND
D23	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND
D24	Pasteurized 7DSL	ultra processed	NG	ND	NA	-
D25	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND
D26	Pasteurized 7DSL	ultra processed	NG	ND	NA	-
D27	Pasteurized 7DSL	ultra processed	NG	ND	NA	-
D28	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND
D29	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND
D30	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND
D31	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND
D32	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND
D33	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND
D34	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND
D35	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND
D36	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND

Table S1A. contd.

D37	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND
D38	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND
D39	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND
D40	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND
D41	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND
D42	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND
D43	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND
D44	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND
D45	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND
D46	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND
D47	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND
D48	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND
D49	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND
D50	Pasteurized 7DSL	ultra processed	NG	ND	NG	ND
O1	Pasteurized Organic	UHT	NG	ND	NA	-
O2	Pasteurized Organic	UHT	NG	ND	NA	ND
O3	Pasteurized Organic	UHT	NG	ND	NG	ND
O4	Pasteurized Organic	UHT	NG	ND	NA	-
O5	Pasteurized Organic	UHT	LNF	-	NG	ND
O6	Pasteurized Organic	UHT	LNF	-	NG	ND
O7	Pasteurized Organic	UHT	NG	ND	NG	ND
O8	Pasteurized Organic	UHT	NG	ND	NG	ND
O9	Pasteurized Organic	UHT	NG	ND	NA	-
O10	Pasteurized Organic	UHT	NG	ND	NA	-
O11	Pasteurized Organic	UHT	NG	ND	NG	ND
O12	Pasteurized Organic	UHT	NG	ND	NG	ND
O13	Pasteurized Organic	UHT	NG	ND	NG	ND
O14	Pasteurized Organic	UHT	NG	ND	NG	ND
O15	Pasteurized Organic	UHT	NG	ND	NG	ND
O16	Pasteurized Organic	UHT	NG	ND	NG	ND
O17	Pasteurized Organic	UHT	NG	ND	NG	ND
O18	Pasteurized Organic	UHT	NG	ND	NG	ND
O19	Pasteurized Organic	UHT	NG	ND	NG	ND
O20	Pasteurized Organic	UHT	NG	ND	NG	ND
Total	**270**			**40 predicted *E. coli***		**67 predicted *S. aureus***

Table S1A. contd.

LF	**Lactose fermenter**
LNF	**Lactose non-fermenter**
NG	**No growth**
NA	**Non aureus**
ND	**Not determined**
Macconkey	
LF	Red non mucoid colonies
LNF	White/colorless colonies
EMB	
+	Purple coloured colonies with green metallic sheen
-	Purple mucoid colonies
Mannitol	
+	Yellow colonies with yellow halo.
-	Pink colonies with pink halos
Baired parker (BP)	
+	Grey-black shiny convex 1-1.5 mm diameter (18 hours) up to 3 mm (48 hours) narrow white entire margin surrounded by zone of clearing 2-5mm
-	Brown colonies (Colonies which do not form the black pigmentation)



Sample source		Number of collected samples
Raw	Raw Cow milk milk	50
	Raw Buffalo milk milk	50
Pateurized	Pasteurized 6 MSL	50
	Pasteurized 3 MSL	50
	Pasteurized 7 DSL	50
	Pasteurized Organic milk	20
Total number of collected samples		270

MSL Month Shelf life

DSL Day Shelf life

Table S1B. Biochemical identification of purified ***E. coli*** isolates.

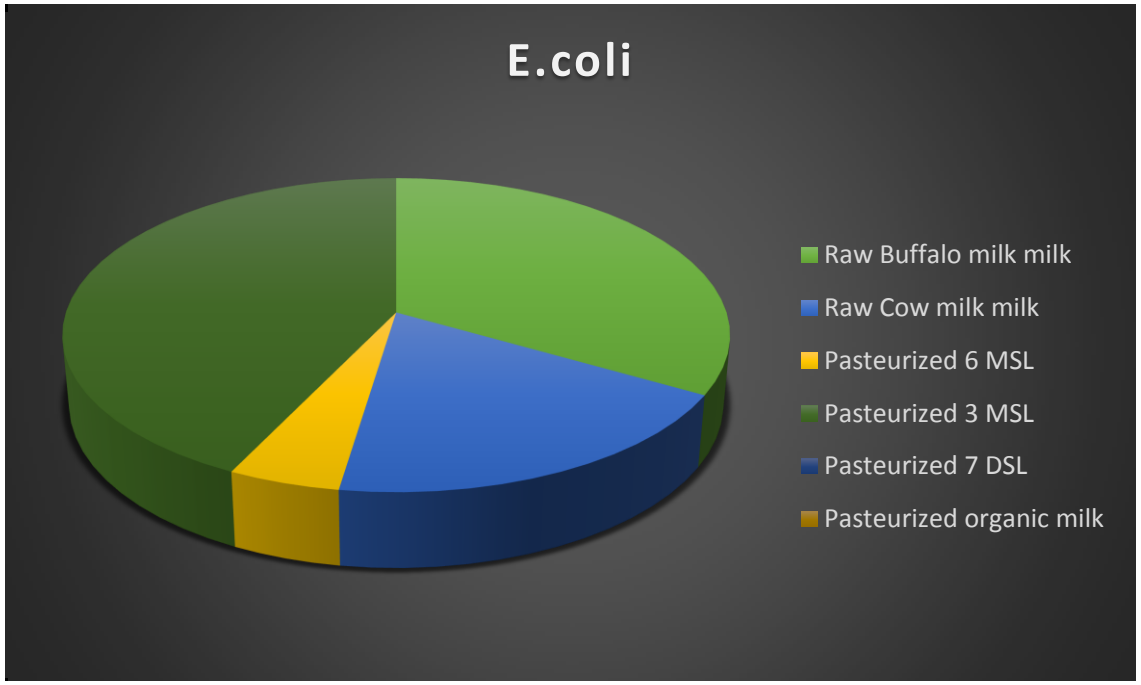
Sample code	Source	Indole test	MR test	VP test	Citrate test	TSI test	isolate code	Interpretation
B1	Buffalo milk	+	+	-	-	+	15	*E. coli*
B7	Buffalo milk	+	+	-	-	+	23	*E. coli*
B8	Buffalo milk	-	-	+	+	-	12	*Enterobacter aerogens*
B10	Buffalo milk	+	+	-	-		E16	*E. coli*
B11	Buffalo milk	-	-	+	+	+	21	Not *E. coli*
B13	Buffalo milk	-	-	+	+	+	13	Not *E. coli*
B14	Buffalo milk	-	-	+	+	+	14	Not *E. coli*
B20	Buffalo milk	-	+	+	+	+	11	Not *E. coli*
B22	Buffalo milk	-	-	+	+	+	9	Not *E. coli*
B24	Buffalo milk	-	+	+	+	+	40	Mixed
B31	Buffalo milk	+	-	+	+	+	22	Not *E. coli*
B41	Buffalo milk	+	+	-	-	+	E25	*E. coli*
B42	Buffalo milk	+	+	-	-	+	E26	*E. coli*
B47	Buffalo milk	+	+	-	-	+	E24	*E. coli*
B48	Buffalo milk	+	+	-	-	+	E39	*E. coli*
C10	Cow milk	-	+	+	+	-	17	Hafnia species
C14	Cow milk	+	-	+	+	+	19	Not *E. coli*
C15	Cow milk	-	+	+	+	-	31	Mixed
C17	Cow milk	+	-	+	+	+	8	Not *E. coli*
C21	Cow milk	+	-	+	+	-	18	Not *E. coli*
C23	Cow milk	+	+	-	-	+	E32	*E. coli*
C41	Cow milk	+	+	-	-	+	E30	*E. coli*
C46	Cow milk	+	+	-	-	+	E27	*E. coli*
C47	Cow milk	+	+	-	-	+	E29	*E. coli*
C48	Cow milk	+	-	+	+	-	28	Not *E. coli*
S12	Pasteurized 6MSL	-	-	+	+	+	1	*Enterobacter agglomerans*
S13	Pasteurized 6MSL	+	+	-	-	+	E41	*E. coli*
T1	Pasteurized 3MSL	-	-	+	+	-	3	mixed
T3	Pasteurized 3MSL	+	-	+	+	-	34	Not *E. coli*
T5	Pasteurized 3MSL	+	+	-	-	+	E35	*E. coli*
T6	Pasteurized 3MSL	+	+	-	-	+	E2	*E. coli*
T7	Pasteurized 3MSL	+	+	-	-	+	E37	*E. coli*
T11	Pasteurized 3MSL	+	+	-	-	+	E7	*E. coli*

Table S1B. contd.

T18	Pasteurized 3MSL	+	+	-	-	+	E5	*E. coli*
T19	Pasteurized 3MSL	+	+	-	-	+	E38	*E. coli*
T20	Pasteurized 3MSL	+	+	-	-	+	E6	*E. coli*
T21	Pasteurized 3MSL	+	+	-	-	+	E4	*E. coli*
T22	Pasteurized 3MSL	+	-	+	+	-	20	Not *E. coli*
T23	Pasteurized 3MSL	-	-	+	+	+	36	*Enterobacter aerogens*
T29	Pasteurized 3MSL	+	+	-	-	+	E33	*E. coli*
Total	**40**							**21 *E. coli***

Triple sugar iron (TSI) test	Butt	Slope	H2S
+	Acid/Gas	Acid	-

Indole test	
+	red color change
-	no color change
Methyl Red (MR)	
+	stable red color
-	yellow color
Voges-Proskauer (VP) test	
+	pink-red color on the surface of the medium 15 minutes to one hour after the addition of the reagents.
-	yellow color on the surface of the medium
Citrate test	
+	colour change of medium to blue
-	no colour change of medium



Types of milk samples	***E. coli***
Raw Buffalo milk milk	7
Raw Cow milk milk	4
Pasteurized 6 MSL	1
Pasteurized 3 MSL	9
Pasteurized 7 DSL	0
Pasteurized organic milk	0
Total	21

Table S1C. Biochemical identification of purified *S.aureus* isolates.

