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Full Length Research Paper

Diversity of MRSA SCC*mec* elements in Pretoria region of South Africa: The problem of variation in the assigned SCC*mec* types by different multiplex-polymerase chain reaction (PCR) methods and a call for an African consensus

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The SCC*mec* element is one of the recommended targets for MRSA characterization and several multiplex-PCR SCC*mec* typing methods have been developed over the past years. However, there are no data on the consistency of the SCC*mec* types in clinical isolates as detected by these methods. Using different previously published, commonly used M-PCR methods, this report describes the diversity of SCC*mec* elements in MRSA isolates in the Pretoria region of South Africa and the discrepancies observed in the assigned SCC*mec* types. Different SCC*mec* types were assigned to the same clinical MRSA isolates. The discrepancies included the assignment of composite SCC*mec* types [(SCC*mec* II and SCC*mecury*) 20.7% (40/193)] and [(SCC*mec* type II+IVc) 22.3% (43/193)] to some of the clinical MRSA isolates. Summarily, the combination of the result of the M-PCR methods showed that the MRSA genotypes circulating in the healthcare facility studied potentially carried SCC*mec* types I, II, IV (subtypes IVa, IVb and IVd) and V. No SCC*mec* types III or VIII was detected among the isolates. At least 25.91% of SCC*mec* type IV was detected in this study, thus corroborating previous findings of the global encroachment of MRSA strains into the hospital settings. The associated epidemiological significance of these observations is discussed and we also call for an African consensus SCC*mec* typing method in order to allow effective epidemiological data comparison across the countries.

Key words: MRSA genotype, SCC*mec* elements, multiplex-polymerase chain reaction (PCR), variation.

INTRODUCTION

Staphylococcus aureus is a virulent bacterial pathogen which is responsible for infections seen in healthcare and

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community settings (Kim, 2009). Infections caused by MRSA were previously associated with healthcare settings [Healthcare-associated MRSA (HA-MRSA)] but the emergence of community-associated MRSA (CA-MRSA) worsened the health challenges associated with MRSA (Moussa et al., 2012). Epidemiological history shows that, the CA-MRSA differed from the HA-MRSA in various ways: (i) the lack of traditional risk factors associated with MRSA among patients, (ii) a susceptibility pattern with resistance to few antimicrobial agents and (iii) the inclusion of specific virulence factors such as the Panton Valentine leucocidin (PVL) genes (Weber, 2005; Lo et al., 2011). In addition, a previous study have shown that CA-MRSA and HA-MRSA are demographically, clinically, and microbiologically different (Naimi et al., 2003). However, recent reports now show that the clinical definition of CA-MRSA and HA-MRSA (based on disease on-set, risk-factors and possession of PVL gene) are becoming blurred (David et al., 2010; Prosperi et al., 2013). A study by Peterson et al. (2012) showed that demographics including the disease on-set and the associated risk-factors are not consistent with the genotypic classification of CA-MRSA and HA-MRSA.

The *S. aureus* genome includes a mobile genetic element [staphylococcal cassette chromosome *mec* elements (SCC*mec*)] that carries the determinant for beta-lactam resistance encoded by *mec*A (IWG-SCC, 2009) and *mec*C (Paterson et al., 2013). Earlier reports indicated that the HA-MRSA strains harbor primarily SCC*mec* type I, II, III or VI (Naimi et al., 2003), while CA-MRSA carries the SCC*mec* type IV, V, VII, or VIII and are resistant to only β-lactam antibiotics and sensitive to non-β-lactam antibiotics (Daum et al., 2002). The possibility of transfer of the antimicrobial resistance determinant (the SCC*mec*) between CA-MRSA and HA-MRSA isolates in healthcare and community settings necessitates accurate and reliable methods for the detection and identification of these strains (Song et al., 2011). Moreover, the lack of healthcare associated risk factors for the definition of CA-MRSA as prescribed by the Centers for Disease Control and Prevention (CDC) (Morrison et al., 2006) was not sufficient in defining the emergence of CA-MRSA- and HA-MRSA-associated infection in the community and the association of CA-MRSA strains with healthcare-associated infections (O'Brien et al., 1999; Saiman et al., 2003). This led to the use of molecular typing tools (based on SCC*mec* element) for the classification of MRSA (Daum et al., 2002; Naimi et al., 2003). Several methods for SCC*mec* typing have been developed and have been previously validated and characterized using MRSA strains with known SCC*mec* elements. These methods were designed in response to new epidemiological and genomic information. For an extensive review of structure of the SCC*mec* element in *S. aureus,* refer to the work of Shore et al. (2013). An in-depth description of the molecular basis for the SCC*mec* typing and other typing methods have been previously reviewed (Stefani et al., 2012). Consequently, a brief description of the regions targeted by the primer sets/SCCmec typing methods investigated in this report and their limitations in detecting SCC*mec* types are thus presented: the primer sets of Oliveira and de Lencastre (2002) targets the upstream and downstream of *mec*A complex incorporating the cassette chromosome recombinase (*ccr*) allele AB. The Oliviera and de Lencastre (2002) method described SCC*mec* type V as type III and did not consider the differentiation between type IV subtypes. An updated version of the Oliviera and de Lencastre (2002) method focused on the detection of SCC*mec* type IV (Milheiriço et al., 2007) by amplifying regions within the *ccr*AB allotypes, five polymorphic J1 regions and a new J1 region that was detected in EMRSA-15 clone. The multiplex PCR (M-PCR) primer sets designed by Zhang et al. (2005) focused on the identification of types 1-4 using the *mec* and *ccr* elements and the subtypes designation are based on the junkyard region. Five isolates were not typable by this method however, Oliveira and de Lencastre (2002) method designated those isolates as SCC*mec* type III while four isolates identified as types I or II were designated as type II by the Oliveira and de Lencastre (2002) method.

It is noteworthy that these two methods showed a 100% agreement in typing control strains. An updated version (Zhang et al., 2012) of the previous Zhang and colleagues' (2005) method was later reported. This improvement addressed the following: (a) detection of SCC*mec* type II strains that lack the *kdp*E gene; (b) SCC*mec* type III lacking the J1 region; (c) detection of subtype IVc by targeting the J1 region and subtype IVe by targeting the J3 region; (d) differentiation of the SCC*mec* VIII and II. Despite this updated method, 4.5% (24/533) of the isolates were not typable.

Boye et al. (2007) developed a method to differentiate between HAMRSA from CA-MRSA carrying SCC*mec* types IV and V thereby, preventing the mistyping of SCC*mec* type V as type III. Six (1.92%) of the isolates tested by this method were not typable. However, four of the six isolates were typable using the Oliveira and de Lancaster (2002) method. The McClure et al. (2010) method focused on the detection of SCC*mec* type VIII by amplifying regions within the class A *mec* gene and type IV *ccr* gene complexes using five PCR targets.

In an effort to incorporate more variable regions within the *S. aureus* genomic make up, Kondo et al. (2007) described a method that included the *ccr* genes, *mec* class A-C, open reading frame of J1 region, transposons *Tn554* and Ψ*Tn554* in the J2 regions and plasmids *PT181* and *Pub110* in the J3 regions. Despite the extensive coverage of the variable regions 93/99 MRSA control strains could be assigned by this method while the *ccr* genes of six *mec*A positive strains could not be defined by this method. The authors reported that the M-PCR reported did not conflict with previous methods by Oliveira and de Lancaster (2002) and Zhang et al. (2005).

While the complete review of the various SCC*mec* typing methods is not the primary scope of this report, a snap shot of the adoption frequency of the methods (discussed in this report) by different laboratories indicates that there is no uniform method or criteria for the use of a particular method (Table 1).

To circumvent the inherent limitations of individual SCC*mec* typing methods, five published M-PCR based SCC*mec* typing were combined in order to determine the diversity of SCC*mec* elements in the Pretoria region of South Africa and to observe the differences in the SCC*mec* types assigned by methods that detect the same range of known SCC*mec* types.

MATERIALS AND METHODS

MRSA sample source and total bacterial DNA purification

One hundred and ninety three (193) MRSA isolates were obtained from the Diagnostic Laboratory, Department of Medical Microbiology, University of Pretoria Tshwane Academic Division, National Health Laboratory Service. The MRSA isolates were sub-cultured on Blood agar plates (Oxoid, England) at 37°C for 18 to 24 h to obtain single colonies for Gram-staining in order to confirm the purity. Genomic DNA was purified from the 193 MRSA isolates using the ZR Fungal/Bacterial DNA MiniPrep (Zymo Research, Thermo Scientific, USA), according to the manufacturer's instructions. Ethical approval for this study was obtained from the Research Ethics Committee of the University of Pretoria (protocol number S189/2010 and S175/2011).

Multiplex-PCR assays for the designation SCCmec types

Five commonly described SCC*mec* typing methods in the literature were investigated. Multiplex PCR (M-PCR) reactions using specific primers were employed as previously described for each of the methods tested (Table 1) and the genomic DNA from a CA-MRSA strain (ATCC CA05) served as a positive control in the M-PCR assays. The samples were reconfirmed using the *S. aureus* specific primers (McClure et al., 2006). The M-PCR amplicons were electrophoretically separated at 100 V/cm in a 1% MetaPhor^{TM} agarose gel (Lonza, Rockland, USA) containing 5 µl of ethidium bromide (10 mg/ml) (Promega, Madison, USA) and visualized using an Ultra Violet light box (DigiDoc, UVP product, Upland, California). The assignments of SCC*mec* types were performed as previously described for individual methods (Table 2).

RESULTS AND DISCUSSION

All the 193 previously determined MRSA samples were reconfirmed using the *S. aureus* specific primers (McClure et al., 2006). The 16S rRNA and the *mec*A gene were detected in all the samples tested. However, variations were observed in the proportion of samples designated as a specific SCC*mec* type or untypable by each SCC*mec* typing method assessed (Table 3). The electrophoretic pattern of the M-PCR amplicons used for the assignment of the SCC*mec* types is shown in supplementary material (Figure S1-S7). Table 3 shows that methods1 and 3 were able to designate equal number and same set of MRSA isolates as SCC*mec* I (3.1%) and SCC*mec* II (9.33%). The number of isolates assigned as SCC*mercury* by methods 1, 2 and 3 were different, with method 3 designating 61.14% (118/193) of the isolates as SCC*mercury* followed by method 1 {(41.5% (80/193)} and method 2 {16.1% (31/193)}. The number of MRSA isolates designated as SCC*mec* I, II and SCC*mercury* by method 2 did not correspond to any of the other methods tested. Method 4, an updated version of method 2 designated 10 additional MRSA isolates as SCC*mec* II, giving a total of 35.75% (69/193) SCC*mec* type II MRSA isolates as compared to method 2 which assigned SCC*mec* type II to 30.7% (59/193) to the isolates. Method 2 was able to subtype the same set of isolates [SCC*mec* type IVa, 1.03% (2/193); SCC*mec* type IVb, 0.52% (1/193); SCC*mec* type IVd, 24.4% (47/193)] designated as SCC*mec* type IV [25.91% (50/193)] by Method 3. Moreover, one isolate was designated as SCC*mec* type V by Method 3.

The rest of the MRSA isolates were designated as composite SCC*mec* types. These included SCC*mec* type II+SCC*mercury,* 20.7% (40/193) assigned by method 1; SCC*mec* type II+IVc, 22.3% (43/193) and 26.42% (51/193) assigned by method 2 and 3 respectively. As SCC*mercury* was detected by methods 1, 2 and 3 and also in composite SCC*mec* type detected by method 1, it is possible that the SCC*mercury* is carried in separate plasmid within the bacterial cell. No SCC*mec* type III or type VIII was detected by the methods 4 and 5, respectively. The proportion of untypable MRSA isolates was 4.7% [(9/193); (Method 2)] and 37.82% {(73/193); (Method 4)}.

Table 4 shows the number of clinical MRSA isolates that were designated the same SCC*mec* type by different M-PCR methods. Methods 2 and 4 assigned SCC*mec* type II to 30.57% (59/193) of the same set of MRSA isolates. This was the highest number of isolates designated the same SCC*mec* type by the different methods investigated. Moreover, about 26% (50/193) of the same isolates were assigned SCC*mec* type IV by methods 2 and 3, while 15.54% (30/193) of the MRSA isolates were designated as SCC*mecury* by methods 1, 2 and 3.

These observations indicated that the assessed M-PCR methods were able to assign a specific SCC*mec* type to the same MRSA isolates, most of the remaining isolates were designated different SCC*mec* types by the methods investigated. In a separate experiment, an attempt to categorize the SCC*mec* types defined by each SCC*mec* typing method in this study revealed that there was no specific distribution pattern of SCC*mec* type(s) among the pulsed field gel electrophoresis derived pulsotypes (data not shown) suggesting that there was no specific association between the chromosomal DNA content of the MRSA isolates and the SCC*mec* type assigned by the methods evaluated.

Table 1. List of primers for six SCC*mec* typing methods including *S. aureus* confirmation.

