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Full Length Research Paper

Study on prevalence and genetic discrimination of methicillin-resistant *Staphylococcus aureus* (MRSA) in Egyptian hospitals

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Methicillin-resistant *Staphylococcus aureus* (MRSA) continues to be a global problem in infection control. The highest proportions of MRSA are reported by Jordan, Egypt and Cyprus investigators, where more than 50% of the invasive isolates are methicillin-resistant. The aim of this work was to study the prevalence, antibiotic sensitivity and genetic discrimination of MRSA in Egypt. Microbiological identification was done using Gram stain, catalase, coagulase and mannitol fermentation along with biochemical identification by analytical profile index (API) tests. Molecular identification was conducted by the polymerase chain reaction (PCR) targeting 16S ribosomal RNA and the *nuc* genes. Additionally, identification of methicillin-resistant *S. aureus* (MRSA) was performed by the amplification of 310 bp of the *mec*A gene. Antibiograms were performed for all isolates. Only 73 isolates out of 166 were oxacillin resistant. The percentage of resistant isolates to erythromycin, rifampicin, vancomycin, Ofloxacin, gentamycin, Amoxicillin clavulanic acid, ciprofloxacin, chloramphenicol, trimethoprim sulfamethoxazole, teicoplanin and tetracycline were 58, 32.50, 2.4, 45.18, 37.9, 39.7, 23.5, 21.6, 40.3, 0 and 39.1%, respectively. MRSA isolates were subdivided into eight biotypes according to their resistance pattern. Random amplification of polymorphic DNA (RAPD) and repetitive sequence DNA (REP) were performed on samples representing each biotype.

Key words: Methicillin-resistant *Staphylococcus aureus* (MRSA), nuclease, *mec*A, 16S rRNA, random amplified polymorphic DNA (RAPD), polymerase chain reaction (PCR).

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) continues to be a global problem in infection control. MRSA may be present on human skin (particularly the scalp, armpits and groins) as well as in the nose and throat, and less commonly in the colon and urine, but its presence does not always mean infection (Kresser, 2012). MRSA has the ability to survive from days to weeks on environmental surfaces in healthcare facilities.

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Table 1. Distribution of clinical isolates and its gender from different hospitals.

Hospital		Gender		Total
		Male	Female	
Alhoodalmarsod	Count	26	18	44
	% within Hospital	59.10	40.90	100.00
Banha	Count	13	0	13
	% within Hospital	100.00	0.00	100.00
El Hussain	Count	8	10	18
	% within Hospital	44.40	55.60	100.00
ElsayedGalal	Count	7	4	11
	% within Hospital	63.60	36.40	100.00
KafrElshiekh	Count	3	4	7
	% within Hospital	42.90	57.10	100.00
Kasralaini	Count	31	30	61
	% within Hospital	50.80	49.20	100.00
Sheikh zayed	Count	7	5	12
	% within Hospital	58.30	41.70	100.00
Total	Count	95	71	166
	% within Hospital	57.20	42.80	100.00

It is capable of withstanding a wide range of temperatures, humidity and exposure to sunlight and is resistant to desiccation. These properties make it able to contaminate a large variety of hospital items, e.g. chairs, mattresses, bed frames and computer keyboards (Bhalla et al., 2004; Lu et al., 2008). The primary site of infection is the nasal inner wall opposite the nostril wing otherwise known as the anterior nares or vestibulum nasi (Williams et al., 2000; Wertheim et al., 2005). Most of the countries in the Mediterranean region are experiencing a surge in MRSA infections (Borg, 2007). The primary site of infection is the nasal inner wall opposite the nostril wing otherwise known as the anterior nares (Williams et al., 2000; Wertheim et al., 2005). MRSA infections result in higher mortality, greater lengths of hospital stay and increased cost compared with methicillin sensitive *S. aureus* (MSSA) infections (Engemann et al., 2003). Patients with MRSA bacteremia have a mortality of 1.78%, three times higher than with MSSA bacteremia (Wang et al., 2011). MRSA show resistance to a wide range of antibiotics, thus limiting the treatment options to few agents, such as vancomycin and teicoplanin. It is therefore important to keep the prevalence of MRSA carriage and MRSA infections as low as possible (Wertheim et al., 2005). The aim of this work is to study the prevalence of MRSA in Egyptian hospitals, isolation and identification of MRSA from different infection sites, different hospitals and different hospital units around Egypt and further identification of MRSA using PCR. It also includes studying the antibiotic sensitivity of MRSA and grouping of the isolates according to their resistance.

MATERIALS AND METHODS

Sample collection

Clinical isolates recovered from blood, skin, pus, sputum, urine, nasal swab, throat swab and surgical wound were collected from different Egyptian hospital laboratories: Kasr Al-Aini, Al Sayed Galal, Al Hussain, AlHoud Almarsood, Banha, Al Sheikh Zayed and Kafr Al Sheikh as shown in Table 1. A total of 166 isolates were collected.

Isolation and identification of staphylococci

The swab specimens were inoculated on nutrient agar and incubated at 37°C for 24 to 48 h; thereafter the colonies were streaked with sterilized wire loap on mannitol salt agar so as to obtain discrete colonies. The plates were incubated for 24 h at 37°C under aerobic conditions after which the cultured plates were examined recording the appearance, size, colour, and morphology of the colonies.

Gram stain reaction, catalase test and coagulase test were carried out. Isolates that were Gram-positive cocci, catalase

Table 2. Interpretive standards for *S. aureus* according to CLSI guidelines 2014.

Antimicrobial agent	Zone diameter interpretive (nearest mm)		
	Sensitive (S)	Intermediate (I)	Resistant (R)
Rifampin	S ≥20	17--19	R ≤16
Amoxycillin/clavulinic	S ≥20	-	R≤19
Ciprofloxacin	S ≥21	16-20	R ≤15
Chloramphenicol	S ≥18	13-17	R ≤12
Trimethoprim Sulphamethoxazole	S ≥16	11-15	R≤ 10
Ofluxacin	S ≥18	15-17	R ≤14
Tetracyclin	S ≥19	15-18	R ≤14
Erythromycin	S≥ 23	14-22	R ≤13
Gentamicin	S ≥15	13-14	R ≤12
Vancomycin	S ≥15	-	R ≤14
Teicoplanin	S ≥14	11-13	R ≤10
Oxacillin	S≥13	-	R≤10

positive, and coagulated human plasma were considered *S. aureus* in this study (Chigbu and Ezeronye, 2003).

Preparation and examination of gram stained films of the collected isolate

Gram stain was performed according to Isenberg (1992).

Growth on mannitol salt agar

Isolates which proved to be from *Staphylococcus* species were subcultured on mannitol salt agar plates. *S. aureus* ferments mannitol changing the medium colour from red to yellow (Pumipuntu et al., 2017).

Biochemical tests

Catalase test

The catalase test involves the addition of hydrogen peroxide to a culture sample or agar slant. If the bacteria in question produce catalase, hydrogen peroxide will be hydrolyzed and oxygen gas will be evolved. A positive test was denoted by evolution of gas resulting in bubbles formation (Kloos and Schleifer, 1986).

Coagulase test

Colonies from different samples were inoculated onto 0.5 ml of rabbit plasma diluted by 1/10 using saline and incubated at 37°C for 1 to 4 h. A positive test was denoted by clot formation in the test tube after chosen time intervals (Sperber and Tatini, 1975).

Analytical profile index (API)

API staph (Bioumerieux, Inc., Marcy E'toile, France) was used to confirm identification of *S. aureus* with the test performed as described by Radebold and Essers (1980).

Antibiotic sensitivity test

166 staphylococci strains were examined *in vitro* against 12 different antibiotics. The antibiotic discs were obtained from Oxoid, UK including: Rifampicin, Amoxicillin clavulanic, Chloramphenicol, trimethoprim Sulfamethoxazole, Ofloxacin, Tetracycline, Erythromycin, Gentamicin, Vancomycin, Teicoplanin and Oxacillin. Approximately 10^8cfu/ml bacterial inoculums were prepared in 5 ml Muller-Hinton broth and inoculated on nutrient agar plates after incubation at 37°C overnight. 3 to 5 isolated colonies were picked from the plate containing the test organism. The turbidity was adjusted the same as the McFarland No. 0.5 standard. Thereafter, swab was streaked onto the surface of the Mueller-Hinton agar (3 times in 3 quadrants) and left for 5 to 10 min to dry the surface of agar. The chosen drug-impregnated discs were placed onto the surface of the inoculated agar plate which was then inverted and incubated at 35°C for 18 to 24 h (Drew et al., 1972).

Interpretation of antibiogram

According to CLSI, the results of antibiogram were interrelated as shown in Table 2.

PCR amplification

Extraction of chromosomal DNA

This was carried out using the traditional method. A total of 5 ml in 24 h broth culture isolates was centrifuged for 10 min at 12000 rpm. The pellet was washed twice by 50 µl distilled water. The cell suspension was heated directly at 100°C for 10 min in a heat block to break the cell membranes and then cooled in the refrigerator for 5 min. Finally, the cell suspension was centrifuged for 5 min at 12000 rpm and the supernatant containing chromosomal DNA was collected.

Amplification of 16SrRNA, nuc, mecA, RAPD, REP1R and REP2 genes

The reaction mixtures were prepared following the protocols described in the Mastermix pcr kits. Thereafter, PCR was performed in a thermal cycler using the following settings; Stage 1 (x1): initial denaturation at 94°C for 5 min; Stage 2 (x35): secondary denaturation at 94°C for 30 sec, annealing at 55°C for 1 min and extension at 72°C for 1 min; Stage 3 (x1): a final extension at 72°C for 10 min. Other changes made for amplification of specific genes

Table 3. Temperature and time conditions of the primers during PCR for each of the tested genes.

Gene	Primary denaturation (5 min)	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension (min)
16SrRNA	94°C	94°C 30 s	55°C 1 min	72°C 1 min	35	72°C 10 min
nuc	94°C	94°C 30 s	55°C 45 s	72°C 45 s	35	72°C 10 min
mecA	94°C	94°C 30 s	50°C 30 s	72°C 40 s	35	72°C 10 min
RAPD	94°C	94°C 1 min	32°C 1 min	72°C 2 min	35	72°C 12 min
REP1R	94°C	94°C 1 min	38°C 1 min	72°C 2 min	35	72°C 12 min
REP2	94°C	94°C 1 min	46°C 1 min	72°C 2 min	35	72°C 12 min

are as stated in Table 3.

Statistical methods

Statistical analysis was done using IBM© SPSS© Statistics version 22 (IBM© Corp., Armonk, NY, USA). Numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test or Fisher's exact test was used to examine the relation between qualitative variables. For quantitative data, comparison between two groups was done using Student's t-test. All tests were two-tailed. P-value <0.05 was considered significant.

RESULTS

Isolation and identification of staphylococcal isolates

A total of 166 clinical isolates were recovered from blood (12 isolates), throat swabs (3 isolates), nasal swabs (2 isolates), wound (53 isolates), pus (9 isolates), sputum (15 isolates), urine (16 isolates) and skin (56 isolates).

The number of clinical isolates from each gender (male and female), collected from each infection site (blood, throat swabs, pus, skin, surgical wound, urine, nasal swab and sputum) is shown in Table 4.

Table 4. Distribution of clinical specimens.

Clinical specimen	No. of isolates		Total
	Male	Female	
Blood	6	6	12
Skin	31	25	56
Surgical wound	35	18	53
Pus	5	4	9
Sputum	9	6	15
Urine	4	12	16
Throat swab	2	1	3
Nasal swab	2	0	2
Total	-	-	166

Morphological characteristics

Gram staining and microscopical examination of the recovered isolates revealed that 166 isolates were Gram positive cocci arranged in bunch. *Staphylococci* found as gram-positive bacteria occur in microscopic clusters resembling grapes. They form large round golden-yellow colonies.

Growth on mannitol salt agar (MSA)

All isolates grown on MSA were mannitol fermenters and are presumed to be *S. aureus*.

Biochemical tests

Catalase and coagulase tests showed that all recovered isolates were catalase and coagulase positive.

Identification of *Staphylococcus aureus* using API staph

Based on the above tests, all clinical isolates were presumptively identified as *S. aureus.* The identity of these isolates was confirmed using API Staph kits.. All tested isolates were identified as *S. aureus* and gave positive results with GLU, FRU, MNE, MAL, LAC, TRE, MAN, NIT, PAL, VP, SAC, NAG, ADH and URE, however showing negative results with XLT, MEL, RAF, XYL and MDG. Identification was made using the Analytic Profile Index with API Staph (bioMerieux) system. The pattern of the reactions are obtained and coded into 7-digit numerical profile. All 166 *S. aureus* isolates gave analytic profile index of 6736153. Upon performing API on isolates, they were all *S. aureus.* All API numerical profiles for 166 isolates showed very good identity of *S. aureus* similar to each other in the test results.

Antimicrobial susceptibility of the isolates to different antibiotics

Antibiogram for isolates

Patterns of susceptibility to antimicrobial agents are used

Table 5. Susceptibility profile of *Staphylococcus aureus* isolates to various antibiotics.

Antimicrobial agent	Concentration (µg/disc)	*Staphylococcus aureus* (166 isolates)					
		Sensitive (S)		Intermediate (I)		Resistant (R)	
		NO.	%	NO.	%	NO.	%
Rifampin	5	107	64.4	5	3.01	54	32.5
Amoxycillin/clavulinic	20/10	100	60.24	Zero	0	66	39.7
Ciprofloxacin	5	121	72.89	6	3.6	39	23.49
Chloramphenicol	30	121	72.89	9	5.42	36	21.6
Trimethoprim sulphamethoxazole	1.25+ 23.75	93	56.02	6	3.6	67	40.3
Ofluxacin	5	88	53.01	3	1.8	75	45.18
Tetracyclin	30	98	59.03	3	1.8	65	39.1
Erythromycin	15	96	57.83	6	3.6	64	38.55
Gentamicin	10	101	60.84	2	1.2	63	37.9
Vancomycin	30	162	97.5	Zero	0	4	2.4
Teicoplanin	30	166	100	Zero	0	Zero	0
Oxacillin	5	93	56.02	Zero	0	73	43.9

for typing because staphylococci isolates can be broken down into separate groups. The susceptibility of *S. aureus* isolates to different antibiotics commonly used for *S. aureus* infections, namely, Rifampicin, Amoxicillin clavulanic ciprofloxacin, Chloramphenicol, Trimethoprim sulfamethoxazole, Ofloxacin, Tetracycline, Erythromycin, Gentamycin, Vancomycin, Teicoplanin and Oxacillin was determined.