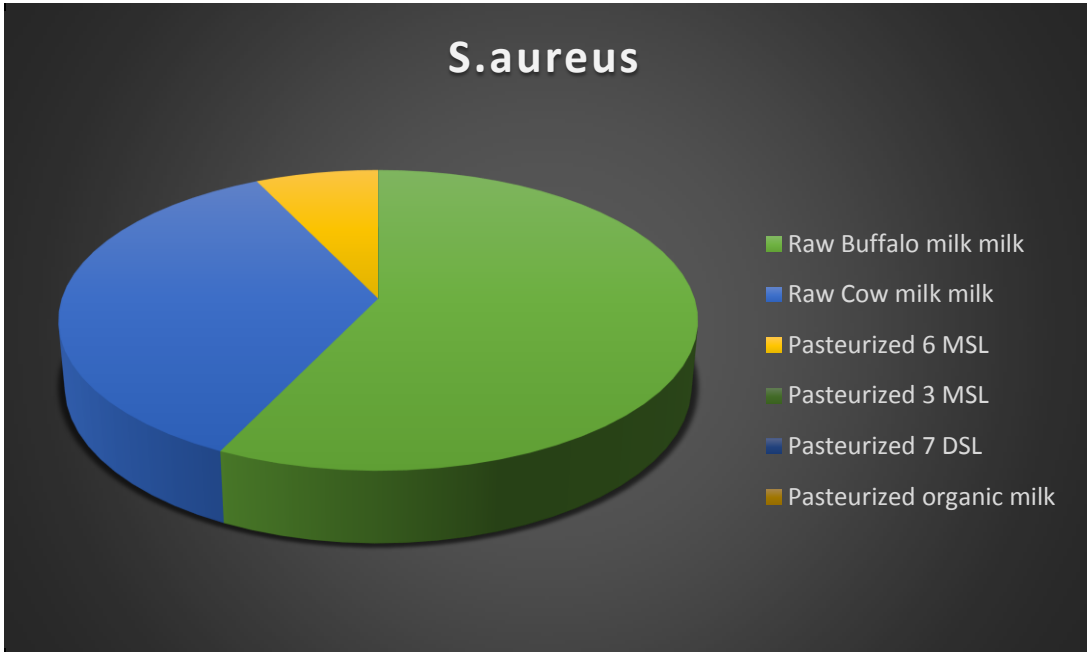
Sample code	Source	Coagulase test	DNase test	Isolate code	Interpretation
B1	Buffalo milk	-	-	46	Not *S. aureus*
B2	Buffalo milk	+	+	S67	*S. aureus*
B3	Buffalo milk	-	-	78	Not *S. aureus*
B4	Buffalo milk	-	-	65	Not *S. aureus*
B5	Buffalo milk	-	-	104	Not *S. aureus*
B6	Buffalo milk	-	-	49	Not *S. aureus*
B7	Buffalo milk	-	-	79	Not *S. aureus*
B8	Buffalo milk	-	-	69	Not *S. aureus*
B9	Buffalo milk	+	+	S57	*S.aureus*
B10	Buffalo milk	-	-	72	Not *S. aureus*
B11	Buffalo milk	-	-	45	Not *S. aureus*
B12	Buffalo milk	-	-	44	Not *S. aureus*
B13	Buffalo milk	-	-	54	Not *S. aureus*
B14	Buffalo milk	-	-	42	Not *S. aureus*
B15	Buffalo milk	-	-	70	Not *S. aureus*
B16	Buffalo milk	+	+	S48	*S.aureus*
B17	Buffalo milk	+	+	S68	*S.aureus*
B18	Buffalo milk	+	+	S61	*S.aureus*
B20	Buffalo milk	-	-	71	Not *S. aureus*
B22	Buffalo milk	-	-	47	Not *S. aureus*
B24	Buffalo milk	+	+	S81	*S.aureus*
B26	Buffalo milk	-	-	58	Not *S. aureus*
B28	Buffalo milk	-	-	88	Not *S. aureus*
B29	Buffalo milk	-	-	83	Not *S. aureus*
B31	Buffalo milk	-	-	43	Not *S. aureus*
B34	Buffalo milk	-	-	62	Not *S. aureus*
B36	Buffalo milk	-	-	112	Not *S. aureus*
B37	Buffalo milk	+	+	S119	*S.aureus*
B41	Buffalo milk	-	-	116	Not *S. aureus*
B42	Buffalo milk	+	+	S113	*S.aureus*
B46	Buffalo milk	-	-	117	Not *S. aureus*
B47	Buffalo milk	-	-	115	Not *S. aureus*

Table S1C. Contd.

C4	Cow milk	-	-	56	Not *S. aureus*
C7	Cow milk	+	+	S55	Not *S. aureus*
C8	Cow milk	-	-	85	Not *S. aureus*
C9	Cow milk	-	-	99	Not *S. aureus*
C11	Cow milk	-	-	110	Not *S. aureus*
C13	Cow milk	+	+	S93	*S.aureus*
C14	Cow milk	-	-	96	Not *S. aureus*
C15	Cow milk	-	-	63	Not *S. aureus*
C16	Cow milk	-	-	108	Not *S. aureus*
C17	Cow milk	-	-	77	Not *S. aureus*
C18	Cow milk	-	-	50	Not *S. aureus*
C19	Cow milk	-	-	97	Not *S. aureus*
C20	Cow milk	-	-	73	Not *S. aureus*
C21	Cow milk	-	-	132	Not *S. aureus*
C22	Cow milk	+	+	S95	*S.aureus*
C23	Cow milk	-	-	82	Not *S. aureus*
C24	Cow milk	+	+	S75	*S.aureus*
C25	Cow milk	-	-	122	Not *S. aureus*
C26	Cow milk	-	-	74	Not *S. aureus*
C28	Cow milk	-	-	123	Not *S. aureus*
C29	Cow milk	-	-	124	Not *S. aureus*
C39	Cow milk	-	-	120	Not *S. aureus*
C40	Cow milk	-	-	121	Not *S. aureus*
C48	Cow milk	+	+	S118	*S.aureus*
S16	Pasteurized 6MSL	+	+	S94	*S.aureus*
T3	Pasteurized 3MSL	-	-	64	Not *S. aureus*
T4	Pasteurized 3MSL	-	-	66	Not *S. aureus*
T8	Pasteurized 3MSL	-	-	91	Not *S. aureus*
T9	Pasteurized 3MSL	-	-	84	Not *S. aureus*
T10	Pasteurized 3MSL	-	-	102	Not *S. aureus*
T11	Pasteurized 3MSL	-	-	105	Not *S. aureus*
T16	Pasteurized 3MSL	-	-	111	Not *S. aureus*
T17	Pasteurized 3MSL	-	-	51	Not *S. aureus*

Table S1C. Contd.

T18	Pasteurized 3MSL	-	-	53	Not *S. aureus*
T25	Pasteurized 3MSL	-	-	103	Not *S. aureus*
D1	Pasteurized Organic	-	-	80	Not *S. aureus*
Total	**67**	**14**	**14**		
Coagulase					
+	Clumping				
-	No clumping				
DNase					
+	Clear zone precipitate around the test organism				
-	No Clear zone around the test organism				



Types of milk samples	***S. aureus***
Raw Buffalo milk milk	8
Raw Cow milk milk	5
Pasteurized 6 MSL	1
Pasteurized 3 MSL	0
Pasteurized 7 DSL	0
Pasteurized organic milk	0
Total	14

Vol. 13(28), pp. 609-618, October, 2019
DOI: 10.5897/AJMR2019.9179
Article Number: 8F6E4DF62134
ISSN: 1996-0808

http://www.academicjournals.org/AJMR

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African Journal of Microbiology Research

Full Length Research Paper

Isolation and characterization of potential phosphate solubilizing bacteria in two regions of Senegal

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Received 24 July, 2019; Accepted 3 September, 2019.

Many soils of the inter-tropical regions are P-deficient because of their high fixing power and low P content. Rock phosphate resources used to produce the phosphate fertilizers are exhausted and chemical fertilizer are causing environmental degradation. This issue raised the question of sustainability of fertilization and subsequently has enhanced the interest in the use of microorganisms as biofertilizers. The aim of this study is to isolate and characterize potential P solubilizing bacteria (PSB) from two P deficient agricultural regions in Senegal. Twelve potential PSB were selected and further screened for other plant growth promoting traits (Indole-3-acetic acid (auxin) and siderophore production) and characterized by 16S rDNA sequencing. All the isolates produced auxin and seven of them produced siderophore. DNA sequencing showed that five isolates were affiliated to the genus *Bacillus,* four to the genus *Staphylococcus,* two to the genus *Microbacterium* and one isolate showed high similarities with members of the genus *Burkholderia.* The selected bacteria will further be tested on some plants to assess their biofertilization potential.

Key words: 16S rDNA, indole-3-acetic-acid (IAA), phosphate solubilizing bacteria (PSB), siderophore.

INTRODUCTION

Early studies proved the existence of a group of soil free living bacteria stimulating plant growth, which was called plant growth promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1978). Since, then, mechanisms for stimulating plant growth were described. PGPR may produce various compounds, including growth regulators

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(phytohormones), siderophores, and organic acids. Some are able to fix atmospheric nitrogen, solubilize phosphorus and produce antibiotics to suppress harmful rhizobacteria (Sureshbabu et al., 2016). These mechanisms either directly affect the metabolism of plants or improve the adaptive capacity of plants to acquire other nutrients from the soil (Santoro et al., 2015). PGPR that are able to mineralize soil phosphorus are called phosphate solubilizing bacteria (PSB). Phosphate solubilization is considered to be most important attribute of plant growth promoting rhizobacteria (Kloepper et al., 1989).