Primer	Oligonucleotide sequence (5'- 3')	Target gene	Amplicon Size (bp)	Reference
Staph 756F	-AACTCTGTTATTAGGGAAGAACA-			
Staph 756R	-CCACCTTCCTCCGGTTTGTCACC-	16S rRNA	756	Primers for *Staphylococcus aureus* confirmation
MecA1-F	-GTAGAAATGACTGAACGTCCGATAA-			McClure et al. (2006)
MecA2-R	-CCAATTCCACATTGTTTCGGTCTAA-	*mec*A	310	
CIF2-F2	-TTCGAGTTGCTGATGAAGAAGG-			
CIF2-R2	-ATTTACCACAAGGACTACCAGC-	SCC*mec* I	495	
KDP-F1	-AATCATCTGCCATTGGTGATGC-			
KDP-R1	-CGAATGAAGTGAAAGAAAGTGG-	SCC*mec* II	284	(Method 1)
RIF5-F10	-TTCTTAAGTACACGCTGAATCG-	SCC*mec*		Oliveira and De Lencastre (2002)
RIF5-F13	-GTCACAGTAATTCCATCAATGC-	III	414	
MECA P4	-TCCAGATTACAACTTCACCAGG-			
MECA P7	-CCACTTCATATCTTGTAACG-	*mec*A	162	
Type I-F	-GCTTTAAAGAGTGTCGTTACAGG-			
Type I-R	-GTTCTCTCATAGTATGACGTCC-	SCC*mec* I	613	
Type II-F	-CGTTGAAGATGATGAAGCG-			
Type II-R	-CGAAATCAATGGTTAATGGACC-	SCC*mec* II	398	
Type III-F	-CCATATTGTGTACGATGCG-			(Method 2)
Type III-R	-CCTTAGTTGTCGTAACAGATCG-	SCC*mec* III	280	Zhang et al. (2005)
Type IVa-F	-GCCTTATTCGAAGAAACCG-			
Type IVa-R	-CTACTCTTCTGAAAAGCGTCG-	SCC*mec*IV a	776	
Type IVb-F	-TCTGGAATTACTTCAGCTGC-			
Type IVb-R	-AAACAATATTGCTCTCCCTC-	SCC*mec*IV b	493	
Type IVc-F	-ACAATATTTGTATTATCGGAGAGC-			
Type IVc-R	-TTGGTATGAGGTATTGCTGG-	SCC*mec*IV c	200	
Type IVd-F	-CTCAAAATACGGACCCCAATACA-			
Type IVd-R	-TGCTCCAGTAATTGCTAAAG-	SCC*mec*IV d	881	
Type V-F	-GAACATTGTTACTTAAATGAGCG-			
TypeV-R	-TGAAAGTTGTACCCTTGACACC-	SCC*mec* V	325	
MecA147-F	-GTGAAGATATACCAAGTGATT-			
MecA147-R	-ATGCGCTATAGATTGAAAGGAT-	*mec*A	147	
B-F	-ATTGCCTTGATAATAGCCYTCT-			
α3-R	-TAAAGGCATCAATGCACAAACACT-	*ccr*A2-B	937	
*ccr*CF-F	-CGTCTATTACAAGATGTTAAGGATAAT-			(Method 3)
*ccr*CR-R	-CCTTTATAGACTGGATTATTCAAAATA-	*ccr*C	518	Boye et al. (2007)
1272-F1	-GCCACTCATAACATATGGAA-			
1272-R1	-CATCCGAGTGAAACCCAAA-	IS1272	415	

Table 1. Contd.

5R*mec*A-F	-TATACCAAACCCGACAACTAC-	*mec*A-IS431	359	Boye et al. (2007)
5R431-R	-CGGCTACAGTGATAACATCC-			
Type II-F2	-TAGCTTATGGTGCTTATGCG-	SCC*mec* II,VIII	128	(Method 4) Zhang et al. (2012)
Type II-R2	-GTGCATGATTTCATTTGTGGC-			
Type III-F5	-TTCTCATTGATGCTGAAGCC-	SCC*mec* III, IIIA	257	
Type III-R6	-GTGTAATTTCTTTTGAAAGATATGG-			
mecI-F	-CCCTTTTTATACAATCTCGTT-	*mec*I	147	
mecI-R	-ATATCATCTGCAGAATGGG-			
ccr4-Fd	-ATCGCTCATTATGGATACYGC-	ccr4	428	(Method 5) McClure et al. (2010)
ccr4-R2	-CAAAACAACCTTTTCTATAACG-			
SCCRP62A	-CAATATTGATTTCCTTCATCGTTTACCTCC-	SCC*mec*VIII	1957	
SCC-CI	-GAGCATCATAAGAAGCAATTTTATGTTACGC-			
nuc1	-GCGATTGATGGTGATACGGTT-	nuc	279	
nuc2	-AGCCAAGCCTTGACGAACTAAAGC-			
mecA147-F	-GTGAAGATATACCAAGTGATT-	*mec*A	112	
mecA112-R	-ATCAGTATTTCACCTTGTCCG-			

Table 2. Number of citations of some SCC*mec* typing methods as observed in Google scholar database.

Method	Number of citations in peer reviewed articles	Number of citation since 2014 till date*
Oliveira and de Lencastre (2002)	1223	91
Zhang et al. (2005)	635	84
Boye et al. (2007)	132	28
Milheirico et al. (2007)	310	58
McClure et al. (2010)	212	38
Zhang et al. (2012)	6	5

*Google Scholar access date: April 25th 2015.

A spectacular instance of misassigned ST398-SCC*mec* III MRSA isolates that took about two years to be reassigned as SCC*mec* type V has been previously reported (van Loo et al., 2007; Jansen et al., 2009) Such incidence would include a redesignation of the isolates from SCC*mec* III to SCC*mec* V based on the molecular typing criteria (Ito et al., 2001, 2004). This report showed that at least 25.91% of the MRSA isolates was of SCC*mec* type VI and correlates with a number of recent reports which have indicated an increase in the number of infections associated with SCC*mec* type IV, V, VII or VIII in the hospital setting (Magilner et al., 2008; David et al., 2010) including the presence of the different genotypes in specific environments (Marchese et al., 2009). Although, the overall epidemiological picture presented in these reports may still be biologically relevant based on the general pattern observed across the different countries involved, the estimated statistics may be misrepresented due to the lack of a unified standard method for SCC*mec* classification.

The need for standardization of SCC*mec* typing and genotype designation is evident by a number of reports including: (i) the continuous blurring of the clinical and genetic distinctions between CA-MRSA and HA-MRSA (David et al., 2010; Prosperi et al., 2013) (ii) the probability that CA-MRSA isolates might displace HA-MRSA in future and become the most prevalent strains in clinical settings (Popovich et al., 2008) and (iii) the likelihood for the eventual co-existence of the two MRSA genotypes based on epidemiological modeling (Kouyos et al., 2013). Therefore, the lack of a consensus typing method will make it difficult to predict the actual genetic changes and evolution of the SCC*mec* elements in *S. aureus*. A standard and consensus typing method will

Table 3. Summary of the proportion of SCC*mec* types detected among the 193 clinical MRSA isolate tested by the M-PCR typing methods assessed in this study.

*SCC*mec* types and subtypes	Oliveira and De Lencastre (2002) {types I to III} Method 1	Zhang et al. (2005) {types I to V & subtype IVa to IVd} Method 2	Boye et al. (2007) {types I to V} Method 3	Zhang et al. (2012) {types II & III} Method 4
SCC*mec* type I	3.1% (6/193)	0.52% (1/193)	3.1% (6/193)	-
SCC*mec* type II	9.33% (18/193)	30.7% (59/193)	9.33% (18/193)	35.75% (69/193)
SCC*mercury*	41.5% (80/193)	16.1% (31/193)	61.14% (118/143)	-
SCC*mec*type IV	-	-	25.91% (50/193)	-
SCC*mec*type IVa	-	1.03% (2/193)	-	-
SCC*mec*type IVb	-	0.52% (1/193)	-	-
SCC*mec*type IVc	-	-	-	-
SCC*mec*type IVd	-	24.4% (47/193)	-	-
SCC*mec* type V	-	-	0.52% (1/193)	-
SCC*mec* type II+SCC*mercury*	20.7% (40/193)	-	-	-
SCC*mec* type II+IVc	-	22.3% (43/193)	26.42% (51/193)±	-
Not typeable	-	4.7% (9/193)	-	37.82% (73/193)#

*****Not all the primer sets described in the original methods were tested in this study. Therefore, data shown are those of the primers tested for each method. See Table 2 for details of the primers tested in each method. Methods 4 and 5 shown in Table 2 did not detect any SCC*mec* type III and VIII in this study. ND: Not detected. This indicates the non-detection of expected SCC*mec* types detectable by the primers used in this study. # The number of isolates that were not typeaple by the Zhang et al. (2012) method was derived by deducting the number of isolates that were already assigned other SCC*mec* types not covered by this method. ±Result obtained using a single-target PCR method by Boye et al. (2007) assigned a composite SCC*mec* type (SCC*mec* type II+IVc) to 26.42% (51/193).

ensure accurate epidemiological assessments within and across different countries and effective management and control of MRSA infections.

Currently, the classification of SCC*mec* elements in *S. aureus* is based on the combination of *mec* and *ccr* genes which have variations upon which the different classes of SCC*mec* elements are inferred (IWG-SCC, 2009).

The multiplex PCR method described by Kondo et al. (2007) attempts to improve the accuracy of detection by an initial PCR identification of the *mec* and cassette chromosome recombinases (*ccr*) types followed by identifying the genes in the "joining regions" (J-regions). Accordingly, sequence variations in the joining regions are then used to classify SCC*mec* I-V. There is an ongoing effort to test the performance of this method on clinical MRSA isolates from a number of African countries, in our laboratory. Despite this continuous improvement, consensus criteria for choosing a typing method for SCC*mec* typing is required.

Based on the variations observed in the designation of SCC*mec* types by various methods targeting different sites and genes within the SCC*mec* elements, it is obvious that the designation of SCC*mec* types across different laboratories around the world may not be in synchrony. This is epitomized in the fact that laboratories across the globe adopt different SCC*mec* typing methods (Table 1).

A recent review indicated that SCC*mec* typing was recommended as one of the methods for the monitoring of the molecular epidemiology of MRSA at national and international levels (IWG-SCC, 2009). The current study presents one of the challenges in the practicality of such endeavor. A more detailed study primarily designed to compare all published SCC*mec* typing methods on MRSA strains with known SCC*mec* sequence information would be required to make informed decision on a consensus M-PCR characterization of the SCC*mec* element.

While the SCC*mec* elements described to date include types I-XI, this study focused on the SCC*mec* types I-V and VIII based on our laboratory dataset on the prevalence of the SCC*mec* types in Pretoria, South Africa. This work attempts to paint a practical picture of the difficulties encountered in low income laboratories that are still using M-PCR for MRSA genotyping and hence focuses on the mostly reported M-PCR methods as presented in Table 2. Therefore, not all reported M-PCR methods could be covered for an in-depth comparative study.

In conclusion, this report shows the differences in the assigned SCC*mec* types by the different M-PCR methods as observed in our laboratory. The fact that in spite of the extensive coverage of the variable regions as observed for each method, some clinical isolates could not be SCC*mec*-typed in the original reports by the authors of

these methods was also highlighted.

The M-PCR detection of composite SCC*mec* types in clinical MRSA isolates (SCC*mec* II + SCC*mecury* and SCC*mec* type II+IVc)) was also reported. A plan is underway to investigate the whole genome sequence of these isolates in order to confirm this finding. From the above discussion, a number of questions thus arise: Is the inability to type clinical strains by SCC*mec* method attributed to different structural types or rearrangement and/or recombination of known SCC*mec* elements? Is there still a clinical-epidemiological relevance of HA-MRSA and CA-MRSA differentiation using SCC*mec* element, considering the reported blurring of the distinction (Peterson et al., 2012) between these two categories? If yes, do we have a consensus algorithm for making this distinction? Is SCC*mec* element still a reliable tool for typing MRSA isolates as previously suggested (IWG-SCC, 2009) taking into consideration the variations in the nucleic acid content of this element and the associated discrepancies in identification? While it is obvious that diagnostic microarray, sequencing of SCC*mec* elements and whole genome sequencing are among the modern methods of choice that may resolve this problem, majority of the laboratories in low income countries of Africa are still not able to afford the routine use of these methods. As a way forward, the adoption of a consensus method in South Africa and Africa in general is recommended, in order to allow effective epidemiological data comparison.

Limitation of the study

This report was based on empirical observations of real-world scenarios in the laboratory and therefore was not designed to effectively compare and contrast the individual methods mentioned. Such experiments will include the use of well characterized ATCC strains of MRSA and all published SCC*mec* typing algorithms. However, the results are useful as a basis for an agreement on a consensus SCC*mec* typing method in Africa.

Conflict of interests

The authors declare that there is no conflict of interest in relation to the content of this report.

