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Table of Contents: Volume 12 Number 4 28 January, 2018

ARTICLES

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Full Length Research Paper

Direct identification of yeasts from blood culture by MALDI-TOF mass spectrometry

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This study aimed to identify yeasts directly from positive blood cultures by MALDI-TOF mass spectrometry. This was a 5-month prospective study (February to June, 2015) carried out on bottles of blood cultures from in-patients at the Hôpital de l'ARCHET at the CHU, Nice. Positive blood culture broth was analysed by MALDI-TOF mass spectrometry using Sodium Dodecyl Sulphate (SDS) as the lysis buffer. Out of 64 samples, 51 (79.6%) were identified by MALDI-TOF mass spectrometry at the species level and 12 strains (18.7%) at the genus level. The isolated yeasts fell into six species: *Candida albicans* 37.2%, *Candida glabrata* 31.4%, *Candida parapsilosis* 15.7%, *Candida tropicalis* 5.9%, *Candida krusei* 5.9%, and *Candida lusitaniae* 3.9%. In comparison, 95.3% of species were identified from cultured colonies, the main ones being *C. albicans* (37.1%), *C. glabrata* (30.7%) and *C. parapsilosis* (16.1%). Identification of yeasts from blood culture bottles by MALDI-TOF mass spectrometry is a fast, reliable technique. However, analysis of colonies remains the best technique for identification and for antifungal imaging in order to refer the patient to the most appropriate therapy.

Key words: Yeasts, blood cultures, identification, MALDI-TOF, mass spectrometry.

INTRODUCTION

Invasive fungal infections incur high rates of morbidity and mortality (Seifert, 2009), but prompt initiation of appropriate therapy is associated with a favourable prognosis. Since there is often no clear clinical picture, biological tools, particularly blood cultures, play a major role in the diagnostic strategy (Sepharin, 2015).

Blood cultures are the standard diagnostic tools for bacteraemia and fungaemia (Ferroni et al., 2011). However, identifying species that have grown in a blood culture broth requires culture on a solid medium prior to antifungal imaging, which delays identification until 24 to 48 h after detection of positivity, delaying in turn prescription of a targeted therapy (Ferroni el al., 2010, 2011; Lavergne et al., 2013). For these reasons, attention was focussed on blood cultures and rapid identification of the yeasts responsible for fungaemia by MALDI-TOF mass spectrometry.

Microorganisms can be rapidly identified by MALDI-TOF mass spectrometry analysis of the macromolecules of the different bacteria, notably proteins, from colonies

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isolated on solid medium. By adapting this technology to identify microorganisms cultured in liquid medium, it is possible to identify species immediately, thereby speeding up diagnosis (Bougnoux, 2013).

The aim of this study is to avoid the culture stage and identify yeasts directly from positive blood cultures using the Bruker Microflex LT MALDI-TOF Biotyper mass spectrometry system. A significant amount of time would be gained in implementing the most effective therapy, compared with identification by mass spectrometry from colonies isolated after spotting on Sabouraud chromogenic medium.

MATERIALS AND METHODS

Clinical samples

The isolates analysed in this prospective study were recovered from clinical positive blood cultures in the Mycology Department of L'Archet Hospital in Nice, France, during a 5 month period. The arm of the study aimed to compare the performance of yeast colonies identification by MALDI-TOF mass spectrometry (Bruker Daltonik GmbH, Bremen, Germany) with an SDS-based (Sodium Dodécyle Sulfate SDS, Gen-Apex, Prolabo 500 g) lysis direct identification.

The majority of blood samples were collected in BACTECTM Mycosis-IC/F culture vials (BD Diagnostics, Le Pont de Claix, France). For pediatric blood specimens of less than 3 ml, samples were collected in BACTEC Ped PlusTM/F culture vials (BD Diagnostics). These culture vials were loaded in a BACTECTM FX (BD Diagnostics) automat, which alerts when significant microbial growth is detected. When a vial was flagged positive, a small sample was examined microscopically, and if yeasts or mycelium were observed, the original samples will be submitted to the two identification procedures performed in parallel: identification of colonies obtained after solid medium subculture and SDS lysis-based direct identification.

Identification by MALDI-TOF MS

Identification by MALDI-TOF mass spectrometry was carried out using yeast colonies obtained after 48 h incubation on Sabouraud gentamicin, chloramphenicol and CHROMagar media (Becton Dickinson, Le Pont de Claix, France) at 30°C. Protein extraction was carried out according to the manufacturer's instructions, with one to three colonies being removed using a 1 µl loop and suspended in 300 µl distilled water (Water HPLC, Prolabo BDH, Fontenay-sous-Bois, France) and 900 µl absolute ethanol (70%; CarloErba SDS, Val de Reuil, France).

To perform the SDS lysis-based technique, 1.8 ml of positive blood culture was transferred into a 2-ml microtube. After a centrifugation step of 13 000 *g* for 2 min, the supernatant was removed, the pellet was resuspended in 1.8 ml of sterile distilled water and the microtube was centrifuged at 13000 *g* for 2 min. Next, the supernatant was removed and the pellet was resuspended in 1.8 ml of 0.1% SDS in sterile distilled water and incubated for 10 min. After another wash step, the pellet was resuspended in a mixture of 900 µl ethanol and 300 µl sterile distilled water.

Protein extraction was performed identically for the two protocols. After a centrifugation step of 13000 rpm for 2 min, the supernatant was removed and the pellet was air-dried at room temperature for 10 min. Next, the pellet was resuspended in 50 µl of 70% formic acid (Sigma-Aldrich, Lyon, France) and incubated for 5 min. The same volume of 100% acetonitrile (Prolabo BDH) was then added to the sample. The mixture was incubated for 5 min and then centrifuged for 2 min at 13000 rpm. Subsequently, 1.2 µl of the supernatant was placed into duplicate on a target spot on a metallic plate (Anchorchip 96-spot; Bruker Daltonics, Inc.) and dried. Matrix solution (alpha-cyano-4-hydroxycinnamic acid; Bruker Daltonics, Inc; 1.2 µ l of a concentration of 10 mg/ml) was then added to the fungal protein extract and redried. The Microflex mass spectrometer is used routinely for yeast and bacterial isolate identification. As recommended by the company, *Escherichia coli* isolate is generally employed as a positive control but no yeast control was recommended. For standardization purposes a negative control (all reagents without a yeast isolate) and positive controls prepared from reference isolates (*Candida krusei* CBS 5314) was included in each set of analyses.

The samples were analysed using a Microflex LT Instrument (Bruker Daltonics GmbH, Bremen, Germany). The resulting spectra were analyzed using MALDI Biotyper version 3.0 software (Bruker Daltonics GmbH), which contains the 4111-entry reference version of the Bruker Daltonics database. The spectra generated for each yeast were compared to the reference spectra of all yeasts in this database. The identification scores were used as recommended by the manufacturer: ≥2 indicating species identification, while 1.7-1.9 = identification of a genus. Two successive scores between 1.7 and 1.9 with the same species allowed identification of the species, whereas a score <1.7 corresponding to different species did not allow any species identification. In the case of non-identification of the species, a second analysis was carried out after a new extraction, as described previously.

Statistical analysis

The statistical analysis of data was made from EPI INFO 6.04 software. The tests used were the Chi^2 test (χ^2) and Fisher's exact test at the risk of 5%. P-value<0.05 was considered to be statistically significant.

RESULTS

A total of 64 isolates belonging to six species and a single genus as *Candida* were identified using both techniques. There was, however, a discrepancy in the results: 51 (79.6%) strains were identified directly from the blood culture broth by MALDI-TOF mass spectrometry at the species levels, while 12 (18.7%) strains were identified at the genus level, whereas 95.3% of strains (62) were identified from cultured colonies at the species level and 4.6% (3 strains) at the genus level. Only one strain (1.6%) could not be identified from the broth. The yeast species were mainly *Candida albicans* 37.2% (19/51), *Candida glabrata* 31.4% (16/51), *Candida parapsilosis* 15.7% (8/51), *Candida tropicalis* 5.9% (3/51), *C. krusei* 5.9% (3/51) and *Candida lusitaniae* 3.9%. Two strains of a Gram-positive bacterium, *Staphylococcus epidermidis* were found associated with two strains of *C. lusitaniae*, all of which were identified at the genus level. The species isolated from the colonies were identical to those identified from the broth (Tables 1 and 2). Also, a mixed culture was found and was able to identify two yeasts from its colony: *C. glabrata* and *C. parapsilosis*,

Table 1. Identification of yeast grown in blood culture bottles.

Species	Good identification at the species level [N=51]	Identification at genus level [N=12]	No identification [N=1]
	n (%)		
C. albicans	19 (37.2)	3 (25)	0
C. glabrata	16 (31.4)	3 (25)	0
C. parapsilosis	8 (15.7)	3 (25)	0
C. tropicalis	3 (5.9)	1 (8.3)	0
C. krusei	3 (5.9)	0	0
C. lusitaniae	2 (3.9)	0	0
C. lusitaniae + *S. epidermidis*	0	2 (16.7)	0
No identification	0	0	1 (100)

N means total number of identification; n represents the number front of the percentage. *C: Candida, S: Staphylococcus.*

Table 2. Identification of yeast grown on solid media.

Species	Good identification at the species level [N=62]	Identification at genus level [N=3]	No identification [N=1]
	N (%)		
C. albicans	23 (37.1)	1 (33.3)	0
C. glabrata	19 (30.7)	0	0
C. parapsilosis	20 (16.1)	1 (33.3)	0
C. tropicalis	4 (6.5)	0	0
C. krusei	3 (4.8)	0	0
C. lusitaniae	3 (4.8)	1 (33.3)	0
C. lusitaniae +*S. epidermidis*	0	0	0
No identification	0	0	0

which were identified with a score greater than 2. Subjecting the unidentified yeast to a second extraction, a species of *C. albicans* was able to be isolated. There was a discrepancy in the result of one sample: one species of *C. grabata* isolated in the blood culture broth was identified as *C. albicans* by mass spectrometry of the colony, and this would need to be resolved by sequencing. MALDI-TOF spectrometry from cultures is a much more sensitive procedure than identification from broth, as 95.4% of strains were identified at the species level with the former, compared with 79.6% with the latter. The difference, however, is not statistically significant (χ^2=0.0041, P<0.05).

DISCUSSION

Several studies have been carried out on direct identification of yeasts from positive blood culture bottles using various protocols with varying results (Buchan et al., 2012; Drancourt, 2010; Ferreira et al., 2011; Idelevich et al., 2014; Lavergne et al., 2013; Marinach-Patrice et al., 2010; Yan et al., 2011).

While some authors have had very low success rates with fungi (Buchan et al., 2012; Idelevich et al., 2014), others have reported excellent levels of identification with different sample processing methods (Ferroni et al., 2010, 2011; Idelevich et al., 2014; Lavergne et al., 2013; Marinach-Patrice et al., 2010; Yan et al., 2011).

In our previous study, where we used 0.1% sodium dodecyl sulphate (SDS) as the lysis buffer, a level of identification was obtained at the species level of 79.6%. Idelevich et al. (2014) identified 62.5% of species, Jamal et al. (2013) identified 50%, both using the Bruker Sepsityper kit. Ferroni (2010), on the other hand, used saponin as the lysis buffer for 20 *Candida* strains and identified all species (100%), while Yan et al. (2011) identified 100% of species using the Sepsityper kit preceded by two additional washing steps. In their study, Lavergne (2013) and Marinach-Patrice (2010) used the same protocol as ours and were able to rapidly and precisely identify the main *Candida* species, thus avoiding the need for culture on a specific medium. In a second study carried out by Pulcrano et al. (2013), on *Candida* non-*albicans* with 0.5% SDS, all 82 strains were identified at the species level with a score greater than 2.

In contrast to these results, Buchan (2012) and Ferreira (2011) failed to identify any positive yeasts at the species level using the Sepsityper kit. In our series, only one genus of yeast, *Candida*, was isolated unlike Yan et al. (2011), who found yeasts belonging to two genera: *Candida* and *Cryptococcus.* Pulcrano et al. (2013) identified yeasts of the genera *Saccharomyces* and *Lodderomyces* in addition to the genus *Candida*.

C. albicans is the *Candida* spp. most frequently found in human pathology and was the most common species (37.2%) in our series, as in several other studies (Bassetti et al., 2006; Ferroni et al., 2010 ; Seraphin, 2015 ; Yan et al., 2011). In his thesis, Sepharin (2015) identified 96.6% of *C. albicans*, while Yan et al. (2011) identified 66.7%. The other species were *C. parapsilosis* (19%), *C. tropicalis* (11.9%) and *Cryptococcus neoformans* (2.4%). Ferroni et al. (2010) identified 10 species of *C. albicans* out of 20 *Candida* spp*.* Numerous other species of non-*Candida* yeasts and *Candida* non-*albicans* were also identified by Pulcrano et al. (2013), these being 53 species of *C. parapsilosis*, 11 species of *C. glabrata*, 8 species of *C. tropicalis*, 6 species of *Candida guillermondii*, 1 species of *Lodderomyces elongipsorus* and 1 species of *Saccharomyces cerevisiae*. *C. albicans* is the species most frequently responsible for invasive candidiasis, although according to Bille et al. (2012), other non-*albicans* species, including *C. tropicalis*, *C. parapsilosis*, *C. glabrata* and *C. krusei*, are becoming increasingly common. According to Arnold et al. (2010), fungal infections affect the length of hospital stay and increase treatment costs, so it is very important to correctly identify the fungi causing infection in order to promptly administrate targeted treatment. The results of the present study indicate that with sample processing and MALDI-TOF Biotyper analysis, microorganisms can be rapidly and reliably identified directly from bottles of positive blood cultures in 45 min. Jamal et al. (2013) reported an average time of 35 min, Feronni et al. (2010), 20 min.

The main advantage is the 1 to 2 days gained in putting in place the most appropriate therapy for patients with potentially life-threatening diseases (Yan et al., 2011). It was not possible to identify several yeast species in the same broth in the case of yeast associations, as many other studies have (Ferroni et al., 2010, 2011, Marinach-Patrice et al. 2010). On the other hand, it was possible to identify at the genus level two cases of co-infection with *Staphylococcus epidermidis*. In all cases, the yeast associations were identified from the colonies. With this technique, it was possible to carry out antifungal imaging with the aim of testing the susceptibility of the different species.

Conclusion

Identification of yeasts from blood culture bottles by MALDI-TOF mass spectrometry is a fast and reliable technique, with which it was possible to identify 79.6% of yeast species. However, identification from colonies remains the best technique for identification and for carrying out antifungal imaging in order to refer the patient to the most appropriate therapy.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Phenotypic characteristics of rhizobial and non-rhizobial isolates recovered from root nodules of chickpea (*Cicer arietinum* L.) grown in Ethiopia

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Fifty endophytic bacteria recovered from surface sterilized root nodules of chickpea (*Cicer arietinum* L.) grown in Ethiopia were authenticated using three chickpea cultivars (Natoli, ICC-4918 and ICC-5003). These isolates were characterized based on morphological and phenotypic traits. The test isolates were divided into two: bacteria nodulating chickpea (27 isolates, Group I] and endophytic non-nodulating bacteria (23 isolates, Group II]. Twenty isolates from Group I were able to re-infect and form symbiosis with two chickpea cultivars, while the remaining seven isolates re-infected only one chickpea cultivar. The result demonstrated that: a number of the tested isolates were able to produce siderophore, showed variations in terms of their resistance to different antibiotics, grew at pH ranging between 5 and 9.5, tolerated salt concentration as high as 2.5% and grew at a temperature as high as 40ºC, indicating the existence of a wide physiological diversity among themselves. Dendrogram construction indicated the existence of four clusters when 78% similarity level was used as cut-off point. Such diversity among the tested isolates showed the presence of diverse rhizobial and non-rhizobial isolates within chickpea nodules. Further investigation aimed at identifying the types of endophytic bacteria, their invading mechanism, and varietal preferences for nodule formation is recommended.

Key words: BNF, Chickpea, Endophytic, *Mesorhizobium,* non-nodulating, rhizobia.

INTRODUCTION

Ethiopia is the largest producer of chickpea in Africa, and the sixth largest producer in the world (Kimurto et al., 2013). The total area of chickpea in Ethiopia has increased from 168,000 – 230,000 ha over the past decade (Central Statistical Agency - CSA, 2014), and the actual yield of chickpea in Ethiopia is below the potential

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yield (Bejiga and Daba, 2006; Keneni et al., 2011a). However, efforts have been made to improve chickpea cultivars with respect to several desirable attributes including yield, tolerance to different diseases/infestation (Ahmed and Ayalew, 2006; Keneni et al., 2011a) and drought (Anbessa and Bejiga, 2002), thus many improved cultivars have been released in Ethiopia (Shiferaw and Teklewold, 2007). However, these efforts alone could not improve the productivity of chickpea in the country. Therefore, the benefits of its biological nitrogen fixation ability with rhizobia should be exploited and integrated together with the above efforts being made so far to enhance the productivity of chickpea in the country and beyond. As a first step to such an approach, we thus isolated and characterized (based on different morphophysiological features) endophytic bacteria (nodulating and non-nodulating) from nodules of chickpea, with the aim to select the best nitrogen fixing rhizobial strains, but also non-nodulating strains having desirable properties for plant growth.