Phosphorus (P) plays an important role in plant physiology and is frequently the prime limiting factor for plant growth in terrestrial ecosystems (Bunemann et al., 2011). Phosphate is the second most important element for mineral nutrition of plants and by far the least mobile and available to plants in most soil conditions (Hinsinger, 2001). Phosphate is present in the soil at levels of 400 to 1,200 mg kg^{-1}; however, soluble P concentrations in soil are generally very low, at levels of 1 mg kg^{-1} or less (Goldstein, 1994). The poor availability of soil inorganic phosphate is due to the large reactivity and retention of these phosphate ions with other metals (Fe, Al, Ca) (Rodriguez and Fraga, 1999; Hinsinger, 2001).

Application of P-containing fertilizers is common for stimulating crop yields. However, repeated applications of phosphate fertilizers affect environment, microbial diversity and can lead to loss of soil fertility and consequently lower crop yields (Gyaneshwar et al., 2002). Thus, it is a great challenge to search for strategies that may alleviate detrimental effects of current intensive farming practices that use chemical fertilizers. An attractive alternative to the phosphatic fertilizers is the use of PSB as biofertilizer that have been shown to enhance plant growth and improve P availability in the soils (Pereira and Castro, 2014). Nevertheless, plant growth enhancement may also be related to other PGP traits that may act in synergy with P solubilization like indol-3-acetic acid and siderophore production (Pereira and Castro, 2014). Moreover, plant growth enhancement seems to be related not only to P solubilization but also to other PGP traits, like indol-3-acetic acid and siderophore (Pereira and Castro, 2014). Inoculation success also is related to the persistence of the introduced strain, that is, its ability to establish high population levels and to live as a continuing member of the soil microflora even in the absence of plant (Lupwayi et al., 2006). Introduced bacteria are not always competitive with native soil microbial communities (Herrmann and Lesueur, 2013), as they have to compete for niches and nutrients in new environmental conditions.

With the aim to develop a biofertilizer adapted to soils in major cultivation areas of Senegal, PGPR were isolated from P-deficient soils in two agricultural regions (Kaffrine and Kolda). PSB were first isolated from rhizosphere soil and from non-rhizospheric soils, screened for other plant growth promoting traits (indole-3-acetic acid (IAA) and siderophore production) and characterized at the molecular level by 16S rDNA sequencing.

MATERIALS AND METHODS

Soil sampling

Rhizospheric soils and non rhizhospheric soil (bare soil) were collected in Senegal from two sites (Kolda: 12°50'N - 14°50'W and Kaffrine: 13° 57'N- 15° 35'W) (Figure 1). Bare soils were sampled from the top 20 cm soil free of litter. Rhizospheric soils were sampled from roots of *Guiera senegalensis*, *Piliostigma reticulatum* and *Dichrostachys glomerata*. Roots with adhering soil were put in a plastic bag, shaken with hands for 5 min to collect rhizospheric soil and removed. Seventy-six (76) samples were carefully collected in bags and stored at 4°C temperature for the isolation of bacterial strains (PSB). Table 1 indicates the P level in collected soils, showing that even total P content is low.

Isolation and characterization of PSB

For isolation of PSB, 10 g soil samples were suspended in 90 ml of NaCl buffer. A serial dilution assay was carried out in 0.9% NaCl buffer (NaCl: 4.38 g/500 ml, KH_2PO_4: 0.135 g/500 ml; Na_2HPO_4+ $2H_2O$ or Na_2HPO_4: 0.284 g/500 ml) solution. An aliquot of 0.1 ml of each dilution (10^{-2}, 10^{-3} and 10^{-4}) was spread onto Petri plates containing Pikovskaya (PKV) agar (Pikovskaya, 1948). The composition of PKV medium was ($g.l^{-1}$): glucose: 10.0; $Ca_3(PO4)_2$: 5; $(NH_4)_2SO_4$: 0.50; KCl: 0.20; $Mg_2SO_4 \cdot 7H_2O$: 0.010; $Mn_2SO_4 \cdot H_2O$: 0.0001; $Fe_2SO_4 \cdot 7H_2O$: 0.0001, yeast extract: 0.50; pH was adjusted to 7.0. The plates were incubated at room temperature (28°C) for 7 days. Colonies showing a clear zone around the colony was considered as P-solubilizer. The P-solubilizers were purified by repeated streaking and stocked for further use.

Biochemical characterization of PSB isolates

Phosphate solubilization

An aliquot of 0.1 ml of each PSB culture preserved was placed on Pikovskaya's agar (PA) (Petri dish) (Pikovskaya, 1948). The plates were incubated at room temperature (28°C) for 7 days. The solubilization zone was determined by subtracting the diameter of bacterial colony from the diameter of total zone. Solubilization index (SI) was calculated using the formula:

$$SI = \frac{\text{Colony diameter} + \text{halozone diameter}}{\text{Colony diameter}}$$

Solubilization of tri-calcium phosphate was quantified in Pikovskaya's broth. Each flask containing 75 ml medium (PVK) was inoculated with 500 µl of bacterial culture (three replicates were performed for each isolate) and incubated at 28 ± 0.1°C at 140 revolutions per minute ($rev.min^{-1}$) for 4 days in incubator. Simultaneously, a non-inoculated control (free PVK medium) was also kept under similar conditions. Cultures were harvested by centrifugation at 13,000 g for 10 min. The soluble P expressed as $mg.l^{-1}$ in bacterial isolates was quantified by the colorimetric method of Olsen and Sommers (1982).

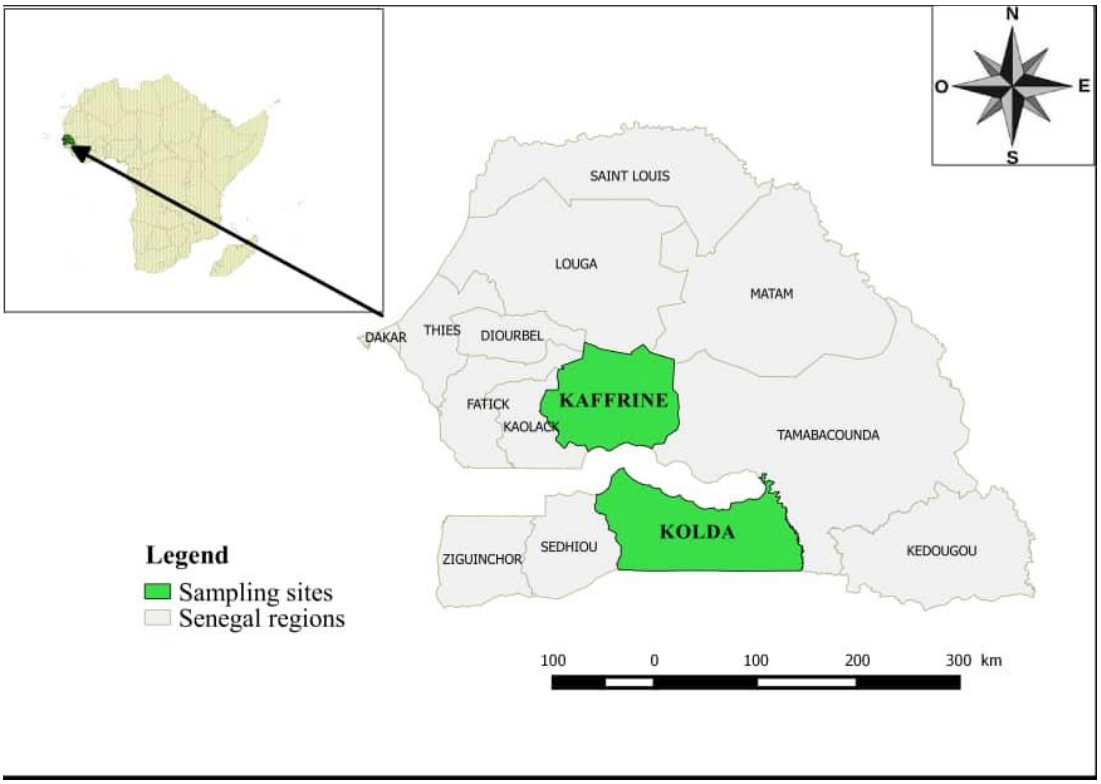

Figure 1. Localization of the two sites.

Table 1. Phosphorus in soils.

Site	Total P	P Olsen
	ppm	
Kolda	60.50	nd
Kaffrine	63.45	5.29

Total P: Total phosphorus, P Olsen: assimilable phosphorus, nd: not determined.

Screening of indole-3-acetic acid (IAA)-producing bacteria

For detection of IAA production by PSB strains, Luria-Bertani solid medium (LBT) enriched with L-tryptophan (1 g l^{-1}) was prepared and flowed into the dishes (Bric et al., 1991). A nitrocellulose membrane was placed directly on the LBT medium and inoculated with the isolates using a loop. The Petri dishes were then incubated at 28°C, for 2 to 4 days (the time required for the colonies to reach a diameter of 2 mm). A 9 cm Whatman qualitative filter paper (No. 2) was impregnated with 2.5 ml of Salkowski's solution (30.8 ml of water, 19.3 ml of 96% pure H_2SO_4 sulfuric acid and 0.6 g of trichloride of iron) (Gordon and Weber, 1951). The nitrocellulose membrane, showing growths of colonies were dropped on a filter paper impregnated with Salkowski's reagent. Bacteria that synthesized IAA were identified by the formation of a characteristic red halo that surrounds the colony.