An antibiogram was performed for all isolates which showed the characteristics of *S. aureus*. The percentage resistance (% R) shows the degree of multiresistance of the isolate. The distribution of antibiotic resistance within *S. aureus,* either sensitive or resistant to Oxacillin was shown on Table 5. The isolates which showed resistance to oxacillin were considered MRSA as shown in Table 7, while isolates sensitive to oxacillin were considered MSSA as shown in Table 8. In many cases, the resistance to certain antibiotics exceeded 40% of the isolates Table 6. The percentage resistance to Erythromycin was 58%, Rifampicin (32.50%), Vancomycin (2.4%), Ofloxacin (45.18%), Gentamycin (37.9%), Amoxicillin clavulanic acid (39.7%), Ciprofloxacin (23.5%), Chloramphenicol (21.6%), Trimethoprim sulfamethoxazole (40.3%), Teicoplanin (0%), and Tetracycline (39.1%) as previously shown in Table 5.

The percentage resistance of MRSA against each antibiotic from different classes which in many cases exceeded 70% resistance to Oxacillin was found to be 100% while resistance to Teicoplanin was 0% and Vancomycin was 5.5%. All isolates were sensitive to Teicoplanin with R% equal to 0% while 43.7% isolates remained resistant to Oxacillin and were considered as MRSA. These results are summarized in Table 6. On the other hand, 56.3% of the *S. aureus* isolates were oxacillin sensitive (MSSA) and their antibiogram results are summarized in Table 8. Based on the results of antibiotic sensitivity, 73 isolates were assumed to be MRSA (43.7% of the *S. aureus* isolates). Total number of MRSA isolates versus total number of MSSA isolates collected from each clinical specimen (blood, throat swabs, pus, skin, surgical wound, urine, nasal swab and sputum) is shown in Table 9. All isolates obtained from both nasal and throat swab were Oxacillin resistant according to the results of antibiogram.

Table 6. Percentage resistance (R %) of *S. aureus* against each antibiotic.

Antibiotic	*S. aureus* R% (No. of isolates)
Rifampin	32.50 (54)
Amoxycillin/Clavulinic	39.7(66)
Ciprofloxacin	23.5 (39)
Chloramphenicol	21.6 (36)
Trimethoprim sulphamethoxazole	40.3 (67)
Ofluxacin	45.18 (75)
Tetracyclin	39.1 (65)
Erythromycin	58 (96)
Gentamicin	37.9 (63)
Vancomycin	2.4 (4)
Teicoplanin	0
Oxacillin	43.7 (73)

Antibiotypes

MRSA isolates were subdivided into eight biotypes according to the resistance pattern as shown in Table 13. One sample from each biotype was taken as representative as shown in Table 14.

Biotype 1 includes isolates resistant to Oxacillin, Gentamicin, Erythromycin, Tetracycline, Ofloxacin,

Table 7. Percentage resistance (R%) of MRSA against each antibiotic.

Antibiotic	MRSA R% (No. of isolates)
Rifampin	76 (56)
Amoxycillin/Clavulinic	84.70 (62)
Ciprofloxacin	52.70 (39)
Chloramphenicol	52.70 (38.4)
Trimethoprim sulphamethoxazole	81.90 (60)
Ofluxacin	97.22 (71)
Tetracyclin	83.33 (61)
Erythromycin	76.30 (56)
Gentamicin	83.33 (61)
Vancomycin	5.50 (4)
Teicoplanin	0
Oxacillin	100 (73)

Table 8. Percentage resistance (R %) of MSSA against each antibiotic.

Antibiotic	MRSA (R %)
Rifampin	1 (1)
Amoxycillin/Clavulinic	5.37 (5)
Ciprofloxacin	1.00 (1)
Chloramphenicol	1.00 (1)
Trimethoprim sulphamethoxazole	9.60 (9)
Ofluxacin	5.37 (5)
Tetracyclin	5.44 (6)
Erythromycin	9.60 (9)
Gentamicin	3.22 (3)
Vancomycin	0.00
Teicoplanin	0
Oxacillin	0

Trimethoprim sulfamethoxazole and Amoxicillin clavulanic.

Biotype 2 includes isolates resistant to Gentamicin, Tetracycline, Ofloxacin, Trimethoprim sulfamethoxazole, Chloramphenicol, Ciprofloxacin, Rifampin and Oxacillin.

Biotype 3 includes isolates resistant to Gentamicin, Erythromycin, Tetracycline, Ofloxacin, Trimethoprim sulfamethoxazole, Amoxicillin clavulanic, Rifampin and Oxacillin.

Biotype 4 includes isolates resistant to Oxacillin, Gentamicin, Erythromycin, Tetracycline, Ofloxacin, Chloramphenicol, Ciprofloxacin, Amoxicillin clavulanic and Rifampin.

Biotype 5 includes isolates resistant to Oxacillin, Erythromycin, Ofloxacin, Trimethoprim sulfamethoxazole, Chloramphenicol, Ciprofloxacin, Amoxicillin clavulanic and Rifampin.

Biotype 6 includes isolates resistant to Oxacillin, Gentamicin, Tetracycline, Ofloxacin, Trimethoprim sulfamethoxazole, Amoxicillin clavulanic and Rifampin.

Table 9. Distribution in clinical specimens.

Sample	No. of isolates		Total	%MRSA
	MRSA	MSSA		
Blood	7	5	12	58.33
Skin	19	38	57	33.9
Surgical wound	28	25	53	52.8
Pus	5	4	9	55.5
Sputum	8	6	14	53.5
Urine	1	15	16	6.25
Throat swab	3	0	3	100
Nasal swab	2	0	2	100
Total	73 43.90%	93 56.10%	166	

Biotype 7 includes isolates resistant to Oxacillin, Gentamicin, Erythromycin, Tetracycline, Trimethoprim sulfamethoxazole, Chloramphenicol, Ciprofloxacin and Amoxicillin clavulanic.

Biotype 8 that includes isolates resistant to Oxacillin, Vancomycin, Gentamicin, Erythromycin, Tetracycline, Ofloxacin, Trimethoprim sulfamethoxazole, Amoxicillin clavulanic and Rifampin.

Polymerase chain reaction (PCR)

The 166 *S. aureus* isolates were subjected to genotyping (*S. aureus* identity confirmation and detection of antibiotic resistance genes).

16s RNA

Identification of the 166 isolates as *Staphylococcus* was confirmed by PCR amplification of *16S rRNA*. The results of amplification of 791bp of *16SrRNA* using the forward primer (CCTATAAGACTGGGATAACTTCGGG) and reverse primer (CTTTGAGTTTCAACCTTGCGGTCG) are shown in Figure 1. All isolates produced a single band at 791 bp size. The 166 isolates were therefore confirmed to be *S. aureus* (Mason et al., 2001) as shown in Figure 1.

Nuc gene

The presence of *Nuc* gene in 166 isolates was confirmed by PCR amplification. Amplification of 395 bp of *NUC* gene were done for all 166 isolates aimed at further confirmation of *S. aureus* using forward primer (ATATGTATGGCAATCGTTTCAAT) and reverse primer

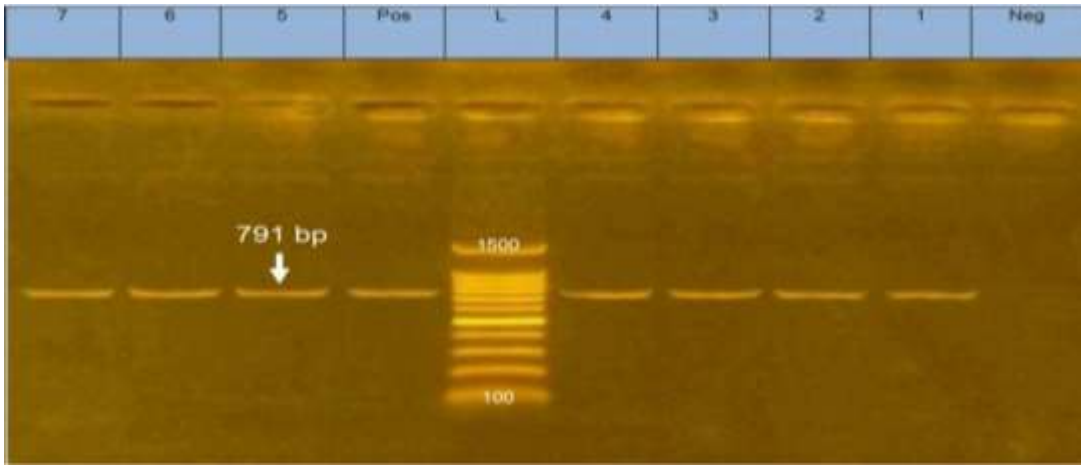

Figure 1. Agarose gel photo documentation of conventional PCR on genetic material extracted from *S. aureus* strains as a molecular typing for detection of 16Sr RNA gene using forward primer: CCTATAAGACTGGGATAACTTCGGG and reverse primer CTTTGAGTTTCAACCTTGCGGTCG. Lane L: molecular weight marker (100 –1500 bp). Lanes 1-7: positive samples *S.aureus*16Sr RNA gene with amplicon size of 791 bp. Lane Neg: negative control. Lane Pos.: positive control.

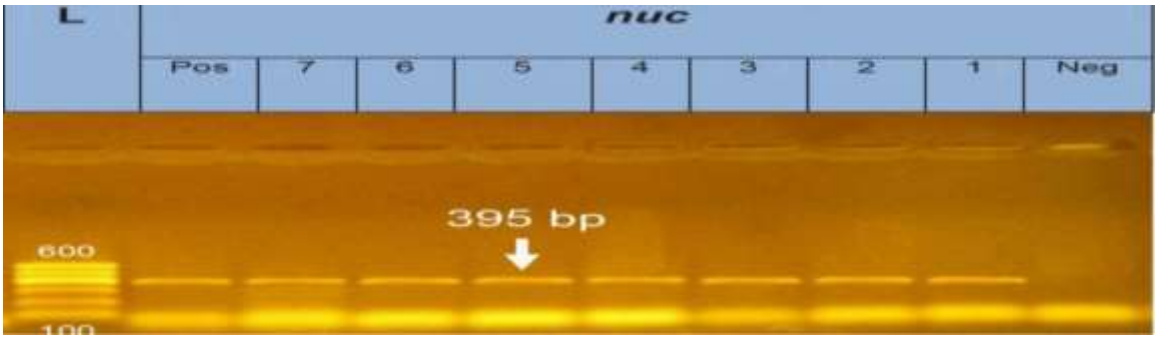

Figure 2. Agarose gel photo documentation of conventional PCR on genetic material extracted from *S. aureus* strains as a molecular typing for detection of *nuc* gene using forward primer: ATATGTATGGCAATCGTTTCAAT and reverse primer: GTAAATGCACTTGCTTCAGGAC . Lane L: molecular weight marker (100 – 600 bp). Lanes 1-7: positive samples nuc gene with amplicon size of 395 bp. Lane Neg: negative control. Lane Pos.: positive control.

(GTAAATGCACTTGCTTCAGGAC).166 clinical isolates contain the *NUC* gene and single band at 600 bp (Gao et al., 2011) as shown in Figure 2.

mecA gene

The presence of antibiotic resistance *mecA* gene in 166 clinical isolates was confirmed by PCR amplification. Amplification of 310bp *mec*A gene was carried out using forward primer (GTA GAA ATG ACT GAA CGT CCG ATA A) and reverse primer (CCA ATT CCA CAT TGT TTC GGT CTA A) (McClure et al., 2006). Only 73 MRSA samples shows positive results for *mec*A single band at 310 bp as shown in Figures 3, 4 and 5.

Random amplification of polymorphic DNA (RAPD)

RAPD was carried out for only one clinical isolate from each biotype as shown in Table 14. Each representing sample was randomly amplified against primers A1, A2, A3, A4, A5 and A6. The results obtained are shown in Figures 6, 7, 8, 9, 10 and 11.

i. RAPD using primers A1 and A2 are shown in Figure 6.
ii. RAPD using primers A3 is shown in Figure 7.
iii. RAPD using primers A4 is shown in Figure 8.
iv. RAPD using primers A5 is shown in Figure 9.
v. RAPD using primers A6 is shown in Figure 10.
vi. RAPD using primers: A1, A2, A3, A4, A5 and A6 against sample 30 (antibiotype 8) are shown in Figure 11.

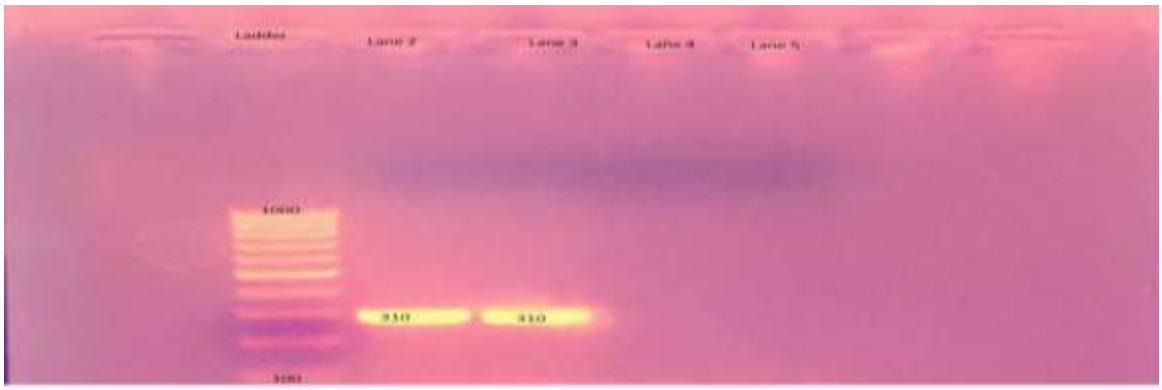

Figure 3. Agarose gel photo documentation of conventional PCR on genetic material extracted from *S. aureus* strains as a molecular typing for detection of *mecA* gene using forward primer : GTA GAA ATG ACT GAA CGT CCG ATA A and reverse primer CCA ATT CCA CAT TGT TTC GGT CTA A . Lane L: molecular weight marker (100 – 1000 bp). Lanes 2, 3: positive samples MRSA mecA gene with amplicon size of 310 bp., Lane 3,4: negative samples MSSA *mec*A.