Chickpea forms symbiotic nitrogen-fixation association with *Mesorhizobium* species (Jarvis et al., 1997; Nour et al., 1995). *Mesorhizobium* strains naturally vary in their nitrogen fixing capacity and adaptation to prevailing environmental stresses (L'Taief et al., 2007). Despite high specificity of the legume-*Rhizobium* interaction and the selective nodule environment, the presence of non-nodulating endophtes has been reported; for example, the investigation of *Bacillus* from soybean (Bai et al., 2002), *Agrobacterium* from tropical grass (de Lajudie et al., 1999), 1999), *Klebsiella* from groundnut, clover and bean (Ozawa et al., 2003) and *Pseudomonas* from acacia and soybean (Hoque et al., 2011; Kuklinsky-Sobral et al., 2004), *Shinella* from *Milletia ferruginea* ((Degefu et al., 2013), and non-symbiotic endophytic bacteria from several legumes growing in Ethiopia (Aserse et al., 2013). In most of the aforementioned investigations, it was reported that the non-nodulating endophtic bacteria were shown to have plant growth promoting characteristics (PGP), by mechanisms other than biological nitrogen fixation such as production of Indole-3-Acetic Acids (IAA), sidrophore production and phosphate solubilization. In Ethiopia, except for few studies (Jida and Assefa, 2012; Tena et al., 2017), several undertakings were devoted to breeding of chickpea for adaptation to several stressors including, among others, drought and pest infestations (Ahmed and Ayalew, 2006; Anbessa and Bejiga, 2002; Keneni et al., 2011a; Keneni et al., 2011b). However, in other areas where chickpea is commonly grown, several studies have been conducted on characterization of rhizobia nodulating chickpea and screening of best nitrogen fixing strains for inoculant development (Küçük and Kivanc, 2008; L'Taief et al., 2007; Maâtallah et al., 2002). Nevertheless, except for few studies, similar exhaustive studies are lacking in Ethiopia. This therefore calls for a research aimed at isolation and characterization of endophytic bacteria harboring nodules of chickpea growing in Ethiopia using various morphophysiological features. This study, while having significance on rhizobial biodiversity conservation, will also contribute to sustainable utilization of the nodules' microsymbionts in chickpea production in the country and beyond.

MATERIALS AND METHODS

Chickpea root nodules were collected by the research team of the Debre Zeit Agricultural Research Center during the 2014 cropping season. These nodules were collected from the East, West, and North Shewa zones and West Harerge zone in Ethiopia. Collections were made from randomly selected farmers' fields when most chickpea crops were at flowering stage. The randomly selected fields were on average 15 km apart. These areas have an altitude ranging between 1876 (East Shewa, Ada'a) and 2659 m (North Shewa, Enewari) above sea level. The GPS reading and specific locations are indicated in Table 1. Five healthy plants were uprooted from farmers' field and the nodules were detached from roots aseptically using 70% ethanol and flames for sterilization. Thereafter, the root nodules were put into the vials and filled with silica gel and cotton. The nodules were brought to the Soil Microbiology Laboratory at Hawassa University College of Agriculture for detailed characterization.

Isolation of the chickpea nodule bacteria was made on yeast extract manitol agar (YEMA) medium using the procedure mentioned by Vincent (1970). After a week's incubation, a single colony of chickpea bacteria were isolated and transferred to a new plate. Pure colonies of rhizobia and other endophytic non-rhizobial isolates were transferred onto YEMA slant, for further analysis. Authentication test of the presumptive 50 rhizobial isolates was done in order to distinguish the symbiotic nature of rhizobia from other endophytic isolates. The 3 genotypes used for the authentication test were Natoli, ICC-4918 and ICC-5003. Each of these cultivars were allowed to grow in a Leonard jar and inoculated with 1 ml of isolate broth containing about 10^7 CFU/ml in YEMA broth media. The authentication was replicated 3 times for each chickpea cultivar. The non-inoculated control is taken as a proof of authentication (Maâtallah et al., 2002; Somasegaran and Hoben, 1994). Isolates were evaluated for temperature, pH and salt resistance in YEMA media. The temperature test was made by allowing isolates to grow at temperatures ranging from 5 to 45°C, with an interval of 5°C (8 levels). The pH tolerance capacity of each isolate was tested by growing them on an acidic (pH 4.0, 4.5, 5.0) and alkaline (pH 8.0, 8.5, 9.0, 9.5, 10.0, 10.5 and 11.0) media of YEMA respectively, and adjusted with 1 N HCl or NaOH solutions (Amarger et al., 1997). The ability of the isolates to grow at different levels of salt concentration were determined by inoculating each isolate on the YEMA media containing 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5% of NaCl (w/v) (Amarger et al., 1997).

Carbon Source Utilization (CSU) capacity of isolates was determined following the standard procedure described elsewhere (Somasegaran and Hoben, 1994). Nine carbon sources, namely: D-glucose, D- fructose, D-arabinose, D-xylose, maltose, D-sucrose, Trehalose, glycerol and sorbitol were used in the test. Carbohydrates were prepared as 10% (w/v) solution in water. The carbohydrates-free medium, which was essentially similar to YEMA medium, was modified by reducing the yeast extract to 0.05 g/L. Nitrogen Source Utilization (NSU) was determined using different amino acids as mentioned elsewhere (Amarger et al., 1997). Intrinsic Antibiotic Resistance (IAR) was made using the procedure mentioned elsewhere (Somasegaran and Hoben, 1994). Two levels of 4 antibiotics were used for the IAR test. These antibiotics were Ampicillin (5 and 10 $\mu g.ml^{-1}$), chloramphenicol (5

Table 1. Test isolates used, their geographical origins, associated GPS readings and group designation in the present study.

Classification	No.	Zone	District	Latitude, North	Longitude, East	Altitude (m)	Isolates Code
	1	West Shewa	Ambo	08'58"983'	037'01"885'	2428	ICRE01
	2	West Shewa	Dendi	09'01"400'	038'10"763'	2205	ICRE02
	3	West Shewa	Dendi	09'01"400'	038'10"763'	2205	ICRE03
	4	West Shewa	Dendi	09'01"400'	038'10"763'	2205	ICRE04
	5	West Shewa	Dendi	09'01"387'	038'10"854'	2205	ICRE05
	6	West Shewa	Dendi	09'01"387'	038'10"854'	2205	ICRE06
	7	West Shewa	Dendi	09'00"525'	038'17"143'	2155	ICRE07
	8	North Shewa	Enewari	09'52"187'	039'10"697'	2659	ICRE08
	9	West Harerge	Hirna	09'16"692'	041'09"858'	2326	ICRE09
	10	North Shewa	Enewari	09'52"187'	039'10"697'	2659	ICRE10
	11	North Shewa	Enewari	09'52"187'	039'10"697'	2659	ICRE11
	12	Addis Ababa	Akaki	09'53"730'	038'49"283'	2196	ICRE12
	13	Addis Ababa	Akaki	09'53"730'	038'49"283'	2196	ICRE13
Group I	14	Addis Ababa	Akaki	08'47"634'	039'16"628'	2282	ICRE14
	15	Limu	Mojo	08'41"091'	039'10"754'	2012	ICRE15
	16	East Shewa	Ada'a	08'46"365'	039'00"354'	1910	ICRE16
	17	East Shewa	Ada'a	08'41"396'	039'02"655'	1876	ICRE17
	18	West Harerge	Chiro	09'03"815'	040'54"350'	2254	ICRE18
	19	West Harerge	Chiro	09'10"156'	041'02"446'	2212	ICRE19
	20	West Harerge	Chiro	09'10"156'	041'02"446'	2212	ICRE20
	21	West Harerge	Tulo	09'10"680'	041'03"245'	2184	ICRE21
	22	West Harerge	Tulo	09'10"680'	041'03"245'	2184	ICRE22
	23	West Harerge	Tulo	09'10"680'	041'03"245'	2184	ICRE23
	24	West Harerge	Hirna	09'15"189'	041'07"356'	2032	ICRE24
	25	West Harerge	Hirna	09'15"189'	041'07"356'	2032	ICRE25
	26	West Harerge	Kulubi	09'25"391'	041'09"858'	2371	ICRE26
	27	West Harerge	Hirna	09'16"692'	041'09"858'	2326	ICRE27
	1	East Shewa	Ada'a	08°46.411'	038°59.681'	1930	ICNRE01
	2	East Shewa	Ada'a	08°45.991'	038°59.821'	1939	ICNRE02
	3	East Shewa	Gimbichu	08°48.264'	039°00.166'	1977	ICNRE03
	4	East Shewa	Gimbichu	08°48.501'	039°00.203'	1953	ICNRE04
	5	East Shewa	Gimbichu	08°48.780'	039°00.246'	2206	ICNRE05
	6	Finfine Zuria	Legedadi	08°50.998'	039°01.942'	2419	ICNRE06
	7	Finfine Zuria	Menagesha	08°59.610'	039°05.623'	2497	ICNRE07
	8	West Shewa	Ambo	10°35.019'	038°56.067'	2526	ICNRE08
	9	West Shewa	Dendi	10°37.665'	038°54.133'	2472	ICNRE09
	10	West Shewa	Dendi	10°42.420'	038°54.133'	2474	ICNRE10
	11	East Gojam	Bichena	09°58.745'	038°55.188'	2123	ICNRE11
Group II	12	East Gojam	Bichena	09°59.045'	038°55.546'	2313	ICNRE12
	13	East Gojam	Debre work	09°63.010'	038°55.598'	2089	ICNRE13
	14	A/Ababa	Akaki	08°59.817'	038°56.167'	2110	ICNRE14
	15	Finfine Zuria	Gelan	08°58.010'	038°46.073'	2012	ICNRE15
	16	East Shewa	Ada'a	08°40.512'	038°59.310'	1960	ICNRE16
	17	East Shewa	Ada'a	08°39.571'	038°59.435'	1950	ICNRE17
	18	East Shewa	Ada'a	08°33.215'	038°59.214'	1949	ICNRE18
	19	East Shewa	Liben	08°37.012'	038°49.345'	2027	ICNRE19
	20	East Shewa	Ada'a	08°36.358'	038°56.139'	1900	ICNRE20
	21	West Shewa	Ambo	08'59"433'	037'48"022'	1934	ICNRE21
	22	West Shewa	Ambo	08'57"089'	037'55"071'	2225	ICNRE22
	23	West Shewa	Ambo	08'57"089'	037'55"071'	2225	ICNRE23

Table 2. Twenty eight nodule forming rhizobacteria isolates on 3 chickpea seedlings (Natoli, ICC-5003, ICC-4918).

S/N	Chickpea cultivars	Isolates that infected only one variety	Isolates that infected 2 varieties (Natoli, ICC-5003)	Isolates that infected 2 varieties (ICC-5003, ICC-4918)	Isolates that infected 2 varieties (Natoli, ICC-4918)	Isolates that infected all 3 varieties (Natoli, ICC-5003)
1	Natoli	ICRE19, ICRE20, ICRE27	ICRE11, ICRE14, ICRE16, ICRE17, ICRE18, ICRE22, ICRE23	---	ICRE07, ICRE09, ICRE15, ICRE21, ICRE26	ICRE01, ICRE02, ICRE03, ICRE24, ICRE25
2	ICC-5003	ICRE08, ICRE12		ICRE04, ICRE05, ICRE13	---	
3	ICC-4918	ICRE06, ICRE10, ICRE16	---		ICRE07, ICRE09, ICRE15, ICRE21, ICRE26	
Total		8	7	3	5	5

and 10 µg.ml^{-1}), erythromycin (5 and 10 µg.ml^{-1}) and streptomycin (10 and 50 µg.ml^{-1}). The stock solution of each antibiotic was prepared as described before (Zhang et al., 1991). Resistance was determined as resistant (R), intermediate (I) (if any), and susceptible (S) after 5 days of growth.

Intrinsic Heavy Metal Resistance (IHMR) (Al, Cu, Zn, Mn, Pb and Co) was determined on TY agar containing 5.0 g Trypton, 3.0 g yeast extract, 0.87 g $CaCl_2$, 12 g agar and 1000 ml distilled water as described in previous investigation (Zhang et al., 1991). Isolates grown on succinate solution medium (SM) (K_2HPO_4 6 g/L, KH_2PO_4 3 g/L, $MgSO_4.7H_2O$ 0.2 g/L, $(NH_4)_2$ SO_4 1 g/L, succinic acid 4 g/L) pH adjusted to 7.0, were made ready for siderophore production following the procedure described elsewhere (Schwyn and Neilands, 1987). Indole Acetic Acid (IAA) production capacity of each isolate was studied using the log phase culture of each isolate. Exponentially grown cultures of each isolate were incubated separately on nutrient broth medium supplemented with 5 mm L-tryptophan (100 µg/ml) for 72 h (Bric et al., 1991). Mixtures were incubated for 25 min and were observed for the development of pink color. Amylase production test was made on starch agar plates as described before (Cappuccino and Sherman, 2008). The isolates were streaked on starch agar plates and incubated at 28°C. Amylase production was detected by flooding the plates with iodine solution (Hols et al., 1994). Cellulase production test was made on carboxyl methyl cellulose agar, as mentioned before (Bhatt and Vyas, 2014) by flooding with an aqueous solution of Congo red (1% w/v). Cluster analysis of phenotypic variables was worked out using a similarity coefficient, by the un-weighed pair group method with the average (UPGMA) clustering, using NTSYspc21 version software.

RESULTS AND DISCUSSION

Isolation of root nodule bacteria

A total of fifty endophytic bacteria from nodules of chickpea growing in diverse localities in Ethiopia (Table 1) were used for this study of which twenty-seven (54%) isolates were able to re-infect roots of chickpea cultivars. Out of the 27 isolates, 20 (74%) were able to re-infect a minimum of 2 chickpea cultivars seedlings, while 7 (25.9%) isolates re-infected only one chickpea cultivar. The 27 isolates capable of re-infecting chickpea seedlings were the true rhizobial isolates and were designated as Group I. However, the isolates had their own cultivar preference. The remaining 23 (46%) isolates that were not able to re-infect seedlings to form nodules with any of the tested chickpea cultivars were designated as Group II (endophytic non-rhizobial bacteria) (Table 1). Both nodulating isolates and endophytic non-nodulating bacterial isolates were characterized based on their physiological and biochemical properties. The investigation of both nodulating and non-nodulating endophtic bacteria from nodules of chickpea in the present study is becoming a common place phenomena, since several studies have also reported similar results from nodules of diverse legume species (Aserse et al., 2013; Degefu et al., 2013; Gurtler et al., 1991; Liu et al., 2005; Mahdhi et al., 2008). The reason why nodulating members were present in nodules of chickpea is obvious, as they provide the host plant with fixed nitrogen through biological nitrogen fixation process. With respect to the non-nodulating endophytes, they could also promote plant growth by mechanisms other than biological nitrogen fixation as evidenced in several in vitro investigations (Alikhani and Yakhchali, 2010; Alikhani et al., 2006; Aserse et al., 2013; Mahdhi et al., 2008). However, the current available reports from different findings are inconclusive, thus the comprehensive mechanisms as to how they offer growth to host plant remains to be seen.

As indicated in Table 2, only 5 isolates were capable of re-infecting and forming nodules on all 3 chickpea cultivars. Two alternate cultivars of Natoli and ICC-5003, ICC-5003 and ICC-4918, and Natoli and ICC-4918, formed root nodules in symbiosis with 7, 3 and 5 isolates, respectively (Table 2). The 27 isolates that exhibited the ability to re-infect chickpea seedlings had their own varietal preference. It has been well demonstrated that nodule formation and biological nitrogen fixation is a function of the genotypes of legumes and rhizobia (Giller et al., 2013), which corroborates our present findings. The 3 chickpea cultivars Natoli, ICC-5003, and ICC-4918, were capable of forming root nodules in symbiosis with

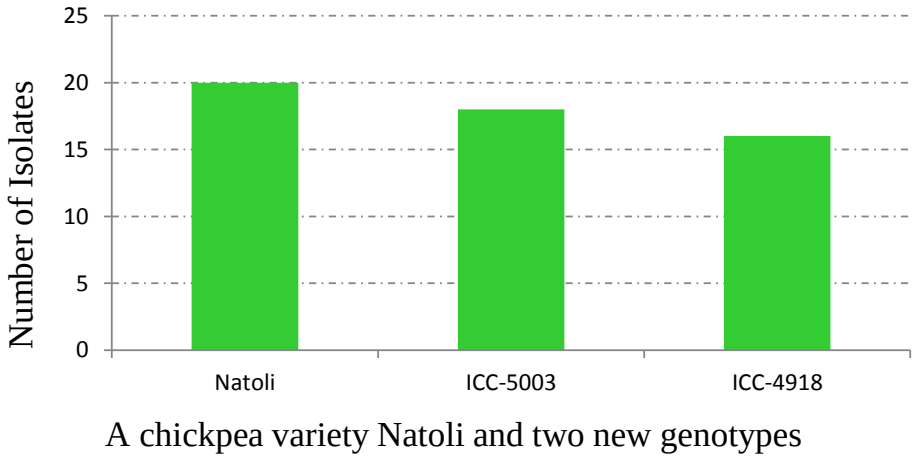

Figure 1. Number of isolates capable of re-infecting and forming nodule on different chickpea cultivars.

20, 17 and 16 isolates, respectively (Figure 1).