The production of IAA was quantified following the method of Salkowski (Gravel et al., 2007). The isolates were first cultured in Tryptic Soy Broth (TSB) for 24 h at 28°C, then 30 µl of the pure culture was inoculated into test tubes containing 3 ml of Luria Bertani (LB) medium supplemented with 1 $g.l^{-1}$ L- tryptophan. The test tubes containing the bacterial isolates were incubated for 5 days with shaking (200 $rev.min^{-1}$) at 28°C. The determination of the IAA concentration was carried out by the addition of 100 µl of the Salkowski solution [30.8 ml water, 19.3 ml of pure sulfuric acid H_2SO_4 96% and 0.6 g of Iron (III) chloride] (Gordon and Weber, 1951) to 100 µl of the culture supernatant which had been previously centrifuged for 20 min at 13,000 g. After 20 min incubation at room temperature, the optical density at 535 nm was recorded. IAA production is indicated by the presence of a pinkish color. The following dilutions: 10, 25; 100, 200 and 400 $ng.ml^{-1}$ of IAA (Sigma I-2886) was used to establish a standard curve (r^2 = 0.9997). This range of dilutions was prepared from a 10^{-3} M auxin stock solution by diluting 17.5 mg of auxin in 1 ml of absolute ethanol and then adjusting the volume to 100 ml with sterile demineralized water (Gupta et al., 2014). The amount of IAA produced was expressed as $ng.ml^{-1}$ by comparison with the standard curve.

Siderophore assay

The production of siderophore in liquid and solid medium was tested in King B medium and the Chrome azurol-S (CAS) following the methodology described by Schwyn and Neilands (1987) and modified by Milagres et al. (1999). The King B medium was prepared and mixed with CAS in the following proportions: 100 ml of CAS + 900 ml of King B. After solidification, the plates were inoculated with the pure culture of bacteria (30 µl) and incubated at 28°C for 4 days. The presence of an orange-yellow halo around the strain is described as positive for the production of siderophore. This color change is due to the transfer of ferric ions from the CAS to the siderophore.

The siderophore production of isolates was also tested by the method of Ribeiro and Cardoso (2012). This involves inoculating tubes containing 3 ml of liquid KB medium with isolates and incubating for 7 days at 28°C under constant agitation. After this period, 1 ml of the culture was added to 1.5 ml microtubes and centrifuged for 5 min at 14,000 g. Then 100 µl of the supernatant from each culture was added to a microplate well containing 100 µl from the reagent chrome azurol S (CAS) and incubated for 30 min. The orange or yellow coloring of the medium indicates the production of siderophores by bacteria. The uninoculated KB medium was used as negative control. Absorbance was read at 630 nm. The following equation was used to calculate the percent activity of siderophore produced.

$$\%\ Siderophore\ units = \frac{Ar - As}{As}\ X\ 100$$

where Ar is the absorbance of reference (CAS assay solution + uninoculated media) and As is the absorbance of the sample (CAS assay solution + cell-free supernatant).

Molecular identification of PSB

DNA extraction

The identification of PSB was done by 16S rDNA gene sequencing. The genomic DNA of PSB was extracted by the E.Z.N.A. Bacterial DNA kit of OMEGA bio-tek (400 Pinnacle Way Suite 450 Norcross, GA, 30071 USA) was according to instructions of the manufacturer.

PCR amplification of bacterial 16S rDNA

The variable region (V3) of the 16S rDNA gene was targeted for the identification of PSB bacterial strains. The gene encoding the 16S subunit ribosomal DNA (rDNA) is mostly used because of its structure highly conserved in all bacteria which is very useful for the identification of universal primers (Hillis and Dixon, 1991). Universal bacterial primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'- AAGGAGGTGATCCAGCCGCA-3') (Weisburg et al., 1991) were used for amplification of the V3 region. The PCR reaction mixture was composed of 0.25 µl dNTP (10 mM each dNTP), 2.5 µl $MgCl_2$ buffer (25 mM), 0.22 µl GoTaq (X5), 5 µl GoTaq buffer (5 U/µl), 1 µl of the DNA sample, 1.25 µl (of each) Primers (dD1 and rD1 at 10 µM) and sterile H_2O to reach 25 µl (final volume per sample). The following cycling conditions were used: a first denaturation phase (5 min at 94°C) was followed by 35 cycles of 94°C for 30 s (denaturation), 55°C for 1 min (Hybridization) and 72°C for 1 min (Elongation), and finally a final elongation step at 72°C for 5 min. The PCR amplification was carried out using a Gene Amp PCR System 9700 thermocycler (Applied Biosystem).

Sequencing and phylogeny analyses

The PCR products were sequenced by GENEWIZ (USA). The sequences were compared with National Center of Biotechnology Information-USA (NCBI) database using BLAST method. The evolutionary history was inferred by the Maximum Likelihood method based on the Kimura 2-parameter model with 600 bootstraps. Phylogenetic analysis was conducted using MEGA 7.0.

Statistical analysis

Determination of significant differences between strains for quantitative PGP production was performed by using one-way analysis of variance (ANOVA) and non-parametric test. Fisher's and Kruskal Wallis's paired multiple comparison post-hoc tests with the software XLSTAT (XLSTAT 2016 Addinsoft, France) was carried out if the difference between the treatments was significant. Least significant differences (LSD) were calculated at the 5% level.

RESULTS

Selection of PSB and biochemical characterization of PS activity

PSB isolation and solubilization index (SI)

After 7 days of incubation at room temperature (28°C), bacterial isolates producing transparent halos on PVK solid medium were considered as PSB. A total of 12 strains solubilizing phosphate were obtained (Table 2). One strain was obtained from non-rhizospheric soil (SN1) and the other 11 from rhizospheric soil of the followed species: *P. reticulatum* (PR3, PR4 and PR5), *G. senegalensis* (GS9, GS10, GS12, GS13, GS14, GS16 and GS17) and *D. glomerata* (DG7).

The SI determined ranged from 11.61 to 21.40 mm between the isolates (Table 2). Eleven (11) isolates exhibit a SI higher than 15 mm. The lowest SI was recorded in isolate GS10 with 11.61 mm. The highest SI was found with GS17 (21.40 mm), DG7 (20.40 mm) and GS14 (20.35 mm).

P-solubilization in liquid culture

When cultivated in liquid PVK medium, strains showed high variation for their ability to solubilize tricalcium phosphate, from 53.54 to 423.41 $mg.l^{-1}$ (Figure 2). The isolate GS17 originating from rhizosphere soil of *G. senegalensis* in Kaffrine solubilized significantly higher phosphate than all other bacterial strains. The lowest solubilization capacity was obtained from GS9 (53.54 $mg.l^{-1}$). The solubilization capacity was not correlated to the origin of the strain.

IAA production

All the bacterial isolates induced a red halo surrounding

Table 2. Origin, solubilization index, indole acetic acid (IAA) and siderophore detection.

Isolates code	Sites	Soil origin	Solubilization index (SI, mm)	IAA production	Siderophore production
SN1	Kolda	Non-rhizospheric	19,40^{bc} (±1.22)	+	+
PR3	Kolda		17,23^{e} (±0.34)	+	-
PR4	Kolda	*Piliostigma reticulatum*	12.76^{g} (±0.50)	+	+
PR5	Kolda		17.71^{de} (±1.40)	+	-
DG7	Kolda	*Dichrostachys glomerata*	20.40^{ab} (±0.56)	+	-
GS9	Kaffrine		19.03^{bcd} (±0.28)	+	+
GS10	Kaffrine		11.61^{gh} (±0.44)	+	+
GS12	Kaffrine		16.56^{ef} (±0.63)	+	+
GS13	Kaffrine	*Guiera senegalensis*	15.22^{f} (±0.78)	+	-
GS14	Kaffrine		20.35^{ab} (±1.45)	+	+
GS16	Kaffrine		18.01^{cde} (±0.40)	+	-
GS17	Kaffrine		21.40^{a} (±0.77)	+	+

(-) Not detected; (+) production. Means of three replicates followed by different letters differ by Fisher's test (P = 5%).

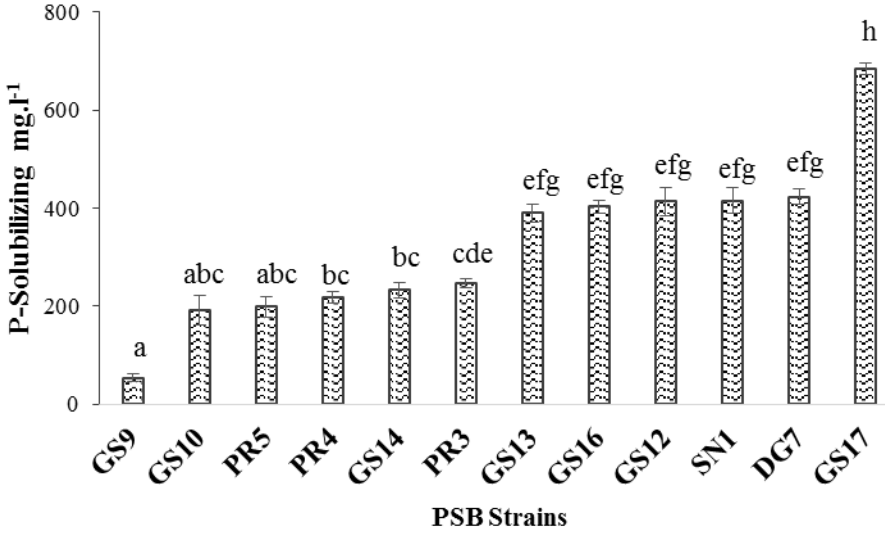

Figure 2. Phosphate solubilization by phosphate solubilizing bacteria (PSB) strains in PVK liquid culture medium. Means of three replicates followed by different letters differ by Kruskal Wallis's test (P=5%). The bars represent the standard deviations of the mean.

the colony, showing IAA activity **(**Table 2**)**. Quantitative estimation of IAA production was made by comparison with standard curve. Production of IAA by the isolates ranged from 47.94 to 248.02 µg.ml^{-1} (Figure 3). The highest concentration (248.02 µg.ml^{-1}) was produced by the isolate GS12 in liquid LB broth (Figure 3). The lowest IAA production was shown by PR3, GS16, DG7 and GS17.