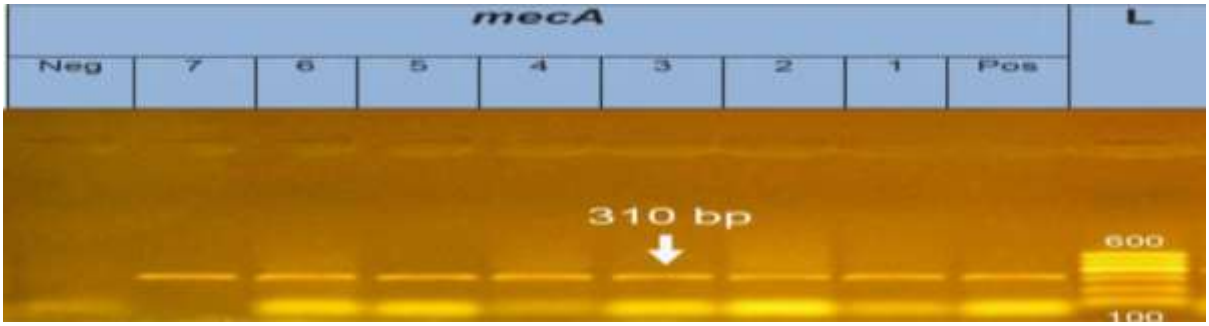

Figure 4. Agarose gel photo documentation of conventional PCR on genetic material extracted from *S. aureus* strains as a molecular typing for detection of *mec*A gene using forward primer: GTA GAA ATG ACT GAA CGT CCG ATA A and reverse primer CCA ATT CCA CAT TGT TTC GGT CTA A . Lane L: molecular weight marker (100 – 600 bp). Lanes 1-7: positive samples MRSA mec A gene with amplicon size of 310 bp. Lane Neg: negative control. Lane Pos.: positive control.

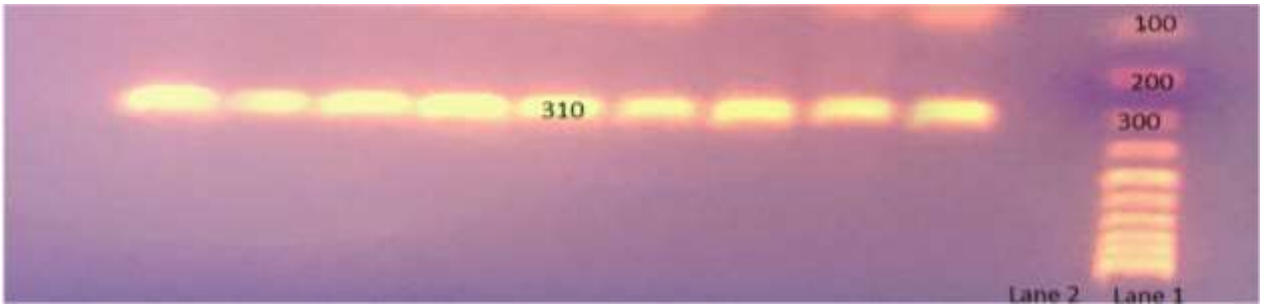

Figure 5. Agarose gel photo documentation of conventional PCR on genetic material extracted from *S. aureus* strains as a molecular typing for detection of *mecA* gene using forward primer : GTA GAA ATG ACT GAA CGT CCG ATA A and reverse primer CCA ATT CCA CAT TGT TTC GGT CTA A . Lane L: molecular weight marker (100 – 600 BP). Lanes 1-7: positive samples MRSA *mecA* gene with amplicon size of 310 bp. Lane Neg: negative control. Lane Pos.: positive control.

Repetitive sequence DNA (REP)

REP was performed for isolates 69, 23, 164, 83, 74, 56 and 88 using REP primers REP1 and REP2.

i. REP using primer REP1 and REP2 with 69, 23, 164, 74, 56 and 88 and representing biotypes 1, 2, 3, 4, 5, 6

Figure 6. Agarose gel electrophoresis of RAPD PCR with A1 and A2.

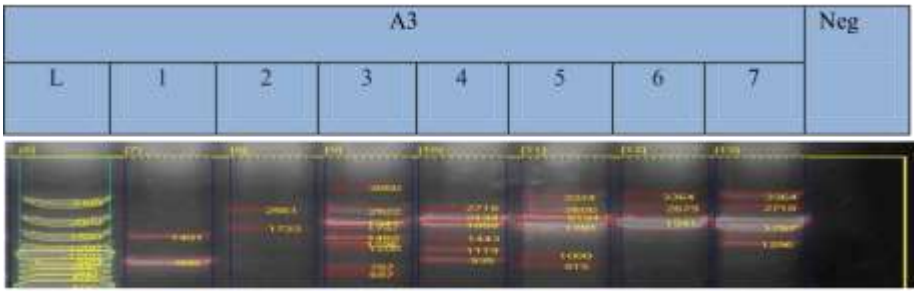

Figure 7. Agarose gel electrophoresis of RAPD PCR with A3.

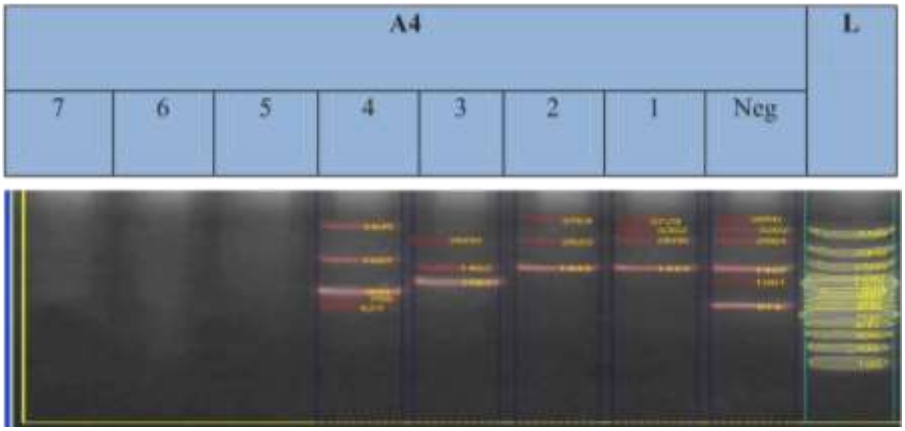

Figure 8. Agarose gel electrophoresis of RAPD PCR with A4.

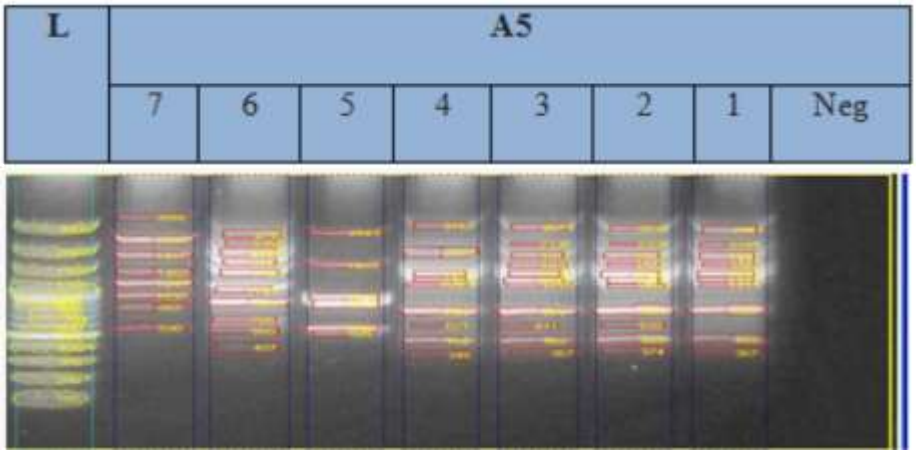

Figure 9. Agarose gel electrophoresis of RAPD PCR with A5.

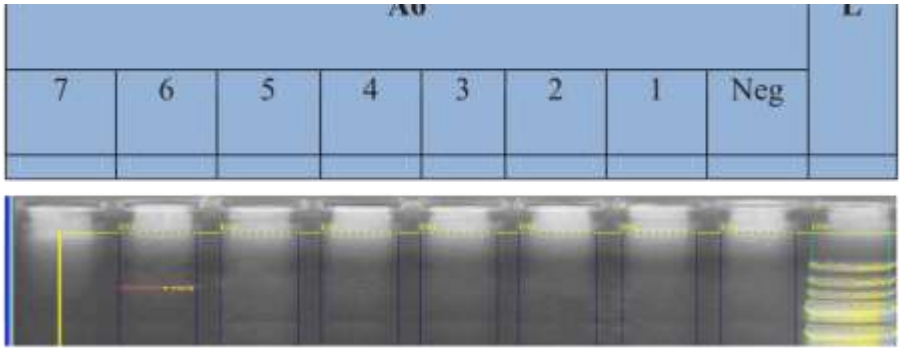

Figure 10. Agarose gel electrophoresis of RAPD PCR with A6.

Figure 11. Sample 30 with primers A1 to A6.

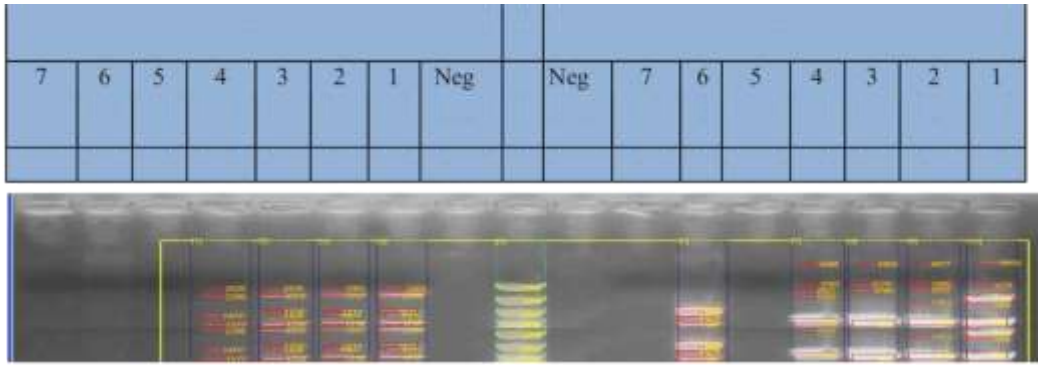

Figure 12. Agarose gel electrophoresis of Repetitive sequence DNA was done for isolate 69, 23, 164, 83, 74, 56 and 88 using primers REP1and REP2.

Figure 13. Agarose gel electrophoresis for isolate 30 using REP1 and REP2 primers.

and 7 respectively results are shown in Figure 12.
ii. Repetitive sequence PCR for isolate 30 representing antibiotype 8 is shown in Figure 13.

Statistical analysis

Relationships between different factors with MRSA were

Table 10. Relation between MRSA and site of infection.

Variable			Site of infection								Total
			Blood	Nasal swab	Pus	Skin	Sputum	Throat swab	Urine	Wound	
MRSA	S	Count	5	0	4	39	6	0	15	25	94
		% Site of infection	41.7	0.0	44.4	68.4	42.9	0.0	93.8	47.2	56.6
	R	Count	7	2	5	18	8	3	1	28	72
		% Site of infection	58.3	100.0	55.6	31.6	57.1	100.0	6.3	52.8	43.4
Total		Count	12	2	9	57	14	3	16	53	166
		% Site of infection	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

No p-value because of small number of cases within subgroups so we combined both throat and nasal swabs in one group.

Table 11. Relation between MRSA and site of infection.

Variable			Site of infection							Total
			Blood	Pus	Skin	Sputum	Throat and nasal swab	Urine	Wound	
MRSA	S	Count	5	4	39	6	0	15	25	94
		% within infection site 1	41.7	44.4	68.4	42.9	0.0	93.8	47.2	56.6
	R	Count	7	5	18	8	5	1	28	72
		% within infection site 1	58.3	55.6	31.6	57.1	100.0	6.3	52.8	43.4
Total		Count	12	9	57	14	**5**	16	53	166
		% within infection site 1	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Fisher's Exact Test with p-value<0.001, no p-value because of small number of cases within subgroups.

Table 12. Relation between MRSA and hospitals.

Variable			Hospital							Total
			Alhoodalmarsod	Banha	El Hussain	ElsayedGalal	KafrElshiekh	Kasralaini	Sheikh zayed	
S. aureus	S	Count	32	7	11	5	2	29	8	94
		% within Hospital	72.7	53.8	61.1	45.5	28.6	47.5	66.7	56.6
	R	Count	12	6	7	6	5	32	4	72
		% within Hospital	27.3	46.2	38.9	54.5	71.4	52.5	33.3	43.4
Total		Count	44	13	18	11	7	61	12	166
		% within Hospital	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

presented as follows: MRSA and site of infection (Tables 10 and 11); MRSA and gender (Table 15), MRSA and hospitals (Table 12) and relation of hospitals with infection site (Table 16).

DISCUSSION

S. aureus is a ubiquitous bacterial species capable of colonizing and cause infections in a wide range of hosts. It is the cause of serious infections in humans and number one cause of hospital-associated infections. A high proportion of these infections are caused by Methicillin-resistant *S. aureus* (MRSA) which became highly prevalent in hospitals worldwide.

The evolution of MRSA has paralleled penicillin-resistant *S. aureus* from 1940s. MRSA is now pandemic, with dissemination of HA-MRSA clones from 1960s, CA-MRSA clones from 1990s, and LA-MRSA clones from 2000s. Although epidemiological data from separate

Table 13. Subdivision of MRSA into eight biotypes.

Antibiotype	Teicoplanin	Rifampin	Amoxycillin/ Clavulinic	Ciprofloxacin	Chloramphenicol	Trimethoprim Sulphamethoxazole	Ofluxacin	Tetracyclin	Erythromycin	Gentamicin	Vancomycin	Oxacillin
1	S	S	R	S	S	R	R	R	R	R	S	R
2	S	R	S	R	R	R	R	R	S	R	S	R
3	S	R	R	S	S	R	R	R	R	R	S	R
4	S	R	R	R	R	S	R	R	R	R	S	R
5	S	R	R	R	R	R	R	S	R	S	S	R
6	S	R	R	S	S	R	R	R	S	R	S	R
7	S	S	R	R	R	R	S	R	R	R	S	R
8	S	R	R	S	S	R	R	R	R	R	R	R

Isolates within the same group showed the same resistance pattern against different antibiotics.

Table 14. The representing sample for each biotype.