Bacterial growth and morphological characteristics

On the basis of their generation times, 18% of the tested isolates belong to Group I, and 6% of test isolates circumscribed within Group II were fast growers with generation time ranging between 1.8 and 2.8 h. 74% of the test isolates (comprising the two groups) were found to be slow growers (with generation time exceeding 3 h and reaching as high as 9 h). Isolate ICRE13 was an extra-slow grower with a generation time that exceeded 9 h. This is consistent with earlier studies of similar nature, reporting that chickpea is being nodulated with rhizobia comprising fast, slow and extra-slow growers. For example, chickpea rhizobia have previously been shown to possess both fast and slow growing strains (Chakrabarti et al., 1986). It was reported that moderately slow growing rhizobia *Mesorhizobium mediteraneum* is specific but also the natural symbionts of chickpea (Nour et al., 1995). Furthermore, in another study of similar nature, 22% of the isolates from chickpea were fast growers, 32% slow growers and 46% extra-slow growing bacteria with a generation time of more than 9 h (Maâtallah et al., 2002). With the exception of few test isolates including ICRE06, ICRE08, ICRE09, ICRE13, ICRE16 and ICRE17 from Group I, and ICNRE01, ICNRE09, ICNRE16, ICNRE17, ICNRE19 and ICNRE20 from Group II, the majority of the tested isolates did not absorb Congo red. This is a distinctive character of rhizobia as already reported elsewhere (Somasegaran and Hoben, 1994) (Table 3).

All strains from both groups were found to have circular colonies with regular borders, flat in elevation, creamy in color, showing intermediate to high production of mucus. 70% of the test isolates (Groups I and II) were characterized with large mucoid texture, whereas, 24% of the isolates appeared as large watery colonies. The colony diameter of all the test isolates within the two defined groups ranged between1.8 and 9.8 mm except for isolates ICRE07 and ICRE22, which showed a colony diameter less than 1 µm. 38% of the tested isolates included within the two groups were shown to produce gum when plated on YEMA media. The majority of the tested isolates produced yellow color on BTB medium indicating that they are acid producers (Vincent, 1970), except isolates ICRE18, ICRE22, ICRE25 and ICRE26 that produced blue color on BTB medium (Table 3). Most of the tested isolates (representing Groups I and II) did not grow on BCP medium except 3 isolates (ICRE09, ICRE17 and ICRE20) from Group I, and 2 isolates (ICNRE04 and ICNRE14) from Group II (Table 3). In an earlier study, it has been well documented that rhizobia were unable to grow on BCP media (Somasegaran and Hoben, 1994). However, this research finding contradicts previous reports of Somasegaran and Hoben (1994). In agreement with this study findings, other studies reported that rhizobial could also grow well on BCP medium indicating the capability of the isolates to use glucose as sole carbon source (Küçük et al., 2006).

Physiological and biochemical tests

pH tolerance

All the isolates (both Groups I and II) tolerated a pH of 8, whereas 86% of both groups grew at a pH of 5.5 (Table S1). This is consistent with the findings of other studies on rhizobia from chickpea growing in Ethiopia (Jida and Assefa, 2012), who reported that all tested isolates from nodules of chickpea grew well in moderately acidic pH (5.5) to neutral pH and slightly alkaline pH (8.0). As indicated in Figure 2, 51% of isolates were tolerant to a lower pH of 4. Very few isolates (5%) were able to resist

Table 3. Isolate code, Congo red absorption on CR-YEMA media (described as either S=slightly absorbed the CR-YEMA medium or No= did not absorb CR-Medium), colony color on BTB (described as Y=for yellow, B=for blue) and growth on BCP (described as '+'=for growth, '-'=for no growth), gum production ('+'=produced gum, '-'=did not produce gum) and mean generation time (in hours) of the tested isolates.

#	Isolate code	CR-YEMA	BTB	BCP	Colony Diameter (mm)	Gum Production	Mean Generation Time
1	ICNRE01	S	Y	-	5.1	-	3.8
2	ICNRE02	No	Y	-	1	-	6.8
3	ICNRE03	No	Y	-	0.7	-	3.2
4	ICNRE04	S	Y	+	3.3	-	2.2
5	ICNRE05	No	Y	-	3.9	+	3.3
6	ICNRE06	No	Y	-	1.6	+	2.1
7	ICNRE07	No	Y	-	1.4	-	3.5
8	ICNRE08	No	Y	-	1.9	-	6.2
9	ICNRE09	S	Y	-	1.2	+	7.1
10	ICNRE10	No	Y	-	3.5	+	3.7
11	ICNRE11	No	Y	-	4.9	+	5.6
12	ICNRE12	No	Y	-	5.1	+	4
13	ICNRE13	No	Y	-	5.1	+	3.7
14	ICNRE14	No	Y	+	5.5	-	2.6
15	ICNRE15	No	Y	-	3.1	-	3
16	ICNRE16	S	Y	-	3	-	1.9
17	ICNRE17	S	Y	-	3.6	+	5.5
18	ICNRE18	No	Y	-	1.4	+	3.2
19	ICNRE19	S	Y	-	4.5	-	5.2
20	ICNRE20	S	Y	-	5.8	-	2.1
21	ICNRE21	No	Y	-	5.6	-	4.3
22	ICNRE22	No	Y	-	2.3	-	2.8
23	ICRE01	No	Y	-	3	+	1.8
24	ICRE02	No	Y	-	3	-	2.6
25	ICRE03	No	Y	-	1.7	-	3.8
26	ICRE04	No	Y	-	1.7	-	4
27	ICRE05	No	Y	-	2	-	5.1
28	ICRE06	S	Y	-	2.9	+	1.9
29	ICRE07	No	Y	-	0.7	-	3.2
30	ICRE08	Yes	Y	-	3.4	-	4.5
31	ICRE09	S	Y	+	3.3	-	2.2
32	ICRE10	No	Y	-	1.4	-	4.8
33	ICRE11	No	Y	+	1.5	-	4.9
34	ICRE12	No	Y	-	4.9	-	4.7
35	ICRE13	No	Y	-	2.3	-	4.6
36	ICRE14	No	Y	-	2.1	+	4.8
37	ICRE15	S	Y	-	4.8	+	9.8
38	ICRE16	S	Y	-	1.5	+	3.6
39	ICRE17	S	Y	-	2.3	+	1.8
40	ICRE18	No	B	-	3.5	-	5.1
41	ICRE19	No	Y	-	1	-	3.3
42	ICRE20	No	Y	+	4.1	-	4.9
43	ICRE21	No	Y	-	1.5	+	2.5
44	ICRE22	No	B	-	0.9	-	2.3
45	ICRE23	No	Y	+	5	-	6.2
46	ICRE24	No	Y	-	2.6	-	3.9
47	ICRE25	No	B	-	2	-	6.4
48	ICRE26	No	B	-	3.5	-	2.8
49	ICRE27	No	Y	-	5.3	+	3.1
50	ICRE28	No	Y	-	2	+	3.7

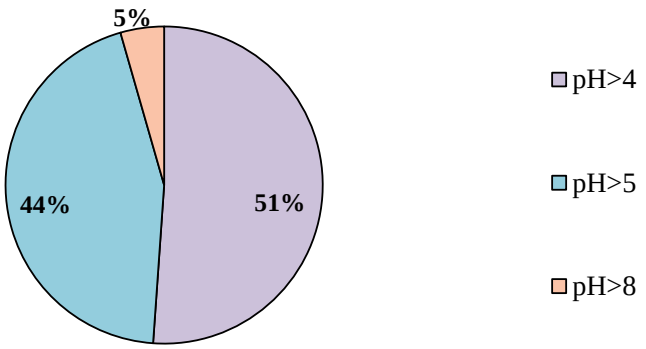

Figure 2. Proportion of chickpea nodule endophytes tolerance to different pH values.

a pH of 8 to 10. 30% of Groups I and II isolates grew at pH value of 4, whereas 72% of Groups I and II test isolates were able to tolerate a pH of 10.5. The pH can be a major limiting factor for growth of a number of microorganisms in the soil (Brockwell et al., 1991). Considering pH as one of the parameters influencing the survival and growth of microorganisms in the soils, the tested strains with broad range of pH preference could have a selective advantage over those requiring narrow pH ranges. Nevertheless, the survival and growth of those strains remains to be seen under field conditions with contrasting pH values before developing them into inoculant.

Salt tolerance

All the tested isolates (Groups I and II) were able to grow on YEMA containing 0.5% of NaCl, but showed steady decrease in growth as the concentration of salt kept increasing. As indicated in Table S1, the test isolates showed variations to high NaCl concentration. For example, 76% of Group I isolates grew at a salt concentration of 1% NaCl. These results are in agreement with other findings who reported 75% of the tested rhizobial inoculants grew well with 1% NaCl (Jida and Assefa, 2012). All isolates were tolerant to the lowest level of NaCl concentration. Of the tested isolates, only ICRE25 and ICRE28 from Group I and ICNRE11 and ICNRE22 from Group II were able to tolerate NaCl concentration of 4%. Only one isolate from Group I (that is, ICRE08) was found to grow at a NaCl concentration of 5%. This osmo-tolerant isolate may be considered as a potential candidates for inoculant development for localities where salinity is a problem, as salinity is reported to decrease the efficiency of rhizobium legume symbiosis (Bouhmouch et al., 2001; Hashem et al., 1998).

Temperature tolerance

High soil temperatures in tropical and subtropical areas are a major problem for biological nitrogen fixation of legume crops, since temperature affects almost all stages of legume-rhizobia symbiosis (Hungria and Vargas, 2000; Zahran, 1999). High root temperatures strongly affect bacterial infection and N_2 fixation in several legume species. For most rhizobia, the optimum temperature range for growth in culture is 28 to 31°C, and many are unable to grow at 37°C (Graham, 1992). Tolerance of the isolates to different temperature levels were tested and are presented in Table S1. 40% of the test isolates tolerated temperatures up to 40°C. Fifteen isolates were tolerant to the lower level of temperature (5°C). In the present study, all Groups I and II isolates grew at a temperature of 20-30°C, whereas 32% of isolates (10 isolates from Group I and 6 isolates from Group II) grew at a temperature of 5°C. The results of this study are consistent with previous findings (Berrada et al., 2012; Zahran, 1999), which showed that rhizobia are mesophiles, and can grow at temperature values ranging between 10 and 37°C. 42% of isolates (12 from Group I and 9 from Group II) were able to grow at a temperature of 40°C. Similarly, growth was reported for both *M. ciceri* and *M. mediterraneum* type strains, which are the natural symbiont of chickpea, at a temperature of 40ºC (Nour et al., 1995). Therefore, it can be suggested that the thermophilic isolates investigated in this study might be the presumptive candidates for inoculant development in areas under extreme temperatures. However, the in vitro results reported in the present study cannot be conclusive, thus those strains should further be screened with the aim to investigate their performance under field conditions.

Carbon source utilization

66% of the chickpea isolates were able to utilize above 80% of carbon sources tested (Table S1). 22% of Group I isolates were able to utilize all carbon sources, while 30% of Group II isolates were able to utilize all carbon sources. 66% of isolates (comprising the two groups) were able to utilize fructose. These are in line with the findings of others from Ethiopian casess who reported that isolates were capable of utilizing fructose as their sole carbon source (Keneni et al., 2010). In another study, it was reported that the slow growing rhizobia grow best on pentose as their carbon source and fast growers usually grow best on glucose or sucrose as their sole carbon source (Somasegaran and Hoben, 1994). However, in the present study, preference for a carbon source seems not to be influenced by being fast or slow growing rhizobia. Concordant with this, different carbon sources could not discriminate the fast and slow growing isolates (Hameed et al., 2004).

Nitrogen source utilization

Similar to the CSU, 66% of the chickpea isolates were

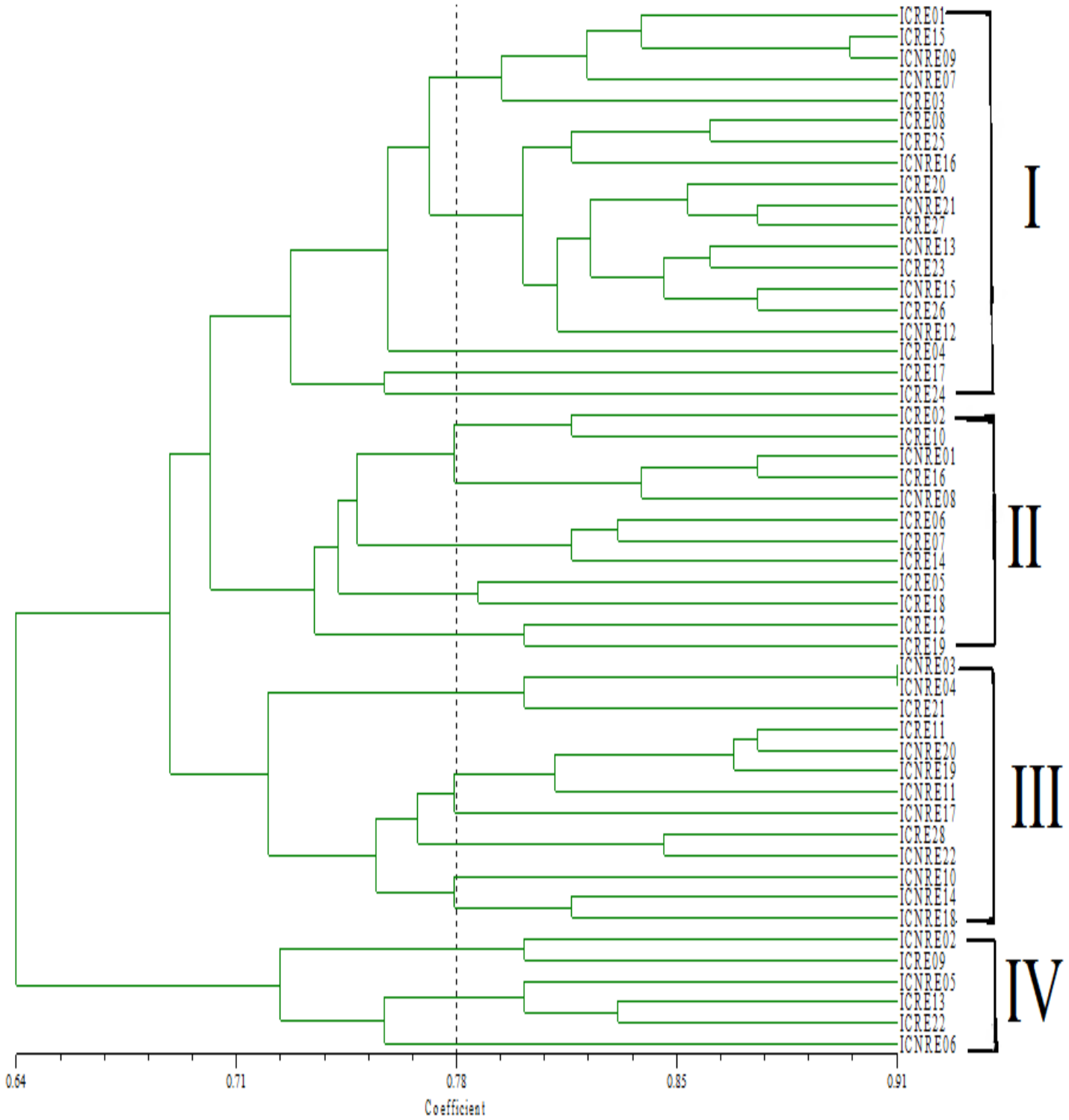

Figure 3. Dendrogram highlighting the phenotypic similarities among non-nodulating and rhizobia nodulating chickpea growing in Ethiopia.

able to utilize above 80% of nitrogen sources (Table S1). The isolates were capable of utilizing amino acids as their sole nitrogen source. All the isolates (comprising the two groups) catabolized L-proline. Two isolates, ICNRE13 and ICNRE15, catabolized all tested amino acids. 96% of Group I and Group II were able to utilize L-Asparagine. Contrary to the findings of this study, it was reported that none of the rhizobia strains utilized the amino acid L-

asparagine (Zhang et al., 1991). In the present study, chickpea rhizobia exhibited diversity in utilizing different N sources. Hence, the finding of this study agreed with other reports of others who reported that many amino acids were found to serve as sole nitrogen sources to rhizobia isolates (Küçük et al., 2006). The ability of the tested isolates to utilize a wide range of amino acids as sole nitrogen source could be considered as a desirable trait for bacterial survival and growth in areas where nitrogen source is a limiting factor.

Intrinsic antibiotic resistance (IAR)

All endophytic bacteria were assessed for their IAR pattern against two doses of Ampicillin, Chloramphenicol, Erythromycin, and Streptomycin. As indicated in Table S1, all the tested isolates (comprising the two groups) were resistant to Erythromycin at a concentration of 5 µg/ml. Only isolates ICNRE22 (Group II) and ICRE03 (Group I) were resistant to 10 µg/ml Neomycin and 10 µg/ml Kanamycin, respectively. Streptomycin, Neomycin and Kanamycin were found to be the most potent antibiotics that allowed the growth of few isolates. The pattern of antibiotics resistance has been used to identify diversity among strains of rhizobia (Somasegaran and Hoben, 1994). The isolates in this study showed variation in their resistance to the tested antibiotics. Similarly, great variations among chickpea rhizobia with respect to IAR pattern were detected in other studies (Küçük and Kivanc, 2008; Maâtallah et al., 2002). It has been reported that fast-growing strains are more sensitive to antibiotics than slow-growing rhizobia (Maâtallah et al., 2002). Conversely, in this study, the tested isolates comprising of fast, intermediate and slow growing members, showed a wide range of behavior with regard to antibiotics. It can be suggested that, the tested isolates with high resistance to different antibiotics could be selected as a candidate for inoculant development for localities where antibiotics production by different soils dwelling microorganisms are a common phenomenon.