Siderophore production

Incubation in King B and Chrome-azurol-S (CAS) solid medium showed seven isolates with yellow coloring both in solid and liquid medium, demonstrating their capacities to produce siderophores (Figure 4 and Table 2). Quantitative estimation of siderophore using Chrome-azurol-S (CAS) liquid assay revealed that SN1 is the

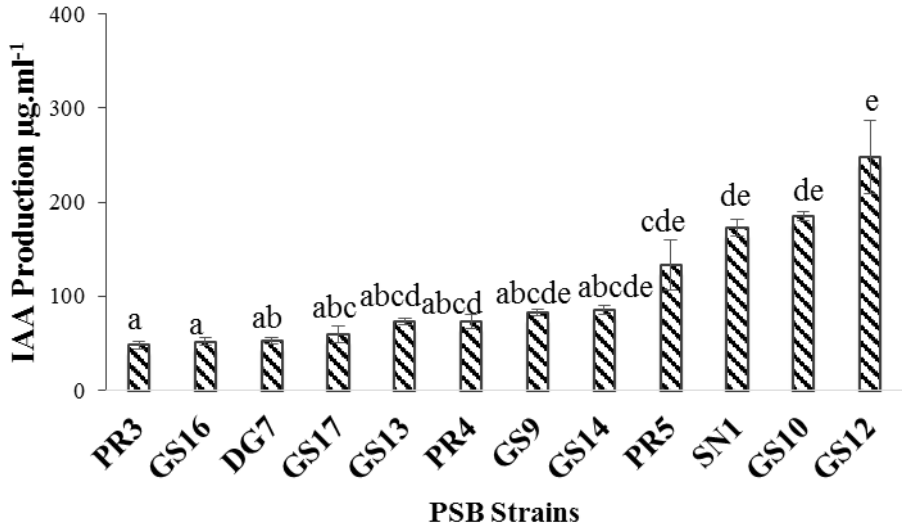

Figure 3. Production of indole-3-acetic acid (IAA) by phosphate solubilizing bacteria (PSB) in LB liquid culture medium after 5 days of incubation. Means of three replicates followed by different letters differ by Kruskal Wallis's test (P = 5%). The bars represent the standard deviations of the mean.

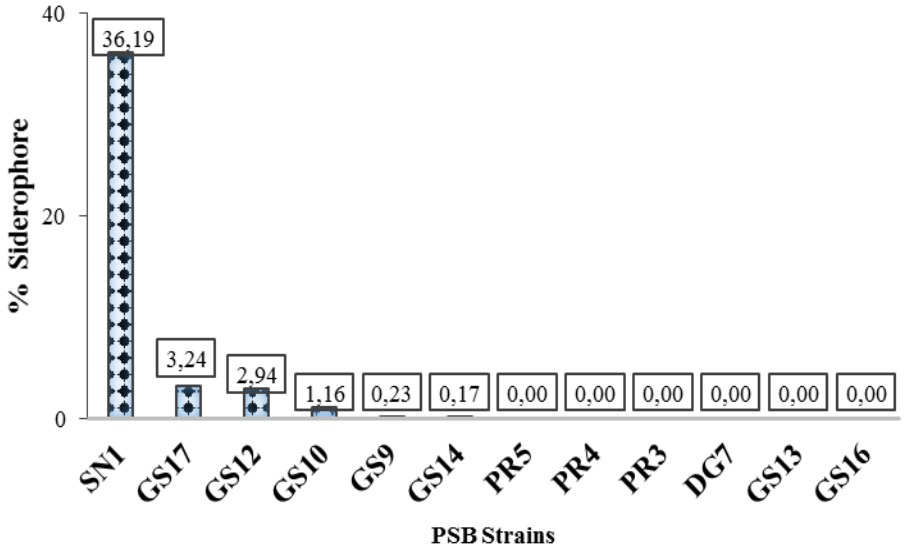

Figure 4. Siderophore production rate by phosphate solubilizing bacteria (PSB) strains in King B and CAS medium.

highest siderophore rate (36.19%) (Figure 4). Minimum siderophore production (0.17 and 0.23%) was found in isolates GS14 and GS9.

Molecular identification

Analysis of 16S rDNA gene sequences using data available in GenBank showed that five strains belong to genus *Bacillus*, four to genus *Staphylococcus,* two to genus *Microbacterium and* one to genus *Burkholderia* (Table 3 and Figure 5). Among the seven PSB isolated from *G. senegalensis* rhizosphere in Kaffrine region, four were *Bacillus* species, two isolates presenting a single nucleotide difference clustered with *Microbacterium maritypicum*. The remaining isolate (GS14) presented 100% sequence similarities to strains isolated from Kolda either from non rhizospheric soil (SN1) or from the

Table 3. Strains molecular identification. Sequence size (kilobasepairs: kb) percent similarity and accession number of sequences.

Isolate	Size of the sequence 16S rDNA (Kb)	Accession number	Strains Identification	% Similarity
GS12	1.067	MK209023	*Bacillus aryabhattai strain B8W22*	100
GS13	0.970	MK209024	*Bacillus cereus* strain ATCC 14579	100
PR3	0.916	MK209017	*Bacillus tropicus* strain MCCC 1A01406	100
GS9	0.925	MK209021	*Bacillus zhangzhouensis* strain MCCC 1A08372	100
GS10	0.998	MK209022	*Bacillus subtilis* strain IAM 12118	100
PR5	0.999	MK209019	*Burkholderia cepacia* strain ATCC 25416	99
GS16	0.929	MK209026	*Microbacterium maritypicum* strain DSM 12512	99
GS17	0.973	MK209027	*Microbacterium maritypicum* strain DSM 12512	99
GS14	1.017	MK209025	*Staphylococcus gallinarum* strain VIII1	99
PR4	1.039	MK209018	*Staphylococcus gallinarum* strain VIII1	99
DG7	1.035	MK209020	*Staphylococcus gallinarum* strain VIII1	99
SN1	0.995	MK209014	*Staphylococcus gallinarum* strain VIII1	99

rhizosphere of *D. glomerata* (DG7) and showed two base differences with one strain isolated from *P. reticulatum* rhizosphere (PR4); these four strains presented either 1 or 2 base substitutions with *Staphylococcus gallinarum* strain VIII1. Finally, the two remaining Kolda isolates recovered from the rhizosphere of *P. reticulatum* were clustering either with several *Bacillus* spp. including *B. cereus* with which they shared 100% sequence similarities (PR3) or with a beta-proteobacterium belonging to the genus *Burkhloderia* (PR5).

DISCUSSION

Origin and identification of PSB

This study is the first step of a project aiming to develop PGPR based biofertilizers adapted to main agricultural regions of Senegal. Our approach is based on a primary isolation of P solubilizing bacteria from P-deficient soils, in two main agricultural regions of Senegal (Kolda and Kaffrine). Thus, the entry point was the capacity of bacteria for P solubilization using TCP-based medium that were further analyzed for other PGPR traits. Most PSB were isolated from rhizospheric soil of shrubs found locally (*G. senegalensis* in Kaffrine; *P. reticulatum*, *D. glomerata* in Kolda). This result confirms those of de Abreu et al. (2017), which showed that PSBs are ubiquitous in soils. Of the 12 strains isolated in different areas, 11 were isolated in rhizospheric soil and only one was recovered from non-rhizospheric soil. This result suggests that rhizospheric soils are more likely to harbor PSB than non-rhizospheric or bulk soil. Similar results were reported by Baliah et al. (2016) who found abundant populations of PSB in rhizosphere soil compared to non rhizospheric soil. Indeed, the secretion of carbohydrates and amino acids from roots enhances the growth and multiplication of bacterial species and constitutes a biotope suitable for microorganisms growth (Bertin et al., 2003). Phosphate solubilizing bacteria are known to be abundant in the rhizospheric soils of various plants (Ashok et al., 2012), but their presence varied considerably according to plant species (Reyes et al., 2007). According to Marschner et al. (2004)**,** abundance and diversity of microorganisms in the rhizosphere are likely to be related to plant species due to differences in root exudation and rhizodeposition. In this study, more PSB were obtained from *G. senegalensis* and *P. reticulatum* rhizospheric soils*.* These species are known to be very useful in the maintenance of soil fertilization through root exudates and litter inputs (Wezel et al., 2000; Diakhate, 2016).

Strains belonging to the genera *Bacillus, Microbacterium, Staphylococcus* and *Burkholderia* were identified in this study. Those strains have already been found to be PSB in other studies (Rodriguez and Fraga, 1999). According to some authors (Bouizgarne, 2013; Kumar et al., 2016), those genera are particularly effective P-solubilizers. In the present study, we found that the majority of isolated strains belong to the genus *Bacillus*, however, the greatest ability to solubilize phosphate was found in strains belonging to the genus *Microbacterium*.