Biotype	Representing isolate	Age	Clinical specimen	Hospital	Gender
1	69	30	Urine	Kasr Elaini	M
2	23	29	Wound	Kasr El-Aini	M
3	164	15	Wound	Kasr Elaini	M
4	83	33	Skin	Kasr Elaini	F
5	74	13	Wound	Kasr Elaini	F
6	56	59	Blood	Elhhod Elmarsod	F
7	88	40	Blood	Sayed Galal	M
8	30	25	Wound	Sayed Galal	M

studies are often not comparable owing to differences in study design and populations sampled, with the highest rates (>50%) reported in North and South America, Asia and Malta, intermediate rates (25 to 50%) are reported in China, Australia, Africa and some European countries [e.g. Portugal (49%), Greece (40%), Italy (37%) and Romania (34%)]. Other European countries have generally low prevalence rates (for example, Netherlands and Scandinavia). High morbidity and mortality rates are associated with MRSA because of the development of multidrug resistance. *S. aureus* strains with the tendency to accumulate additional resistance determinants have resulted in the formation of multiple-antibiotic resistant MRSA strains. These strains show resistance to a wide range of antibiotics, thus limiting the treatment options to few agents, such as Vancomycin and Teicoplanin (Peacock et al., 2005; Perez-Roth et al., 2001). To compound this problem further, *S. aureus* has ability to form biofilms making it difficult to be eradicated from the infected host. They are reported to display susceptibilities towards antibiotics and biocides which are 10 to 1000 times, less than the equivalent populations of free-floating planktonic bacteria (Sasirekha et al., 2012).

Table 15. Relation between MRSA and gender.

Variables			Gender		Total
			Male	Female	
S. aureus	S	Count	53	41	94
		% within gender	55.8	57.7	56.6
	R	Count	42	30	72
		% within gender	44.2	42.3	43.4
Total		Count	95	71	166
		% within gender	100.0	100.0	100.0

Pearson Chi-square test (p-value = 0.801) = 0.063

Table 16. Relation between hospitals and site of infection.

Parameter			Hospital							Total
			Alhood almarsod	Banha	El Hussain	Elsayed Galal	Kafr Elshiekh	Kasr alaini	Sheikh zayed	
Infection.site1	Blood	Count	4	2	2	1	0	2	1	12
		% Hospital	9.10	15.40	11.10	9.10	0.00	3.30	8.30	7.20
	Pus	Count	1	2	1	0	2	3	0	9
		% Hospital	2.30	15.40	5.60	0.00	28.60	4.90	0.00	5.40
	Skin	Count	15	3	7	4	2	24	2	57
		% Hospital	34.10	23.10	38.90	36.40	28.60	39.30	16.70	34.30
	Sputum	Count	3	0	1	0	1	7	2	14
		% Hospital	6.80	0.00	5.60	0.00	14.30	11.50	16.70	8.40
	Throat and Nasal swab	Count	1	0	0	2	0	2	0	5
		% Hospital	2.30	0.00	0.00	18.20	0.00	3.30	0.00	3.00
	Urine	Count	5	1	1	1	0	6	2	16
		% Hospital	11.40	7.70	5.60	9.10	0.00	9.80	16.70	9.60
	Wound	Count	15	5	6	3	2	17	5	53
		% Hospital	34.10	38.50	33.30	27.30	28.60	27.90	41.70	31.90
Total		Count	44	13	18	11	7	61	12	166
		% Hospital	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

No p-value because of small number of cases within subgroups.

A number of different classes of antimicrobials have been mentioned and both overall institutional use and individual patients' use of antimicrobials increases the risk of MRSA. Many control measures need to be established to decrease the fast spreading of MRSA infections. MRSA has become a significant clinical pathogen due to three factors:

i. An intrinsic pathogenicity mediated by specified (and often unique) virulence factors.
ii. High frequency of nosocomial dissemination and acquisition within the healthcare environment.
ii. Limited therapeutic options.

Usually, the etiological diagnosis is dependent on isolation of the bacterium from the focus of infection or blood cultures. In some cases, access to the focus may be difficult or dangerous or cultural confirmation may be hampered by ongoing antimicrobial therapy. Also, serological assays for *S. aureus* infections are of limited value because of the insufficient diagnostic sensitivity and specificity.

Detection of MRSA by a variety of procedures has been associated with decreasing levels of infection and prevention of the transmission of these microorganisms (Loureino et al., 2000). It is clear that, the sooner a MRSA infection is diagnosed and the susceptibility to antimicrobial agents established, the sooner the appropriate therapy and control measures will be initiated. Laboratory diagnosis and susceptibility testing are crucial steps in controlling and preventing MRSA infections.

In our study, a total of 166 clinical isolates were recovered from blood (12 isolates), throat swabs (3 isolates), nasal swabs (2 isolates), wound (53 isolates), pus (9 isolates), sputum (15 isolates), urine (16 isolates) and skin (56 isolates) specimens, collected from different hospitals, different units and different infection sites. Isolation and identification were done according to Bergey's Manual of Systematic Bacteriology (Holt et al., 1993). Gram stain, growth on specific media and biochemical tests were the tools used for identification. The 166 isolates showed characteristics commonly known of *S. aureus*.

The antibiogram susceptibility pattern of these isolates was investigated to screen and determine the resistance profile for Oxacillin and other antibiotics. Upon performing antibiogram for staphylococcal isolates against Oxacillin discs, only 73 isolates representing 43.9% of the *S. aureus* isolates were Oxacillin resistant whereas resistance to other antibiotics was variable among the collected isolates and these results agree with results of Borg et al. (2007) who found that overall median MRSA proportion was 39% (interquartile range: 27.1 to 51.1%). This percentage was lower than that found by Idil and Aksöz which was 56.5%.

It was speculated that the prevalence of MRSA infections especially in health care units was rapidly increasing all over the world. Overall, the median MRSA proportion was 39% (interquartile range: 27.1 to 51.1%). The highest proportions of MRSA were reported by Jordan, Egypt and Cyprus, where more than 50% of the invasive isolates were methicillin-resistant (Borg et al., 2007). This was in agreement with the results shown in this study, since 73 out of 166 *S. aureus* isolates collected from different laboratories were methicillin resistant.

The present study shows that the prevalence rate of MRSA is higher in males (44/73), which represent 60.27% than in females (29/73) that represent 39.7%. In 2009, Rahman et al. (2011) have also reported a greater percentage of MRSA in males (58%) than in females (42%) in Peshawar. In Riyadh and Saudi Arabia, Baddour et al. (2006) have also reported a greater frequency of MRSA in males (64.4%) than in females (35.6%). Similarly, Tiemersma et al. (2004) have reported a higher rate of MRSA prevalence in males than females. However, India Sharma and Mall (2011) have reported greater rate of prevalence MRSA in females 14/25 (60.86%) than males 9/25 (39.13%).

MRSA rates were highest among clinical specimens from throat and nasal swabs (100%) followed by blood (58%), pus (55.5%), sputum (53.5%), surgical wound (52.8%) and Skin (33.9%). Our observations suggest that the throat and nose are important port of entry of MRSA. On the other hand, MRSA rates were lowest among clinical specimens from urine and skin. In a previous study by Wuduren et al. (1994), it was reported that the respiratory tract is an important port of entry.

Regarding the age of the patients, the occurrence of *S. aureus* was higher among patients in the age group 21 to 40 years. Mulla et al. (2007) also reported that *S. aureus* was commonly isolated from patients in age group 21 to 30 years.

The antibiotics used in this study are known to be efficacious against bacteria through different mechanisms. Penicillin, cephalosporin and carbapenems belong to the family of antibiotics called beta-lactams. These antibiotics work by disrupting the synthesis of the cell envelope in growing cells, inactivating the penicillin-binding proteins thus inhibiting the synthesis of bacterial cell wall.

Antibiotics such as Gentamicin, Tetracycline, Erythromycin and Clindamycin work by inhibiting protein synthesis in bacteria (Salyer and Whitt, 2005). Rifampin belongs to the family of antibiotics which prevents bacterial growth by inhibiting the RNA polymerase and halting the bacteria protein synthesis (Kohanski et al., 2010).

Vancomycin works much like penicillin as it inhibits the synthesis of bacterial cell wall. Another commonly prescribed antibiotic against MRSA is Trimethoprim sulfamethoxazole. This antibiotic inhibits the necessary cofactors for bacterial DNA synthesis (Salyer and Whitt, 2005; Kohanski et al., 2010). The antibiogram performed on methicillin-resistant *S. aureus* is further evidence on

the multi-resistance of MRSA, where the distribution of antibiotic resistance among *S. aureus* isolates, whether sensitive or resistant to Oxacillin was great.

Vancomycin and Teicoplanin showed excellent therapeutic activity against MRSA. No resistant MRSA strain was detected against Teicoplanin in this study, while 5.5% of the strains showed resistance to Vancomycin. Similar results have been obtained for Teicoplanin in previous studies (Perwaiz et al., 2007; Hussain et al., 2005).

The percentage resistance of the total *S. aureus* isolates (166 isolates) to Oxacillin and Erythromycin was 43.7 and 38.55%, respectively, which was higher than Rifampin (32.5%) and Vancomycin (2.4%), but less than Tetracycline (39%). In our study, the prevalence of multidrug resistance in *S. aureus* isolated from different clinical samples was investigated. The results demonstrated that, sensitivity to most of the antibiotics tested among MSSA was significantly higher than MRSA. Rifampicin resistance percentage was 76 and 1% for MRSA and MSSA, respectively; Amoxicillin clavulanic 84.7 and 5.4%; Ciprofloxacin 52.7 and 1%; Chloramphenicol 52.7 and 1%; Trimethoprim sulfamethoxazole 81.9 and 9.6%; Ofloxacin 97.22 and 5.37%; Tetracycline 83.33 and 5.44%; Erythromycin 76.3 and 6%; Gentamicin 83.33 3 and 22%; Vancomycin 5.5 and 0%; Teicoplanin 0 and 0%; and Oxacillin 100 and 0%. These results are in agreement with those reported by Quinn et al. (2002) who proved that, MRSA either produce potent toxins or resist to a wide range of antibiotics. Karska et al. (2010) also showed that MRSA is a multidrug-resistant microorganism and a principal nosocomial pathogen worldwide.

The research showed that, despite the fact that there was an increase in the rate of incidence of MRSA, 100% susceptibility to Teicoplanin and 94.5% to Vancomycin were revealed. Sensitivity of MRSA to Teicoplanin was the same as found by Abbadi et al. (2013), which was 100% .On the other hand, we found that sensitivity to Vancomycin was 94.5% while in Abbadi et al. (2013) it was 100%.

In the present study, MRSA was grouped into eight biotypes on the basis of antibiotic susceptibility profiles and 58.33% were resistant to at least seven types of the antibiotics. In addition, 20.5% of MRSA belonged to biotype 1 followed by biotype 4(17.8%), 5(16.4%), 2(15%), 3(12.3%), 6(9.5%), 8(5.4%) and 7(2.7%). All the biotypes were resistant to Oxacillin and sensitive to Teicoplanin.

Determination of susceptibility or resistance using phenotypic tests is a gold standard against which newer technologies are compared in terms of performance, cost and ease to use. Unfortunately, these methods have their limitations, which are not discriminating enough, highly dependent on growth conditions and mostly due to phenotypic differences between strains from the same species. For these reasons, methods based on molecular techniques have been developed to stop the spread of MRSA (Giammarinaro et al., 2005).

Early detection of MRSA from clinical specimens enables appropriate antimicrobial therapy with an extensive use of antibiotics over the last 50 years which has led to the emergence of bacterial resistance and the dissemination of resistance genes among pathogenic organisms (Malathi et al., 2009; Méndez et al., 2000).

Molecular methods for detecting resistance would impact more directly on patient care which would be valuable infection-control tools by rapid and accurate identification of staphylococci and their resistant types. Thus, it helps in confirming patients infected by resistant bacteria. Rapid and reliable detection of methicillin-resistant *S. aureus* (MRSA) is a prerequisite for the initiation of effective infection control measurements in order to restrict dissemination of this pathogen. Clearly, rapid detection of a specific resistance mechanism in a molecular test would initially allow clinicians, to avoid potentially inappropriate treatment options (Woodford and Sundsfjord, 2002).

One of the strategies to identify MRSA is multiplex PCR, developed for simultaneous amplification of methicillin resistance gene, *mecA* and one of the *S. aureus*-specific genes, such as coa, gyrA, holB (SA442), femA, femB or *nuc* gene, encoding for *S. aureus* specific thermonuclease. In a comparative study, Brakstad et al. (1989) used a multiplex PCR targeting *mec A* and *nuc* which report a 100% agreement with conventional identification methods. This approach is generally applied for the identification of subcultures of MRSA in routine diagnostic microbiological laboratory.

Other protocols were directed toward the specific detection of *S. aureus* and focused on amplification of genes found only in that species. Specific examples include the genes encoding nuclease (*nuc*) and Staphylococcal 16S rRNA (Mason et al., 2001).

MRSA is primarily mediated over production of PBP2a, an additional altered penicillin-binding protein with low affinity for beta-lactam antibiotics. The *mecA* gene and structural determinant encoding PBP2a is considered a useful molecular marker of putative methicillin resistance in *S. aureus* (Brakstad et al., 1992). Similarly, the *nuc* gene which codes thermonuclease is considered specific for *S. aureus* and was chosen as a target gene for *S. aureus* identification.

In the present study, all *S. aureus* isolates were *nuc* positive, while non *S. aureus* isolates were *nuc* negative, showing that the gene is available and specific which agrees with the results of Gao et al. (2011)

This study thus reveals the isolation and biochemical characterization of MRSA as well as detection of *mec-A gene* using PCR. MRSA sensitivity was checked against different antibiotics from different classes. Similar study was conducted by Abd El-Moez et al. (2011) in Egypt with results that agree with Hudson (1994) and Cookson (1998) who proved that, treatment of *S. aureus* infections

may be complicated by multiple antibiotic resistances and specific virulence factors, causing temporary or long-lasting carriage. The findings is also in line with Quinn et al. (2002) and Abd El-Moez et al. (2011), who branded MRSA of being a critical pathogen responsible for great morbidity and mortality especially among immuno-suppressed cases.

MRSA strains obtained from various clinical samples and collected from different hospitals, were characterized phenotypically by susceptibility testing and genotypically using RAPD-PCR and REP-PCR methods for each biotype. It was found that, there was no significant association between genotypes obtained from RAPD and REP-PCR. This agrees with the results obtained by Idil and Aksöz (2013).

Molecular analysis performed in the present study was necessary for assessing feasibility of the PCR approach for identification of *S. aureus* multiple drug resistant strain. Simultaneous identification of *S. aureus* and detection of methicillin resistance using PCR technique with its reproducibility and discriminating capacity has been shown to be an excellent technique for MRSA detection. PCR was applied for the amplification of a sequence of *mecA* and *nuc* genes using two primers targeted at each gene. All strains of *S. aureus* and MRSA clinical isolates were confirmed for the presence or absence of *mec*A and *nuc* genes by PCR. PCR results revealed that, 43.7% of the tested strains carried *mec-A* gene at 310 bp fragment while all of MRSA (100%) carried *mec*A gene. These results are higher compared to those obtained by Abd El-Moez et al. (2011), who had a percentage of 85.7% and reported that, identification of MRSA by drug susceptibility tests alone presented a serious problem because numbers of clinical *S. aureus* isolates have border line resistant to methicillin. Hence quick and accurate amplification for the detection of *mec-A* gene is necessary.