Indole acetic acid (IAA)

Results for Indole Acetic Acid (IAA) production suggest only 33% of these isolates were able to produce it. The Indole Acetic Acid produced by rhizobia accelerates elongation of root hairs and lateral roots, thus improving crop growth (Okon and Kapulnik, 1986). Several researchers demonstrated that rhizobia are capable of producing IAA (Etesami et al., 2009; Sridevi and Mallaiah, 2007). 30% of chickpea isolates in this study produced IAA (Table S1). In another study of similar nature, it was reported that 61.6% of the tested isolates produced IAA (Trivedi et al., 2011), while 12 isolates (out of the 16 tested isolates) were shown to produce IAA (Rani et al., 2011). Endophytic bacteria (nodulating and non-nodulating ones) within the root nodules of legumes were reported to have several desirable attributes, for example, the ability to promote plant growth (Alikhani and Yakhchali, 2010) through the synthesis of phytohormones such as IAA.

Intrinsic heavy metal resistance

In the present study, 74% of tested isolates (comprising the two groups) were resistant to $MnSO_4$, whereas 24% of them were resistant to Pb (COOH) (Table S1). Fifty and 26% of isolates (comprising the two groups) were resistant to Cu and Zn, respectively. The significant importance of resistance of heavy metals to isolates indicates their ability to survive in soil contaminated with heavy metals as described elsewhere (Alikhani and Yakhchali, 2010).

Enzymes production

The isolates were tested for amylase, catalase and Cellulase production. 92% of the isolates (26 from Group I and 20 from Group II) were able to produce amylase. 78% of Groups I and II isolates were able to produce catalase. 66% of the isolates (20 from Group I and 13 from Group II) were able to produce Cellulase (Table 3). These findings are in line with another study of similar nature (Bhatt and Vyas, 2014), who reported production of amylase, catalase and cellulose by chickpea isolates. Furthermore, it was also reported that 64% isolates from chickpea were able to produce cellulase on carboxymethylcellulose (CMC) medium (Bhagat et al., 2014).

Siderophore production

Siderophores are known to bind to the available form of iron (Fe^{3+}) in the chickpea rhizosphere thus making it unavailable to the phytopathogens and consequently protecting plant health (Wani and Khan, 2013). 90% of the tested isolates (25 from Group I and 20 from Group II) exhibited positive results for siderophore production test (Table S1), which is similar to the work of others (Bhagat et al., 2014), who reported 88% of chickpea isolates were able to produce siderophores. Additionally, in other studies, it was reported that Mesorhizobium species recovered from nodules of chickpea produced siderophore (Küçük and Kivanc, 2008; Raychaudhuri et al., 2005). The fact that the test strains were able to produce siderophore was an indication that those strains may protect their host by outcompeting pathogens, thus enhancing the growth of the host plant.

Cluster analysis

The result of the cluster analysis performed on the 50 test isolates recovered from root nodules of chickpea growing in diverse chickpea growing localities in Ethiopia for 74 phenotypic traits, is shown in Figure 3. All the isolates examined were grouped into 4 (designated as I-IV) clusters, when 78% similarity level was used as a cutoff point.

Isolates in cluster I were characterized by surviving pH values ranging between 4 and 10.5. None of them in this cluster survive salt concentration greater than 3.5 except one isolate (ICNRE13) belonging to Group II. In this cluster, seven isolates (3 from Group I and 4 from Group II) were capable of surviving higher temperatures of 40°C. All Groups I and II isolates in cluster I were sensitive to Neomycin and Kanamycin. Ten of Group I isolates in cluster I showed phosphate solubilization. All Groups I and II isolates in cluster I were able to produce siderophore. None of Group I isolates in cluster II were capable of growing at pH 4 whereas, all of them grew at pH 5 - 9.5 except ICRE12 which failed to survive at pH 9.5. All Groups I and II isolates in cluster II were capable of surviving salt concentrations up to 2%. Only isolate ICRE16 of cluster II was able to grow at a temperature of 45°C. Almost all of Groups I and II isolates in cluster II were highly sensitive to Neomycin, Chloramphenicol, Streptomycin and Kanamycin. Isolate ICRE19 solubilized phosphate and isolate ICRE07 produced IAA. In addition, all Groups I and II isolates within cluster II were able to produce catalase and cellulase, while all isolates except ICRE08 and ICRE14 were able to produce Amylase in this cluster. In cluster III, all Groups I and II isolates were resistant to 2% salt concentration, whereas 76.4% of isolates (comprising the two groups) were able to grow at 3% salt concentration. All isolates except ICNRE11, ICNRE15 and ICNRE20 were able to grow at a temperature between 15 and 35°C. None of the isolates in this cluster were able to grow at 45°C.

The fourth cluster contained 5 isolates. The growth of isolates in cluster IV was limited to pH values 5.5 and 8. None of the isolates (comprising the two groups) in cluster IV were able to survive at pH values less than 5.5 and greater than 8, except isolate ICRE13 that was able to grow at pH value 8.5. All Groups I and II isolates in cluster IV were unable to tolerate salt concentration of more than 1%.

Isolates ICNRE05 and ICRE13 were unable to grow at salt concentration of 1%, but they were able to survive temperatures between 10 and 40°C in cluster IV. With regard to intrinsic antibiotic, all Groups I and II isolates in cluster IV were highly sensitive to Neomycin, Kanamycin, Streptomycin and Chloramphenicol at all tested concentrations. All Group I isolates in cluster IV were capable of resisting $MnSO_4$. Isolates ICRE13 and ICRE22, were able to produce siderophore and solubilize phosphate.

Conclusion

This study revealed enormous diversity among the tested isolates. Rhizobial and non-nodulating endophytic bacteria harboring the chickpea root nodule collections were almost equal in proportion. Nodulating group, in this study showed desirable physiological characteristics that could be further verified under field condition to develop inocula for chickpea. Although the non-nodulating endophytic bacteria (Group II in the present study) harboring root nodules of chickpea could not infect the chickpea to produce nodules, they showed very positive physiological characteristics that could accelerate seedling emergence, promoting plant establishment under adverse conditions. However, results from the present study are inconclusive, thus detailed molecular mechanisms revealing their effects and functions in plant growth need to be comprehensively defined. Taken together, the existence of enormous diversity among the test isolates reveal the presence of diverse rhizobial and non-rhizobial isolates within the nodules of chickpea in the study sites. However, more exploration and characterization is required to unearth unidentified but existing endophytic bacteria resident within the nodules of chickpea growing in Ethiopia.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Table S1. Physiological and biochemical characterization of endophytic isolates.

Isolates	pH tolerance	Salt (%) tolerance	Temp. (^{o}c) tolerance	C- Sources Utilized (%)	N-sources Utilized (%)	Antibiotics resistance		Intrinsic Heavy Metals Resistance	Enzymes production			IAA production	PO_4 solublization	Siderophore production
						5%	10%		Amylase	Catalase	Cellulase			
ICRE01	4.5-11	0.5-2	10-30	91	91	Ne, Ch, Er,	Er, St,	Al, Cu	+	-	+	+	+	+
ICRE02	5.5-10.5	0.5-2	10-30	73	82	Er	Er	Mn, Al, Zn	+	+	+	-	-	+
ICRE03	4-10.5	0.5-3	10-40	91	91	Er, Ka	Er, Ka	Mn, Co, Cu	+	-	+	-	+	+
ICRE04	4-11	0.5-2	10-35	73	73	Ch, Er, Ka	Ch, Er	Pb, Mn, Al, Co	+	+	+	+	-	+
ICRE05	5-10.5	0.5	20-40	54	82	Er	Er	Pb, Mn, Al,	+	+	+	-	-	+
ICRE06	4.5-11	0.5	15-30	73	63	Ch, Er	Ch, Er	Mn	+	+	+	-	-	+
ICRE07	4.5-10.5	0.5	15-35	72	54	Er	Er	Mn, Al, Co	+	+	+	+	-	+
ICRE08	4-10.5	0.5-2.5	10-40	91	73	Ne, Ch, Er	Er, Str	Mn	+	+	+	+	-	+
ICNRE01	4.5-10.5	0.5-2	10-40	91	91	Er	Er, Ka	Pb, Mn, Al	-	+	+	-	-	-
ICNRE02	5-5.5	0.5-1	10-30	54	73	Er	Er	Mn	+	+	-	-	-	+
ICRE09	8	0.5-1	20-30	63	73	Er	Er	Mn, Al, ,Cu	+	-	+	-	-	+
ICNRE03	5.5-10	0.5-2	5-40	73	91	Er	-	Al, Cu	+	+	-	+	+	+
ICNRE04	5.5-10	0.5-2	5-40	82	82	Er	-	Mn, Co, Cu	+	-	-	+	+	+
ICRE10	5-10	0.5-2	10-30	91	82	Er	Er	Mn, Al, Co, Zn	+	+	+	-	-	+
ICRE11	8-10	0.5-4	5-40	91	73	Er	Er	Mn, Al, Cu, Zn	+	+	+	-	-	+
ICNRE05	5.5-8	0.5	10-40	82	91	Er	-	Pb, Mn, ,Cu,	-	+	-	+	-	-
ICRE12	5-9	0.5-2	15-35	73	82	Er	Er	Pb, Zn, Co	+	+	+	-	-	+
ICNRE06	5.5-9	0.5-2.5	5-35	82	91	Er	-	Cu, Al, Co	+	+	+	-	-	+
ICRE13	8-8.5	0.5	10-40	100	91	Er	-	Pb, Mn, Co,	+	-	+	-	+	+
ICRE14	5-10.5	0.5	10-30	73	82	Er	-	Mn, Co	-	+	+	-	-	-
ICRE15	4-11	0.5-2	35-40	91	91	Er, Ka	Er	Mn, Al, Co	+	-	-	+	+	+
ICNRE07	4-11	0.5-2.5	15-35	100	91	Er	Er	Mn, Zn	+	+	+	-	+	+
ICNRE08	5-11	0.5	10-35	100	73	Er	Er	Pb, Mn, Al	-	-	+	-	+	-
ICNRE09	4-11	0.5-2	10-30	100	82	Er	Er	Mn, Co	+	-	+	+	-	+
ICRE16	5-10.5	0.5	10-45	100	91	Er	Er	Mn, Al	+	+	+	-	-	+
ICRE17	4-10.5	0.5	15-45	91	63	Er	Er	Mn,Al	+	+	-	+	+	+
ICRE18	5-9.5	0.5	15-35	63	63	Er	Er	Pb, Al, Cu, Zn	+	+	+	-	-	+
ICRE19	5-10.5	0.5-1.5	15-35	100	63	Er	Er, Str.	Pb, Al, Co, Zn	+	+	+	-	+	+
ICRE20	4-10.5	0.5-2	5-40	91	82	Ne, Ch, Er	Er,	Mn, Cu, Al,	+	+	+	+	-	+
ICNRE10	5.5-10.5	0.5-4	5-30	36	73	Er.	Er.	Mn, Cu Co,	+	+	+	-	-	+
ICRE21	5-10.5	0.5-2	5-40	63	82	Ch, Er.	Er.	Mn	+	+	-	+	-	+
ICNRE11	4-10.5	0.5-5	5-40	82	91	Er,	Er,	Pb, Cu, Co	+	+	-	+	-	+
ICRE22	5.5-8	0.5-1	5-30	100	91	Er,	Er	Pb,Mn,cu,co,	+	+	-	-	+	+
ICNRE12	5-10.5	0.5-1	5-30	91	82	Ne, Er,	Er, Str.	Mn,cu Al,Zn	+	+	-	-	+	+
ICNRE13	5-10.5	0.5-4	5-35	91	100	Ch, Er,	Ch Er,	Al, Mn,	+	+	-	-	+	+
ICNRE14	8-11	0.5-3.5	10-35	73	91	Er	Er	Cu, Al,	+	+	+	-	+	+
ICNRE15	4.5-11	0.5-1.5	5-40	100	100	Ch, Er,	Ch, Er,	Mn, Cu, Al,	+	-	+	-	+	+
ICRE23	4-11	0.5-3	1o-30	91	91	Ch, Er,	Ch, Er	Cu, Al	+	+	+	-	+	+
ICRE24	4-11	0.5	5-30	100	54	Ch,Er	Ch,Er	Mn, Al, Co	+	-	+	-	+	+
ICRE25	4-11	0.5-2	10-35	91	63	Ne Ch, Er	Ch, Er	Cu, Al,	+	+	-	-	-	+
ICNRE16	4-11	0.5-3.5	10-45	91	82	Ne, Ch, Er	Ch, Er	Pb, Mn, Cu,	+	+	-	-	+	+
ICNRE17	4.5-10	0.5-4	10-35	73	82	Er	Er	Al, Zn, Co	+	+	-	+	+	+
ICNRE18	5-8.5	0.5	15-35	54	82	Er	Er	Pb, Mn, Zn,	+	+	+	-	+	+
ICNRE19	5.5-8.5	0.5-4	5-30	100	91	Er	Er	Mn, Al, Co	+	+	-	-	+	+
ICNRE20	5.5-10.5	0.5-4.5	5-40	91	82	Er	Er	Co	+	+	+	-	+	+
ICNRE21	4.5-10	0.5-2	10-35	100	91	Ne, Ch, Er	Ne, Er, C	Cu	+	+	+	-	-	+
ICRE26	4.5-10.5	0.5-2	10-40	91	91	Ne, Ch, Er	Er, Ch	Mn, Cu, Al,	+	+	-	-	+	+
ICRE27	4.5-10.5	0.5.2	10-30	100	91	Ne Ch, Er	Ch, Er	Pb, Cu, Zn,	+	+	-	+	-	+
ICRE28	4-10.5	0.5.4.5	5-40	91	63	Ch, Er	Ch, Er	Mn, Al	+	-	+	-	-	+
ICNRE22	4-10.5	0.5-4.5	5-35	100	82	Ne, Er	Ne, Er	Mn, Al	+	+	+	-	-	+

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Full Length Research Paper

Nosocomial infections are still a major concern in peri-urban polyclinics in Ghana

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The study assessed potential sources of nosocomial infections as well as the knowledge of healthcare workers about these infections in a peri-urban polyclinic of Ghana. Swabs were taken in duplicates from beds in the wards, door knobs, sinks, nurses' desks, taps handles, delivery beds, wound dressing rooms, and door and flashing handles of lavatories. After overnight incubation, the samples were serially diluted two times and the third diluent was used in culturing unto plate counting agar, MacConkey agar, blood agar and incubated at 35±2°C for 24 h. The plates were read for colonies and isolated colonies were identified. A simple random method was used to sample the respondents from the various departments in the Polyclinic. Structured questionnaires were administered to solicit their knowledge or understanding of possible causes of nosocomial infections and their perception of the efficacy of the cleaning processes employed in the facility as well as measures put in place to protect healthcare workers from these infections. Data collected showed that *Bacillus spp.* was the predominant bacterium isolated contributing 64.3% of the total isolate. Out of the 24 pathogenic organisms forming 35.7% of the total organisms isolated, *Staphylococcus aureus* (94.8%) formed the majority and *Pseudomonas aeruginosa* (5.2%) was the least isolated. There was no significant difference between the number of isolates recorded before cleaning and after cleaning (P > 0.05) with almost the same number and type of organisms isolated in both cases. Apart from the orderlies who demonstrated little knowledge on nosocomial infections, all the other healthcare workers exhibited adequate knowledge of nosocomial infections. The seemingly high percentage of pathogenic isolates from our study site indicates a high potential risk of nosocomial infections in peri-urban polyclinics.

Key words: Fomites, infections, *Staphylococcus aureus*, *Bacillus spp., Pseudomonas aeruginosa.*

INTRODUCTION

The spread of nosocomial infections serves as a major source of worry for managers in healthcare practice, particularly in developing countries where the health care system is already overstretched (Bello et al., 2011).

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Although the infection is most prevalent among patients on admission, healthcare workers also tend to act as potential routes for these pathogenic agents. Hospitals provide a favorable transmission pathway for the spread of nosocomial infections, owing partly to poor infection control practices among health workers on one hand and overcrowding of patients in most clinical settings on the other (Samuel et al., 2010). Today, most infections acquired in the hospitals are caused by microorganisms which are common in the general population (*Staphylococcus aureus*, *coagulase negative Staphylococci, Enterococci, Enterobacteriaceae*), in which they cause no or milder disease than among hospital patients (Brooks et al., 2007).

In Ghana, however, a similar study by Tagoe et al. (2011) isolated a high percentage of *S. aureus* and *E. coli* as well as *Bacillus spp*. on fomites at the Volta Regional Hospital which indicated a high potential risk of nosocomial infections in that hospital. The case in peri-urban facilities could be worse due the number of patients visiting such facilities (Tagoe et al., 2011). The study therefore sought to investigate potential sources and knowledge of the nosocomial infections in the Ashaiman Polyclinic, a peri-urban hospital in Ghana.