Even though many studies reported the isolation of PSB strains using TCP-based medium, it is important to note that, others are questioning the effectiveness of TCP- based medium to assess the capacity of bacteria for P-solubilization (Bashan et al., 2012, 2013). Other media and P sources such as Metal-P (Al-P, Fe-P, Ca-P) are recommended by those authors, depending on the type of soil and the end use of the targeted bacteria: calcium phosphate (including natural phosphates) for alkaline soils, and iron or aluminium phosphates for acidic soils. These screens would probably maximize the chances of selecting the most effective strains able to contribute to the phosphate nutrition of plants but would

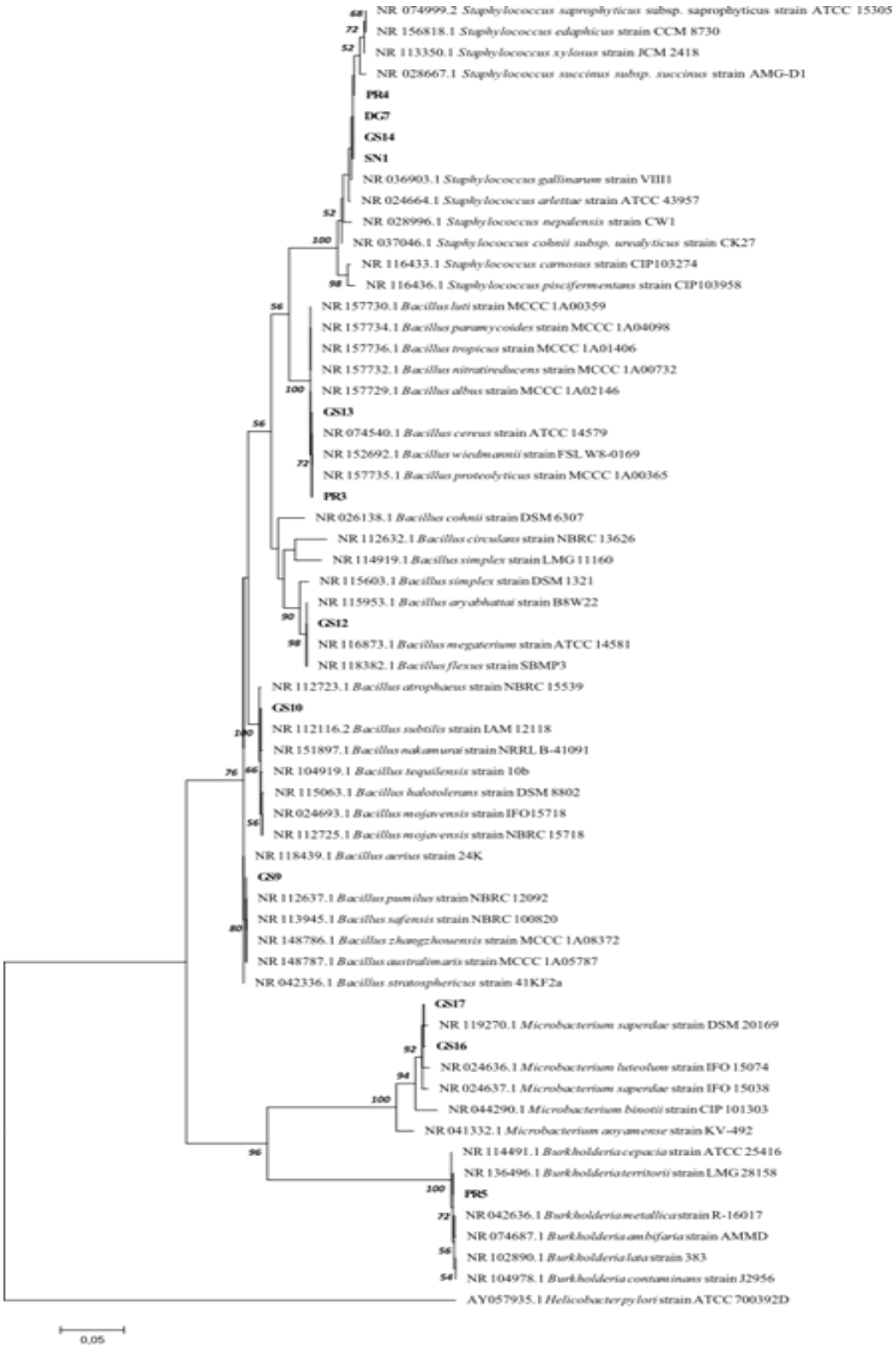

Figure 5. Molecular phylogenetic analysis by Maximum Likelihood method showing the genetic relationships among 12 phosphate solubilizing bacteria isolates and other related species of the genus. The percentage of trees (calculated using 600 bootstraps) in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 77 nucleotide sequences. All positions with less than 95% site coverage were eliminated. There were a total of 879 positions in the final dataset. The sequences of *Helicobacter pylori* strain ATCC 700392D is used as outgroup.

reduce significantly the number of potential P solubilizing strains, and consequently our chance when exploring other PGRP traits.

PGP characters of strains

In the present study, it was found that the capacity to solubilize inorganic P varied considerably between the isolated strains and was independent of their geographical origin. We also found variations among strains belonging to the same genus or species. Similar results were found on 193 isolates (Solanki et al., 2018) selected from the rhizosphere of chickpea, mustard and wheat showing large variations in P solubilization independently of their origin. Moreover, some authors who worked on *Pseudomonas fluorescens* strains isolated from various agricultural fields also indicated that significant variations may also exists within a single bacterial species (Browne et al., 2009). To further investigate the potential PGP of isolated strains, IAA and siderophore productions were tested under *in vitro* conditions.

IAA is a phytohormone produced in large quantities by many PGPR (Vessey, 2003). Some authors report that 80% of rhizobacteria can synthesize IAA (Gupta et al., 2015). In the present study, all selected bacteria had the ability to produce IAA. However, IAA production varied greatly among strains as shown previously by Shahab et al. (2009). According to Walpola and Arunakumara (2016), IAA production by microbial isolates varies greatly among different species and strains and depends on the availability of substrate(s). The production of IAA is also influenced by the culture conditions or the developmental stage (Mirza et al., 2001). Strains belonging to *Azospirillum* (Dobbelaere et al., 1999), *Rhizobium*, *Microbacterium*, *Sphingomonas*, and *Mycobacterium* genera (Tsavkelova et al., 2006) are among the most active IAA producers. In this study, high production of IAA was found in a strain belonging to the genus *Bacillus* (GS12: MK209023), suggesting that other genera may have great potential. Indeed, high IAA production was also found in *Bacillus simplex* and *Paenibacillus polymyxa* species (Erturk et al., 2010).

Another important PGP character is the production of siderophore. Siderophore are small organic molecules produced by microorganisms under iron-limiting conditions that enhance iron uptake capacity (Gouda et al., 2018). Iron is essential for the growth of soil microorganisms. The major strategy to acquire iron is the production and utilization of siderophore (Chaiharn et al., 2008). Microbial siderophore enhance iron uptake by plants that are able to recognize the bacterial ferric-siderophore complex (Dimkpa et al., 2008). In this study, seven isolates were positive for siderophore production in King B and CAS solid media. The rhizobacteria that can produce siderophore could compete for iron with soil borne pathogens and may act as biocontrol agents (Chaiharn et al., 2008).

Conclusion

In a context of climate changes, low fertility of soils and an increasing world population, there is a need to develop sustainable agricultural practices. PGPR represent a real option for crop improvement and protection. Here, the first step of a project aiming at developing a biofertilizer constituted of PGPR was achieved. Globally from the 12 isolates studied, seven exhibit high phosphate solubilizing capacity, and produce IAA and siderophore. These represent good candidates for plant growth stimulation. Finally, rhizobacteria that produce siderophores could also compete for iron with soil borne pathogens. These PGPR could also participate in the protection of the plant and thus represent promising biofertilizers adapted to Senegalese soils.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors thank the West Africa Agricultural Productivity Program (WAAPP) and Fonds National de Recherches Agricoles et Agroalimentaires (FNRAA) which supported this work.

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Vol. 13(28), pp. 619-625, October, 2019
DOI: 10.5897/AJMR2019.9210
Article Number: 5012CA262136
ISSN: 1996-0808

http://www.academicjournals.org/AJMR

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African Journal of Microbiology Research

Full Length Research Paper

High frequency of Torque Teno virus (TTV) among Egyptian hemodialysis patients

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Received 14 September, 2019; Accepted 9 October, 2019.

Very little reports are yet available for the infection rate of Torque Teno virus (TTV) among hemodialysis patients in Upper Egypt. Thus, the aim of this study was to assess the frequency and the possible genotypes of TTV in chronic renal failure patients undergoing dialysis. This cross-sectional study was carried out between August 2016 and February 2017 in three haemodialysis units in Minia, Egypt. Blood samples were collected for serological detection of Hepatitis B Virus (HBV) and Hepatitis C Virus (HCV) and for detection of TTV. Nested polymerase chain reaction (PCR) was used to detect TTV. Isolates genotypes were identified by sequencing of the N22 region and phylogenetic analysis was also performed. Out of 100 dialysis patients, 76 were TTV positive (52 males and 24 females), with no significant association with gender. TTV was significantly more common among young adults than in older patients. Increased period of haemodialysis posed a high risk for acquiring TTV: Odd Ratio (OR) = 1 and 95% Confidence Interval (CI)= 1.01-1.03. No association was noted between TTV infection and either HCV or HBV infection. Genogroup 1, especially genotype 2 was the most frequently found type in hemodialysis patients. TTV is vastly predominant among Egyptian haemodialysis patients with no significant association with HCV or HBV. Further analyses are recommended to associate the renal failure outcome with the virus load.

Key words: Torque Teno virus, dialysis, phylogenetic biogeography.

INTRODUCTION

Torque Teno virus (TTV) was initially isolated from a Japanese patient having post-transfusional non A-G hepatitis (Nishizawa et al., 1997). The virus is a ubiquitous negative stranded DNA virus, which was classified as a member of Anelloviridae family (King et al., 2011). The virus is thought to be a hepatotropic virus, which has various modes of transmission (Focosi et al., 2016). According to sequence analysis, TTV is classified into

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seven genogroups including many genotypes (Hsiao et al., 2016), of which genotype 1 is the most prevalent (Diniz-Mendes et al., 2004). The virus is globally distributed with variable seropositivity rates according to geographical location (Vasilyev et al., 2009; Spandole et al., 2015b; Amen et al., 2018). TTV commonly infects patients who are exposed to blood transfusion as thalassemic and haemodialysis patients as well as intravenous drug users (Akbari et al., 2018).