Additionally, detection of *mec-A* gene by PCR is extremely important for appropriate treatment of MRSA. These results agree with Vannuffel et al. (1995) who indicated that, MRSA has become a major nosocomial pathogen not only in tertiary care hospitals but also in chronic care facilities. The results further agree with those of Anderson and Weese (2006) who found that conventional identification of MRSA requires between 24 to 48 h after sampling and recommended rapid and sensitive method of identification as PCR for detection of *mecA* gene which codes for the drug resistant penicillin-binding protein 2a(PBP2a) or 2(PBP2). Klotz et al. (2005) reported an increase in the frequency of MRSA as an important causative agent of nosocomial infections worldwide, in spite of optimal hygienic conditions.

It was established that, there is no significant association between genotypes obtained from RAPD and REP PCR and antibiotypes profiles vice versa. In the cases of randomly amplified polymorphic DNA (RAPD) assay and a repetitive element sequence-based PCR (rep-PCR) analysis, the major drawback relates to insufficient standardization and low laboratory-to-laboratory reproducibility (Deplano et al., 2000; Van Belkum, 1994).

PCR-based techniques, identified for typing of many bacteria including Staphylococci have contributed significantly to recent advances in tracking the spread of these strains (Stephan et al., 2004). Randomly amplified polymorphic DNA (RAPD)-PCR is one of these methods, based on the use of short oligonucleotide primers with a random sequence which is designed without any prior sequence information concerning the target DNA.

As an alternative to this approach, amplification of highly conserved regions using primers leads to differentiation of the DNA fingerprints (Hyytiä, 1999). A technique called Repetitive element sequence based (REP)-PCR, in which primers derived from REP sequences were used, relies on the amplification of regions between non-coding repetitive sequences (repetitive DNA sequences) and is used for fingerprinting of isolates (Saulnier, 1993). REP PCR for MRSA isolates in this study produced bands with different intensities ranging from 0.15 to 3 kbp.

Another technique used for fingerprinting isolates is RAPD PCR (random amplification of polymorphic DNA) in which knowing the target DNA is not prerequisite. It produces a set of amplification products characteristic for each isolate (Saleki, 2002).

Therefore, implementation of preventive measures is seriously recommended for the control and prevention of increasing MRSA infections. “Cleanliness is next to Godliness”. Environment should be kept clean and hands should be regularly washed with soap and detergents.

Colonized/infected patients should be properly isolated/ treated. Vancomycin and Teicoplanin showed best chemotherapeutic activity against MRSA infections in this study, but their prescription should be kept limited, followed by antibiotic sensitivity tests.

Conclusion

MRSA is a serious problem facing hospitals all over Egypt which needs more attention. So, further studies were recommended as detection of virulence genes and require sequence analysis to methicillin resistance genes which make these strains capable of being multiple multidrug resistances.

Characterization of the antibiotic resistance and sensitivity pattern against different antimicrobials revealed that MRSA was highly sensitive to Teicoplanin (S% was 100%) and Vancomycin (S% was 97.5%), while antibiotic resistance other than Oxacillin was found in 43.9% of the isolates showing multiple drug resistance.

Classification of MRSA samples according to resistance pattern into eight biotypes (then RAPD and REP PCR) were done for a sample from each biotype to

check for genetic relatedness. Molecular techniques were found to be more rapid, highly sensitive and detect non-viable organisms while culture was found to be 100% specific for detection of MRSA.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Paediatric tuberculosis in a low burden setting of Saudi Arabia: Drug and multidrug resistance patterns

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In this study, the infection of young children with *Mycobacterium tuberculosis* and drug-resistant *M. tuberculosis* in a mass gathering area in Al-Madinah Al-Munawwarah, was investigated and discussed. All the children, 15 years old and younger, who were referred to the central tuberculosis laboratory in Al-Madinah between January 2012 and December 2014 were included in this study. Among a total of 622 registered new cases, 68 (10.9%) were children, males were 40 (58.8%) while 28 (41.2 %) were females. All the children were vaccinated with Bacillus Calmette-Guérin (BCG) within their first week of birth. Sixty (88.2%) children were infected with *M. tuberculosis*, whereas 8 (11.8%) had non tuberculous mycobacteria (NTM). Clinically, pulmonary tuberculosis was confirmed in 20 (29.4%) cases, whereas the remaining 48 (70.6%) had extra pulmonary tuberculosis. Multidrug-resistant *M. tuberculosis* (MDR) was isolated from 3 (4.4%) cases, all of whom were younger than five years; one with pulmonary and two with extrapulmonary tuberculosis. All the isolated MDR organisms belonged to the *M. tuberculosis* complex. The rates of mono-resistance to isoniazid (H), streptomycin (S), ethambutol (E) and pyrazinamide (Z) were 5.9, 1.5, 5.9 and 8.8%, respectively. No case was registered as mono-resistant to rifampin (R). The prevalence of childhood tuberculosis in the current study area is higher than the globally estimated rate. Since all the cases were new, MDR-TB was mostly due to infection with originally MDR strains.

Key words: Mycobacteria, polymerase chain reaction, resistance, antibiotics, antimicrobials.

INTRODUCTION

Tuberculosis represents the leading cause of death from a single infectious agent, ranking above HIV/AIDS. Moreover, it is the ninth leading cause of death worldwide. According to the WHO, 10.4 million cases of TB were present in 2016; an estimated 1.3 million deaths occurred amongst HIV-negative patients, and an additional 0.374 million deaths occurred amongst HIV-ositive patients (WHO, 2017). Most cases (90%) were adults, and 65%

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were males. Regarding drug resistance, 0.6 million new cases with resistance to rifampicin (RRTB) were registered in 2016, of which 0.49 includedmultidrug-resistant TB (MDR-TB) (WHO, 2017).

The prevalence of tuberculosis amongst children is always underestimated (Starke, 2002) because of several factors, the most important of which is difficulty in diagnosis (Hailu et al., 2014). Children are not usually considered a significant source of disease transmission; thus, they are neglected by national control programmes (Marais and Pai, 2007).

Multidrug-resistant tuberculosis is defined as TB caused by *Mycobacterium tuberculosis* which is resistant to both rifampicin and isoniazid (WHO, 2008). According to the WHO, the rate of MDR-TB is increasing; because children represent 20% of TB-infected population in high-burden countries (Marais et al., 2006a). It is likely that the prevalence of MDR-TB amongst children is also increasing. The most probable source of infection amongst children is their household contacts; however, routine investigations during surveillance in areas with low resources and high burden of tuberculosis are rare (Hill and Ota, 2010; Hwang et al., 2011). The transmission of drug resistant bacilli from close contact is also a key explanation of MDR-TB in children (Shah and Chilkar, 2012).

The diagnosis of paediatric TB is difficult with regard to microbiological tools because this disease can only be detected in approximately 2 to 40% of all radiologically confirmed pulmonary TB cases (Zar et al., 2005), and it rarely increases above 50% unless extensive disease is present (Marais et al., 2006b); therefore, the treatment of children is usually based on symptoms and radiology, presuming drug-susceptibility (WHO, 2006). In general, physicians do not employ treatment options for MDR-TB without confirmation via microbiological tools because of the prolonged treatment duration and the adverse effect of drugs that might require hospitalisation.

Recent reports regarding the prevalence and incidence of TB in the Kingdom of Saudi Arabia (KSA) have shown variable rates in different regions of the country (8.5 to 23.1%) amongst Saudi nationals; however, the percent has increased in the Makkah region (38%) amongst non-Saudis (Abouzeid et al., 2012; Gleason et al., 2012). Improvements in healthcare have reduced the incidence of TB; however, its prevalence remains high primarily because of the large populations generated by the Hajj and Omarah events (Shibl et al., 2013).

Regarding MDR in KSA, the 2013 WHO estimates revealed previously treated and new case rates of 5.5 and 0.51%, respectively. Several studies conducted throughout KSA showed 14-20% resistance rates to first-line drugs and 1-44% resistance rates to MDR-TB (Al-Rubaish et al., 2001; Abu-Amero, 2002; Al-Hajoj et al., 2007). This study aimed to investigate the prevalence of tuberculosis and MDR Mycobacterium tuberculosis among children in Al-Madinah Al-Monawwarah, KSA.

MATERIALS AND METHODS

Study type, population and sampling

This study included all newly confirmed tuberculosis patients of age 15 years and below (n = 68/622; 10.9%), who were referred to the central tuberculosis laboratory in Al-Madinah Al-Munawwarah between January 2012 and December 2014. Informed consent documents were offered to their parents. Basic demographic data were collected.

Sputum specimens were collected from some of the pulmonary tuberculosis enrolled patients and processed using standard procedures. In patients with extrapulmonary tuberculosis, fine needle aspirates were collected; in other cases, gastric lavage samples were collected from infants.

Isolation and identification of mycobacterium

Ziehl-Neelsen stain (ZN)

ZN staining was performed on all collected specimens using standard measures. Following absolute alcohol fixation, carbolfuchsin was poured over a smear and heated gently until boiling for 5 min and then washed off with water. Then, 20% sulphuric acid was added, left for one min and washed off with water. Methylene blue was added for two min, and the sample was again washed with water. Finally, the slides were allowed to air dry, and they were examined under an oil immersion lens (Olympus, Japan) for acid fast bacilli.

Drug-susceptibility testing via BACTEC MGIT 960

Specimens from the 68 patients were cultured in a Mycobacteria Growth Indicator Tube (MGIT) 960 system (Becton Dickinson Microbiology System, USA). The cultures were further investigated for susceptibility to anti-tuberculosis drugs as directed by the manufacturer using an M960 system. The final drug concentrations used for drug susceptibility testing (DST) were E, 5.0 mg/ml; S, 0.1 mg/ml; R, 1.0 mg/ml; H, 0.1 mg/ml; and Z, 25 mg/ml. For each isolate, a growth tube (a growth supplement without a drug) was included as a control to determine the relative growth ratio, and the M960 system was used to report the results of the DST.

GenXpert MTB/RIF assay

An MTB/RIF assay was conducted according to Blakemore et al. (2010) by using GeneXpert (Cepheid, Sunnyvale, CA). Briefly, three volumes of sample reagent were added to one volume of sample, and the container was shaken and kept at room temperature for 15 min. Then, 2 ml of the mixture was transferred to the test cartridge. Samples with suspicious results (those that differed in culture from those in GenXpert or vice versa) were re-checked.

GenXpert negative samples were subjected to further identification using conventional methods including ZN staining, and the follow-up cultures were created on Lowenstein Jensen (LJ) medium to identify NTM (Gangadharam, 1996).

Data analysis

The Statistical Package for Social Sciences (SPSS), version 21.0 (SPSS Inc., Chicago, IL, USA, 2012), was used for all data analyses. A two-tailed P-value of < 0.05 was considered as significant.

Table 1. Age, gender, nationality, disease site and smear results of the study participants.

Character		No.	%
Sex	Male	40	58.8
	Female	28	41.2
Age	≥1	25	36.8
	2-5	17	25.0
	6-15	26	38.2
Nationality	KSA	64	94.1
	Foreigner	4	5.9
Smear result	Positive	24	35.3
	Negative	44	64.7
Site of disease	Pulmonary	20	29.4
	Extrapulmonary	48	70.6

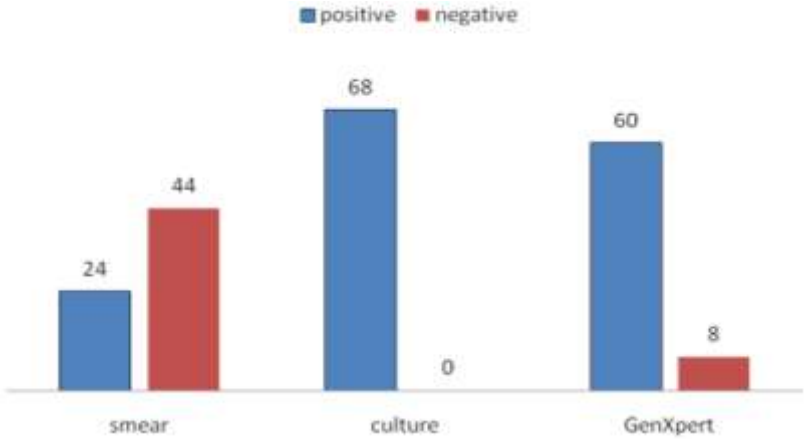

Figure 1. Results of the different diagnostic methods.

RESULTS

Epidemiological findings and risk factors

Between January 2012 and December 2014, 68 children (<15 years old) of 622 cases (10.9%) referred to the tuberculosis reference laboratory in Al-Madinah Al-Munawwarah were enrolled in this study. All cases were confirmed and registered as new, and all received a postnatal BCG vaccination. Regarding nationality, 64 (94.1%) of the patients were Saudis, whereas only 4 (5.9%) were foreign nationals.

This study included 40 males and 28 females, with a male/female ratio of 1.4:1. Despite the lower number of females as compared to males, this difference was not significant. The sample was divided into 3 age groups: 25 (36.8%) infants ≤ 1 year old, 17 (25%) 2- to 5-year-old and 26 (38.2 %) 6- to 15-year-old patients.

Diagnosis was conducted via ZN smear, culture and PCR. Table 1 shows the demographic data of the patients and the smear results, whereas Figure 1 shows the results of the different methods used for diagnosis.

Isolation and identification of *M. tuberculosis*

Positive ZN smears were recorded in 24 (35.3%) cases, with a sensitivity that was significantly lower than the culture ($P < 0.001$). All the cultures tested positive, and 8 of the cultured isolates (11.8%) were rapid growers and tested negative in PCR (GenXpert). The cultures were identified with the aid of biochemical reactions as nontuberculous mycobacteria (NTM) (Table 2) and the remaining 60 (88.2%) were labelled *M. tuberculosis.*

Extrapulmonary tuberculosis

Extrapulmonary tuberculosis (EPTB) was significantly more prevalent than pulmonary tuberculosis ($P < 0.001$),

Table 2. Distribution of NTM among study subjects.