MATERIALS AND METHODS

This study was undertaken at the Ashaiman Polyclinic, a peri-urban health facility in the Ashaiman Municipality of the Greater Accra Region, Ghana. The Polyclinic provides healthcare services to the people of Ashaiman and surrounding communities. The Polyclinic is also situated near the main transport terminal and a market that serves most of the communities around its environs. The study was undertaken between January, 2015 and May, 2015. Samples were taken from the hospital and bacteriological analysis undertaken in the Microbiology Laboratory of the University of Cape Coast.

Sampling

The experimentation technique was used to isolate organisms from fomites of which taps, nurse's desks, door handles, (from wards and lavatories), various theatres, as well as handles of taps, sinks and lavatories of the female ward, labour ward, Out-Patients Department (OPD), laboratory, Lying-in-Ward (Recovery ward) and the circumcision room. These sites were selected based on their contact frequency with both patients and healthcare practitioners. Swabs were taken in duplicates at different times and in two sections from each sampling site of the hospital (just before routine general cleaning and just after cleaning). This was done to evaluate the effectiveness of cleaning as compared to that of the cleaning agents being used. The sterile swabs were put in an ice-pack and transported to the University of Cape Coast for further investigation.

Isolation of organisms

Standard isolation techniques were employed in isolation of organisms. Swabs were immediately transported to the laboratory and incubated in peptone water overnight at 35 ± 2°C to encourage growth as described elsewhere (Tagoe et al., 2011). The samples were serially diluted three times against a McFarland standard and the third diluent was inoculated unto Plate Counting Agar, MacConkey agar and blood agar after incubating overnight. It was then incubated at 35 ± 2°C for 24 h. The plates were observed for colony growth and isolated colonies were identified.

Identification of organisms

Pure isolated colonies were Gram differentiated and then biochemically identified using indole, catalase, citrate, oxidase, Coagulase, and urease tests (Brooks, 2007).

Assessment of health personnel knowledge on nosocomial infection

In the assessment of knowledge of nosocomial infections, 42 health workers in the Polyclinic were randomly recruited. This comprised 30 nurses (of which 5 were ward heads), 5 orderlies or health aids, 5 laboratory workers and 2 physicians. They were each presented with a questionnaire after they consented, to test their knowledge or awareness of possible sources of nosocomial infections in the hospital, the aseptic techniques they employed in their work as well as their impressions and perceptions on the policies put in place to protect them and their patients against nosocomial infection. Responses were categorized as "Good", "Little" and "No knowledge".

Data analysis

The data obtained was analyzed with Microsoft Excel, (2010 version) and presented as graphs showing the relationships between subjects and their frequencies. The MINITAP software version 13 was also used to find the significant difference between the values and the organisms isolated on the various surfaces.

RESULTS

Figure 1 shows bacterial counts at the various sites at the OPD before and after cleaning. The mean bacterial counts were reduced after cleaning of the swab site. Figure 2 illustrates bacterial count at the various sites at the Laboratory before and after cleaning. As shown, the mean bacteria were reduced after cleaning of the swab sites except at the door knob where the mean bacterial count increased after cleaning the swab sites in the laboratory. Figure 3 indicates bacterial counts at the various sites of the labour ward before and after cleaning. As shown, the mean bacterial counts were increased after cleaning of the swab sites in the labour ward. Figure 4 shows bacterial counts at the various sites of the female ward before and after cleaning. As shown, the mean bacterial counts were reduced after cleaning of the swab sites in the female ward. Figure 5 indicates Bacterial counts at the various sites of the Recovery ward before and after cleaning. The mean bacterial count at the various sites of the recovery ward were reduced after cleaning except for the beds where the mean bacterial counts were increased after cleaning in the recovery ward. Figure 6 shows bacterial counts at the various sites of the circumcision room before and after cleaning. The

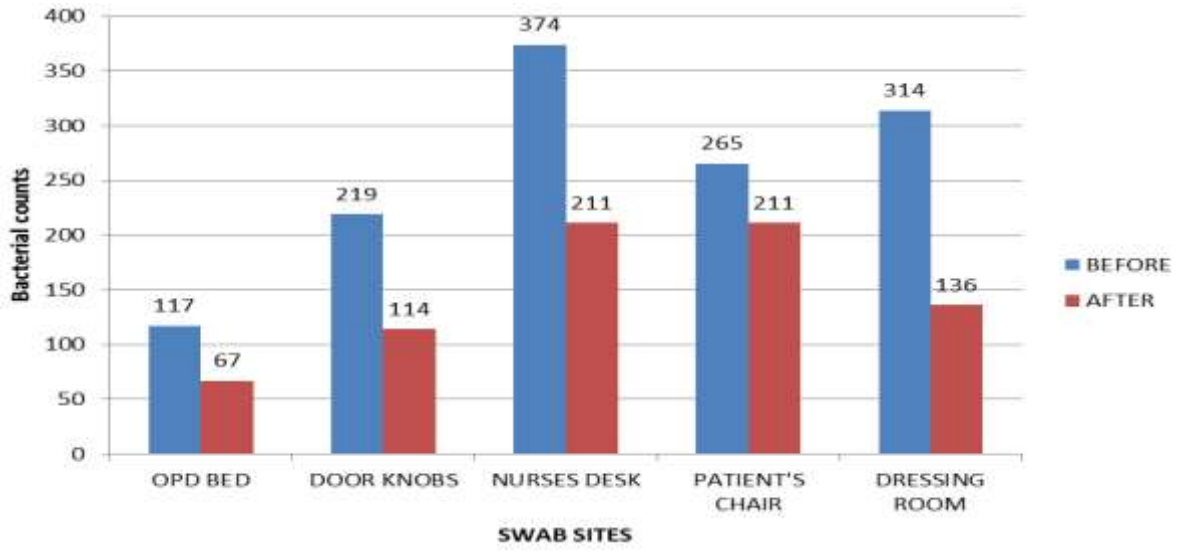

Figure 1. Enumeration of organisms before and after cleaning of the OPD**.**

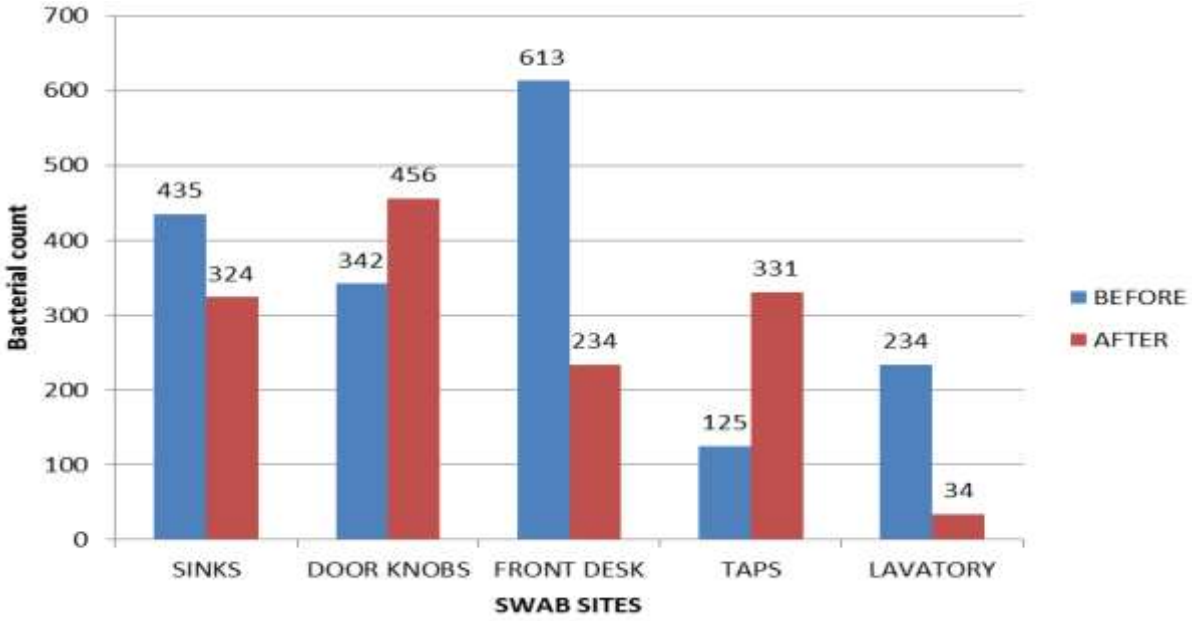

Figure 2. Enumeration of organisms at the various surfaces of the laboratory before and after cleaning

mean bacterial count was almost the same in the various swab sites after cleaning. Figure 7 shows the distribution of pathogenic and non-pathogenic bacteria at the various wards before cleaning. No pathogenic bacteria were recorded in the Out patients' department (OPD) and the laboratory before cleaning. The female and recovery ward recorded equal frequency of pathogenic organisms (*S. aureus*). The labour ward recorded the highest rate of pathogenic infection (*S. aureus* and *P. aeruginosa*). Figure 8 shows the frequency of distribution of isolates in the various wards before and after cleaning of the various swab sites. The frequency of the organisms isolated with the predominant isolate *Bacillus spp.* non-pathogenic (64.3%) and *S. aureus* pathogenic (33.7%) and *P. aeruginosa* (2%) with a significant equal frequency of distribution before and after cleaning. The predominant bacteria in the OPD and laboratory before and after cleaning was *Bacillus* whereas the wards recorded high amount of *S. aureus* before and after cleaning. Figure 9 illustrates the rating of health care workers on the

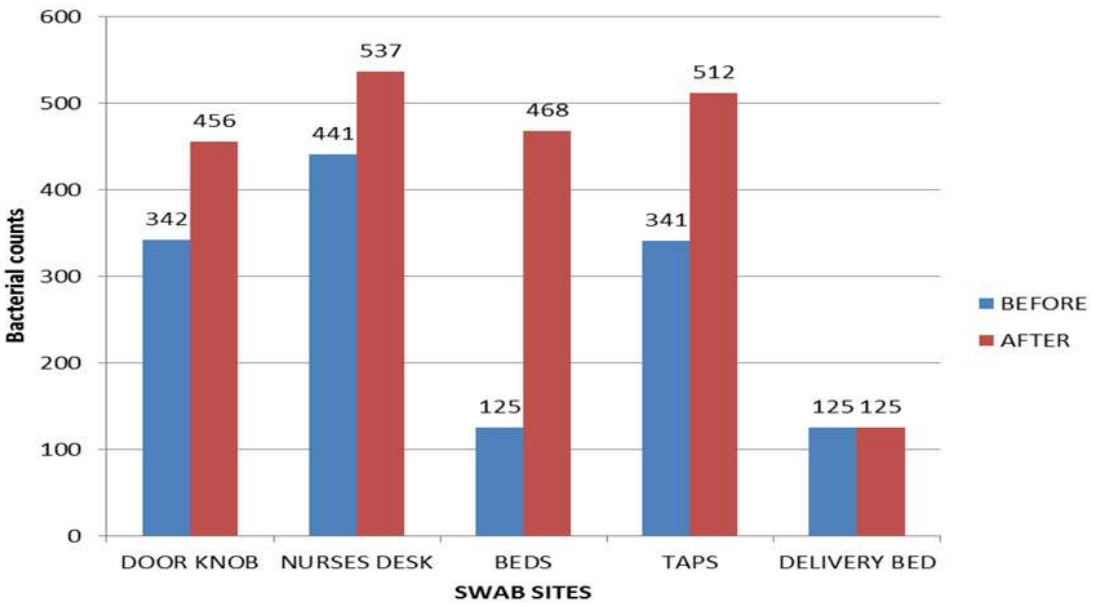

Figure 3. Enumeration of bacteria in the various surfaces of the labour ward before and after cleaning.

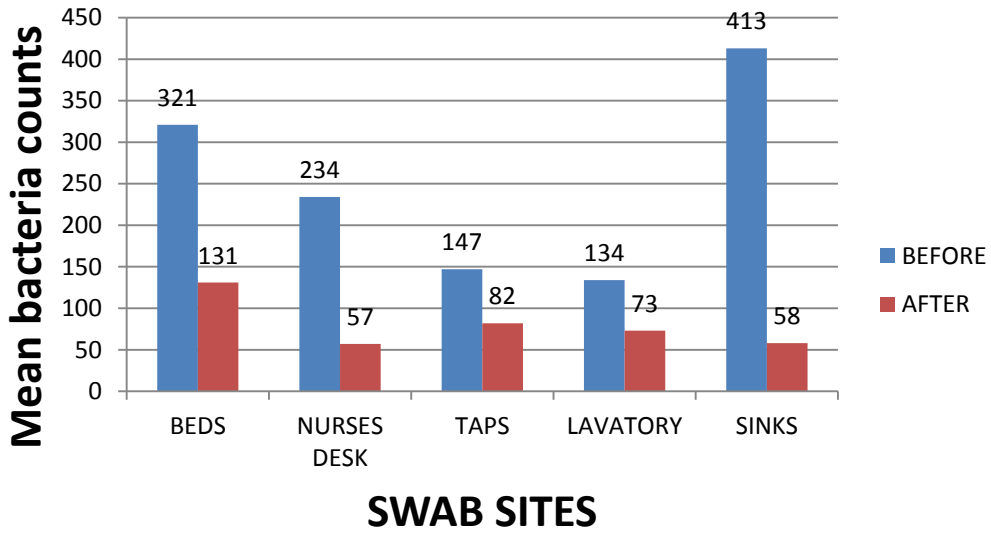

Figure 4. The mean bacterial counts on the surfaces of the female ward before and after cleaning.

knowledge of nosocomial infections. The nurses, laboratory workers, ward heads and physicians had a good knowledge on nosocomial infections whereas the orderlies had no knowledge whatsoever of nosocomial infections.

Figure 10 shows the percentage rating of health workers who have ever acquired nosocomial infection either by suspicion or confirmation by a physician. A greater percentage of the nurses, laboratory workers and orderlies admitted ever acquiring nosocomial infection based on suspicion recording 45, 36 and 30%, respectively whereas a 35, 26 and 30% of the nurses, laboratory workers and orderlies, respectively were confirmed by a physician. None of the doctors reported with nosocomial infection.

Figure 11 shows the rating of the effectiveness of the cleaning sessions by the health workers Majority of the respondents rated the cleaning as either good (40%) or poor (50%). However, 10% of the respondents rated the cleaning as excellent whereas (5%) rated the cleaning as

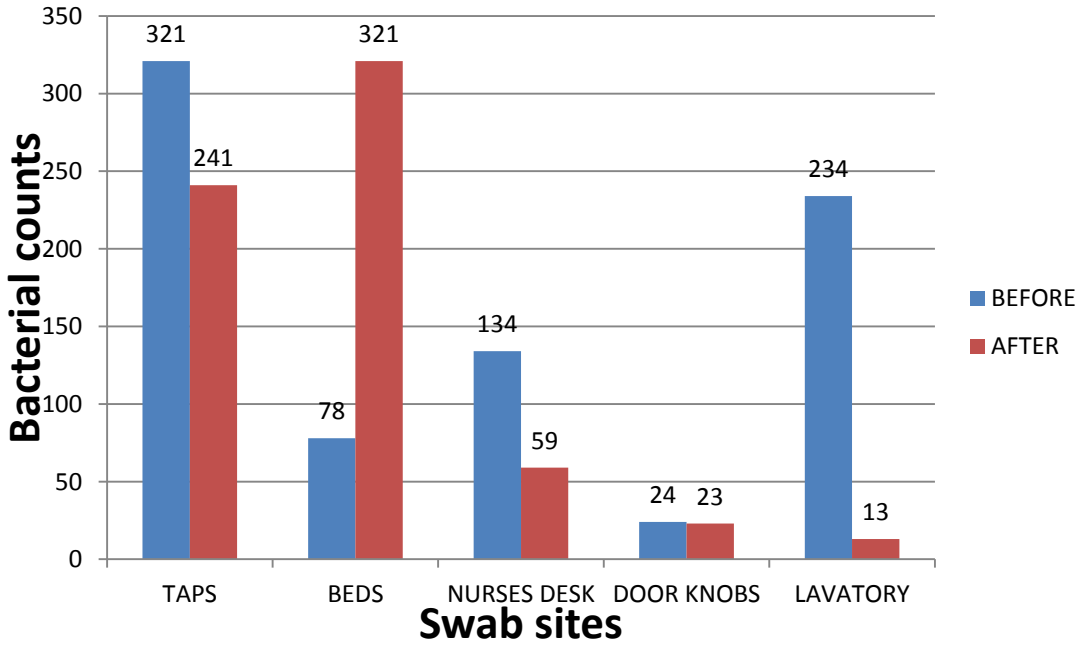

Figure 5. The enumeration of bacteria in the surfaces of the recovery ward before and after cleaning

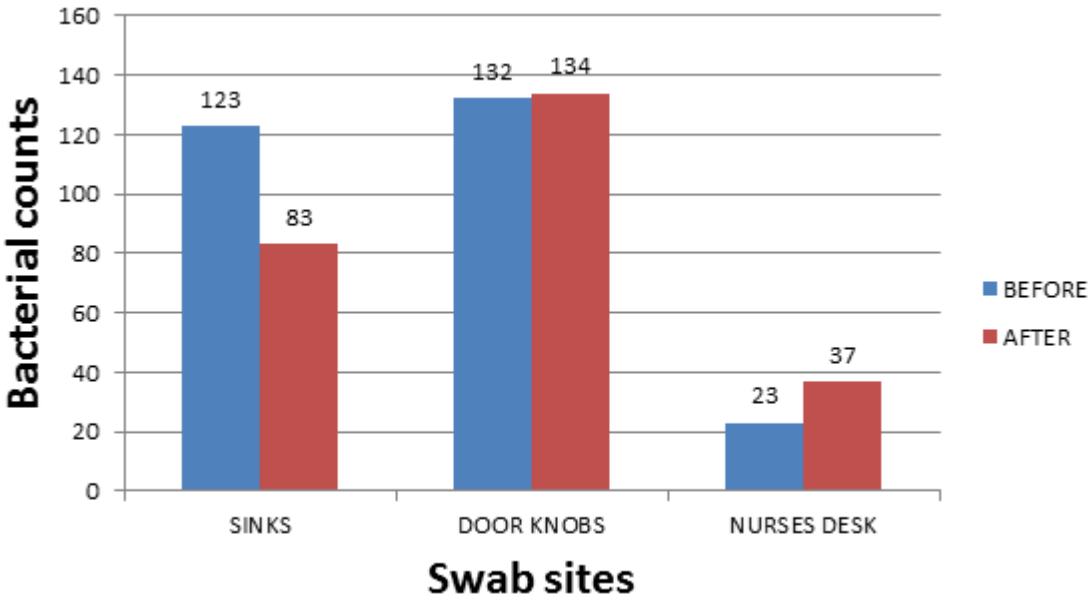

Figure 6. The enumeration of bacteria on the surfaces of the circumcision room before and after cleaning.

very poor. The laboratory workers and the nurses were very much displeased with the cleaning regimes with 60% and 55% rating the cleaning as poor, respectively.