The virus has been considered apathogenic and is even considered as a virus flora, however, its importance arises from its ability to mirror the total capacity of innate and acquired immunity (Chen et al., 2013).

Notwithstanding the countless usefulness of haemodialysis for chronic renal failure patients, it has been associated with many complications; it has been attributed as a high-risk environment for blood-borne transmitted infections, which could be through various sources at the haemodialysis units as the equipment, surfaces and personnel (Zuckerman, 2002). In addition to HCV and HBV, TTV is one of the commonly transmitted viruses in patients undergoing haemodialysis, which frequency is widely variable according to virological, demographic and clinical factors (Brajao de Oliveira, 2015). Few studies have reported the frequency of TTV among various Egyptian cohorts with different infection rates (Gad et al., 2002; Hassoba and Khudyakov, 2005), however, these studies are more than 10 years ago and no reports have described TTV frequency in Minia governorate before.

Furthermore, HCV is endemic in Egypt with reported high incidence in Minia region (Hassuna et al., 2015). Thus, the objectives of this work were to evaluate the frequency of TTV viremia in patients undergoing haemodialysis, to evaluate TTV co-infection rates with HBV and/or HCV and to determine the most frequent TTV genotype.

PATIENTS AND METHODS

This is a cross-sectional study carried out in the period from August 2016 to February 2017. Out of 143 patients admitted to three haemodialysis units (Minia University Hospital, Minia General Hospital and Matai General Hospital, Minia governorate); hundred patients, who agreed to participate in this study, were recruited by convenient and consecutive sampling. The study was carried out per the Helsinki declaration (World Medical, 2001) and was approved by the ethical committee of Science Faculty, Beni Suef University.

History taking

Medical records of patients were reviewed to obtain demographic data such as age, sex, underlying diseases; surgical procedures; and haemodialysis history.

Laboratory investigation

A 5 ml blood sample was withdrawn from the patients at the time of dialysis. Each sample was divided into 2 ml for serum collection for further virological (HCV IgG and HBsAg) and biochemical laboratory tests and 3 mL for detection of TTV in the Microbiology Department, Faculty of Medicine.

Biochemical testing

Alanine amino transferase (ALT) and aspartate transferase levels were assayed on Diatron apparatus (model Pictus B+) according to the manufacturer's kit instructions (BioSystems S.A., Spain). Abnormal values were considered when ≥41 IU/L for ALT and ≥40 IU/L for AST.

HBV and HCV antibodies detection

Antibodies of HCV (IgG) and HBsAg were detected by mini-VIDAS apparatus (Biomerieux) using corresponding kits: VIDAS anti-HCV and anti HBV (bioMerieux, France) and cut-off values were calculated according to manufacturer's instructions.

Detection of TTV

DNA extraction

DNA was purified from whole blood using the High Pure Viral Nucleic Acid Kit (Roche, Switzerland) according to manufacturer's instructions and DNA was stored at -20°C for further processing.

Nested PCR

Nested PCR protocol was applied for each sample to detect TTV DNA ORF1 (N22) region spanning nucleotides (915-2185) of TTV (Maggi et al., 1999).

The following primers were used for the first PCR round: A5430F (5' CAGACAGAGGAGAAGGCAACATG 3') and A5432F (5'CTACCTCCTGGCATTTTACCA3') and the reactions were carried out in 50 µl volumes which comprised 25 µl of Master Mix (DreamTaq Green Master Mix, Thermo Scientific, USA), 1 µl of each primer, 5 µl template DNA and 18 µl of sterile nuclease free water. Amplification included 35 cycles of 30 s of denaturation at 94°C, 30 s of annealing at 58°C and 45 s of elongation at 72°C, followed by 7 min extension at 72°C. Primers for the second PCR round were: A7861 (5' GGMAAYATGYTRTGGATAGACTGG 3') and NG063 (5' CTGGCATTTTACCATTTCCAAAGTT 3'). Same volumes as aforementioned were used and the cycling conditions were similar to those of the first round of PCR except that the number of cycles was 25. The PCR products were separated on 2% agarose gel and were visualized under ultraviolet (UV) light.

DNA sequencing and phylogenetic analysis

Second round amplicons of the nested PCR were purified and sequenced in both directions using ABI 3500 Genetic Analyzer using BigDye® Terminator V 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Raw DNA sequence reads were further trimmed and contigs were assembled using Vector NTI Express® 1.2.0 (Thermo-Fisher Scientific). Obtained TTV sequences were confirmed by comparison to sequences in the Gene Bank library using BLAST tool (Basic Local Alignment Search Tool; NCBI). Multiple sequence alignment (MSA) and phylogenetic evolutionary analysis for the selected obtained sequences and reference sequences of the main genotypes acquired from the database was carried out deploying Mega7 software (Molecular

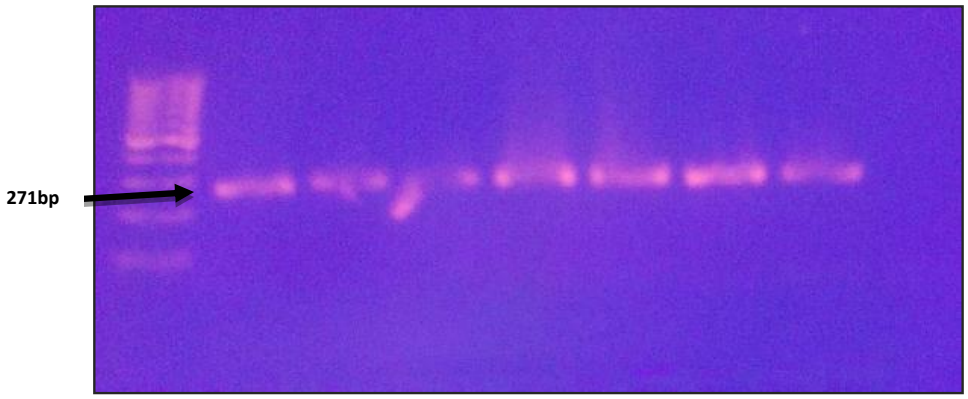

Figure 1. Nested PCR product of the 271bp region of TTV virus.

Table 1. Demographic characteristics and baseline clinical data in TTV-positive and -negative haemodialysis patients.

Variable		Torque Teno Virus (TTV) infection		P-value
		TTV positive (No= 76)	TTV negative (No=24)	
Age		45.7±13.9	52.4±11.8	0.035
Sex	Males (%)	52 (74.3)	18 (25.7)	0.540
	Females (%)	24 (80)	6 (20)	
Period of haemodialysis (months)		72 (3-216)	48 (5-144)	0.098

Evolutionary Genetics Analysis version 7.0) (Kumar et al., 2016). Evolutionary history was evaluated by the Neighbor-Joining method (Saitou and Nei, 1987). The bootstrap consensus tree deduced from 500 replicates was procured to represent the evolutionary history of the analysed taxa (Felsenstein, 1985). The percentage of replicate trees in which the related taxa grouped together in the bootstrap test (500 replicates) was demonstrated alongside the branches. The Maximum Composite Likelihood method was applied for calculating the evolutionary distances and are in the units of the number of base substitutions per site (Tamura et al., 2004).

Statistical analysis

The statistical analysis was carried out using Prism 6 and SPSS 16.0 for Windows®. Data are presented as mean ± standard deviation (SD), median with range for quantitative variables and number, percentages for categorical variables. Student's t-test was used to compare between two independent means for age and other numeric variable were compared by Mann Whitney test. The Chi-square test was used for categorical variables. P value ≤0.5 was considered statistically significant.

RESULTS

Out of 143 patients attending the three hemodialysis centres, only 100 agreed to participate in the study. Most of the dialysis patients were males (70%) with a mean age of 47.3 years (standard deviation - SD: 13.7), who spent a mean time of 71.9 months in dialysis with a SD of ±44.8. Regarding the prevalence of TTV infection: 52 males (73.4%) were positive compared to 24 females (80%), which was statistically non-significant with almost comparable or equal prevalence rates (p=0.540) (Figure 1). The two groups were significantly associated in relation to age, as TTV was more frequent in relatively younger age (Table 1). Forty-six (60.5%) of the dialysis patients with TTV viremic showed HCV seropositivity, while more than 79% of those without TTV viremia were HCV seropositive. Only two of the TTV positive patients possessed HBsAg, while none of TTV negative had the antigen. Longer period of dialysis was considered as a risk factor for TTV positivity (Table 2). The levels of AST or ALT were not significantly different with HCV alone, TTV alone or with both infections (Figure 2).

Five isolates were randomly selected for DNA sequencing and submitted to GenBank with the following accession numbers: KY750543-KY750547. MEGA 7 software was used to carry out multiple sequence alignments (MSA) for the isolated sequences in addition to reference sequences for TTV acquired from GenBank. Four of the isolates were related to genotype 2 (KY750543-KY750546) and only one isolate was closely related to genotype 1 (Figure 3). The sequence identity

Table 2. Binary logistic regression analysis for detection of variables independently associated with TTV infection in hemodialysis patients.