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Full Length Research Paper

Special biochemical profiles of *Escherichia coli* strains isolated from humans and camels by the VITEK 2 automated system in Al-Ahsa, Saudi Arabia

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The VITEK 2 automated system was used for comparing biochemical characteristics of *Escherichia coli* isolates from human urinary tract infections (UTIs) and camel faecal samples for the first time in the study area. Identification by the system to the species level was accurate. Recovery rate of *E. coli* from camel specimens was 26% and for human specimens was 33%. Based on biochemical activities, human and animal strains were distributed into 19 profiles. Biochemical profiles 1 and 2, of the classical *E. coli* activity, comprised 26 camel (50%) and16 human strains (24.2%). The rest human strains (75.8%) were distributed among 10 profiles and 50% camel strains among 7 profiles. *E. coli* O157 was not confirmed as 6.1% human isolates were β-glucuronidase negative but sorbitol positive whereas, 11.5% camel isolates were sorbitol negative and β-glucuronidase positive. The results showed atypical biochemical reactions but no unique biochemical profile number for *E. coli* causing community-acquired UTIs in the study area. Phenotypic similarity between camel and human isolates was demonstrated and implication of camel isolates in environmental contamination is discussed.

Key words: *Escherichia coli*, non-O157, VITEK 2, camel, human, Saudi Arabia.

INTRODUCTION

Escherichia coli is a member of Family Enterobacteriaceae that live as a commensal in the intestinal tract of humans and animals but occasionally may cause infection in the intestinal tract and other body systems. *E. coli* is Gram negative, rod-shaped, non-spore forming, motile with peritrichous flagella or non-motile and about 2.0 µm long and 0.25 - 1.0 µm in diameter. They are able to grow under aerobic and anaerobic conditions. Optimal growth occurs at 35-37°C (Koneman et al., 2005).

Traditionally, biochemical reactions are used for identification and confirmation of bacteria to species level. All Enterobacteriaceae are oxidase negative except *Pleisomonas shigelloides*. *Escherichia* species are

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positive for indole. It ferments dextrose (D-glucose) by producing mixed acids (e.g. lactic, acetic and formic acids) that can then be made visible with the addition of an indicator sensitive to pH change as phenol red or methyl red. *E*. *coli* is catalase positive, oxidase negative and reduces nitrates. There are many other biochemical tests to indicate the presence of *E. coli*. For instance, Voges and Proskauer found a test to detect acetoin and 2,3-butanediol produced when *Klebsiella* and *Enterobacter* ferment glucose. The researchers found that under alkaline conditions, these two compounds oxidize themselves into diacetyl. Diacetyl then reacts with creatine (a guanidine derivative) and appears as a pinkish-red compound, or it reacts with a-naphtol and appears cherry-red in colour (Koneman et al., 2005).

Kingdom of Saudi Arabia (KSA) has a wealth of camel (*Camelus dromedaries*) population that provide milk, meat, wool, hides and skin. In rural areas, a close relationship occurs between camels and their owners.

Extra-intestinal pathogenic *E. coli* (ExPEC) is a diverse *E. coli* pathogenic type with genetic diversity which is reflective of its colonization of widespread ecological niches (Singer, 2015). Among these, urinary tract infections (UTIs) are one of the most common reasons for attendance at primary and secondary healthcare services. There are an estimated 150 million UTIs every year worldwide (Russo and Johnson, 2003). Enterohemorrhagic strains of *E. coli*, especially *E. coli* O157, have emerged as important enteric pathogens in recent years. The group produces a toxin almost identical to that of *Shigella dysenteriae* and this is responsible for the gastroenteritis in man, which ranges in severity from mild to bloody diarrhea and hemorrhagic colitis. Some patients develop hemolytic uremic syndrome (HUS) with anemia and acute renal failure. Some farm animals are infected with *E. coli* O157 without showing signs of the illness, that is, they are sub-clinically infected. Feces from these animals may contain *E. coli* O157 in varying numbers. *E. coli* O157 is generally identified as being a non-sorbitol fermenting, Gram negative rod shaped organism, ranging from 0.7 to 1.5 x 2 to 5 µm in size, oxidase negative, catalase positive and indole positive (ISO, 2001).

Evidence accumulates that *E. coli* populations in the GI tract of the human host changed frequently over time through clonal replacement, but the ecological and genetic reasons for these changes were never clarified (Caugant et al., 1981). Similar observations of a high rate of *E. coli* turnover in the GI tract have also been made in animal populations (Hinton, 1986). Natural selection may increase the frequency of new beneficial mutations as standing genetic variation. It is not yet well understood how different features of population biology or different environmental circumstances affect these adaptive processes (Burke, 2012). Multilocus sequence typing (MLST) provides an efficient genotyping tool for molecular epidemiology analysis. *E. coli* strains with identical MLST profiles (known as sequence types or STs) may possess distinct genotypes. This enables different ecotypic or pathotypic lifestyles. However, STs are not uniform with regard to genetic properties or ecotypic/pathotypic behaviors (Weissman et al., 2012).

The VITEK 2 Automated System (bioMérieux, Marcy L'Etoile, France) is one of the most widely used systems in clinical microbiology laboratories for the identification of bacteria up to species level. The system uses reagent cards that have 64 wells, each with individual substrate for sugar utilization, enzymatic and biochemical tests. Identification cards are inoculated with microorganism suspensions using an integrated vacuum apparatus. A test tube containing the microorganism suspension is placed into cassette and the identification card was placed in the neighboring slot while inserting the transfer tube into the corresponding suspension tube then the vacuum is applied and air is re-introduced into the station, the organism suspension is forced through the transfer tube into micro-channels that fill all the test wells (Pincus, 2006). Basically, it is a colorimetric reading of biochemical reactions of microorganisms. Based on these readings, an identification profile is established and interpreted according to a specific algorithm. Final profile results are compared with the database, generating identification of the unknown organism. Final results are analyzed using a software which is an Advanced Expert System (AES) specifically designed to evaluate the results generated by the VITEK 2 system. Testing is repeated wherever suggested by the AES.

Many bacterial species can be transmitted between animals and humans, either through the food chain, via the environment, or by physical contact. Transmission of extra-intestinal infections by *E. coli* from food animals could be responsible for human infection (Bergeron et al., 2012). Normal bacterial flora in the body of camels may benefit the host; occasionally, may be source of infection. From a public health perspective, camel may act as reservoir for *E*. *coli* infection.

Studies on the phenotypic characteristics of *E. coli* living as commensals in the gut of dromedaries camel are very few. Hence, the goal of this study was to determine phenotypic similarities of *E. coli* isolates from humans and camel. The hypothesis for this investigation is that *E. coli* recovered from fecal samples of camels is a reservoir for *E. coli* causing community-acquired UTI in the study area.

MATERIALS AND METHODS

Animal specimens

Freshly voided feces (200 samples) were collected from camel farms in Al Ahsa Province in sterile containers and transferred immediately in icebox to the laboratory. To prepare the samples, 1 g of fecal sample was dissolved in 9 mL sterile physiological saline for culturing (Manyi-Loh et al., 2014).

Human specimens

The study population consisted of positive cultures of urine samples (therefore no ethical approval or informed consent was required) from female patients diagnosed with UTIs, who had samples sent to the medical diagnostic laboratory of King Fahad Hospital, Al-Ahsa for culture and sensitivity testing. *E. coli isolates* from a total of 200 urine samples were randomly selected to be included in the study.

Laboratory procedures

Camel specimens were streaked onto blood agar (Oxoid, Basingstoke, UK) and MacConkey agar plates (Oxoid). The plates were incubated at 37°C for 24 h. Culture characteristics and microscopic features were observed and recorded for presumptive identification, as described by Koneman et al. (2005).

Human specimens were sub-cultured on blood and MacConkey agar, incubated at 37°C for 24 h and prepared for identification as described by Koneman et al. (2005). Sorbitol MacConkey agar (SMA) (Oxoid) was used to type *E. coli* O157 from the obtained human and animal isolates.

VITEK 2 GN identification procedure

Confirmation of the identification of isolates was performed using the VITEK 2 technique (Valenza et al., 2007).

A bacterial suspension made in 0.45% aqueous NaCl was adjusted to a McFarland standard of 0.5 with a VITEK 2 DensiCheck instrument (bioMérieux). The card for biochemical tests for Gram negative bacterial species which consists of 47 substrates (Table 1) was used. Result interpretation was done by comparing an unknown biochemical pattern to the database of reactions for each taxon and a numerical probability was calculated. Various levels of identification were assigned based on numerical probability calculations as, excellent (% probability 96-99), very good (% probability 93-95), good (% probability 89-92) and acceptable (% probability 85 to 88).

RESULTS

Animal specimens

From camel fresh feces, a total of 200 specimens were examined. *E. coli* in pure culture was isolated and identified by conventional methods. Cultural characteristics on Blood agar and MacConkey agar were studied together with microscopic examination of microbiological smears stained with Gram's stain. Lactose-fermenting colonies on MacConkey agar were suggestive to be *E. coli.*

Hemolytic activity

Five (9.6%) camel strains showed β-hemolysis on blood agar plates. All the isolates were confirmed by VITEK 2 technique. *E. coli* was confirmed from 52 specimens with recovery rate of 26% by the biochemical tests of VITEK 2 technique (Table 2).

Identification of *E. coli* O157 strains

Testing of *E. coli* isolates on SMA, showed six isolates (11.5%) with clear colorless colonies after incubation for 24 h at 35°C. The isolates were considered to be non-sorbitol fermenting and presumptive *E. coli* O157 strains. The rest of the isolates gave pink colonies indicating sorbitol fermentation.

From 200 human UTI specimens, *E. coli* was isolated and confirmed from 66 cases giving a percentage of 33% by the biochemical tests of Viteck 2 technique (Table 3). On SMA, all human strains were sorbitol fermenters.

Hemolytic activity:

A total of 14 (21.2%) human strains showed β-hemolysis on blood agar plates. Based on biochemical activities, all 118 human and camel *E. coli* strains were divided into 19 biochemical profiles. Profiles were different at least in one of the reactions tested.

Biochemical profile 1(P1), showing classical *E. coli* biochemical activities, was represented by 12 human and 16 camel isolates (Table 2). Profile 2 (P2) was represented by 4 human and 10 camel isolates which differed from P1 strains only by being positive for gamma-glutamyl-transferase. Profiles 3 – 12 (P3 – P12) contain human strains and profiles 13 – 19 (P13 – P19) contain camel strains. P3 was negative for D-mannose while P4 was positive for malonate and both contained 4 human strains. P5 was negative for D-maltose, positive for L-proline arylamidase and P6 positive for sucrose and glycine arylamidase. P7 – P9 gave odd reaction to four biochemical tests. P7 (4 strains), had odd reactions to L-lactate alkalinisation, glycine arylamidase, ornithine decarboxylase, O/129 Resistance; P8 (8 strains), odd to L-proline arylamidase, D-tagatose, phosphatase, glycine arylamidase. P9 (4 strains), odd to sucrose, D-tagatose, phosphatase, glycine arylamidase. P10 – P11 gave odd reaction to five biochemical tests. P10 (4 strains), odd to tyrosine-arylamidase, sucrose, L-lactate alkalinisation, succinate alkalinisation, alpha-galactosidase. P11 (6 strains) was odd to tyrosine-arylamidase, sucrose, L-lactate alkalinisation, succinate alkalinisation, O/129 Resistance. P12 (4 strains) was odd to gamma-glutamyl-transferase, D-tagatose, 5-keto-D-gluconate, phosphatase, glycine arylamidase, ornithine decarboxylase and beta-glucoronidase. In camel strains, P13 – P15 gave odd reaction to only one biochemical test. P13 (4 strains), was odd to L-proline arylamidase; P14 (2 strains), was odd to sucrose; P15 (2 strains), odd to phosphatase. P16 – P18 gave odd reaction to two biochemical tests. P16 (4 strains) was odd to sucrose and phosphatase; P17 (4 strains) was odd to L-proline arylamidase and sucrose; P18 (4 strains) was odd to D-maltose and phosphatase. P19 (6 strains) was odd to four biochemical tests: gamma-glutamyl-transferase, D-mannose, L-proline arylamidase and D-sorbitol.

Table 1. VITEK 2 biochemical test substrates and amount per well on the card for Gram negative bacterial species.