Figure 12 shows the percentage ratings of perception of health workers on protection of nosocomial infection. A majority of the workers forming 60% of nurses, 55% orderlies, 40% of the ward heads do not believe the facility protects them from nosocomial infection.

DISCUSSION

The burden of nosocomial infections in public healthcare facilities cannot be overemphasized (Tagoe et al., 2011), particularly in developing countries. The study therefore

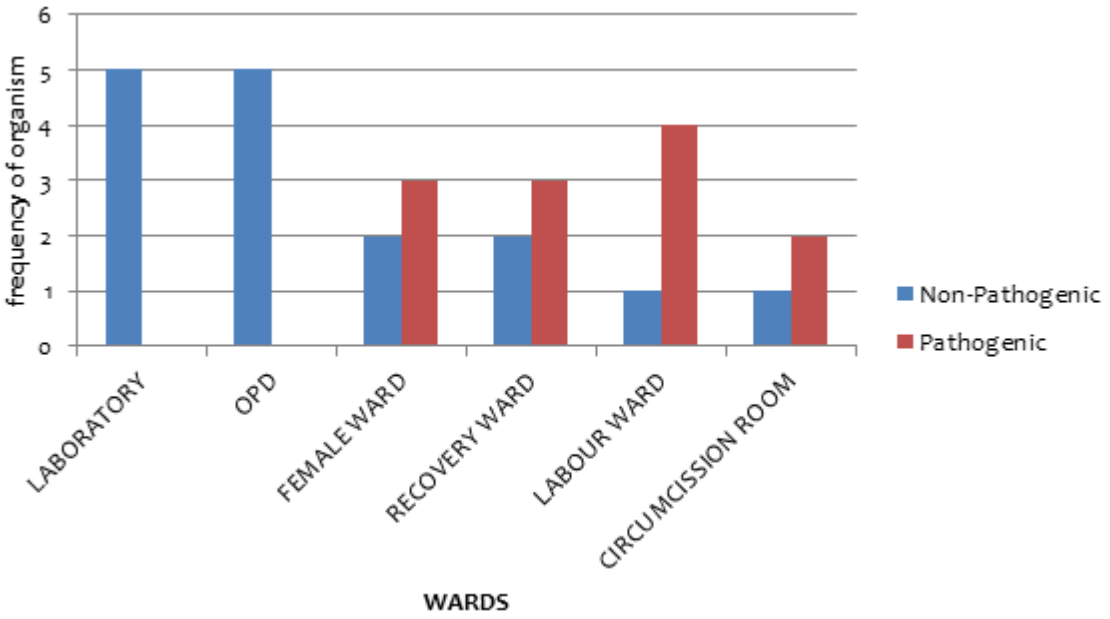

Figure 7. Distribution of pathogenic and non-pathogenic bacteria across the various compartments in the polyclinic

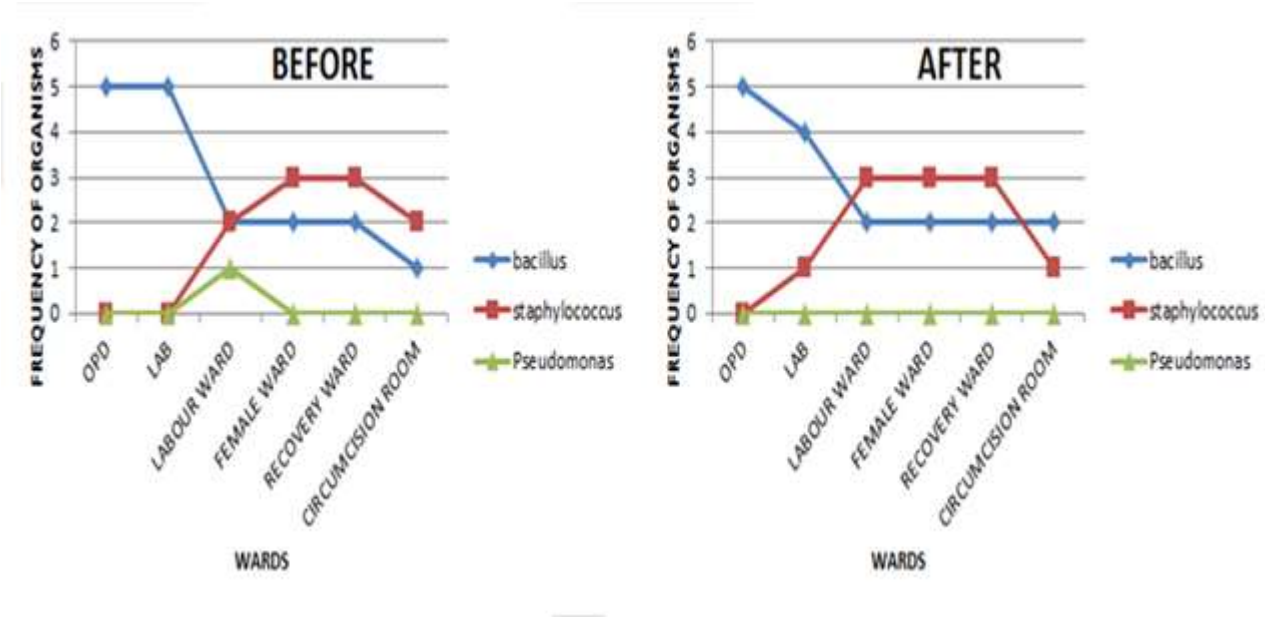

Figure 8. Distribution of isolates in the various wards before and after cleaning.

sought to assess potential sources of nosocomial infections as well as the knowledge of healthcare workers about these infections in a peri-urban polyclinic of Ghana. Results from the study showed that there was a significant growth of bacteria on all the surfaces swabbed (98%). In spite of the fact that majority of the isolated organisms were non-pathogenic (64.3%), it is noteworthy that 35.7% of the pathogenic isolates are of clinical importance and cannot be ignored. In relation to our findings, Ricks et al. (2007) isolated pathogenic bacteria from a hospital ward at a lower percentage of 15%, and this risk had a 10% chance of causing a nosocomial infection (Samuel et al., 2010). Similarly, in this study, the labour ward recorded the highest rate of pathogenic

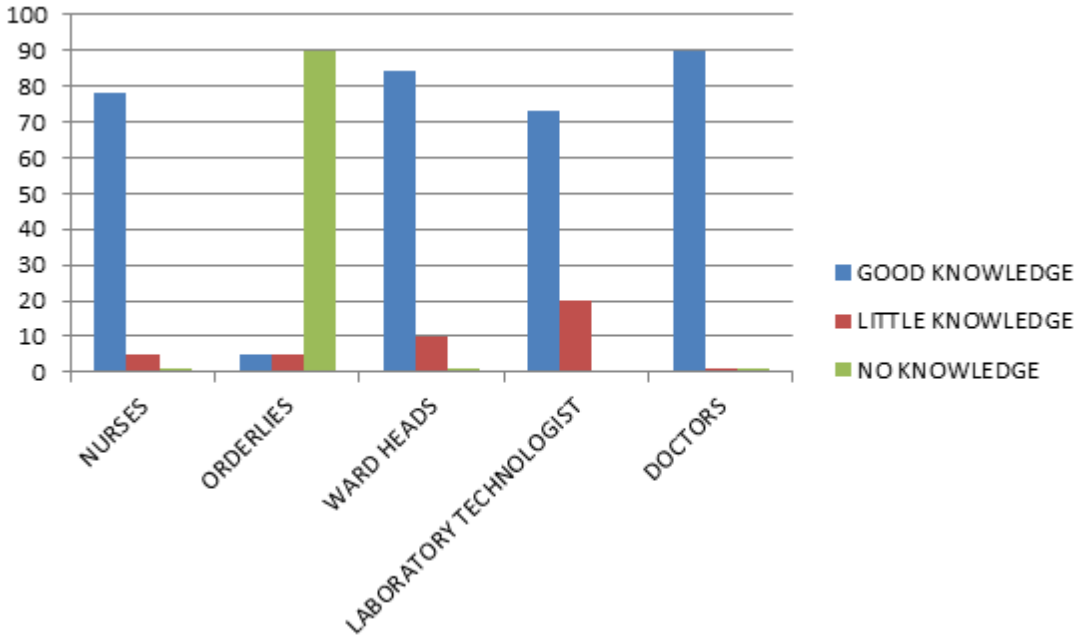

Figure 9. Assessment of the knowledge of health workers on the potential sources of nosocomial infections.

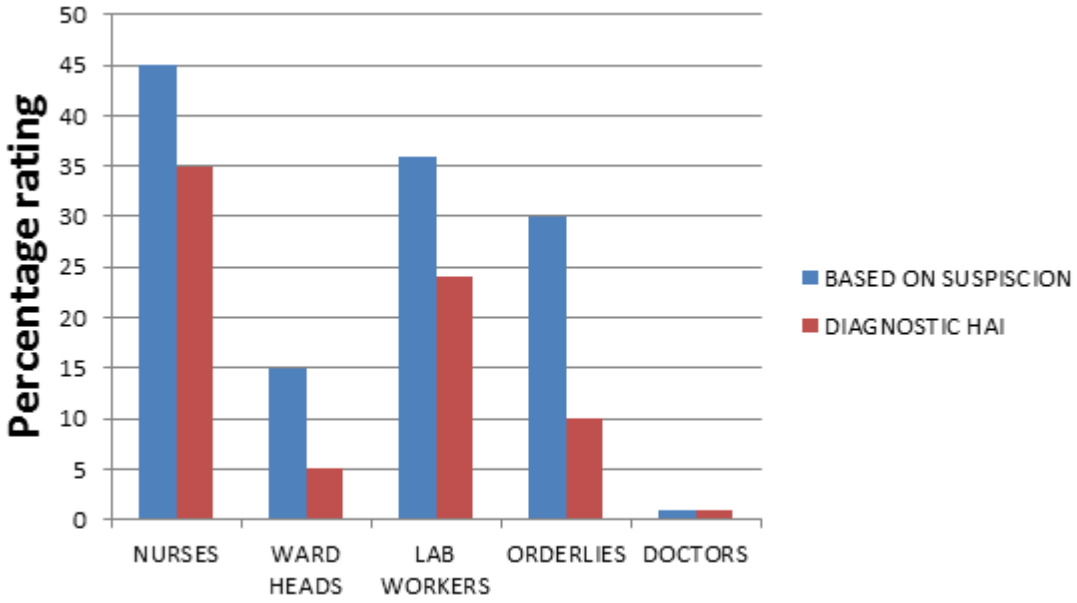

Figure 10. Percentage of workers who have ever acquired a nosocomial infection.

microbes (*S. aureus* and *P. aeruginosa*) before cleaning. This study also discovered that nurses, laboratory workers, ward in-charge's and physicians had a good knowledge about nosocomial infections whereas the orderlies had no knowledge whatsoever of nosocomial infections. Even though about 50% of the respondents rated the effectives of the detergents as good or excellent, there were meaningful bacterial growth at the various swab sites even after cleaning similar to other reports (Alabi and Sanusi, 2012). Interestingly, none of the surfaces recorded "no bacterial growth" before and after cleaning.

The overall distribution of isolates in the various wards before and after cleaning of the various swab sites

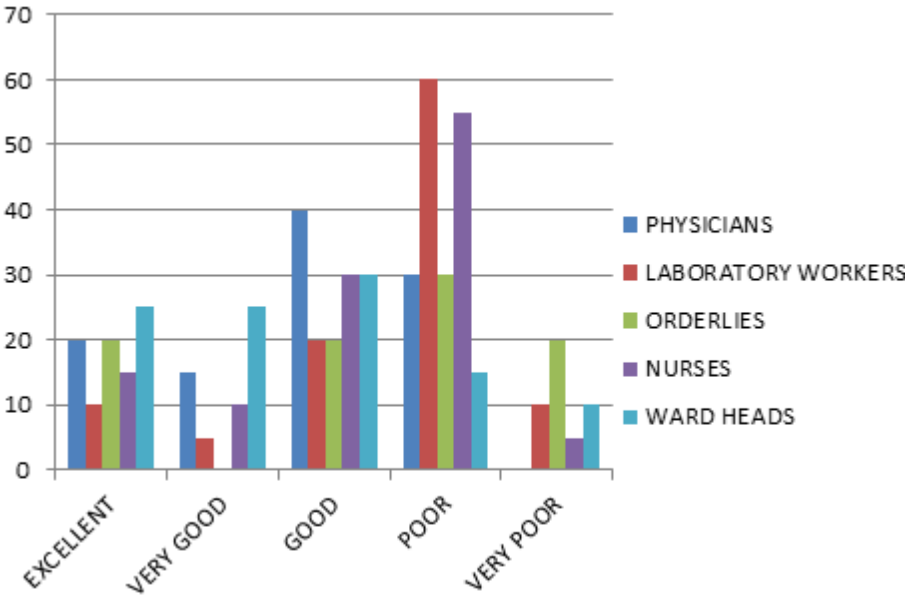

Figure 11. Percentage ratings by various health care workers on the effectiveness of the cleaning in the facility.

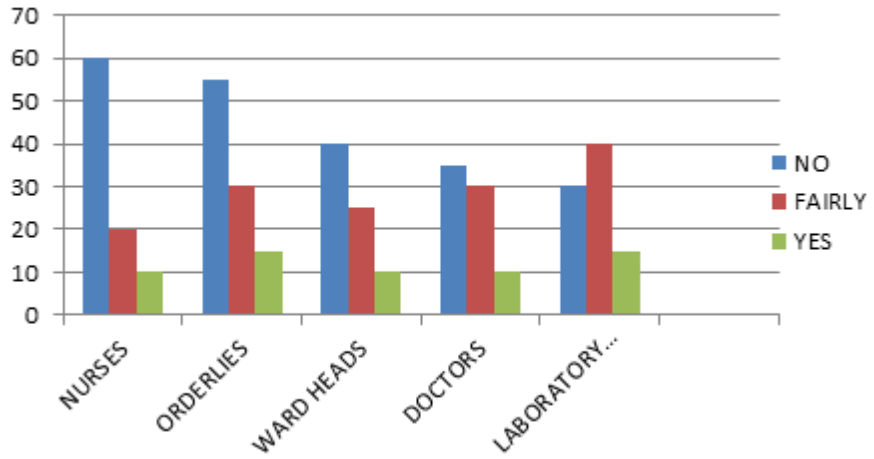

Figure 12. Percentage rating of perception of health care workers on protection from nosocomial infections.

indicated *Bacillus spp.* as the predominant non-pathogenic organism colonizing the sampled surfaces. The colonization of most of the surfaces ditto could be attributed to the fact that *Bacillus spp.* an aerobic, spore-forming gram positive rod bacteria is ubiquitous in nature with their spores able to withstand environmental changes, dry heat and certain chemical disinfectants for moderate periods (Brooks et al., 2007). The high colonization of *Bacillus spp.* is therefore justified especially before cleaning in our study site which is also situated by the main transport terminal and market.

Studies have shown that most hospitals in developing countries have no effective infection control programme due to lack of awareness of the problem, lack of personnel, poor water supply, erratic electricity supply, ineffective antibiotic policies with emergence of multiple antibiotic resistant microbes, poor laboratory backup, poor funding and non-adherence to safe practices by health workers (Samuel et al., 2010; Ismail, 2015). This study however discovered that after cleaning, the number of colonies counted were almost the same as the colonies before cleaning, supporting other findings (Alabi

and Sanusi, 2012). This raises lots of concerns especially when the efficacy of the cleaning processes is compromised by healthcare workers reconstituting detergents before usage.