Character		No.	%
Sex	Male	6	75
	Female	2	25
Age	1 ≥	1	12.5
	2-5	1	12.5
	6-15	6	75
Nationality	SA	8	100
	Foreigner	0	0
Smear result	Positive	1	12.5
	Negative	7	87.5
Site of disease	Pulmonary	6	75
	Extra pulmonary	2	25

which was found in 48 (70.6%) of the cases, whereas only 29.4% had PTB. The distribution of extrapulmonary cases in the sample is shown in Table 3. Samples for extrapulmonary TB included fine needle lymph node aspirates, pus and gastric lavage. Nearly two third of the extrapulmonary cases (31/48; 64.6%) were infants of age range of 1 month - 1 year. In addition, two of the extrapulmonary TB cases (4.2%) were due to NTM (Table 2).

Drug-susceptibility testing

The mono-drug resistance to antibacterial agents considered as the first-line treatment for tuberculosis was determined. Of the 68 isolates, 6 (8.8%) were resistant to pyrazinamide, 4 (5.9%) were resistant to isoniazid, 4 (5.9%) were resistant to ethambutol, and 1 (1.5%) was resistant to streptomycin; however, no mono-resistance was noted against Rifampin. Poly-drug resistance was found in 5 (7.4%) of the isolates, and MDR was found in 3 (4.4%) of the isolates. Table 4 shows the resistance patterns to isoniazid, pyrazinamide, ethambutol, rifampin and streptomycin.

In this study, MDR was diagnosed in 3/68 (4.4%) patients; one was isolated from a five-month-old Saudi male with pulmonary tuberculosis, and the other two were isolated from 2- and 4-year-old Saudi males with extrapulmonary TB. Table 5 presents the distribution of MDR among the study population while Table 6 shows the microbiological and demographic data of these MDR cases.

DISCUSSION

Tuberculosis remains the infectious disease with the highest morbidity rate (WHO, 2017). It is not well recorded amongst children (Starke, 2002) because of the difficulty associated with its diagnosis (Hailu et al., 2014). Children are usually expected to contract the infection from their household contacts (Hwang et al., 2011, Hill et al., 2010).

In this study, 622 patients referred to the central tuberculosis laboratory in Al-Madina were included after giving their informed consent. Among them, 68 (10.9%) children of the age of 15 years and less were present. The percent of paediatric TB found in the current study is higher than the expectations of the WHO, which recorded a 6% infection rate amongst children (WHO, 2014). However, it is well documented that only 50% of paediatric TB cases are recorded (Joshi et al., 2015); moreover, the actual percentage of infected children is approximately 11% (Nelson and Wells, 2004). In this context, this stiudy finding is within the normal range. However, active case finding is recommended through several means including screening of household contacts, providing better motherhood healthcare services and the screening of schoolchildren (Joshi et al., 2015).

In this study, NTM were found in 11.8% of the studied children population. This result differs from those obtained in Italy, where 62.9% of the mycobacterial infections of the head and neck in children were due to NTM (Caruso et al., 2009). This discrepancy might be because of several social, environmental or genetic factors.

The age groups did not significantly differ with regard to susceptibility to the infection. However, children less than one year old comprised 36.8% of the sample, which is more than one-third of the total. Given that the age range of the remaining patients is 2-15 years, this frequency is relatively high. Differences in the immune response that account for the difference in the national history of the disease might explain this high frequency of children less than one year of age (Piccini et al., 2014; Starke, 2004). Before two years of age, the primary infection leads to the rapid onset of the disease with severe symptoms, whereas disease progression is less likely in young children (Marais et al., 2004).

Table 3. Distribution of extrapulmonary TB amongst study participants.

Character		No.	%
Sex	Male	37	77.1
	Female	11	22.9
Age	≥1	31	64.6
	2-5	11	22.9
	6-15	6	12.5
Nationality	KSA	47	97.9
	Foreigner	1	2.1
Smear result	Positive	13	27.1
	Negative	35	72.9

Table 4. Drug susceptibility pattern of *M. tuberculosis* to different first-line anti-TB drugs.

Drug	No.	%
Z	6	8.8
E	2+2	5.9
R	0	0
H	4	5.9
S	1	1.5
R+H	3	4.4
Z+H	5	7.4
Z+S	0	0
H+S	0	0
R+H+S	0	0
Z+H+S	0	0
Z+E+S	0	0
Z+E+R+H	0	0
Z+R+H	0	0
Z+R+H+S	0	0
Z+E+R+H+S	0	0
Total Z	11	16.2
Total E	4	5.9
Total R	3	4.4
Total H	12	17.6
Total S	1	1.5
Poly-resistant	5	7.4
Multidrug-resistant	3	4.4

Z: pyrazinamide, E: ethambutol, R: rifampin, H: isoniazid, S: streptomycin.

In this study, ZN smears were positive only in 35.3% of the study subjects. Because of the difficulty in collecting specimens from children (fine needle use, gastric aspiration and sputum collection, which is not spontaneously induced in children), it is agreed internationally that TB diagnosis is an obscure area of research that generally involves inaccurate and slow diagnoses (Graham et al., 2014; Marais et al., 2013).

In this study, 70.6% of the cases were identified as extrapulmonary tuberculosis while only 29.4% had PTB. Nearly two-thirds of the extrapulmonary cases (31/48; 64.6%) were infants with an age range of 1 month to 1 year. In addition, two of the extrapulmonary TB cases (4.2%) were due to NTM. This result does not match those of previous studies from other parts of the world including the USA, Turkey, Denmark, Italy and South

Table 5. Distribution of MDR TB among study subjects.

Character		No.	%
Sex	Male	3	100
	Female	0	0
Age	1≥	1	33.3
	2-5	2	66.7
	6-15	0	0
Nationality	SA	3	100
	Foreigner	0	0
Smear result	Positive	1	33.3
	Negative	2	66.7
Site of disease	Pulmonary	1	33.3
	Extra pulmonary	2	66.7

Table 6. Demographic data, bacteriological findings and drug susceptibility results of MDR cases.

Age	Sex	Nationality	Type of specimen	Type of TB	Smear	Culture	dst S	dst H	dst R	dst E	dst Z
2 Years	M	KSA	FNA*	EP	NEG	POS	S	R	R	S	S
4 Years	M	KSA	PUS	EP	NEG	POS	S	R	R	S	S
5 Months	M	KSA	FNA*	EP	POS	POS	S	R	R	S	S

*Fine-needle aspirate.

Africa, where PTB was more prevalent than EPTB (Marais et al., 2006c; Peto et al., 2009; Buonsenso et al., 2012; Pekcan et al., 2013; Hatleberg et al., 2014). Because more than two-thirds of the patients in this study had EPTB, this finding alone suggests the possibility of under diagnosing EPTB in the absence of pulmonary evidence of the disease. It is well agreed worldwide that TB remains a major challenge because EPTB has a silent onset and difficult microbiological confirmation measures (Devrim et al., 2014). However, the bright side of EPTB in this study is that most of the cases presented with the least deleterious clinical form, lymphadenopathy. This result corroborates those of other studies (Devrim et al., 2014; Buonsenso et al., 2012). Moreover, none of the cases were recorded as TB meningitis in this study. The low incidence of CNS TB is mostly attributable to the high BCG vaccination rate (Devrim et al., 2014; Abubakar et al., 2013); in fact, almost all of the participants were vaccinated. Another factor that might influence EPTB is age because children less than 5 years old have a greater tendency to develop EPTB than older children (Cruz and Starke, 2010). In this study, 64.6% of children with EPTB were less than 1 year old, and 22.9% were between 2 and 5 years old, for an overall rate of 87.5%. This result corroborates those of other reports (Cruz and Starke, 2010).

The resistance to isoniazid was 5.9%, whereas the total resistance (either to isoniazid alone or combined with another drug) was 17.6%, making this important anti-tuberculosis therapy a target for the highest resistance rate. Isoniazid resistance in children is a worldwide phenomenon with variability in its frequency depending on geographical region (Devrim et al., 2014; Yuen et al., 2013; Dilber et al., 2000). In 2015, the global estimate of isonizid resistance amongst children was 12.1%, with higher rates in the west Pacific, southeast Asia and Europe, with the latter harbouring a rate that exceeds 26.1% (Yuen et al., 2015); thus, the current finding is within the regional and global levels.

The resistance to pyrazinamide in the current study was 11.7%. A retrospective cohort study from the USA revealed a resistance rate of 1.8% amongst the isolates tested with regard to this antimicrobial agent. This rate is much lower than that of the present study; in the American study, however, the target population included all age groups (not only children); although, the authors concluded that an association exists between mono-resistance to pyrazinamide and extrapulmonary TB, they did not find a relationship between resistance to pyrazinamide and mortality or MDR burden (Budzik et al., 2014). The current study partially corroborates these results because no association between resistance to pyrazinamide and MDR was found.

In this study, MDR was diagnosed in 3/68 (4.4%). This percentage is similar to the international value reported by the WHO in 2014 (3.5%) amongst new cases (WHO, 2014). It is also similar to the results of a recent study conducted in the same area (Elhassan et al., 2017) and

other areas throughout KSA (Varghese et al., 2013), although these studies included participants of all age groups. However, this value is lower than the MDR rate amongst children in other parts of the world such as Mexico, where 11.1% of children are diagnosed as having MDR-TB. The authors of that study attributed this finding to several possible factors, the most important of which is drug failure (Parra et al., 2011) which is not applicable in the current study.

Conclusion

Drug resistance can be attributed to several factors including genetic mutations, inadequate treatment, past treatment (Shah and Rahangdale, 2011) or the transmission of drug-resistant strains from close contact (Shah and Chilkar, 2012). Since all the participants were new cases in this study, approximately two-thirds were less than 5 years old, and 94% were Saudi nationals (that is, non-immigrants from countries with high TB burdens), the most likely source of infection is close contact. Hence, case finding and household contact screening might provide an effective control measure for limiting paediatric TB and paediatric MDR-TB in this area.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Identification and molecular phylogeny analysis using random amplification of polymorphic DNA (RAPD) and 16SrRNA sequencing of N_2 fixing tea field soil bacteria from North Bengal tea gardens

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Random amplification of polymorphic DNA (RAPD) amplification genomic DNA of 23 selected laboratory cultures of bacteria using RAPD revealed their polymorphism. Polymerase chain reaction (PCR) amplification of the bacterial 16SrDNA was performed using 704F GTAGCGGTGAAATGCGTAGA and 907R CCGTCAATTCCTTTGAGTTT primer, sequenced and accessed in NCBI (No. KY636356, KY631488, KY 860028, KX587470, KX665547, KY631489, KX608591, KY636360, KY671245, KY631490, KX587469, KY859856, KX665546, KX608590, KX587468, KY859798, KY636357, KY636361, KY 636359, KY631491, KY 859855, KY636358, KY636362) after submitting the contig. FASTA sequence in NCBI database was seen. All most all 23 bacterial strains (viz. TS-1-16, TS-4-23 DJ-1-22, DS-1-20, AS-1-4, DJ-1-24 , DJ-1-10, DJ-1-46) showed strong homology with free living nitrogen fixing soil bacteria, also showing (98 to 100%) identity and E-value of 00 with *Burkholderia* spp, Strain-S-9-19, Str-S-9-15, *SP-2386*, *Stenotrophomonas maltophilla, strn-MM-3-3, Str-D-3,LP-05*, *Bacillus cereus Strn*-FORC021and *Azospirillum* sp *TSH51* gene, having good nitrogen fixing capacity. Phylogenetic tree analysis among the 23 isolates and between the different strains from GenBank showed close similarity. Most of the isolated bacterial strain identified as a member of the genus *Burkholderia* sp, *Stenotrophomonas maltophila, Herbaspirillum* sp, *Acinetobacter johnsonii, Methylobacter, SP-T-20*, *and Bacillus cereus, Azospirillum* sp would be consider to be the most suitable bioferiliser for organic and conventional tea gardens of North Bengal, India.

Key words: Molecular phylogeny, RAPD, 16SrRNA sequencing, free living, N_2 fixing soil bacteria.

Introduction

Tea *Camellia sinensis* (L) O. "Kuntze" of family *Theaceae* is the most commonly used beverage in India and in the world. Tea is an evergreen shrub that mainly grows in tropical and subtropical areas. It is thought to have

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originated in East Asia somewhere between China and Burma. India is the world's second largest tea producer country next to China. In the financial year 2015 to 2016, India has recorded tea production of 1,233 million kg (mn kg) and exports crossing 230 mn kg after 35 years. The top five teas producing countries are China, India, Kenya, Sri Lanka and Turkey (http://www.gktoday.in/blog/key-facts-about-tea-production-in-india/). India has around 563.98 thousand hectares of tea cultivated land (December 2013). Assam is the highest Indian agricultural soil that contains low nitrogen and cellulose, and therefore the self-sustaining free-living nitrogen-fixing micro flora would be of great advantage if their identity is known and their ability is properly exploited. The reduction of chemical fertilizers by the application of biological fertilizers is mainly based on the bacteria involved in nitrogen fixation as one of the suitable steps in sustainable agriculture (Vejan et al., 2016).

The plant growth promoting rhizobacteria (PGPR) microorganisms play beneficial effects on the plant health (Philippot et al., 2013) directly by nitrogen fixation, different phytohormone production, phosphate solubilisation and iron sequestration by siderophore production and indirectly by plant growth stimulation by producing antifungal metabolites preventing different phytopathogens (Glick and Bashan, 1997). Diverse bacterial genera such as *Arthrobacter, Azospirillum*, *Azotobacter*, *Bacillus*, *Pseudomonas*, *Klebsiella*, *Burkholderia*, *Erwinia*, *Flavobacterium*, *Micrococcous*, *Enterobacter*, *Xanthomonas*, *Chromobacterium*, *Serratia* and *Caulobacter* spp have been reported to increase plant growth (Bhattacharyya and Jha, 2012; Bal *et* al., 2013). Rhizospheric *Azospirillum* sp of many grasses and cereals is a well known PGPR all over the world. Presently, N_2 fixing PGPR that have plant stimulation includes the endophytes that is, *Azoarcus*, *Burkholderia*, *Herbaspirillum* spp and *Gluconacetobacter diazotrophicus*, and the rhizospheric bacteria *Azotobacter*sp and *Paenibacillus (Bacillus) polymyxa* (Vessey, 2003).

In recent times, PGPR got more attention and it has been used as potent biofertilizers (Richardson et al., 2009; Compant et al., 2010) as prolonged use of chemical fertilizers is perilous to soil, as well as, human health and also detiorate the crop quality (Islam et al., 2013). Alternative biotechnological approaches are adapted in different agriculture practices to not only increase the crop production and plant growth, but also to maintain soil health. It has been reported that inoculation of *Azospirillum* biofertiliser or liquid near the rhizosphere of tea significantly increased growth. Although, research about PGPR impact on the tea plants is still poorly organized, especially in the Northeast region of India including North Bengal tea growing region. The productivity of tea is decreased remarkably due to intensive application of chemical fertilizers for a prolonged period (Sharma et al., 2014).