Well	Abbreviation	Test substrate	Amount/well (mg)
2	APPA	Ala-Phe-Pro-Arylamidase	0.0384
3	ADO	Adonitol	0.1875
4	PYRA	L-Pyrrolydonyl-Arylamidase	0.018
5	IARL	L-Arabitol	0.3
7	DCEL	D-Cellobiose	0.3
9	BGAL	Beta-Galactosidase	0.036
10	H2S	H2S Production	0.0024
11	BNAG	Beta-N-Acetyl-Glucosaminidase	0.0408
12	AGLTP	Glutamyl Arylamidase pNA	0.0324
13	dGLU	D-Glucose	0.3
14	GGT	Gamma-Glutamyl-Transferase	0.0228
15	OFF	Fermentation/Glucose	0.45
17	BGLU	Beta Glucosidase	0.036
18	dMAL	D-Maltose	0.3
19	dMAN	D-Mannitol	0.1875
20	dMNE	D-Mannose	0.3
21	BXYL	Beta-Xylosidase	0.032
22	BALAP	Beta-Alanine- Arylamidase pNA	0.0174
23	PROA	L-Proline Arylamidase	0.0234
26	LIP	Lipase	0.0192
27	PLE	Palatinose	0.3
29	TyRA	Tyrosine-Arylamidase	0.0276
31	URE	Urease	0.15
32	dSOR	D-Sorbitol	0.1875
33	SAC	Saccharose/Sucrose	0.3
34	DTAG	D-Tagatose	0.3
35	dTRE	D-Trehalose	0.3
36	CIT	Citrate (Sodium)	0.054
37	MNT	Malonate	0.15
39	5KG	5-Keto-D-Gluconate	0.3
40	ILATK	L-Lactate alkalinisation	0.15
41	AGLU	Alpha-Glucosidase	0.036
42	SUCT	Succinate alkalinisation	0.15
43	NAGA	Beta-N-Acetyl-Galactosaminidase	0.0306
44	AGAL	Alpha-Galactosidase	0.036
45	PHOS	Phosphatase	0.0504
46	GLYA	Glycine Arylamidase	0.012
47	ODC	Ornithine Decarboxylase	0.3
48	LDC	Lysine Decarboxylase	0.15
53	IHISA	L-Histidine assimilation	0.087
56	CMT	Coumarate	0.126
57	BGUR	Beta-Glucoronidase	0.0378
58	O129R	O/129 Resistance (comp.Vibrio.)	0.0105
59	GGAA	Glu-Gly-Arg-Arylamidase	0.0576
61	IMLTA	L-Malate assimilation	0.042
62	ELLM	Ellman	0.3
64	ILATA	L-Lactate assimilation	0.186

DISCUSSION

In the present study, identification of *E. coli* by the Vitek 2 technique was excellent or very good. Crowley et al. (2012) in an evaluation study of the technique, concluded that the VITEK 2 GN identification method is an acceptable automated method for the rapid identification of Gram-negative bacteria.

Based on biochemical activities, *E. coli* human strains were divided into 12 biochemical profiles and camel

Table 2. Biochemical profiles of *E coli* isolates of profiles 1 and 2 from camel faecal samples and human urinary tract infection specimens from Al Ahsa Province, KSA.

Code	Reagent	Profile 1	Profile 2
2	APPA	-	-
3	ADO	-	-
4	PYRA	-	-
5	IARL	-	-
7	DCEL	-	-
9	BGAL	+	+
10	H2S	-	-
11	BNAG	-	-
12	AGLTP	-	-
13	dGLU	+	+
14	GGT	-	+
15	OFF	+	+
17	BGLU	-	-
18	dMAL	+	+
19	dMAN	+	+
20	dMNE	+	+
21	BXYL	-	-
22	BALAP	-	-
23	PROA	-	-
26	LIP	-	-
27	PLE	-	-
29	TyRA	+	+
31	URE	-	-
32	dSOR	+	+
33	SAC	-	-
34	DTAG	-	-
35	dTRE	+	+
36	CIT	-	-
37	MNT	-	-
39	5KG	-	-
40	ILATK	+	+
41	AGLU	-	-
42	SUCT	+	+
43	NAGA	-	-
44	AGAL	+	+
45	PHOS	+	-
46	GLYA	-	-
47	ODC	+	+
48	LDC	+	+
53	IHISA	-	-
56	CMT	+	+
57	BGUR	+	+
58	O129R	+	+
59	GGAA	-	-
61	IMLTA	-	-
62	ELLM	+	+
64	ILATA	-	-
		Human	Human
	Source/	12 (18)	4 (6.1)
	No. (%)	Camel	Camel
		16 (30.8)	10 (19.2)

strains in 9 profiles. P1 and P2 which contain biochemical reactions of classical *E. coli* isolates, were represented by 24.1% human and 50% camel strains (Table 2). Percentage variation between human and camel strains could be explained by the fact that camel isolates were obtained from apparently healthy animals. However, it has been established that domestic animals are the natural reservoirs of *E. coli* and the uncontrolled release of faeces results in the presence of these bacteria in the environment (Capriole et al., 2005). Pathogenic *E. coli* has been recovered from water, sewage, vegetables and sprout (Fremaux et al., 2008; Miko et al., 2013; Scharlach et al., 2013).

All human and camel strains, in the present study, were urease negative although human isolates were obtained from cases of UTI. It needs further work to detect whether this phenotype offers any pathological advantage to isolates of UTI.

E. coli O157 strains are β-glucuronidase and sorbitol negative. β-Glucuronidase appears to be a confirmed character to differentiate between *E. coli* O157 and non-O157 strains, however, the sorbitol fermentation is more questionable (Leclercq et al., 2001). In the present study, four human isolates (6.1%) were β-glucuronidase negative but sorbitol positive whereas, three camel isolates (11.5%) were sorbitol negative and β-glucuronidase positive. It is worth mentioning that Vitek 2 system gives results of testing for 24 h; some strains of *E. coli* may ferment sorbitol after 48 h of incubation. As for human isolates, being sorbitol positive, throws doubt for confirmation as O157 strains. Furthermore, *E. coli* O157 has not been reported from UTIs in humans. In another study, Leclercq et al. (2001) reported that negativity of β-glucuronidase was fairly frequent (17.9%) among non-O157 serotypes.

In the present study, P4 was positive for malonate and identified from 4 human strains. This is in disagreement with other studies which reported that all *E. coli* tested strains were negative for malonate (Farmer et al., 1985; Ewing, 1986; Leclercq et al., 2001; Koneman et al., 2005), however, Krieg and Holt (1984) reported positive reaction in a range of 0 to 1% in the strains.

The results of the present study suggest that *E. coli* of profile number P12 consisted of four human strains which were negative for ornithine decarboxylase and β-glucuronidase. Other reports indicated that approximately 30% of *E. coli* clinical isolates were ornithine decarboxylase negative (Leclercq et al., 2001; Koneman et al., 2005). P18 and P19 of camel strains did not ferment D-maltose and D-mannose. It was reported that about 2% *E. coli* isolates are D-maltose and D-mannose negative (Koneman et al., 2005).

In the present study, it was demonstrated that 9.6% camel and 21.2% human strains were β-hemolytic. This indicates that some camel isolates could be potential pathogens. Other investigators reported that 10% of *E. coli* isolates from UTI in humans were hemolytic (Bhattacharyya et al., 2015).

Table 3. Biochemical profiles 3 to 19 of *E coli* Isolates from camel faecal samples and human urinary tract infection specimens from Al Ahsa Province, KSA.

Code	Reagent	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18	P 19
2	APPA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	ADO	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	PYRA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	IARL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	DCEL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	BGAL	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10	H2S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	BNAG	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-
12	AGLTP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	dGLU	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
14	GGT	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
15	OFF	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
17	BGLU	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18	dMAL	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+
19	dMAN	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
20	dMNE	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
21	BXYL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
22	BALAP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
23	PROA	-	-	+	-	-	+	-	-	-	-	+	-	-	-	+	-	+
26	LIP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
27	PLE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
29	TyRA	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+
31	URE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
32	dSOR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
33	SAC	-	-	-	+	-	-	+	+	+	-	-	+	-	+	+	-	-
34	DTAG	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-	-	-
35	dTRE	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
36	CIT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
37	MNT	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
39	5KG	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
40	ILATK	+	+	+	+	-	+	+	-	-	+	+	+	+	+	+	+	+
41	AGLU	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
42	SUCT	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+
43	NAGA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
44	AGAL	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
45	PHOS	-	-	-	-	-	+	+	-	-	+	-	-	+	+	-	+	-
46	GLYA	-	-	-	+	+	+	+	-	-	+	-	-	-	-	-	-	-
47	ODC	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+
48	LDC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
53	IHISA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 3. Contd.

Code	Reagent	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18	P 19
56	CMT	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
57	BGUR	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
58	O129R	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+
59	GGAA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
61	IMLTA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
62	ELLM	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
64	ILATA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Human Camel	H	H	H	H	H	H	H	H	H	H	C	C	C	C	C	C	C
	No. (%)	4(6.1)	4 (6.1)	4 (6.1)	4 (6.1)	4 (6.1)	8(12.1)	4 (6.1)	4 (6.1)	6(9.1)	4 (6.1)	4 (7.7)	2(3.8)	2(3.8)	4 (7.7)	4 (7.7)	4 (7.7)	6(11.5)

Results of the current study show that 50% of camel strains and 25% of human strains displayed biochemical activities of classical *E. coli*. The rest of the strains could not be assigned to a single profile, half of camel isolates were distributed into 7 profiles and three-quarters of human isolates were distributed into 10 profiles. This is interesting as there are no previous studies on biochemical characterization of *E. coli* camel isolates in the study area. All the tested strains should be considered as non-O157 *E. coli.* More work is needed to investigate more E. coli strains from sick camels and human UTIs for phenotypic and genotypic characteristics from the study area.

Conclusion

E. coli isolates from community-acquired UTIs in the study area belongs to the group, *non-O157 E. coli* which showed similarity with camel faecal isolates. Biochemical activities indicated that half camel and quarter human strains were classical as non-O157 *E. coli,* the remaing strains displayed deviation in some biochemical reactions.

The rest of the strains could not be assigned to a single profile, half of camel isolates were distributed into 7 biochemical profiles and three-quarters of human isolates were distributed into 10 profiles.

VITEK 2 identification system for Gram negative bacterial species is helpful in biochemical confirmation of *E. coli* isolates especially for differentiation of *E. coli* O157 and non-O157.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Outbreak of *Burkholderia cepacia* complex associated with contaminated liquid soap for hospital use: A case study

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***Burkholderia cepacia* complex (BCC) is gram negative bacteria which causes different types of human infections including catheter-associated infections and respiratory tract infections especially in immunocompromised patients. This study was conducted to decide the source, to prevent the possible hospital outbreak of *BCC* and to determine the epidemiological source. Eleven cartridge of liquid soaps were purchased by Near East University Hospital, Nicosia. Liquid soaps were cultured on 5% sheep blood agar and Eosine Methilene Blue Agar at 35°C in 24 h. Colonies which suspected were confirmed by Phoenix 100. Patient blood was cultured in BD Bactec Plus Aerobic culture bottles. BCC strains and patient blood sample were identified by using two different polymerase chain reaction (PCR) methods. The liquid soaps were contaminated with BCC. A few days later, in the blood culture of the 35-year-old Turkish Cyptiot male patient, BCC was identified. The molecular results indicated that the BCC strain isolated from the patient blood culture was identical with the strain isolated from the liquid soaps. The molecular epidemiological studies showed that the patient blood culture strain and the liquid soap strain was in the same cluster. It was suggested that the liquid soaps should always be analyzed and then distributed to the hospital departments.**

Key words: *Burkholderia cepacia* complex (BCC), liquid soap, prevent.

INTRODUCTION

*Pseudomo*nas cepacia was first described by the William Burkholder as a plant pathogen which caused the onion rot in 1950 (Mahenthiralingam, 2008; Dolan, 2011). However, when the molecular analysis was executed, this species were categorised to a new genus, *Burkholderia* (Mahenthiralingam, 2008). After 16S rRNA analysis, results indicated that, *B. cepacia* could be associated with *Burkholderia cepacia* complex (BCC) as they were related

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related species (Torbeck, 2011). BCC is a non-fermenting gram negative bacteria which causes different types of human infections including catheter-associated infections and respiratory tract infections, especially in immuno-compromised patients (Lee, 2013). BCC also causes endocartitis, wound infections, catheter-related urinary infections, intravenous bacteremia and foot infections (Torbeck, 2011). BCC is an avirulent bacterium for many healthy people and is usually related with pneumonia in cystic fibrosis patients (Hua, 2013). However, if *BCC* infection is seen in healthy people, physician should give an attention. BCC can be potential microorganisms that cause hospital oubreaks as it generally dissipates at hospital environment (Lee, 2013). Hospital outbreaks have commonly been caused by the contaminated sources such as disinfectant products, intravenous solutions or medical devices (Kuzumoto, 2011). Studies reported that BCC has been usually found in contaminated nebulizer solutions, intravenous solutions, mounthwash, hospital sinks, antiseptics (Lee, 2013), pharmaceuticals cosmetics, preservative products (Torbeck, 2011), tap water, and reusable temperature probes (Dolan, 2011).

The outbreak of *BCC* among 250 bed hospital in Nicosia, Cyprus of North in November 2013 was described and prevented. The epidemiological investigation was started by the Infection Control Unit. At the end of the investigation, the source of this contamination was located. Molecular typing of the samples obtained from the patient and environmental isolates were performed by DiversiLab and Pulsed Field Gel Electrophoresis (PFGE).

MATERIALS AND METHODS

Identification of the problem

At the Near East University Hospital, as a routine process before the liquid soaps are distributed to the hospital departments, samples are analyzed and report of the results are discussed by the Infection Control Unit.

In November 2013, 11 cartridge of liquid soaps were purchased by Near East University Hospital, Nicosia. Among 4 liquid soaps, samples were taken randomly for culture analysis by the infection control nurse before distrubuted to the hospital departments. BCC were identified in the liquid soap at the end of the culture analysis. After one week, BCC bacteremia was identified from the blood culture of one non-cystic fibrosis patient. Three experts including two infection control doctors and an infection control nurse made an investigation about the outbreak.