The fomites at the OPD and the laboratory recorded the highest number of *Bacillus spp.* at the various swab sites recording 62.5% of the *Bacillus spp*. and this is comparable to another study (Weinstein and Hota, 2004) which reported that taps usually have the most pathogenic isolates but the least total bacterial colonization. In a similar study in an urban hospital in Ghana, Tagoe et al. (2011) reported that the predominant isolates in the laboratory was *Bacillus spp*. except for the lavatory. Their finding associated the laboratory's periodic disinfection of the working benches and tables as part of daily quality control measures even after cleaning as a contributing factor to the colonization of these surfaces. Interestingly the present data also reports a similar finding in our setting. The predominant pathogenic organism isolated were *S. aureus* (94.8%) and *P. aeroginosa* (5.2%). This finding is confirmed by Ducel *et al (*2002) who grouped *E. coli,* and *S. aureus* among the predominant organisms that are isolated in the hospitals and among the leading causes of nosocomial *infection*. We report that across the wards, the predominant isolate was *S. aureus* recording 40%, *P. aeroginosa* 10% and *Bacillus spp.* 50%. The labour ward recorded 60% of *S. aureus on* the door knobs and beds and 5.55% of *P. aeroginosa* on the delivery bed. Findings from our study reveal that the labour ward in comparison to other areas posed the highest risk of nosocomial infection. This situation is quite alarming since the labour ward supports women in child delivery and even expectant mothers are housed for some time prior to delivery; so *S. aureus* colonizing the surfaces of beds and door knobs could be detrimental to the health of the mother, baby and healthcare workers as well.

The recovery ward recorded *S. aureus* as the predominant and only pathogenic bacteria. These isolates were found on the taps, nurse desks and the lavatories comparable to the sites swabbed in the female ward. In 2004, Hota found out that taps usually have the most pathogenic isolates but the least total bacterial colonization. This is because the handles of the taps come into contact with detergents most often hence the sites are being washed. These sites are likely to contain bacteria that are resistant to the detergent or one that was left before the swabbing was done. Knowledge on nosocomial infections by the professionally trained health care workers (nurses, doctors, ward managers and biomedical scientist) in our study was generally high as reported in a similar study (Parmeggiani et al., 2010). As expected, it was also observed that orderlies who are least trained amongst the array of health care workers, demonstrated little or no knowledge of nosocomial infections and this situation is worrying especially since they are chiefly involved in routine cleaning processes.

There is evidence that contaminated surfaces contribute to the spread of nosocomial infections (Otter et al., 2013). Similarly, in our study, the efficacy of cleaning regimes in the polyclinic were deemed to be poor as about half of our respondents rated the effectiveness of the cleaning as poor, showing their dissatisfaction with the cleaning regimes in the polyclinic. The nurses and the laboratory workers were particularly most dissatisfied (55 and 60%, respectively) amongst our study participants. Interestingly, similar observations were also made in the respondent's perception of whether they were protected against nosocomial infections. Again, about half of the participants reported they believe they have little or no protection against nosocomial infections from this study.

Conclusion

This study shows clearly that the main non-pathogenic organism present on fomites at the polyclinic is *Bacillus spp.* whereas the main pathogenic organism present were *S. aureus* and *P. aeruginosa* with *S. aureus* forming majority of the pathogenic bacteria isolated. The cleaning sessions at the polyclinic had no meaningful difference in the number of bacteria isolated before and after cleaning was done. Apart from the orderlies who demonstrated little knowledge on nosocomial infections, all the other healthcare workers exhibited adequate knowledge of nosocomial infections. In this wise, it is recommended that orderlies be given special and adequate training especially on nosocomial infections so as to keep them well informed and educated on issues concerning hospital acquired infections for the benefits of the patients, facility and the workers as a whole.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Quorum sensing: An imperative longevity weapon in bacteria

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Bacterial cells exhibit a complex pattern of co-operative behaviour as shown by their capacity to communicate amongst each other. Quorum sensing (QS) is a generic term used for bacterial cell-to-cell communication which secures survival of its species. Many QS bacteria produce and release autoinducers like acyl-homoserine lactone-signaling molecules to regulate cell population density. Different species of bacteria utilize different QS molecules to regulate its gene expression. A free-living marine bacterium, *Vibrio harveyi,* uses two QS system to control the density-dependent expression of bioluminescence (*lux*), commonly classified as sensor and autoinducer system. In *Pseudomonas aeruginosa*, QS not only controls virulence factor production but also biofilm formation. It is comprised two hierarchically organised systems, each consisting of an autoinducer synthetase (*LasI*/*RhlI*) and a corresponding regulator protein (*LasR*/*RhlR*). Biofilms produced by *Pseudomonas,* under control of QS, are ubiquitous in nature and contribute towards colonizations in patients of cystic fibrosis. Other organisms like *Haemophilus influenzae* and *Streptococcus* also utilize QS mechanism to control virulence in otitis and endocarditic decay. Overall, QS plays a major role in controlling bacterial economy. It is a simple, practical and effective mechanism of production and control. If the concentration of enzyme is critical, bacteria can sense it and perform a prompt activation or repression of certain target genes for controlling its environment. This review focuses on the QS mechanisms and their role in the survival of few important bacterial species.

Key words: Quorum sensing (QS), quorum sensing peptides (QSPs), auto-inducer 1 (AI-1), auto-inducer 2 (AI-2), acyl homoserine lactone (AHL).

INTRODUCTION

Quorum sensing (QS) in bacteria regulates gene expression in response to changes in cell density (Tomasz et al., 1965). The mechanism of QS helps to produce, release and respond to autoinducers and is observed in both gram-positives like *Streptococcus pneumoniae* and gram-negative bacteria e.g. *Vibrio fischeri* and *Vibrio harveyi* (Tomasz et al., 1965; Nealson et al., 1970). Essentially, QS phenomenon gives rise to important phenotypes of biofilm formation, virulence and swarming motility (Wynendaele et al., 2013). In gram positive bacteria, this is driven by quorum sensing peptides (QSPs) while in gram negatives, it is achieved

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by acylated homoserine lactones (AHLs) (Miller et al., 2001). The QSPs can stimulate two-component system by directly binding to the transcription factor, which further stimulates changes in target gene expression (Schauder et al., 2001; Jimenez et al., 2014).

QS in clinically relevant bacteria like *Staphylococcus epidermidis* and *Enterococcus faecalis* is presented by biofilm formation and with expression of pathogenicity-related extracellular protease (Krämer et al., 2009; Nishiguchi et al., 2009). In some species of *Streptococcus,* namely *S. pneumoniae* and *Streptococcus gordonii*, QS is controlled by competence-stimulating peptides (CSPs) (Havarstein et al., 1997). In general, the gram-negative bacteria seem to use AHLs as autoinducers, while the gram-positives use peptide-based signaling systems (Montgomery et al., 2013). QS is also seen in non-clinically relevant organisms like halophiles (eukaryotic algae), acidophiles (*Ferroplasma acidarmanus*), thermophiles (*Thermotoga maritima*), psychrophiles (*Pseudoaltermonas haloplanktis*), piezophiles (*Shewanella benthica*) and archaeas (*Methanothrix*) (Das Sarma et al., 2006). These organisms have been found to contain many genes related to biofilm formation and motility but no *LuxR* or *LuxS* homologues were identified (Baker et al., 2010; Nichols et al., 2009; Medigue et al., 2005; Bodor et al., 2008).

Inhibition of QS mechanism can be important strategy for combating bacterial pathogenicity (Kumar et al., 2013). QS mechanism can be disrupted using small molecules, monoclonal antibodies and receptor antagonists (Thoendel et al., 2010; Dong et al., 2007). Drugs like ambuic acid and RNA III Inhibiting Peptide (RIP) have been seen to inhibit QS mechanism (Nakayama et al., 2009; Nakayama et al., 2007). Some anti-autoinducer monoclonal antibodies have also been found to hinder QS in *Staphylococcus aureus* (Park et al., 2007). In *Escherichia faecalis*, QS was found to be inhibited by Siamycin I (Shojima et al., 2014). Cultivation of *V. fisheri* produces large quantities of luciferase possibly by QS mechanism, which has been beneficial in population's survival by combating environmental threats (Tomasz et al., 1965). This article focuses on QS mechanisms in some important bacteria and its role in their survival. Table 1 compares QS in these important bacterial species.

QS in *V. harveyi*

There are two parallel quorum-sensing systems in *V. harveyi* which can detect either autoinducer 1 (AI-1) or autoinducer 2 (AI-2) signaling molecules (Engebrecht et al., 1983). These QS bacteria produce and release AHL molecules which can affect a signal transduction cascade to change the organism's behavior (Engebrecht et al., 1983). These lactone molecules have various acyl chain lengths, saturation degrees and modifications at third carbon of acyl chain, which upon interaction with a signal, can activate or inhibit *LuxR* homologues, thereby controlling number of biological functions like biofilm formation, bioluminescence and virulence (Engebrecht et al., 1983). The two QS systems in *V. harveyi* control the density-dependent expression of bioluminescence composed of a sensor and a cognate autoinducer (Bassler et al., 1995). Both systems, each with two sensors responding to AI-1 and AI-2, are integrated via a shared regulatory protein to control the light emission (Bassler et al., 1994; Freeman et al., 1999). The AI-1 is identified as hydroxybutanoyl-L-homoserine lactone whose synthesis is depended on *luxL* and *luxM* genes (Cao et al., 1989). The sensor proteins are two component adaptive regulatory proteins which are regulated by a phosphorylation-dephosphorylation mechanism (Bassler et al., 1993, 1994). Surette et al. (????) have done some extensive work in understanding production of AI-2 in *V. harveyi*. In one of their experiments, a library of wild-type *V. harveyi* BB120 genomic DNA was transformed into *E. coli* strain DH5α to understand functions of AI-2 production (Michael et al., 1999). From 2,500 clones, five DH5α clones resulted in a 300-fold stimulation. Furthermore, 962 *E. coli* strains harboring Tn5 insertions in pBB2929 were tested for the loss of the ability to produce AI-2 and four did not produce AI-2. All of the four transposon insertions were found to be in the same 2.6-kb *HindIII V. harveyi* genomic DNA and only one ORF (*LuxSV.h.* gene) was identified to produce AI-2. Addition of culture fluids from the control Tn5 insertion strain induced 780-fold luminescence in the reporter, whereas culture fluid from the *luxSV.h.::Tn5* insertion strain did not induce the expression of luminescence in the reporter concluding that all the null mutants in *luxSV.h.* eliminate AI-2 production. An intricate dependence of *LuxSV.h.* gene and AI-2 production was observed.

QS in *Salmonella typhimurium*

Similar to *V. harveyi, S. typhimurium* LT2 also produces similar AI-2 whose activity is maximal in mid-exponential phase, as detected by autoinducer production assay. This is degraded when the bacteria enter stationary phase (Surette et al., 1998). AI-2 production is influenced by several factors like logarithmic growth, preferred carbon sources, low pH and high osmolarity, and factors like carbon source, neutral pH, and low osmolarity induce degradation (Surette et al., 1999). The signal production and degradation was further found to be depended on the amount of protein synthesis (Surette et al., 1999). The gene responsible for AI-2 production was identified by random mutations in *MudJ* transposon (Maloy et al., 1996). One *MudJ* insertion mutant was identified from 10,000 mutants that lacked detectable AI-2 in culture

Table 1. Comparison of QS in important bacterial species.

Feature	*Vibrio harveyi*	*Salmonella typhimurium*	*Escherichia coli*	*Pseudomonas aeruginosa*	Reference
Molecules	AI-1, AI-2, Acyl-homoserine lactone (AHL)	AI-2, AHLs	AI-2	2-heptyl-3-hydroxy-4-quinolone (PQS), diketopiperazines	Diggle et al. (2007)
Phenotypic effect	Biofilm formation, Bioluminescence and virulence	Virulence	Virulence	Biofilm formation, Virulence	Costerton et al. (1999) and Brown et al. (1988)
Affected genes	*luxR, luxL* and *luxM*	*mudJ, sdiA*	*ygaG, luxS, luxSV.h., luxSS.t.,* and *luxSE.c.*	*lasI, rhli, lasR, qscR* and *rhlR*	Barie et al. (1990), Brown et al. (1988) and Maloy et al. (1996)
External influencing factors	Not applicable	Logarithmic growth, preferred carbon sources, low or neutral pH and high osmolarity, and protein synthesis	Temperature, glucose and carbon	Not applicable	Surette et al. (1999) and Surette et al. (1998)
Anti-QS Products	Furanone compounds	Digoxin, Taxol, Artemisin, Vincristine, Vinblastine, Ginkgo, Flavonoids, Phenols, Stilbenes and non-protein amino acids, Southern Florida seaweeds	Oil extract from mature ripe fruit husk of *Aframomum corrorima*, *Nigella sativa*, *Albiza schimperiana* (ASRM) and *Justica schimperiana*	Extracts of *Conocarpus erectus*, *Chamaesyce hypericifolia*, *Callistemon viminalis*, *Bucida buceras*, *Tetrazygia bicolor*, and *Quercus virginiana*	Huber et al. (2003), Bjarnsholt et al. (2005), Choo et al. (2006), Manefield et al. (1999) and Gao et al. (2003)

fluids at mid-exponential phase (Maloy et al., 1996). PCR amplification and sequencing determined this site mapped to *E. coli MG1655* genome corresponding to an open reading frame (ORF) of unknown function denoted as *ygaG* (Blattner et al., 1997). Further, testing of *E. coli* O157:H7 *ygaG* gene and *V. harveyi luxSV.h.* genes in restoration of AI-2 production via complementation assays revealed that *E. coli* and *S. typhimurium* LT2, respectively produced 1.5 and 1.4 times more AI-2 activity than *V. harveyi* (Blattner et al., 1997). Furthermore, sequence comparison of AI-2 production genes from *V. harveyi, E. coli,* and *S. typhimurium* revealed that the translated protein sequences encoded by the *ygaG* ORFs aligned with the translated LuxS protein sequence from *V. harveyi* and that *E. coli YgaG* proteins were found to be 77% identical to *LuxS* from *V. harveyi* (Blattner et al., 1997). The sequence adjacent to the *MudJ* that inactivated the AI-2-production matched perfectly to the fragment *B_TR7095.85-T7* in the *S. typhimurium*. Moreover, it could be complemented to a full AI-2 production by the introduction of either the *E. coli luxSE.c.* gene or the *V. harveyi luxSV.h.* gene (Michael et al., 1999).

It has been observed that *Salmonella* possesses two QS systems (autoinductor AI-2 and acyl-homoserine-lactones), where AI-2 seems most important in cell to cell communication system by regulating *SdiA*, while a counterpart of *LuxR* which activates the genes of SPI-1 genes is involved in virulence (Janssens et al., 2007).

In *Salmonella*, natural products like digoxin, taxol, artemisin, vincristine, vinblastine, Ginkgo, favonoids, phenols, stilbenes and non-protein amino acids have been shown to have some QS activities (Huber et al.,2003; Bjarnsholt et al., 2005; Choo et al., 2006). Although there are number of quorum-quenching enzymes that can hydrolyse AHLs, only halogenated furanones from the red alga *Delisea pulchra* have been shown to have anti-QS activity (Manefield et al., 1999). Some of the Southern Florida seaweeds and few terrestrial plants have also shown such activities (Gao et al., 2003). Preliminary studies have shown the usage of antibacterial drugs. Further exploration of this property may prove beneficial in

treatment of *S. typhimurium* infection which specifically uses QS as survival strategy (Adonizio et al., 2006).

QS in *Escherichia coli*

Certain strains of *E. coli (DH5α)* do not produce AI-2 but can do so if there is an introduction of *luxS* gene from *E. coli O157:H7* strain (Michael et al., 1999). The genes of QS (*luxSV.h., luxSS.t.,* and *luxSE.c.*) are highly homologous to each other and the *LuxS* genes of *E. coli* have been defined as a new family of autoinducer genes (Michael et al., 1999). Identification of the *ygaG* locus in *E. coli* has been associated with a production defect of AI-2, which may occur because of a premature truncation caused by frameshift mutation resulting from the G/C deletion in *ygaG* (Michael et al., 1999).

Complementation studies demonstrate that the AI-2 production defect in *E. coli DH5α* is recessive to in-trans expression of *ygaG* (Michael et al., 1999). Regulation of AI-2 production differs between pathogenic and nonpathogenic strains, where temperature, glucose and carbon source play important roles (Gilson et al., 1995). Moreover, pathogenic *E. coli* strains have been shown to significantly produce more AI-2 than non-pathogenic (Gilson et al., 1995). AI-2 class of autoinducers are novel as *luxS* genes bear no homology to other genes known to be involved in production of HSL autoinducers (Gilson et al., 1995). LuxS protein of *V. harveyi* has also been detected in other organisms like *Haemophilus influenzae, Helicobacter pylori, Bacillus subtilis, Borrelia burgdorferi, Neisseria meningitidis, Neisseria gonorrhoeae, Yersinia pestis, Campylobacter jejuni, Vibrio cholerae, Deinococcus radiodurans, Mycobacterium tuberculosis, E. faecalis* and *Streptococcus pyogenes* (Bassler et al., 1997). AI-2 is, thus, found to be an important target in regulating the transition from a nonpathogenic existence outside a host to a pathogenic existence inside a host and inducing expression of the Type III secretion system contributing to its virulence (Michael et al., 1999).