Variable		Odd Ratio (95% CI)	P-value
Age		0.94 (0.89-0.98)	0.007
Sex	Females	1.0 (reference)	0.2
	Males	1.91 (0.60-6.05)	
HCV and/or HBV	Negative	1.0 (reference)	0.1
	Positive	0.41 (0.12-1.34)	
Duration of hemodialysis (months)		1.01 (1.0-1.03)	0.04

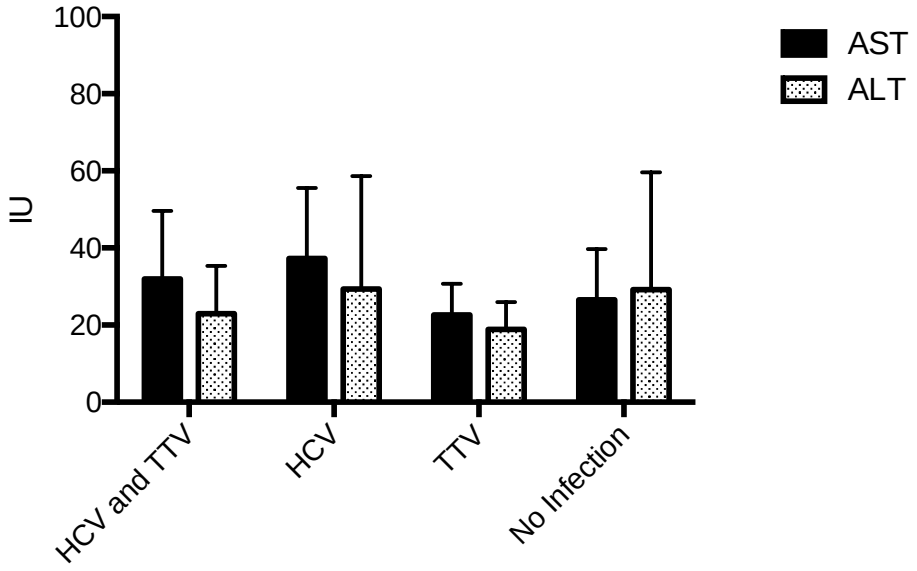

Figure 2. AST and ALT levels in haemodialysis patients. Values are the means±SD.

for isolates obtained from haemodialysis patients was in the range of 66 to 98%.

Green diamonds represent isolates from this study with accession numbers: KY750543-KY750547. Different genotypes were represented as follows: genotype 1: JaBD28, TA278, TX011; genotype 2: PT3, TS003 and NA004; genotype 3: TKB6; genotype 4: TKM1; genotype 5: THEM 1; genotype 6: TFC3155; genotype 7: THEM2; genotype 8: THEM3.

DISCUSSION

Details concerning the prevalence of TTV in Egypt among different groups are deficient. Up to our knowledge, this is the first multi-centered study evaluating the presence of TTV viremia among haemodialysis patients in Upper Egypt. There is a widely variable geographical distribution for TTV infection among dialysis patients, which depends on the detection methods, clinical and demographical status of the grouped patients as well as the size of the study.

The frequency of TTV in this study was 76%, which is comparable with Abou-Dounia et al. (2007) whose study was the only one carried on haemodialysis patients in Egypt; nevertheless, it was in Lower-Egypt. Moderately lower frequencies are found in various countries; ranging between 9.3 and 60.9%, which is possibly due to the earlier defined variances between the studied groups specially detection methods (Chan et al., 2000; Kheradpezhouh et al., 2007). A state of endemicity could also explain the high prevalence of TTV in this study as well as some studies carried out in Japan and India (Utsunomiya et al., 1999; Irshad et al., 2010).

Except for age, none of the tested demographic factors (sex or period of dialysis) were associated with the high

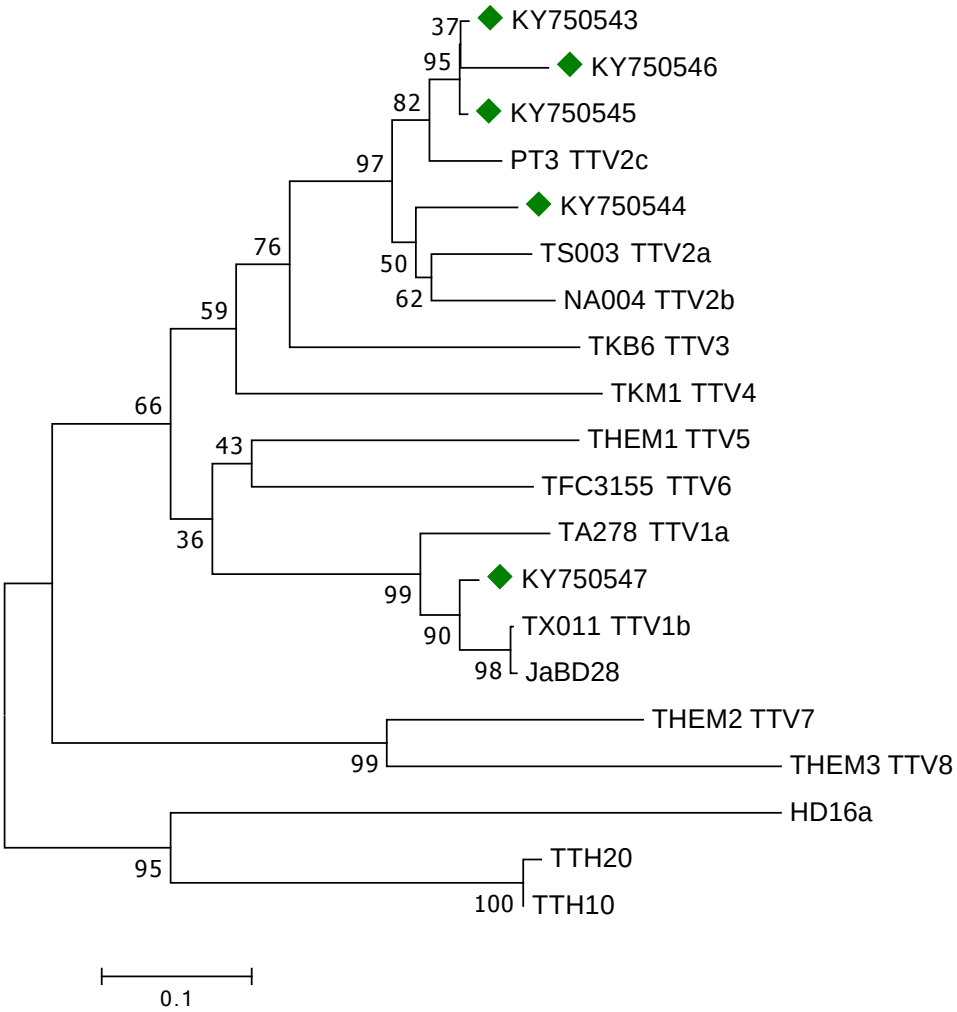

Figure 3. A phylogenetic analysis for the ORF1 region of the TTV. The evolutionary history was inferred using the Neighbour-Joining method using TTV isolates and reference strains.

frequency of TTV, which was in agreement with several studies (Martinez et al., 2000; Kheradpezhouh et al., 2007). An interesting finding was the increased frequency of TTV viremia with younger age, which could be due to possible clearance of the virus with age. Longer period of dialysis poses a risk for acquiring TTV infection, which perhaps could be due to longer exposure duration in addition to the progressive state of immune-deficiency with time in dialysis patients. Furthermore, dialysis techniques also markedly present additional hazard factors for infection in such group of patients (Jalali et al., 2017).

There is a big debate regarding the involvement of TTV in different pathologies especially liver pathology with assumptions that the virus cannot produce the pathology alone (Spandole et al., 2015a); hence, this study evaluated liver enzymes: ALT and AST as biomarkers for liver damage (histological data were not available for this study) in patients with HCV alone, TTV alone or with both infections. No significant difference was detected in either AST or ALT levels in any of the groups, which concurs with the postulation that TTV is a commensal virus and only certain genotypes and genogroups are associated with liver pathology (Peng et al., 2015; Hazanudin et al., 2019). Further studies are required to evaluate the titre of the virus and its relation with possible renal or hepatic damage as some studies suggested the potential of TTV-induced hepatic damage at certain titres (Simonetta et al., 2017).

Concurrence between TTV and hepatitis viruses has been markedly studied in many reports (Al-Qahtani et al., 2016; Najafimemar et al., 2018). In the current study, seropositivity of HCV IgG was relatively high (65%), while HBsAg was found in only two patients (2%). The high frequency of HCV is similar to an earlier study done in the same region in a similar cohort (Elzorkany and Zahran,

2017). In addition, the HBsAg seropositivity was similar to that found in a study carried out on blood donors in the same area (Hassuna et al., 2015), and is also in agreement with another study carried out by Atwa and Wahed (2017) on transfusion-transmitted infections. Regarding the co-infection status of TTV with HCV and HBV, no significant association between TTV and either HCV or HBV, and despite the fact that the three viruses are parentally transmitted, the lack of concordance could be due to the presence of other non-parenteral routes for TTV transmission.

As mentioned earlier, only certain TTV genotypes are associated with known pathologies: renal pathology (Yokoyama et al., 2002), acute respiratory diseases (Maggi et al., 2003), arthritis (Maggi et al., 2007), laryngeal carcinoma (Hettmann et al., 2016) and post transfusion hepatitis (Tanaka et al., 2000). Accordingly, five TTV isolates were randomly selected and sequenced (not all the isolates were sequenced due to very limited resources) with only four of our isolates closely linked to genotype 2 and only one related to genotype 1. This study is the first to show the prevalent genotypes of TTV among dialysis patients in Egypt with no previous data concerning the prevalent TTV genotypes in Egypt, except for one study carried out by our group on thalassemic patients showing genotype 1 to be the most prevalent (Hassuna et al., 2017).

Limitations to this study included sequencing of few samples for genotyping, which is due to lack of funding and also led to the inability to titrate the virus levels.

Conclusion

The frequency of TTV is relatively high among dialysis patients, especially younger dialysis patients with no significant association with either sex, period of transfusion or HCV/HBV infection. Interestingly, genotype 2 was more frequently found than genotype 1 with high relationship between the viruses. Future measurement of the virus titre could help in evaluating the immunological status of dialysis patients.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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