Microbiological testing

Clinical specimens that were sent to the Near East University Hospital Microbiology Laboratory, were then cultured according to their types of material. The BCC was cultured according to standard microbilogical methods. Patient blood (3 to 6 ml) was cultured in BD Bactec Plus Aerobic culture bottles (Becton Dickinson, USA) and incubacted for 7 days at 35°C in Bactec 9120 (Becton Dickinson, USA) with daily visual inspection of the chromogenic indicator for growth. When the blood culture system was alarmed, patient blood samples were cultured using 5% sheep blood agar (Oxoid) and Eosine Methilene Blue Agar (Oxoid). Samples were plated and incubated at 35°C in 24 h. Colonies were counted and microorganims were identified by Phoenix 100 autamated system (Becton Dickinson, USA). Liquid soaps were tested by filtration with a 0.45 µm cellulose membrane filter and cultured on 5% sheep blood agar and Eosine Methilene Blue Agar at 35°C in 24 h. Colonies suspected were confirmed by Phoenix 100 autamated system. For further analysis, 12 BCC isolates were stored at -80°C on porous beads (Microbank, Pro-Lab Diagnostic, Richmond Hill, ON, Canada).

Species determination

BCC strains isolated from liquid soaps as well as one patient blood sample were identified by using two different genotyping methods; DiversiLab and PFGE.

Strain genotyping

BCC strains isolated from liquid soaps products and the patient were genotyping by using PFGE with a modification of the protocol by Shueh et al. (2013). Isolates were digested with *Xba* restriction endonucleases. PFGE was performed using the Bio-Rad Gene Path system (Bio-Rad, Hercules, USA) and run on a 1% agarose gel in 0.5x TBE Buffer at 14°C with a linear ramp time of 5.3 to 52.4 s over a period of 24 h. Afterwards, gels were stained with ethidium bromide and photographed. DiversiLab protocol was used as described by Fluid et al. (2010). Isolates were cultured on blood agar for 24 h at 35°C. Extraction of *B. cepacia* DNA was made with the UltraClean microbial DNA isolaton kit (Mo Bio Laboratories, Inc., Carlsbad, CA) following the manufacturer intructions. Extraction samples were diluted untill the samples were 35 ng/µl. Repetitive sequence-based PCR (REP-PCR) of extracted DNA was made using the DiversiLab Bacterial Kit (bioMérieux, Marcy l'Etoile, France). Briefly, 35 ng genomic DNA, 2.5 U AmpliTaq polymerase, 2.5µl 10× PCR buffer (Applied Biosystems) and 2 µl primer mix were added to the rep-PCR master mix in a total volume of 25 µl per reaction. Thermal cycling parameters were as follows: initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 92°C for 30 s, annealing at 50°C for 30 s and extension at 70°C for 90 s, with a final extension at 70°C for 3 min. Amplified fragments of various sizes and intensities were separated and detected with DNA chip (bioMérieux, Marcy l'Etoile, France) on Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, California). Results were demonstrated as dendrogram (with a Pearson correlation similarity matrix) including a virtual gel image of the fingerprint for each DNA sample. Strain-level discrimination is indicated between samples of the same species with >95% similarity and minor band differences.

RESULTS

In November 2013, 11 cartridge of liquid soaps were purchased by Near East University Hospital, Nicosia. The liquid soaps were at pH 5.5 and the firm accreditation with the ISO 9001-140001-22000, OHSAS 18001 and GMP 22716. 4 liquid soaps randomly selected by Infection Control Nurse. The culture analysis results showed that these liquid soaps were contaminated with BCC. As a result of this report, all 11 liquid soaps were then analyzed. The culture results showed that all liquid soaps

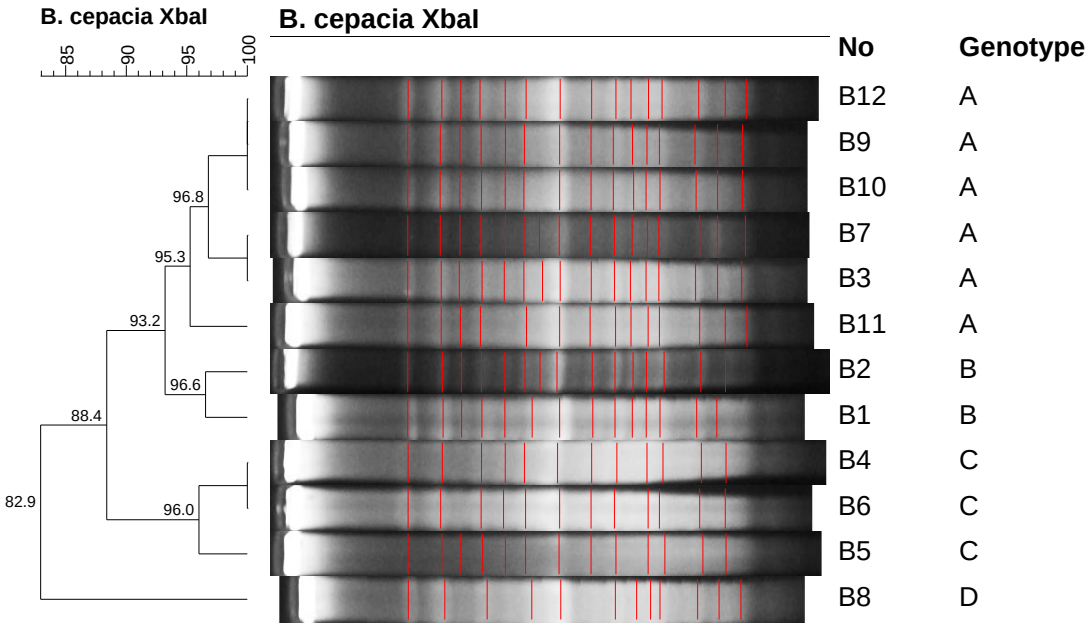

Figure 1. Dendrogram of PFGE patern showing similarities of 12 *B. cepacia* strains. Dice coefficient (threshold ≥%95) and the "unweighted pair-group method with arithmetic mean" (UPGMA) cluster method were used for the dendogram analysis (B3. enviromental sample) Genotyping Pulsed Field Gel Electrophoresis (PFGE).

were contaminated with BCC. The group of these liquid soaps were not distributed before the culture analysis. All BCC strains were sensitive to ceftazidime, cotrimoksazole and meropenem, intermediate to levofloksasine and resistant to ticarcillin/clavulonat according to the Phoenix 100 results.

A few days later, in the blood culture of the 35-year-old Turkish Cyptiot male patient, BCC was identified. The patient is sickle cell anemia and periodically needs hospital caring. He was treated with cotrimoksazole immediately to eradicate the infection.

After it was found that the source of the contamination was the liquid soaps, the Infection Control Commitee showed special attention for the handling of disinfectant equipments. They collected all of the disinfectant bottles from all departments for inhibiting the dissemination of the outbreak.

Molecular and epidemiological studies were performed. Strains were analysed to be genotyping by both DiversiLab and PFGE. Molecular results showed that BCC has 4 different clusters (A, B, C and D). The patient strain was in the A cluster which was larger than the other clusters. DiversiLab result were confirmed with the PFGE result. PFGE (Figures 1 and 2) and DiversiLab (Figure 3) analysis results were shown.

After the source of the contamination was identified, in the discussion with the hospital autohorities, infection control unit decided to eliminate all previously distributed liquid soaps and also abolish the aggrement with the firm. As the precausions were taken, no other BCC contamination was detected throught the hospital.

DISCUSSION

BCC is one of the main microorganism that can be found in enviroment in diverse ranges (Mahenthiralingam, 2008). BCC is important for the healthcare-associated infectious agents in especially hospitalized and immunocomprimised patients (Kuzumoto, 2011). 16S rRNA results indicated that BCC strains are a group at least 17 species and 15 genospecies with high similarity (>97.5%) (Kuzumoto, 2011). Most of the members of this genus are useful in many biotechnological applications such as bioremedication, biological control of the plant diseases, water management and nitrogen fixations (Choh, 2013). BCC is also a bio-pesticide for inhibiting fungal diseases (Kuzumoto, 2011). When BCC is located in manufacturing equipment, components or water used in manufacturing pharmaceutical products, this can be associated with high patient risk (Torbeck, 2011). Although BCC does not live on completely dry surfaces more than one week, it can stay alive in water for months (Torbeck, 2011). BCC were transmitted by contact or aerosol and by environmental acquistion (Peeters, 2008).

Infection Control Unit decided that hand washing is important before contact with patient as hands are the main transmission way of the microorganism (Caetano, 2011). It was indicated that low-level disinfectants like benzalkonium chloride, chlorhexidine and alkyldiaminothyglycene hydrochloride are not appropriate as the contamination has occurred by microorganisms (Oie, 2012). It has been reported that BCC had been alive in commercially manufactured proviodine iodine.

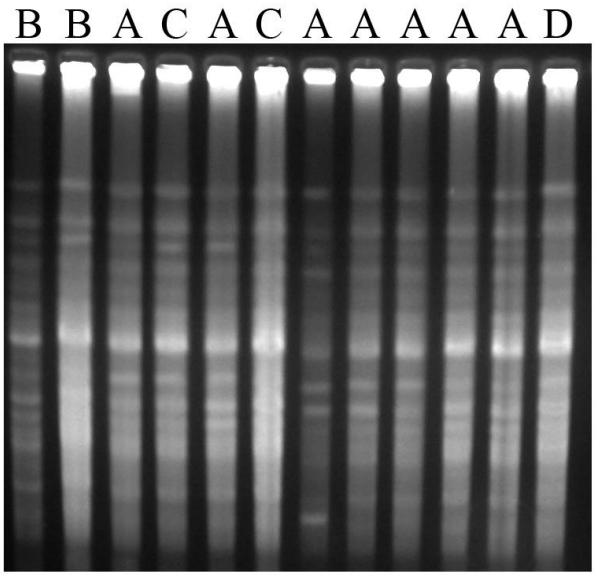

Figure 2. PFGE typing results of 12 *B. cepacia* strains.

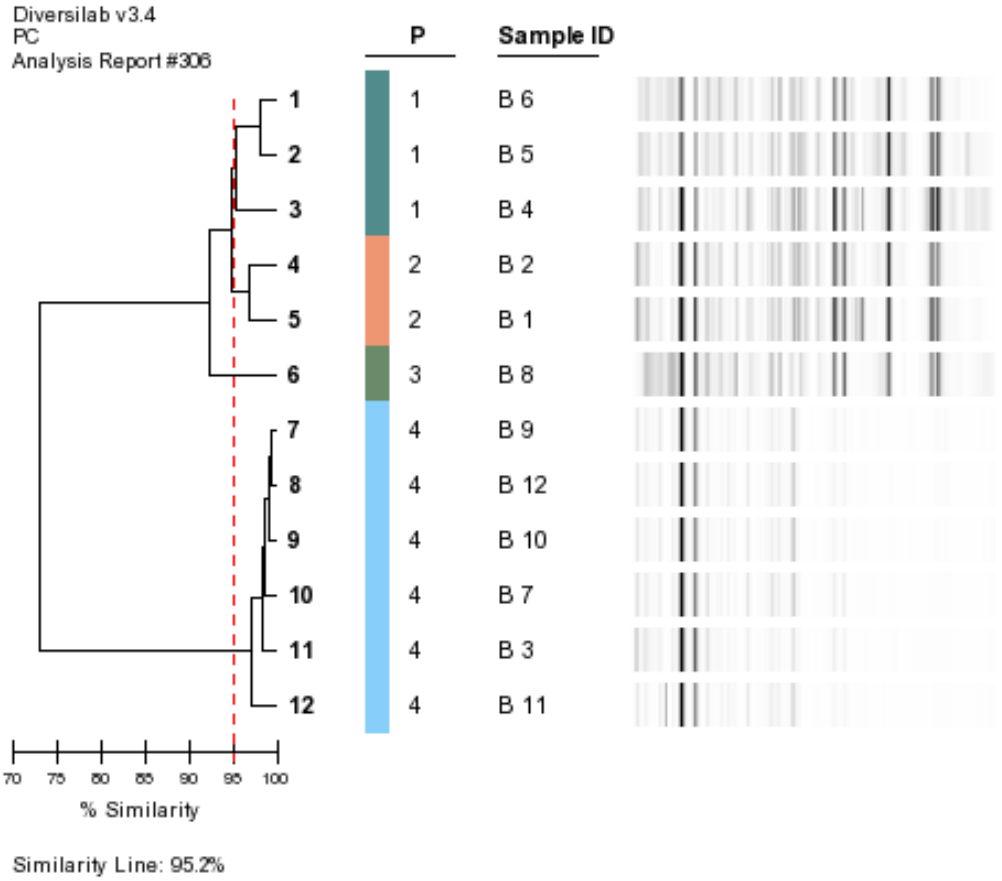

Figure 3. rep-PCR-based dendrogram and virtual gel image fingerprints obtained from 12 *B. cepacia* strains using the DiversiLab system with the bacteriel kit. Pearson's correlation coefficient was used to create a pairwise percentage similarity matrix and the dendogram was derived using UPGMA. An 95 % similarity threshold (vertical line) was chosen for *B. cepacia.* B3; patient, B1-2, B4-12; enviromental samples. Rep-PCR-based DNA fingerprinting by the DiversiLab System.