QS in *Pseudomonas aeruginosa*

In gram-negative bacteria like *Pseudomonas*, QS molecules like 2-heptyl-3-hydroxy-4-quinolone (PQS) and diketopiperazines have been found to be involved in QS phenomenon (Holden et al., 1999). Biofilms are complex communities of microorganisms embedded in a self-produced matrix which can adhere to surface, either inert or alive (Costerton et al., 1999). Biofilm-associated bacteria on implants or catheters can cause chronic infections like cystic fibrosis by *P. aeruginosa* or the endocarditic decay by *Streptococcus viridans* group (Brown et al., 1988). Biofilm grown cells have been found to be 10 to 1,000-fold more resistant to the effects of antimicrobial agents than their planktonic counterparts (Brown et al., 1988). The QS system in *P. aeruginosa* is formed by autoinducer synthetase (*LasI/RhlI*) and a corresponding regulator protein (*LasR/RhlR*). Each system produces its own AHL synthetase (*LasI* and *Rhli*) and its regulating place (*LasR* and *RhlR*). Regulation of genes encoding the exoproducts depends on a signalling system that encompasses at least two sets of *LuxRI* homologues (*LasI* and *LasR*). The second quorum-sensing system of *P. aeruginosa* is controlled by the *LuxRI* homologues, *RhlRI* which activates expression of *rhlAB*, an operon encoding Rhamnosyltransferase, this leads to reduction of surface tension and thereby allowing *P. aeruginosa* cells to swarm over semi-solid surfaces (Barie et al., 1990). Although, the third regulator, *QscR*, is not seen to participate in the synthesis of AHL, more than 400 genes are affected in *Pseudomonas* which is implicated in virulence (Barie et al., 1990). Biofilms have been shown to have resistance to antibiotics including ampicillin, streptomycin, tetracyclines and gentamicin (Barie et al., 1990). The dosage levels to treat biofilms can reach toxic levels; moreover, *P. aeruginosa* can produce extracellular virulence factors such as proteases, haemolysins, exotoxin A, exoenzyme S and pyocyanin which are controlled by QS thereby contributing to its pathogenesis (Barie et al., 1990).

QS in gram-positive bacteria

In many gram-positive bacteria's, QS autoinducers are diverse in sequence and structure, and are interacted with membrane bound two-component signal transduction systems (Havarstein et al., 1995). The cell membrane in gram-positive bacteria's is impermeable to peptides, and they need some specialized transporters for secretion (Havarstein et al., 1995). All these changes prone autoinducers to do posttranslational modifications (Bouillaut et al., 2008). The sensor kinases of two-component systems auto-phosphorylate and the phosphoryl group is then passed from the histidine to a conserved aspartate on a cytoplasmic protein on binding to autoinducers (Simon et al., 2007). Components of QS in many gram-positive bacteria consist of AI, transporter, histidine kinase receptor, and response regulator on one operon (Peterson et al., 2000). Some of the bacteria's known to operate QS in the aforementioned way are *S. pneumonia*, *Bacillus subtilis*, *S. aureus*, *Listeria monocytogenes*, *E. faecalis*, and *Clostridium perfringens* (Ohtani et al., 2009; Riedel et al., 2009; Thoendel et al., 2011). Couple of important gram-positive bacteria's where QS is studied extensively are stated as the following.

QS in *S. aureus*

S. aureus utilizes a canonical two-component QS system

encoded by the *agr* locus (Thoendel et al., 2011). QS in *S. aureus* has four components which are driven by RNAII expression (Thoendel et al., 2009). AI in *S. aureus* is truncated to a 7-9 residues peptide and coupled with cyclization of a five membered peptide ring, which is bounded to a membrane bound histidine kinase AgrC, the autophosphorylation of which transfers the phosphate group to an aspartate on the regulator AgrA (Thoendel et al., 2009). It is synthesized as a precursor from *agr.* The AgrA then binds to the P2 promoter to autoinduce the *agr* operon, the mature AI is then transported out of the cell via transporter AgrB (Thoendel et al., 2009). Apart from P2 activation, the phosphorylated AgrA can also activate the divergently encoded P3 promoter which controls expression of RNAIII encoding the virulence factor δ-hemolysin, which in turn can activate production of α-toxin and repress the expression of *rot*, fibronectin binding proteins and other surface proteins (Novick et al., 1993). Thus, RNAIII acts as both direct and indirect regulator. The virulence in *S. aureus* is also attributed to its biofilm development.

There has been a functional collision between biofilm development and *agr* system, which may have sought to gain time for establishing a mature biofilm community and when there's a time of virulence dispersion, *S. aureus* terminates biofilm production and decreases surface proteins (Boles et al., 2008). The *agr* regulators in *S. aureus* also can respond to extracellular environmental signals like autoinducers. It has been hypothesized that an unknown regulator of *agr* can control RNAIII levels, which in turn causes direct transcription of surface proteins and pigment production to inhibit expression of secreted toxins following extracellular stress (Lauderdale et al., 2009). This may be needed as some of the stress regulons of sigma ensures that *S. aureus* does not undergo QS under conditions when the bacteria must dedicate resources to decrease stress. Another two-component system, SrrA/SrrB has also been seen to control virulence, where overexpression of SrrA/SrrB has been seen to decrease virulence, likely due to inhibition of *agr* expression (Yarwood et al., 2001). Further, hypervariability amongst *agrD* and *agrB* genes leads to the production of one of four different types of *S. aureus* autoinducers depending on the strain (Dufour et al., 2002). The hypervariability has also been observed in *agrC* gene encoding the sensing domain of the AI receptor (Dufour et al., 2002). The type of AI seems important as it determines the stabilization of an inhibitory confirmation of AgrC; this can halt cell-cell signaling and control the infection (Geisinger et al., 2009).

QS in *B. cereus*

B. cereus is important gram-positive bacteria which is closely related to *B. cereus*, *B. anthrasis*, and *B. thuringiensis* and can cause secretion of a variety of hemolysins and toxins (Bottone et al., 2010). QS in *B. cereus* is controlled by a transcription factor PlcR, which binds intracellular AI derived from the PapR protein which is a 48 amino acids long protein containing an amino-terminal signal peptide (Slamti et al., 2002). Another protein, NprB is a secreted neutral protease B which cleaves the pro-AIP PapR into peptides, which can then activate PlcR activity (Slamti et al., 2002). There is also a sequence diversity in the PapR autoinducers classifying this species into four pherotypes (Slamti et al., 2005). When transported back into the cell, the PapR helps bind AI to the transcription factor PlcR, thereby regulating transcription (Slamti et al., 2005). The PlcR interacting with the PapR AIP can control expression of 45 genes regulating enterotoxins, hemolysins, phospholipases, and proteases (Gohar et al., 2008).

Novel therapeutic techniques to target QS

Quorum sensing peptides (QSPs) drive QS phenomenon in gram-positive bacteria (Miller et al., 2001). Targeting QSPs can be an alternative strategy to combat bacterial pathogenicity (Kumar et al., 2013). Therefore, analysis and prediction of QSPs are of immense importance in gram-positive bacteria. A machine learning tool for identification of novel and effective biofilm inhibitory peptides (BIPs) has recently been proved an efficient method of classification (Akanksha et al., 2015). Furthermore, physicochemical properties like aromaticity, molecular weight and secondary structure have also been observed to differentiate QSPs from non-QSPs (Tian et al., 2009). One study utilizes support vector machine (SVM) to extract physicochemical indices, where QSPs are seen to prefer secondary structure conformations (α-helix, coil and β-sheet) similar to QSPs of *S. mutans* with random coil α-helix conformations (Tian et al., 2009; Syvitski et al., 2007). Biofilms in bacteria are known to resist the environmental stresses like biocidal agents, UV damage, metal toxicity and acid exposure (Hall et al., 2004). They can have a spatiotemporal heterogeneity making them 1000 times more resistant to antibiotics (Costerton et al., 1999). Thus, there seems a significant need to develop antimicrobial peptides (AMPs) as prophylactic and therapeutic agents against drug-resistant bacteria and biofilms (Fox et al., 2013). Studies have been conducted to evaluate action of peptides against multiple bacterial species. Machine learning tools have been used to build six SVM and weka-based models trained on 80 biofilm-active AMPs and 88 QSPs (Arun et al., 2016). The dPABBs web server develops a prediction strategy for the identification and optimisation of such anti-biofilm peptides (Arun et al., 2016). Homology-based prediction has been proven to be extremely successful in identifying antimicrobial peptides (Lynn et al., 2004). Other machine learning prediction

tools based on SVM (Lata et al., 2010; Thomas et al., 2010), hidden markov models (Fjell et al., 2007), sequence alignments and feature selection (Wang et al., 2011) have also been effective. Various techniques have been used for network analysis and visualization of QS data in different organisms. A network of potential anti-quorum sensing agents for *P. aeruginosa* was created with information from biomedical ontologies and curated databases (Martín et al., 2017). Some groups have already applied network approaches to study antibiotic resistance in *P. aeruginosa,* while others have tried to extract information types and apply it to the retrieval and curation of research articles in *P. aeruginosa* QS (Hwang et al., 2016). In *V. fischeri*, LuxI is an important component of QS signaling pathway (Engebrecht et al., 1987). Homology modeling is a good way of predicting docking sites and a three-dimensional structure of LuxI and other QS components (Mihăşan et al., 2010). Homology modeling is a method of structure prediction based on amino acid sequence similarity to closely-related known structures (Mihăşan et al., 2010). Groups have tried to utilize such techniques of homology modeling using Phyre2 and GalaxyWEB server (Mohammed et al., 2016). Ultra-high-throughput screening approaches have been utilized for screening around 200,000 compounds for inhibitors of LasR-dependent gene expression (Ute et al., 2006). A theoretical approach has been adopted to build an interactome comprising proteins from *Salmonella* and then analyzing the networks with parameters like centrality and k-core measures (Chandrajit et al., 2014; Chandrajit et al., 2012).

A set of responsible virulent proteins have been identified from published microarray data, which could serve as sensitive predictors and form the foundation for a series of trials in the wet-lab setting. Analysis of protein interaction networks (PINs) has gained importance as one of the promising strategies, where the topology and modularity analysis of the networks have been implied (Pan et al., 2016). Analyses of a PIN starts by determining the number of interacting partners of a particular protein to identify its degree centrality (DC) which correlates with its biological importance. Other important measures like closeness centrality (CC), betweenness centrality (BC) and eigenvector centrality (EC) with a cartographic analysis of identifying the functional modules in the network have been implied to be a useful technique to identify therapeutic targets (Pawar et al., 2017). All these existing and newer *in silico* approaches are promising ways in targeting QS in different bacterial species.

Relationship between QS genes of gram-positive and negative bacteria's

Several groups have tried to explore concept of interspecies communication between gram-positive and negative bacteria. One important and interesting study was recently performed by Rajput et al. (2017) and Akanksha et al. (2017). Here, they have compared gram-positive and negative bacteria group for the presence of putative *LuxI/LuxR* with respect to its conservation in domain, motif, compositions, gene ontology (GO), and taxonomic distribution. A phylogenetic reconstruction of a tree was done to investigate the evolutionary trends in two-component system proteins, *LuxI* and *LuxR* using a Maximum Likelihood (ML) method. As shown in Figure 1, 11 *LuxI* sequences of gram-positive bacteria located with their respective gram-negative bacteria BLAST hits except *Mycobacterium* species *djl-10*. Some of the species used in this study were *Mumia ava*, *Burkholderia*, *Streptomyces purpurogeneiscleroticus*, *Methylobacterium* species *Leaf361*, *Syntrophaceticus* and *Desulfobacterium autotrophicum*. The ML tree for representative *LuxR* sequences of gram-positive bacteria localized with gram-negative bacteria with the exception of two, Streptomycetaceae and Bacillaceae Lactobacillaceae family.

The topological arrangement of six canonical *luxI* and *luxR* genes among these two bacterial species showed that they both are considerably related to each other with fewer differences in amino acids. *Streptomyces purpurogeneiscleroticus* and *Albizia ferruginea* showed similar topology with conserved *LuxI/LuxR* motifs, while the protein of *S. schinkii* was localized in the same clades between two trees. Overall, a significant overlap is seen between these two genes in gram-positive and negative species. Further exploration on likely overlaps in other QS genes and components is very much possible and needs an evaluation.

Conclusion

Bacterial ability to monitor cell density, prior to expressing a phenotype, is due to QS phenomenon. Production and liberation of enzymes by bacteria to reach its adequate concentration is decided by QS which in turn can activate or repress certain target genes. Further, biofilms can help attachment of bacteria to each other and wet surfaces assisted by QS. The knowledge of the molecular mechanisms in QS and biofilms can improve therapeutic approaches. Different bacteria can use QS in different ways. The QS system in *P. aeruginosa* is formed by autoinducer synthetase (*LasI/RhlI*) and a corresponding regulator protein (*LasR/RhlR*). Each system produces its own AHL synthetase (*LasI* and *Rhli*) and its regulating place (*LasR* and *RhlR*). The second quorum-sensing system of *P. aeruginosa* is controlled by the *LuxRI* homologues. In *E. coli*, QS is controlled by *luxSV.h., luxSS.t.,* and *luxSE.c.* genes, while in *V. harveyi* QS is controlled by acyl-homoserine lactone-signaling molecules which can effect a signal transduction cascade

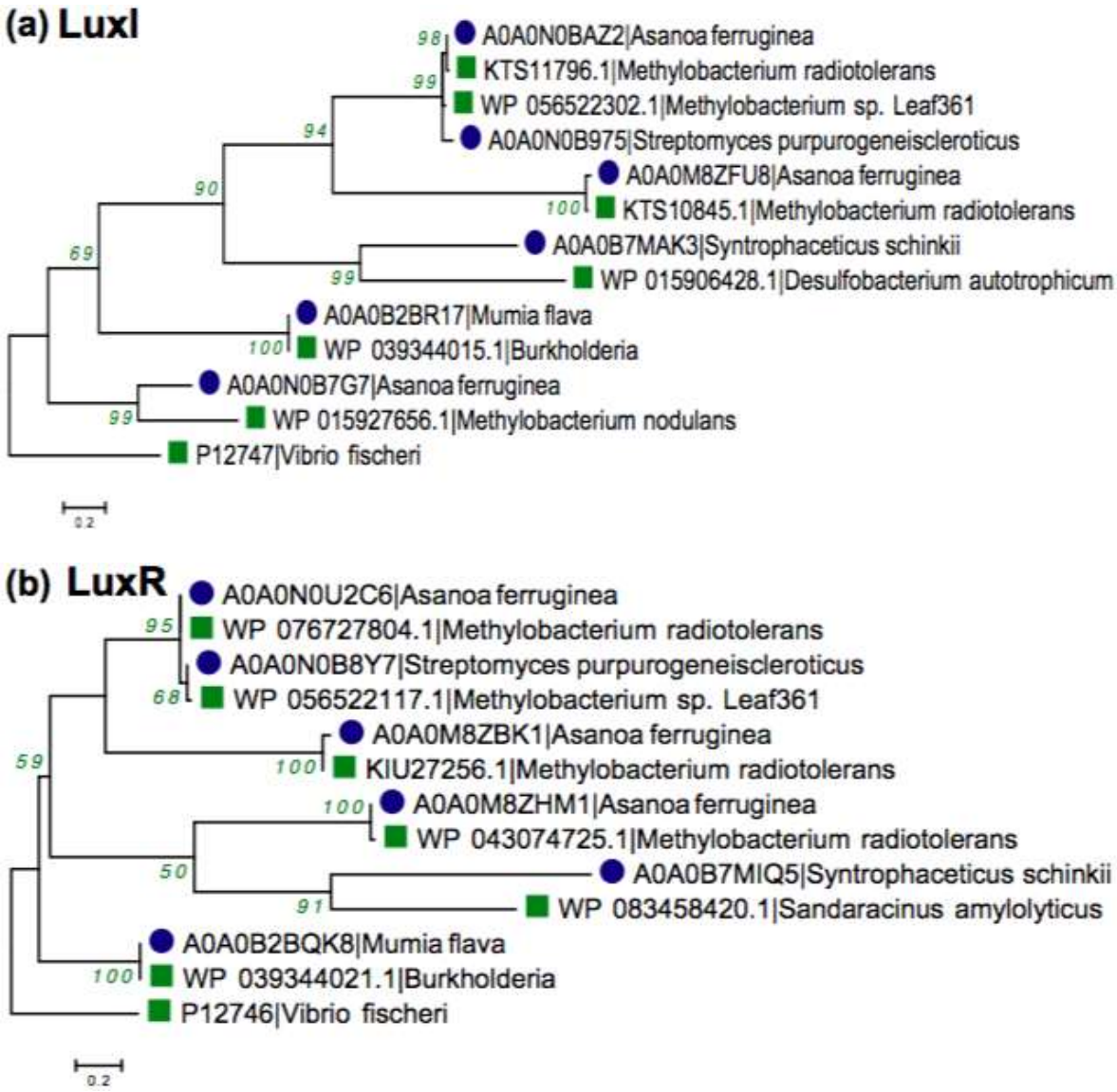

Figure 1. Phylogenetic tree reconstruction using Maximum Likelihood method for gram-positive bacteria and their respective gram-negative BLAST hits (a) *LuxI* containing sequences; (b) *LuxR* containing sequences [Gram-positive bacteria: green colour; Gram-negative bacteria: blue colour].
Source: Reproduced from Rajput et al. (2017).

to change the behavior of organism. In conclusion, a detailed understanding of QS phenomenon can help in manipulation of its behaviour and can shift paradigms of treating bacterial infections.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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