Therefore, there is a growing demand to explore the indigenous micro flora associated with the tea rhizosphere soil not only to reduce the application of chemical fertilizer, but also for the benefit of plant, soil health and the environment. The 16S rRNA represents the right candidate to study bacterial evolution, ecology, phylogenetic relationships among taxa, bacterial diversity and quantification of the relative abundance of taxa of various ranks (Hugenholtz et al., 1998). Whereas, random amplified polymorphic DNA (RAPD) fingerprinting explores genetic polymorphisms (Teaumroong and Boonkerd, 1998) in bacteria. RAPD fingerprinting has been used for strain identification and to determine the genetic diversity within a field population of pink-pigmented facultative methylotrophs (Balachandar et al., 2008), *Rhizobium* isolates (Rajsundari et al., 2009), *Photorabdus* and *Xenorabdus* isolates (Moghaieb et al., 2017).

There was scanty report on molecular identification of free living N_2 fixing PGPR of North Bengal tea gardens of West Bengal. A preliminary investigation on isolation and characterization of free-living soil bacteria from tea gardens of Terai, Dooars and Darjeeling district West Bengal have been carried by the present research group. Morphological and biochemical evaluation of free living N_2 fixing tea rhizospheric and tea soil bacteria of North Bengal tea gardens has also been investigated (Bhaduri et al., 2018).

Keeping the background information, the present study has been undertaken for molecular identification and to understand the genetic diversity of free living N_2 fixing soil bacteria from tea garden soil of North Bengal to be used as biofertiliser.

MATERIALS AND METHODS

Pure cultures of previous study (Bhaduri et al., 2018) were used as experimental sample for this investigation, 23 strains for 16SrRNA analysis and 22 strains for RAPD analysis (El-Fiki, 2006).

Isolation of genomic DNA

Genomic DNA was isolated from selected pure bacterial isolates of three different region sample screened on the basis of salt tolerance, antibiotic resistance total N content, etc. following the cetyl trimethylammonium bromide (CTAB) method (Gomes et al., 2000). The isolated genomic DNA was treated with RNase and then subjected to Agarose Gel (0.8%) electrophoresis to check the purity of DNA.

RAPD

Random Amplification of Polymorphic DNA of selected strains to observe the genetic variability between them was carried out at Xcelris Lab. Ahmedabad, Gujrat using two RAPD primer P1v(5' to 3'): GTG TGT GTG TGT GTG TGT GT, (20) nts., El-Fiki 2006 and P2:OPQ1 (5' to 3'): GGGACGATGG (10) nts (Balachandar et al.,

2008; Rajsundari et al., 2009; Moghaieb et al., 2017). PCR was carried out in a final reaction volume of 25 µl in ABI Veriti Thermal Cycler. Amplification reactions were performed in a 25 µL volume, containing: 20 mM Tris-HCl (pH 8.4), 50 mMKCl, 2.5 mM $MgCl_2$, 200 µM each of dNTPs, 1 µM primer, 30 ng of genomic DNA and 1.5 U of *Taq* DNA polymerase. The reaction mixture was flooded with two drops of mineral oil, initial denaturation for 5 min at 95°C, the amplification, then continued for 35 cycles consisting of 30 s at 94°C, 30 s at 36°C and 60 s at 72°C followed by a 7 min final extension at 72°C. Amplification product was separated by gel electrophoresis on precast 1.2% agarose gel and visualized under ultra-violent (UV) illumination after staining with ethidium bromide and Gel Documented on Gel Documentation System (Lee et al., 2012).

PCR amplification of 16SrRNA gene

PCR amplification of 16SrRNA was performed using 8F AGAGTTTGATCCTGGCTCAG. 1492R ACGGCTACCTTGTTACGACTT and sequencing of isolated bacterial 16SrRNA was performed using 704F GTAGCGGTGAAATGCGTAGA and 907R CCGTCAATTCCTTTGAGTTT primer. PCR amplification conditions: DNA1 µl, 16S Forward Primer 400ng, 16S Reverse Primer 400ng, dNTPs (2.5 mM each) 4 µl, 10X Taq DNA polymerase Assay Buffer 10 µl,Taq DNA Polymerase Enzyme (3U/ µl) 1 µl,Water X µl,Total reaction volume 100 µl. The PCR was conducted at 95°C, 94°C, 50°C ,72°C, 72°C, 5 min, 30 s, 30 s, 1 min 30 s and 7 min respectively for 35 cycles (protocol followed by Xcelris Lab) .

Sequencing of 16S rRNA

The PCR amplicon (1.4 kb approximately) was purified with ExoSap enzymatic purification as per the manufacturer's instruction (ABI). After the purification, the products were subjected to Sanger sequencing using ABI, 3730XL DNA analyzer using BdT v3. 1 chemistry. Each forward and reverse reaction of PCR amplified products were sequenced separately. Forward and Reverse DNA sequencing reaction of PCR amplicons of respective samples was carried out using BDT v3. 1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.

Construction of phylogenetic tree

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura et al. (2004) model. Evolutionary genetics analysis uses maximum likelihood, evolutionary distance, and maximum parsimony methods were conducted in MEGA 5 software (Tamura et al., 2011). The RAPD profile derived phylogeny was performed by Xcelris Lab.

RESULTS AND DISCUSSION

Purified genomic DNA isolated from bacterial strains after resolving in 0.8% agarose gel reveals their good yield and large genome size (Figure not shown).

PCR amplification of 16SrRNA gene and sequencing

Twenty three isolated bacterial genomic DNA was amplified with forward and reverse sequencing primer. PCR amplified fragments of approximately 1.4 kb in size are sequenced.

GenBank accession followed by homology searching

Twenty three (23) GenBank Accession were obtained after submitting the contig FASTA sequence. Most of the 23 isolated bacterial strain demonstrated strong homology with known nitrogen fixing bacteria. The strain TS-1-16 (Accession No. KY636356) displayed 100% homology with *Burkholderia* sp, Strain-S-9-19 (Accession No. KY357337) having nitrogen fixing capacity was ascribed as a member of the genus *Burkholderia*, since our sequence was not full length. TS-4-23 (Accession No. KY631489) showed 100% identity along with *Burkholderia* sp, Str-S-9-15 (Accession No. KX212131) having good nitrogen fixing capacity**.** DS-1-20 (Accession No. KY636360) showed 100% identity with *Stenotrophomonas maltophilla, strn-MM**-3-3,*** (Accession No. KT970988) having nitrogen fixing capacity and the other *Stenotrophomonas* species, hence it is ascribed as a member of the genus *Stenotrophomonas*. DJ-1-22 (Accession No. KY 859855) showed 100% identity with *Burkholderia SP-2386*, (Accession No.JX174263) having good nitrogen fixing capacity. AS-4 (Accession No.KY636361) showed 100% identity with *S. maltophilla, Str-D-3* (Accession No. KM488439) having nitrogen fixing capacity. The bacterial strains DJ-1-24 (Accession No. KY636358) showed 100% identity with *S. maltophilla-LP-05* (Accession No. KT427904), DJ-1-10 (Accession No. KY631491) 99% identity with *Bacillus cereus Strn*-FORC021 (Accession No. CP-014486), DJ-1-46 (Accession No. KY636362) 98% identity and with *Azospirillum*sp *TSH51 gene*, (Accession No. AB508854) having nitrogen fixing capacity, the latter is well known free living nitrogen fixing soil bacteria (Table 1).

Evolutionary genetics analysis and phylogenetic tree

Phylogenetic tree analysis between the 23 isolated strains showed close similarity among the strains. The tree reveals that there are 8 main groups consisting of two closely related strains. The group 1 consists of strains of TS-4-23 and DS-1-16 which resembles the *Burkholderia* sp functioning as a free living N_2 fixer and belonging to PGPR activity (Hayat et al., 2010).

Grou p 2 contained strains of TS-4-12 and DJ-1-46 which resembles the *Burkholderia cepacia* and *Azospirillum* sp *TSH51* gene, which solely functions as a free living N_2 fixer and displaying PGPR activity. Group 3 contained strains of DJ-1-3 and DJ-1-22 which resembles the *Burkholderia* sp different strain which solely functions as a free living N_2 fixer and PGPR activity. Group 4 contained strains of AS-4 and DJ-1-24 which resembles

Table 1. Homology and annotation of 23 GenBank accession of isolated N_2 fixing Bacterial strain.

Strain code	NCBI accession No	Homology	Length (bp)	Annotation	Max score	Total score	E-value	Identical (%)
TS-1-16	KY636356	KY357337	1439	*Burkholderia*sp, Strain-S-9 19,Nitrogen fixation	2658	2658	00	100
TS-3-15	KY631488	KX3500422	1202	*Stenotrophomonasmaltophila* ,Str-F-4-2-35,Nitrogen fixation.	2220	2220	00	100
TS-4-12	KY 860028	KX055886	1309	*Burkholderiacepacia*str-TY-5-SH,Nitrogen fixation	2418	2418	00	100
TS-3-27	KX587470	KY266795	934	Bacterium str-CH-2,Nitrogen fixation.	1447	1447	00	97
TS-4-16	KX665547	KP276241	611	*Herbaspirillumsp*str hz-10,Nitrogen fixiation	2658	2658	16-61	78
TS-4-23	KY631489	KX212131	1186	*Burkholderia sp,*Str-S-9-15 ,Nitrogen fixation	2191	2191	00	100
TS-4-24	KX608591	DQ257427	620	*Acinetobacter johnsonii*, CA-1-14-,Nitrogen fixation	697	697	00	88
DS-1-20	KY636360	KT970988	1473	*Stenotrophomonasmaltophilla, strn-MM-3-3*,Nitrogen fixation	2721	2721	00	100
DS-1-16	KY671245	KF826288	1451	*Burkholderiacenocepacia* , Nitrogen fixation	2680	2680	00	100
DS-1-18	KY631490	KP216607	1412	*Burkholderiaanthina*strn-JT N301, Nitrogen fixation	2189	2189	00	98
DS-1-25	KX587469	KY810619	928	*Burkholderiacenocepcia*, StrKNL-15, Nitrogen fixation	1504	1504	00	97
DS-1-26	KY859856	CP-007747	1105	*Burkholderiacepacia* ,ATCC-25416Nitrogen fixination	1882	1882	00	98
DS-2-8	KX665546	AF131868	552	*Methylobacter,SP-T-20*, Nitrogen fixation	324	324	2e-84	78
DS-2-9	KX608590	EU 705721	742	*Uncultured Ralstonia*sp*, Clone-3P-3-2*, Nitrogen fixation	477	477	2e-130	79
DS-2-10	KX587468	NR114140	918	*HerbaspirillumfrisingenseStr -NBRC 102-522,* Nitrogen fixation.	538	538	1e-148	81
AS-1	KY859798	KT580654	1200	*Stenotrophomonasmaltophilla ,strn*-Can R-47,Nitrogen fixation	2109	2109	00	98
AS-2	KY636357	HM246521	1434	*Stenotrophomonasmaltophilla , strn-6*,Nitrogen fixation	2644	2644	00	100
AS-1-4	KY636361	KM488439	1412	*Stenotrophomonasmaltophilla, Str-D-3*,Nitrogen fixation	2577	2577	00	99
DJ-1-3	KY 636359	KY357336	1432	*Burkholderia Strn-S-9-18*, Nitrogen fixation	2645	2645	00	100
DJ-1-10	KY631491	CP-014486	1531	*Bacillus cereus Strn* FORC021,Nitrogen fixation	2693	2693	00	99
DJ-1-22	KY 859855	JX174263	1428	*Burkholderia SP-2386*, Nitrogen fixation	2638	2638	00	100
DJ-1-24	KY636358	KT427904	1412	*Stenotrophomonas maltophilla-LP-05*, Nitrogen fixation	2593	2593	00	100
DJ-1-46	KY636362	AB508854	1416	*Azospirillumsp TSH51 gene*, Nitrogen fixation	2420	2420	00	98

the *S. maltophilla* different strain which solely functions as a free living N_2 fixer and PGPR activity (Fouzia et al., 2015).

Group 5 contained strains of TS-3-15 and DS-1-20 which resembles the *S. maltophilla* different strain which solely functions as a free living N_2 fixer and PGPR activity. Group 6 contained strains of TS-3-27 and DJ-1-10 which resembles the Bacterium str-CH-2 and *Bacillus cereus strn*-FORC021which solely functions as a free living N_2 fixer and PGPR activity. Group 7 contained strains of TS-4-16 and DS-2-10 which resembles *Herbaspirillum* sp different strain which solely functions as free living as well as endophytic (certain strain) N_2 fixer and PGPR activity in tea plant (Zhan et al., 2016).

Group 8 contained strains of DS-2-8 and DS-2-9 which resembles *Methylobacter, SP-T-20* and uncultured *Ralstonia* sp., *Clone-3P-3-2* which solely functions as a free living N_2 fixer and PGPR activity. The other seven strains are distantly related to these clusters having nitrogen fixing and plant growth promoting activity (Figure 1) (Hayat et al., 2010).