This contaminations cause both infections and pseudo-infections at hospital environment (Weber, 2007). Also, other studies indicated that contaminated quanternary ammonium compound used for the disinfection of the rubber stoppers of blood culture bottles caused pseudo-outbreak of BCC (Weber, 2007). This studies showed that the sources of BCC contamination can vary from medical instruments and disinfectants to alcohol-free mouthwash and body milk (Martin, 2011).

FDA reported that between years 2004-2011, BCC alone was mentioned in 34% of the non-steril recalls including mouthwashes, moist wipes, soaps, sanitizers, nasal products and hair dyes. The BCC contamination is an important situation for the FDA (Sutton, 2012). Lucero et al. (2011) reported that the most mechanism for tranmission, BCC, was the hospital tap water for using the oral and tracheostomy care. Also, Smet et al. (2013) described the first blood-stream infecton from a resource-limited setting from the BCC. Gel-alcohol based products are used by many hospitals for hand washing beacuse of the antimicrobial activity, rapid action, good cutaneous tolerance and easy applications (Caetano, 2011). In Near East University Hospital, Infections Control Units recommends the gel-alcohol based and liquid products for the hand washing. In November 2013, it was shown that all the liquid soaps were contaminated with BCC. The group of these liquid soaps were not distributed before the culture analysis. Although the previously distributed liquid soaps were collected immediately, a few days later, blood culture of a patient was characterized by BCC. This situation implied that previous purchases of liquid soaps might be contaminated with BCC. As the patient is a sickle cell anemia patient, he is an immunocomprised person. Therefore, he is more convenient to the bacteremia than healty people.

BCC is multiple resistant bacteria to many antibiotics such as aminoglycosides, quinolones, polymixsins and β-lactams so that the treatment of BCC is difficult (Choh, 2013). The strain obtained from blood culture was sensitive to cotrimoksazole. The clinician immediately started the treatment with cotrimoksazole. Both strains were analysed with DiversiLab and PFGE for their molecular epidemiology. The DiversiLab was based on REP-PCR. Our DiversiLab results were confirmed by the PFGE which is a gold standart. The advantage of the DiversiLab is that the result in the same day and property of high repeatability. Also, analysis of the results by DiversiLab was easy to perform in routine laboratory.

Therefore, DiversiLab may be used for the bacterial epidemiological analysis to begin early precaution. The molecular epidemiological studies showed that the patient blood culture strain and the liquid soap strain was in the same cluster. These results indicated that the outbreak of the BCC at the Near East University Hospital was prevented. It was suggested that the liquid soaps should always be analyzed and then distributed to the hospital departments.

Conflict of Interests

The authors have not declared any conflict of interests.

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Antibacterial activity *Lactobacillus plantarum* isolated from fermented vegetables and investigation of the plantaricin genes

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***Escherichia coli* and *Staphylococcus aureus* are two food pathogens that cause severe food poisonings. Another problem found on a global level is the continuous increase of antimicrobial resistance in bacteria isolated from food. This study aimed to evaluate the antibacterial activity of *Lactobacillus plantarum* against pathogenic bacteria including *E. coli* and *S. aureus* and to study if *L. plantarum* with antibacterial activity contained the most plantaricin genes or not. A total of 50 lactic acid bacteria isolates (LAB) were evaluated for antibacterial activity and identified plantaricin genes by polymerase chain reaction (PCR) methods. Seven LAB isolates with antibacterial activity against *S. aureus* and *E. coli* were identified as *Lactobacillus* based on morphological physiological and biochemical properties. Using species-specific PCR and 16S rRNA gene sequencing, B0039 was identified as *Lactobacillus paracasei*, other isolates were identified as *L. plantarum*. 3 strains tested positive for all the genes in the *pln*ABCD operon. The *pln*EFI operon was detected in four strains. Genes encoding for the two-peptide *pln*J/K were detected only in 2 strains. Finally, the *pln*G/V was also found in 3 strains of *L. plantarm.* The plantarum gene sequences of B0055 were 97 to 100% similarity with the *L. plantarum* WCFS1. The findings suggest that LAB with bacteriocin genes can be used as an alternative mechanism to control drug resistant foodborne pathogens.**

Key worlds: *Lactobacillus*, antibacterial activity, plantaricin gene, *Staphylococcus aureus*, *Escherichia coli*.

INTRODUCTION

Escherichia coli and *Staphylococcus aureus* are two food pathogens that cause severe food poisonings (Bachir and

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Benali, 2012). Many food-poisoning outbreaks of *E. coli* have been associated with contaminated food, such as beef, pork, chicken and water (Wang, 2008). *S. aureus* may produce a number of toxins, the most important ones with respect to foodborne illness belong to the family of heat-stable staphylococcal enterotoxins (SEs). Another public health concern is associated with the increased incidence of antibiotic-resistant strains isolated from poultry meat (Dan et al., 2015). Due to the widespread use of antimicrobials in chicken and pig growth units, the development of resistant strains that can infect humans via the food chain has increased (Mihaiu et al., 2014). As a result, contamination of pathogenic microorganism is recognized as a potential public health concern. As more bacteria become resistant to traditional antibiotics, this leads to emergence and re-emergence of multidrug-resistant pathogens.

Lactic acid bacteria (LAB) have been used in the production of varieties of fermented dairy, vegetables and meat products for many centuries (Man et al., 2014). Recent research revealed that LAB can produce antibacterial substances including organic acids, hydrogen peroxide, diacetyl, inhibitory enzymes and bacteriocins (Herna´ndez et al., 2005) to inhibit the growth of a wide range of intestinal pathogens (García-Ruiz et al., 2013). Bacteriocins were defined as antimicrobial peptides or proteins have been observed in many genera of bacteria, including many strains of LAB, which are directed mainly to inhibit the growth of related species (Anyogu et al., 2014). *Lactobacillus rhamnosus* has been reported to interact with intestinal epithelium and prevent the internalization of enterohemorrhagic *E. coli* (Moorthy et al., 2007). *Lactobacillus sake* C2 which produced a bacteriocin strongly inhibited *S. aureus* ATCC 63589 and *E. coli* ATCC 25922 was isolated from traditional Chinese fermented cabbage (Gao et al., 2010). Bacteriocins bacST202Ch and bacST216Ch, produced by *Lactobacillus plantarum* strains isolated from Beloura and Chourico, inhibited the growth of a number of Gram-positive and Gram-negative meat spoilage bacteria (Todorov et al., 2010). *L. plantarum* B0105 isolated from traditional Taiwan fermented mustard, was found to produce bacteriocin inhibiting *Streptococcus mutans* BCRC 10793 (Chen et al., 2013). *L. plantarum* ST71KS was isolated from homemade goat feta cheese and displayed a bactericidal effect against *Listeria monocytogenes* strains 603 and 607 (Martinez et al., 2013).

LAB are very important in ensuring the safety of various foods by production of bacteriocins and other antimicrobial substances. Bacteriocins produced by *L. plantarum* are known as plantaricins (Omar et al., 2008). The genetic determinants for plantaricins were reported as shown for strains *L. plantarum* strain C11 (Diep et al., 2003), WCFS1 (Kleerebezem et al., 2003), NC8 (Maldonado et al., 2003) and J23 (Rojo-Bezares et al., 2008). The *L. plantarum* C11 plantaricin cluster contains five operons (Diep et al., 2003). Two of them (*pln*EFI and *pln*JKLR) code for bacteriocins and immunity proteins, the transport operon (*pln*GHSTUV), which is involved in the secretion of the pheromone and bacteriocins; *pln*ABCD for the signal-transducing pathway, and *pln*MNOP containing genes with unknown functions in bacteriocin synthesis (Diep et al., 2003). In this study, LAB isolated from traditional fermented products, were screened for the antibacterial activity and presence of plantaricin genes in strains were also investigated.

MATERIALS AND METHODS

Bacterial strains

Lactic acid bacteria were isolated from traditional fermented mustard and vegetable samples collected from southern areas of Taiwan. The LAB isolates were characterized based on acid production, Gram stain and catalase test were tested. *Lactococcus lactis* subsp. *lactis* (ATCC 11454) and *L. plantarum* (ATCC 14917) with plantaricin genes as control strain, *S. aureus* (BCRC 12653, BCRC 12654, BCRC12658, BCRC13824 and BCRC 13829) and *E. coli* (BCRC 14825, BCRC 15375 and BCRC 41443), used as test microorganisms in determining antibacterial activity were obtained from Bioresource Collection and Research Center (BCRC), Hsin Chu, Taiwan.

Screening antibacterial activity of LAB

For screening the antibacterial activity of LAB isolates, 1% (v/v) of these cultures were inoculated into 50 ml de Man, Rogosa and Sharpe (MRS) broth individually and incubated at 35°C for 24 h without agitation. Bacterial cells were removed by centrifugation (17,000 g, 10 min, 4°C) and the resulting solution were subjected to filtration with 0.22 µm filter, then, the diameters of inhibition zones were measured using the agar diffusion assay method (Anyogu et al., 2014). Overnight test cultures of *E. coli* and *S. aureus* were diluted in saline solution into 10^8 CFU/ml and 100 µl of dilution were inoculated in nutrient agar medium. Briefly, 100 µl of spent cell-free supernatant (SCS) were placed into wells (10.0 mm in diameter) on nutrient agar plates seeded with the test pathogens. After incubation at 35°C for 14 h, the diameter of inhibition zones was determined. The pH of MRS broth was also adjusted to the same value as blank. The antimicrobial activity was lost after treatment with pepsin, indicating a peptide nature (Rojo-Bezares et al., 2007). The bacterial cell-free supernatants were incubated 37°C overnight with or without pepsin (Sigma, St. Louis, Missouri) at a final concentration of 2 mg/ml. *L. lactis* subsp. *lactis* (nisin producing strain, BCRC 11454) was used as the negative control and *L. plantarum* with plantaricin genes (ATCC 14917) as the positive control. As a blank control, aliquots of MRS broth treated as filtered supernatants were used. All of the tests were repeated 3 times. Diameters of inhibition zones were determined. Reduced inhibition zone (mm) = inhibition zone of SCS - inhibition zone of SCS treated with pepsin.

Strain identification

A polymerase chain reaction (PCR) assay was performed using genomic DNA from strains that showed antibacterial activity. Amplification of 16S rDNA sequences by PCR was performed using the primers 27F- AGAGTTTGATCMTGGCTCAG and 1492R-GGYTACCTTGTTACGACTT described by Tanner et al. (2000). For the PCR identification, genomic DNA was extracted using the

Table 1. Plantaricin gene primers and conditions used in this study.

Target	PCR Primers (5'→3')	Annealing temperature (°C)	Amplicon size (bp)
plnA	F: GTA CAG TAC TAA TGG GAG R: CTT ACG CCA ATC TAT ACG	53	450
plnB	F: TTC AGA GCA AGC CTA AAT GAC R: GCC ACT GTA ACA CCA TGA C	51.5	165
plnC	F: AGC AGA TGA AAT TCG GCA G R: ATA ATC CAA CGG TGC AAT CC	49.5	108
plnD	F: TGA GGA CAA ACA GAC TGG AC R: GCA TCG GAA AAA TTG CGG ATA C	53	414
plnEF	F: GGC ATA GTT AAA ATT CCC CCC R: CAG GTT GCC GCA AAA AAA G	53.2	428
plnI	F: CTC GAC GGT GAA ATT AGG TGT AAG R: CGT TTA TCC TAT CCT CTA AGC ATT GG	52.5	450
plnJ	F: TAA CGA CGG ATT GCT CTG R: AAT CAA GGA ATT ATC ACA TTA GTC	51	475
plnK	F: CTG TAA GCA TTG CTA ACC AAT C R: ACT GCT GAC GCT GAA AAG	52.9	246
plnG	F: TGC GGT TAT CAG TAT GTC AAAG R: CCT CGA AAC AAT TTC CCC C	52.8	453
plnN	F: ATT GCC GGG TTA GGT ATC G R: CCT AAA CCA TGC CAT GCA C	51.9	146
plnV	F: CAG TTT ATT GGC AGC AAT CG R: ATC CAC TCC ATC CAA ACA ATC	54	727
plnNC8	F: GGT CTG CGT ATA AGC ATC GC R: AAA TTG AAC ATA TGG GTG CTT TAA ATT CC	60	207

All the plantaricin primers and annealing temperature are followed from Sáenz et al. (2009).

Genomic isolation kit (GeneMark, Georgin, USA) according to the manufacturer's instructions. Genomic DNA concentration was determined spectrophotometrically (Hitachi, U-2800A, Tokyo, Japan). PCR primers were used to amplify a 1484 bp DNA fragment. The reaction mixture contained 10 µl genomic DNA, 2.5 units of *Taq* polymerase (Promega, Madision, WI), 2 µl each of 10 mM dATP, dTTP, dCTP and dGTP, 5 µl of 10 X reaction buffer (10 mM Tris-HCl (pH 8.3 at 25°C) containing 50 mM KCl, 0.01% Triton X-100, 0.01% gelatin, 6.0 mM $MgCl_2$), and 50 pmol of each primers in a final volume of 50 µl. The DNA was denatured at 94°C for 2 min and amplified for 35 cycles at 94°C for 40 s, 45°C for 50 s and 72°C for 50 s. A final extension incubation of 2 min at 72°C was included. Amplification reactions were performed on a thermal cycler (Perkin-Elmer GeneAmp PCR System 2400, Foster city, CA). The PCR products were purified with Gel/PCR DNA fragments extraction kit (Geneaid, Taipei, Taiwan) and sequenced by automated sequencing core laboratory, National Cheng Kung University (Tainan, Taiwan). Sequence homologies were examined by comparing the obtained sequence with those in the DNA data bases (http//www.ncbi.nim.nih.gov/BLAST) (Todorov et al., 2010).

PCR amplification of plantaricin genes

A PCR assay was performed using total genomic DNA from strains that showed antibacterial activity against test pathogens. PCR amplification of plantaricin genes were carried out, the primers and conditions were specified in Table 1 (Sáenz et al., 2009), with initial

Table 2. Antibacterial activities of lactic acid bacteria spent cell supernatants (SCS) against test pathogens.

Strains LAB	Inhibition zones (mm)							
	E. coli			*S. aureus*				
	BCRC14825	BCRC15375	BCRC41443	BCRC12653	BCRC12654	BCRC12658	BCRC13824	BCRC13829
L. plantarum	21.0 ± 0.00	19.3 ± 0.05	26.6 ± 0.5	18.0 ± 0.0	19.0 ± 0.0	19.3 ± 0.5	12.3 ± 0.5	20.0 ± 0.0
L. lactis	16.3 ± 0.2	18.5 ± 0.0	23.8 ± 0.2	-	-	-	-	-
B0013	22.0 ± 1.0	23.8 ± 0.2	29.0 ± 0.5	18.2 ± 0.02	18.0 ± 0.5	15.2 ± 0.2	14.1 ± 0.7	15.5 ± 0.0
B0039	21.5 ± 0.1	22.7 ± 0.2	29.3 ± 0.2	18.5 ± 0.08	17.8 ± 0.2	16.3 ± 0.2	12.8 ± 0.2	16.0 ± 0.5
B0055	22.2 ± 0.6	24.0 ± 1.0	29.0 ± 0.6	14.0 ± 0.05	14.9 ± 0.1	1.68 ± 0.2	15.5 ± 0.5	15.7 ± 0.2
B0105	20.8 ± 0.2	23.1 ± 0.2	29.3 ± 0.2	14.2 ± 0.02	15.1 ± 0.2	14.2 ± 0.2	13.6 ± 0.7	16.5 ± 0.5
B0125	24.0 ± 0.0	25.6 ± 0.5	29.0 ± 0.0	22.0 ± 0.00	26.6 ± 0.5	22.0 ± 0.0	20.0 ± 0.0	24.0 ± 1.0
B0126	20.9 ± 0.3	22.8 ± 0.7	28.0 ± 0.5	15.2 ± 0.05	15.3 ± 1.0	15.5 ± 1.0	12.9 ± 0.1	18.0 ± 0.4
B0134	20.5 ± 0.5	23.5 ± 0.5	28.3 ± 0.5	14.5 ± 0.03	17.4 ± 0.7	14.2 ± 0.2	12.4 ± 0.3	17.5 ± 0.0

*All of the tests are repeated at least 3 times.

denaturation at 94°C for 3 min and 30 cycles of 94°C for 1 min, annealing at an appropriate temperature (Table 1) for 1 min, 72°C for 30 s, and a final extension at 72°C for 5 min. Amplification reactions were performed on a thermal cycler (Perkin-Elmer GeneAmp PCR System 2400, Foster city, CA). The amplification products were loaded onto a 1.8% agarose gel. After electrophoresis in 1X TBE (Tris-Borate-EDTA) buffer, the gel was stained with ethidium bromide before being photographed by ultraviolet illumination. Sequence homologies were examined by comparing the obtained sequence with those in the DNA data bases (http//www.ncbi.nim.nih.gov/BLAST). The PCR products of plantaricin genes of B0055 were purified with Gel/PCR DNA fragments extraction kit (Geneaid, Taipei, Taiwan) and sequenced by automated sequencing core laboratory, National Cheng Kung University (Tainan, Taiwan).

RESULTS

Screening antibacterial activity of LAB

A total of 50 presumptive lactic acid bacteria isolates obtained from traditional fermented mustard and vegetables were determined with phenotypical and physiological tests. Out of the 50 LAB isolates, only 7 isolates showed antagonistic effect against the test pathogens (*S. aureus* and *E. coli*) (Table 2).

The diameters of inhibition zones against *E. coli* and *S. aureus* ranged between 12 and 29 mm. In some cases, antimicrobial activity reduced after addition of pepsin on the SCS of LAB, the reduction of diameters of inhibition zones against *E. coli* and *S. aureus* ranged between 2 and 7 mm (Table 3).

Strains identification

Amplification of 16S rDNA sequence by PCR was performed using the primers described by Tanner et al. (2000). The identification of LAB isolates with antibacterial activity revealed that strain B0039 was identified as *Lactobacillus paracasei* and other strains were identified as *L. plantarum*. The 16S rDNA nucleotide sequences of the test strains were 99 to 100% similarity with the GenBank access number assigned in Table 4.

Amplification of plantaricin genes

In an attempt to determine whether the strains that showed antibacterial activity against test pathogens carried genes for the production of known plantaricins. A PCR assay was performed using total genomic DNA from strains. Several strains carried at least one or more genes of the plantaricin cluster. *L. plantarum* strains B0013, B0055, B0126 tested positive for the *pln*ABCD operon. The complete *pln*EFI operon was detected in *L. plantarum* B0013, B0055 and B0126, while B0105 was detected only *pln*EF. Genes encoding for the two-peptide *pln*J/K were detected only in *L. plantarum* B0055 and B0126. Finally, the *pln*G/V, which is part of a large operon involved in plantaricin export, was also found in *L. plantarum* B0013, B0055, and B0126. Three strains gave all negative plantaricin genes in PCR analyses (Table 5). Among these positive results, B0055 was

Table 3. Effect of pepsin treatment on the antibacterial activity of LAB SCS against indicators.

Strains	Indicator strains							
	E. coli				*S. aureus*			
	BCRC14825	**BCRC15375**	**BCRC41443**	**BCRC12653**	**BCRC12654**	**BCRC12658**	**BCRC13824**	**BCRC13829**
L. plantarum	-	+	++	-	++	++	-+	++
B0013	++	++	++	+++	+++	++	++	++
B0039	++	++	++	++	++	++	+	++
B0055	++	+++	+++	+	++	++	++	++
B0105	++	+++	+++	-	+	+++	++	+++
B0125	++	++	++	+	++	++	+	++
B0126	++	++	++	+	++	+	+	+++
B0134	++	++	++	++	+++	+	++	+++

*All of the tests are repeated at least 3 times. **Reduced inhibition zone (mm) = inhibition zone of SCS - inhibition zone of SCS treated with pepsin. ***The inhibition zones ≤1 mm, 2–3 mm, 4-5 mm and >6 mm, were classified as strains of no -; little +; mild ++; and strong +++ reduction of inhibition zone, respectively.

Table 4. Identification of LAB strains based on 16S rDNA sequence similarity.

Strains of LAB	Strains	GeneBank acc. no.	Similarity (%)
B0013	*Lactobacillus plantarum*	HM058986	100
B0039	*Lactobacillus paracasei*	HM067019	99
B0055	*Lactobacillus plantarum*	HM058986	100
B0125	*Lactobacillus plantarum*	HM058694	99
B0126	*Lactobacillus plantarum*	HM058986	100
B0134	*Lactobacillus plantarum*	HM058986	100

confirmed by sequencing. The plantarum gene sequences of B0055 were 97 to 100% similarity with the GenBank access number assigned in Table 6.

DISCUSSION

Bacteriocins of LAB are active against Gram-positive bacteria, such as LAB (García-Ruiz et al., 2013) and *S. aureus* (Anyogu et al., 2014; Omar et al., 2008; Sebastià et al., 2011). However, plantaricins produced by *L. plantarum* strains had broad spectra of inhibition activity against Gram-negative bacteria including *E. coli* and *Salmonella* enterica (Anyogu et al., 2014; Omar et al., 2008). Strain *L. sakei* C2 producing a bacteriocin strongly inhibited *S. aureus* and *E. coli* (Gao et al., 2010). In our study, seven strains showed antibacterial activity against test pathogens. Rojo-Bezares et al. (2007) indicated that antimicrobial activity was lost after treatment with trypsin, α-chymotrypsin, papaine, protease, proteinase K, and acid proteases, indicating bacteriocin was peptide nature. Gao et al. (2010) indicated that after treatment by all the three kinds of protease, the antimicrobial activity of cell-free supernatant of strain *L. sake* C2 disappeared and the protein nature of this antimicrobial substance produced by strains C2 was verified. In this study, after treatment by pepsin, the inhibition zones of seven strains selected towards the tested pathogens reduced. It indicated that the substance

Table 5. Plantaricin genes detected by PCR from lactic acid bacteria with antibacterial activity.

Strains	Plantaricin gene											
	***pln*A (450 bp)**	***pln*B (165 bp)**	***pln*C (108 bp)**	***pln*D (414 bp)**	***pln*EF (428 bp)**	***pln*I (450 bp)**	***pln*J (475 bp)**	***pln*K (246 bp)**	***pln*G (453 bp)**	***pln*N (146 bp)**	***pln*V (727 bp)**	***pln*NC8 (207 bp)**
L. plantarum	+	+	+	+	+	+	+	+	+	-	+	-
L. lactis	-	-	-	-	-	-	-	-	-	-	-	-
B0013	+	+	+	+	+	+	-	-	+	-	+	-
B0039	-	-	-	-	-	-	-	-	-	-	-	-
B0055	+	+	+	+	+	+	+	+	+	-	+	-
B0105	-	-	-	-	+	-	-	-	-	-	-	-
B0125	-	-	-	-	-	-	-	-	-	-	-	-
B0126	+	+	+	+	+	+	+	+	+	-	+	-
B0134	-	-	-	-	-	-	-	-	-	-	-	-

Table 6. Identification of B0055 plantaricin gene sequences similarity.

Plantaricin gene	Gene ID	Similarity (%)
*pln*A	1064174	98
*pln*B	1061276	100
*pln*C	1061285	97
*pln*D	1064173	100
*pln*E	1064171	99
*pln*F	1061287	99
*pln*I	1061281	99
*pln*J	1064185	98
*pln*K	1064190	98
*pln*G	1061291	99
*pln*V	1061306	99

with antibacterial activity was sensitive to pepsin, where it might be considered that the bioactive compound produced by some strains might be the protein or peptide nature (Rojo-Bezares et al., 2007; Gao et al., 2010). The variation of the antibacterial activity of our test strains are almost similar to those shown by other works on the antibacterial activity of LAB (García-Ruiz et al., 2013; Anyogu et al., 2014; Gao et al., 2010).Previous studies on the microorganisms of traditional fermented vegetables and fruits had shown that *L. plantarum* was dominant among the isolates from fermented mustards (Chen et al., 2013). All test strains were identified as *L. plantarum* except B0039 as *L. paracasei. L. plantarum* is important in many food fermentations as a component of the natural microflora or as a starter culture (Gao et al., 2010; Omar et al., 2008).

Bacteriocin-producing strains of *L. plantarum* have been reported from many vegetable foods such as fermented cereal doughs, wara (Omar et al., 2008), fermented cassava (Kostinek et al., 2005) and fermented mustard (Chen et al., 2013). Bacteriocins of lactic acid bacteria are active against closely related bacteria. However, activity against Gram-negative bacteria has been described in several cases. Omar et al. (2008)showed that bacteriocins *pln*ABCD encode for the signal-transducing pathway. Variations in this operon have also been reported for the gene clusters described in *L. plantarum* from poto poto with only *pln*C being not conserved (Omar et al., 2008) and *L. plantarum* J23 from grape must, with only *pln*D being conserved (Rojo-Bezares et al., 2008). In this study, *L. plantarum* strains, B0013, B0055 and B0126 tested positive for the *pln*ABCD operon, others are not detected. In other words, variations in *pln*ABCD also exist in our strains. Two of *pln*EFI and *pln*J/K code for bacteriocins to inhibit pathogens. Genes encoding for the *pln*EFI and *pln*J/K were detected in *L. plantarum* B0055 and B0126, while *L. plantantum* B0013 only test positive for *pln*EFI. Omar et al. (2008) indicated that the *pln*EFI operon was detected in thirteen isolates from poto poto, while others only tested positive for *pln*EF or *pln*I. From our results, B0105 detected only positive of *pln*EF. Variations in *pln*J/K operon, including the absence of *pln*K and the presence of *pln*J gene have also been reported in *L. plantarum* J23 (Rojo-Bezares et al., 2008). *Lactobacillus* strains from poto poto also reported the absence of *pln*NC8 plantaricins, while *pln*N gene was absent in some strains (Omar et al., 2008). However, the genes encoding for *pln*N and *pln*NC8 were not detected in any strain. Finally, *pln*G and *pln*V gene, which is part of a large operon involved in plantaricin transport operon (Sáenz et al., 2009) were found in *L. plantarum* strains B0013, B0055, B0126 strains. With antibacterial activity, but showed negative in plantaricin gene, these findings might explain that *L. plantarum* may contain other bacteriocin genes that were not detected in this study. However, *L. plantarum* B0125 and B0134 strains which gave negative results for the PCR amplification should be investigated for possible sequence heterogeneity in the plantaricin operons. Plantaricin gene sequences of B0055 were 97 and 100% similarity with the GenBank access number. The bactericidal effect might be from the production of organic acids and/or in combination with the production of bacteriocin (Lin et al., 2008). In our results, antibacterial activity of selected strains might be from carried genes for the production of plantaricins and production of organic acids.