RAPD

RAPD analysis of isolated bacterial genomic DNA reveals a little polymorphism pattern (Figure 2). Among the two primers tested only primer P1 was proper for amplification. The bacterial isolates DS-2-10; DS-2-8; DS-1-25; TS-4-24 gave no response

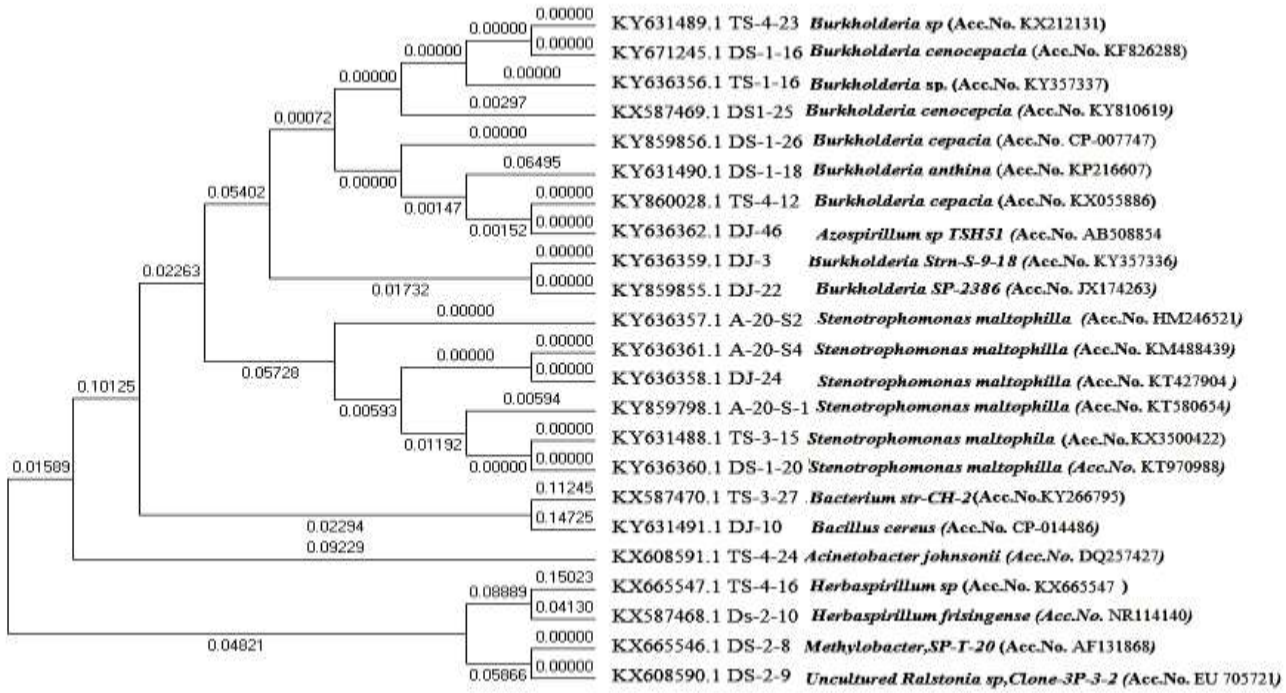

Figure 1. Phylogenetic tree between the 23 isolated bacterial strain showing homology (interrelationship) between them and highest matching with the GenBank Accession in National Center for Biotechnology Information.

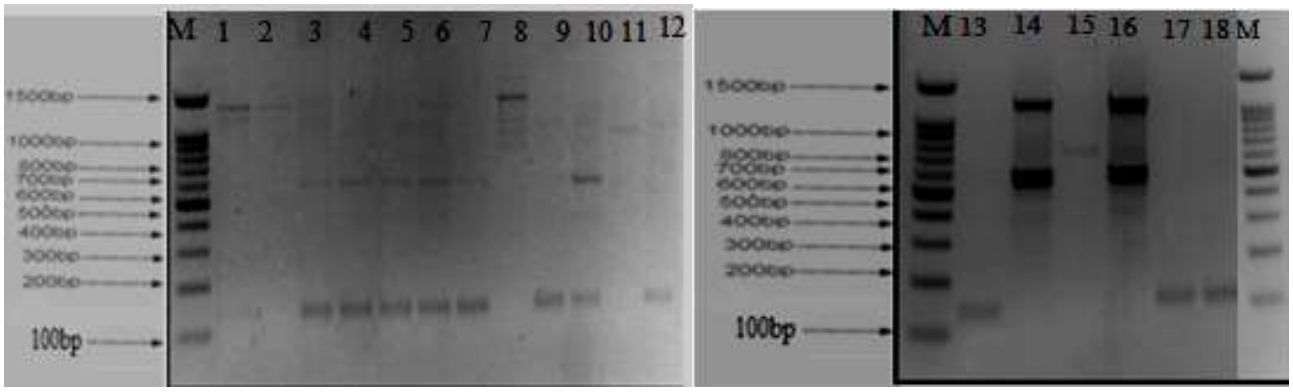

Figure 2. Representative RAPD profile with primer P1, M. 100 bp DNA Ladder of 1.5 kb size; 1. TS-3-15; 2. DJ-1-10; 3. DS-1-20; 4. DS-1-26; 5. DS-1-16; 6. TS-4-12; 7. DS-1-18; 8. DJ-1-22; 9. TS-1-6; 10. TS-4-23; 11. DJ-1-3; 12. AS-1-4; 13. DJ-1-46; 14. DJ-1-24; 15. AS-1; 16. AS-2; 17. DS-2-9; 18. TS-3-27.

at RAPD amplification and rest of the 18 isolates showed amplification. The DNA amplified fragment varied in size ranges from 100 bp to 1.5 kb. The dendrogram result of polymorphic band showed similarity between the organisms as exhibited by 18 strains (Figure 3). The strain DS-1-20 is closely related to the cluster of DS-1-18, TS-4-12, DS-1-16 and DS-1-26. The strain AS-1-4 shows similarity to cluster containing TS-1-6 and TS-4-23, the former is closely related to the cluster formed by TS-3-27, DJ-1-46 and DS-2-9. The strains DJ-1-10 and TS-3-15 are closely related to each other and distantly related to a cluster formed by AS-1-2 and DJ-1-24, the latter two are related to a cluster formed by DJ-1-22 and DJ-1-3. AS-1-1 strain could not produce polymorphism and hence cannot relate to the cluster (Figure 3).

The genus *Burkholderia* comprises of 19 species, which includes soil and rhizosphere bacteria as well as plant and human pathogens (Bevivino et al., 1998; Achouak et al., 1999; Zhang et al., 2000; Balandreau et al., 2001). The aerobic, rod-shaped, endospore producing

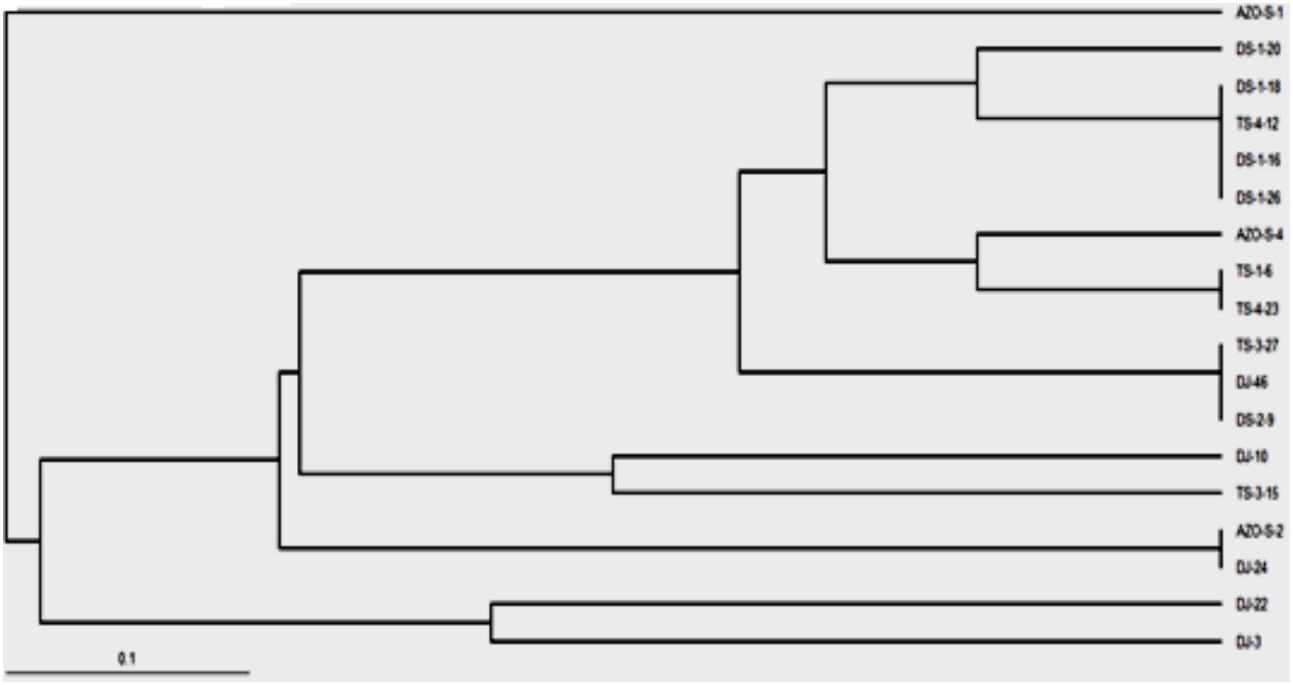

Figure 3. Total homology analysis of 22 Bacterial Genomic DNA derived from RAPD profile showing their genetic relatedness.

genus *Bacillus* is a systematically diverse taxon (Claus and Berkeley, 1986). Gene sequence analyses (16SrRNA) have identified at least 10 phylogenetic groups in the genus Bacillus (Shida et al., 1997a). *Bacillus cereus* AR156 having PGPR activities induces systemic resistance in *A. thaliana* by simultaneously activating salicylate- and jasmonate/ ethylene-dependent signaling pathways which has been established (Niu et al., 2011). *B. cepacia* is recognized for its abilities, to promote maize growth (Bevivino et al., 1998), to enhance crop yields (Chiarini et al., 1998), and to suppress many soilborne plant pathogens (Bevivino et al., 1998; Hebbar et al., 1998; McLoughlin et al., 1992), as well as to degrade diverse pesticides (Daubaras et al., 1996; Mueller et al., 1997).

The genus *Stenotrophomonas* comprises of about eight species. Strains of the most common species, *Stenotrophomonas maltophilia*, have a function that includes beneficial effects of plant growth (Ryan et al., 2009). *S. maltophilia* is an ubiquitous, aerobic, non-fermentative and Gram-negative bacillus that is closely related to the *Pseudomonas* species (Calza et al., 2003). The genus *Stenotrophomonas* has pathogenic effect and they are resistant to certain antibiotics and susceptible to Chlorampenicol which we have already investigated in our previous study. Nahi et al. (2016) studied the effect of herbicide on nitrogenase and N_2 fixing capacity of *Stenotrophomonas maltophilia* (Sb 16). Bacteria of the genus *Azospirillum* (α-subclass of Proteobacteria) are known as plant growth promoting rhizobacteria (Okon, 1994. They were isolated from the rhizosphere of many grasses and cereals all over the world, in tropical as well as in temperate climates (Patriquin et al., 1983). Due to cell shape, growth behavior and habitat within grass roots, genus *Herbaspirillum* were previously thought to be a new *Azospirillum* species. However, RNA-RNA hybridization experiments reveal no relationship with *Azospirillum* spp or *Aquaspirillum itersonii* (Falk et al., 1986). *Herbaspirillum seropedicae*, *Herbaspirillum frisingense* and *Herbaspirillum lusitanum*able are reported to fix nitrogen (Baldani et al., 1986; Kirchhof et al., 2001; Valverde et al., 2003). The endophytic *Herbaspirillum* sp WT00C isolated from the tea plant, seems to have a potential ability to promote tea-plant rooting and budding due to its capability of producing indole-3-acetic acid (IAA), ammonia and siderophores (Zhan et al., 2016). Bacterial species of the genus *Acinetobacter* are ubiquitous in nature (Bergogne-Berezin and Towner, 1996).

In recent years, members of the genus *Acinetobacter* have been isolated from the rhizo- sphere of different plants (Kuklinsky-Sobral et al., 2004; Roberts et al., 2005; Nakayama et al., 2007; Li et al., 2008). In India, *A. indicus* was described for the first time in soil samples collected from hexachlorocyclohexane dump sites (Malhotra et al., 2012). Strains belonging to the genus *Acinetobacter,* and their plant growth-promoting properties have been reported in the literature (Sachdeva et al., 2010). The presence of different species of *Acinetobacter* was worked in the rhizosphere of three agricultural wheat fields of Pune, India. The genetic diversity of *Acinetobacter* species using metagenomics

study in the wheat rhizosphere was assessed by denaturing gradient gel electrophoresis (DGGE) of 16 SrRNA genes PCR products. Plant growth-promoting traits such as nitrogen fixation, siderophore production and mineral solubilization were reported in *in vitro* culture of *Acinetobacter* isolates (Sachdeva et al., 2010). From the perusal of literature it has been revealed that, in India no work has been done with *Acinetobacter* sp in tea field soil for their study related to biofertiliser or PGPR.

Auman et al. (2001) reported *nitrogenous* and utilize N_2 as a nitrogen source by some methane-oxidizing bacteria (methanotrophs). There are two types of methanotrophs - type I and type II. Type II methanotrophs and members of the type I genus *Methylococcus*have been shown to be capable of nitrogen fixation, while type I methanotrophs are not (Dedysh et al., 2000; Murrell and Dalton, 1983; Oakley and Murell, 1988). The genus *Ralstonia* established in 1995 by Yabuuchi et al. (1995) accommodate species previously known as *Alcaligeneseutrophus*, *Pseudomonas solanacearum* and *Pseudomonas pickettii*. *Ralstonia eutropha*isolated from sludge, soil and *R*. *basilensis* from waste-water (Steinle et al., 1998). Chen et al. (2001) isolated several strains of *Ralstonia* from *Mimosa* as a symbiont nitrogen fixer, the most promising one is *Ralstonia taiwanensis*, cells are Gram negative, non spore forming rod shaped and mean cell size which ranges from 0.5 to 0.7 m width and 0.8 to 2.0 m in length (Chen et al., 2001).

Gulati et al. (2011) reported the presence of Gram-negative nitrogen fixing bacteria of α-Proteobacteria genera *Brevundimonas*, *Rhizobium*, and *Mesorhizobium*; γ-Proteobacteria genera *Pseudomonas* and *Stenotrophomonas*; and β-Proteobacteria genera *Azospira*, *Burkholderia*, *Delftia, Herbaspirillum and Ralstonia* associated with the tea roots of Kangra Valley of Himachal Pradesh. The isolated bacterial strain identified as *Burkholderia*sp, *Stenotrophomonas maltophila, Herbaspirillum* sp, *Acinetobacter johnsonii, Methylobacter, SP-T-20*, *and Bacillus cereus, Azospirillum* sp depicts the N_2 fixing as well as PGPR activities as evident from homology searching can be used as potent bioferiliser for organic and conventional tea gardens especially in North Bengal. The present investigation will suggest an insight to the tea growers of North Bengal and researchers as readily established “Bio accelerant” or” Bio fertiliser”.

Conclusion

Since tea is a non-leguminous plant, the search for free living N_2 fixing soil bacteria in tea growing areas is gaining momentum day by day. Few good strains have been identified to be used as a potential N_2 fixer in tea field. The isolated bacterial strain identified as a member of the genus *Burkholderia* sp, *Stenotrophomonas maltophila, Herbaspirillum* sp, *Acinetobacter johnsonii, Methylobacter, SP-T-20*, *and Bacillus cereus, Azospirillum* sp can be the right candidates as potent bioferiliser for organic and conventional tea gardens of North Bengal, India. Hence, it is evident from the homology searching that our isolated strain would be ascribed as a member of the respective genus until and unless DNA-DNA hybridization and other biochemical parameter have been tested. The study reveals a thorough investigation regarding the molecular identification of free-living N_2 fixing bacteria; however, their field application is still needed in further study.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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