Conclusion

LAB isolated from fermented vegetalbes seem to have high potentials for production of antimicrobial substances, and also seem to have variations in the plantaricin genes. In this study, fermented vegetables were found to contain LAB and other pathogens. Since *L. plantarum* strains produce bacteriocins that inhibit food-borne pathogens such as *E. coli* and *S. aureus*, the use of these antimicrobial substances as food additives or use of the bacteriocin-producing strains as starters, might contribute to the production of a safer and healthier traditional fermented product (Omar et al., 2008). The findings in this study suggest that LAB producing bacteriocins can be used as alternate mechanism to inhibit the growth of drug-resistant pathogens.

Conflict of Interests

The authors have not declared any conflict of interests.

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Microbial safety criteria and quality of traditional Egyptian Karish cheese

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Some traditional fermented dairy products like Egyptian Karish cheese and traditional fermented milk (Laban Zeer) are manufactured in farmhouses following traditional techniques using raw milk without heat treatment or addition of selected starter cultures, these products are generally designed as "artisanal". The representative Karish samples were analyzed for their content of total microbial, *Enterobacteriaceae*, yeast, molds, *Staphylococci* coagulase positive, *Salmonella* spp., *Listeria* spp., in addition to detection of *Bacillus cereus, Listeria monocytogenes, Clostridium perfringens* and *Campylobacter* spp. using classical methods. The physico-chemical properties of collected samples showed that the average of protein, fat, moisture and acidity were in accepted range with that reported in literatures and Egyptian standard. Some lactic acid bacteria (*Lactococcus lactis* supsp. *lactis, Lactobacillus delbrueckii* subsp. *lactis* and *Enterococcus feacium)* were isolated and identified from fresh karish cheese using classical methods followed by rep-PCR. *Lactococcus garvieae* was detected in fresh samples using TTGE but could not be detected using classical methods. The results of microbiological analysis showed the presence of high numbers of *Enterobacteriaceae* that ranged from log_{10} 2.6 cfu/g to log_{10} 3.5 cfu/g, *Bacillus* ssp. ranged from log_{10} 4.2 cfu/g to log_{10} 5.6 cfu/g, *Staphylococci* counts are ranged from log_{10} 1.8 cfu/g to log_{10} 2.7 cfu/g, faecal enterococci count ranged from log10 3.5 cfu/g to log10 5.8 cfu/g. Yeasts and molds count ranged from log_{10} 2.7 cfu/g to log_{10} 3.5 cfu/g. *B. cereus, L. monocytogenes, C. perfringens, Shigella, Salmonella, Escherichia coli* β-glucuronidase positive and *Campylobacter* spp. were not detected in all the examined samples. The count of undesirable microorganisms indicated the poor hygienic practice of traditional Karish cheese production; starting with using poor quality of raw milk and processing under uncontrolled environments. This would require using clean raw milk, controlled manufacturing steps and selection of the appropriate starter culture for the fermentation.

Key words: Karish cheese, microbiological analysis, safety.

INTRODUCTION

Karish cheese is traditional soft type cheese commonly made in Egyptian villages at farmhouses. It is an acid

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dairy product and made from defatted milk, the cheese is traditional made by leaving the raw milk for coagulation by natural flora and then collecting the fat layer. The fat in dry matter and the moisture content in Karish cheese should not exceed 10 and 75%, respectively (Egyptian Standard 2000/4-1008).

Microbial communities of traditional fermented dairy products are complex and insufficiently characterized. Microbial diversity is considered essential for the sensory properties of traditional cheeses. However, some members of these complex communities may also constitute a health risk (Montel et al., 2014). Consequently, studying and understanding this particular ecosystem is of great interest from both public health and economic perspectives (Irlinger et al., 2015).

Karish cheese is usually produced under artisanal conditions using raw milk without heat treatment. Numerous microorganisms, including bacteria, yeasts and molds constitute the complex ecosystem in traditional Karish cheese were analyzed using classical methods. The analysis results showed extremely high numbers of *Staphylococcus* ssp. (3.6 $x10^5$ cfu/g), coliform (18.9 $x10^3$ to 6.9$x10^7$ cfu/g); faecal enterococci (8$x10^6$ cfu/g), *Enterobacteriaceae* (in the range of 1.01 x 10^6 to 1.34 $x10^8$ cfu/g) which was reported by El Leboudy (1998), Said and Fahmy (1991), El–Kholy (1989) and Tawfek et al. (1988).

In the last decades, culture-independent techniques were used to study the total microbial communities in fermented dairy products. Results of culture-independent techniques have greatly improved our understanding of their composition, dynamics and activities (Irlinger and Mounier, 2009). When compared with culture-based methods, PCR is faster, more sensitive, more specific and enables detection of sub-dominant populations, even in the absence of a selective enrichment medium and in the presence of other (dominant) populations. Moreover, it allows detection of dead cells or viable but non-cultivable cells (Delbès et al., 2007; Irlinger et al., 2015). The origin, safety and functional role of microbial communities in Karish cheese making are still not well understood. The microbial communities, present on Karish cheese, largely could not be completely defined by classical methods. For this purpose, semi-systematic approach based on genetic profiling by temporal temperature gradient electrophoresis (TTGE) has been used by El-Baradei et al. (2005) to describe the bacterial ecosystem of some samples of Karish cheese, but the analysis of cheese during storage has not been done. So, the aims of this work were to evaluate the lactic acid bacteria (LAB) involved in the cheese fermentation and cheese safety by defection of the pathogenic bacteria. Evaluation of lactic acid bacteria was carried out using both culture dependent (enumeration, isolation and genotypic characterization of bacteria strains on selective medium) and independent (PCR-TTGE analysis) methods. Undesirable microbial groups (e.g., *Bacillus*, coliform and *Enterobacteriaceae)* and pathogenic bacteria (for example, *Staphylococcus aureus* coagulase positive, *Bacillus cereus, Listeria monocytogenes, Clostridium perfringens, Shigella, Salmonella, Escherichia coli* β-glucuronidase positive and *Campylobacter* and) were also analyzed by classical methods. Also, texture profile analysis and physico-chemical properties were also examined.

MATERIALS AND METHODS

Samples

Fifteen (15) samples,of traditional Karish cheese made using raw milk, were collected from local markets in different Governorates of Egypt (Alexandria, Behira, Kafer El Sheick, Domiata, Gharbia). The fresh cheeses were transferred into ice box to the laboratory and stored at refrigerator temperature (5 ± 1°C) for 15 days.

Physico-chemical analysis

Total protein was determined by Kjeldahl (AOAC, 2003) and fat content was measured by Gerber method (AOAC, 2003). Titratable acidity was expressed as percentage of lactic acid content of cheese by weight. pH value was measure in well-mixed ground cheese samples. Moisture analyzer (Mettler Toledo Model HR73) was used to determine the moisture content. Chloride meter (Jenway, England, UK) was used to determine sodium chloride in cheese samples.

Texture profile analysis

Karish cheese cubes (50 x 50 x 50 mm) were prepared and placed in plastic cups, sealed (to prevent dehydration) at temperature of 10 ± 0.5°C prior to analysis. A two-bite penetration test was performed using the Texture Analyzer (TA1000, CNS-Farnell, England) operated at a crosshead speed of 1 mms $^{-}$1 and compressed to 50%. Hardness, cohesiveness, springiness and chewiness were evaluated in triplicate according to the definitions given by Bourne (1978).

Microbiological analysis

Undesirable bacterial group were plated on selective agar media as follows:

1. Brilliant green agar for *Enterobacteriaceae* (ISO 21528-2-2004)
2. Violet red bile agar for coliform group (Difico 1998)
3. Xylose lysine deoxycholate agar (XLD, Biolife, Italy) was used for *Salmonella and Shigella* spp. (Mahon and Manuselis, 1995).
4. Oxford agar (Biolife, Italy) for *L. monocytogenes* (Anon, 1995 and ISO 11290-1/A1:2004).
5. Baird-parker medium for *Staphylococcus* spp. (ISO 11867, 1997)
6. Mannitol-egg yolk-polymyxin agar, (MYP, Biolife, Italy) for *B. cereus* (ISO 7932, 2004).
7. Modified-*Campylobacter* blood free selective agar base (modified-CCDA, Oxoid, England) for *Campylobacter* spp. (ISO 10272-1, 2006).
8. Sulfite-cycloserine agar (SC, Biolife, Italy) for *Clostridium perfringens* (ISO 7937, 2004).
9. Oxytetracycline glucose yeast extract agar for yeast and molds

(O.G.Y.E agar, LAB, United Kingdom) (ISO 6611, 2004).
10. Tryptone bile X-glucuronide agar (TBX, Biolife, Italy) for *E. coli* β-glucuronidase positive (ISO, 16649-1, 2001).

Isolation and identification of lactic acid bacteria by Rep-PCR

Lactic acid bacteria were isolated and pre-identified according to the methods described by El Soda et al. (2003). The isolates were identified using rep-PCR as described by Mohammed et al. (2009). The pellet of each isolate was obtained by centrifugation of 1.6 ml of fresh culture in log phase at 5000 xg for 10 min at 10°C and the then the pellet was washed twice with sterile deionized water and stored overnight at 4°C. Total DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, Wiscon). DNA concentration of each isolate was adjusted to 25 ng/µL, to which 0.3 µM BOXAIR primer (5′CTACGGCAAGGCGACGCTGACG-3′) was added (Biolegio BV, Nijmegen, The Netherlands). Ready-To-Go PCR beads (Amersham Biosciences) that included 200 µM deoxynucleoside triphosphate, 2.5 U puRe Taq DNA polymerase (Amersham Biosciences), 10 mM Tris–HCl pH 9, 50 mM KCl, 1.5 mM $MgCl_2$ and sterile deionised water to reach a final volume of 25µL were also added. PCR amplification was performed in a Flexigene Thermal Cycler (Techne, Duxford, Cambridge, UK) according to the polymerase chain reaction (PCR) programme (De Urraza et al., 2000). PCR products were separated by electrophoresis at 50 V on a 2% (w/v) agarose gel (Amersham Biosciences) and DNA was detected by UV trans-illumination after staining with ethidium bromide (10 mg/mL). The molecular sizes of amplified DNA fragments were estimated by comparison with a 100 bp DNA ladder (Promega) and were photographed using the Digimage System (Major Science, Pan-Chiao City, TaipeiHsien, Taiwan). The Rep profiles were processed using Gel ComparII version 5.00 software (Applied Maths, Kortrijk, Belgium). Pearson correlation coefficient was used and UPGMA was done to cluster the different groups.

Temporal temperature gradient gel electrophoresis (TTGE)

Each Karish cheese sample (5 g) was dissolved in 40 ml of sterile trisodium citrate 2% and homogenized by Ultra-Turrax mechanical blender at 18000 rpm for 1 min until the solution was opaque. 50 mg of Pronase (Protease, from Streptomyces griseus- SIGMA-ALDRICH) and 100 µl of β- mercaptoethanol were added to each sample. This was followed by 3 h incubation at 50°C. Both cheese fats and proteins were removed by centrifugation at 10.000 rpm/15min at 4°C. The pellet of bacteria was washed twice with TES buffer (25 mM tris-HCl, 0.1 M EDTA, 25% (wt/vol) sucrose pH 8) and centrifuged. The cells were resuspended in 480 µl of TE buffer and 120 µl of lysozyme followed by incubation at 37°C for 1 h and centrifugation at 10.000 rpm/10min at 4°C. The supernatant was removed and total DNA was extracted from bacterial cells (pellet) as described using Wizard Genomic DNA purification Kit with the manufacturer's (Promega, Madison, Wisconsin).

Forty five different bacterial species or subspecies of bacteria with low G+C genomes were selected for reference strains setup belonging the genera *Lactobacillus*, *Lactococcus, Enterococcus, Leuconostoc*, *Streptococcus* and *Pediococcus*.

Nested-PCR

The V3 region of the 16S rRNA gen is the substrate for PCR amplification. The extracted DNA was amplified by two successive PCR amplifications as described by Ogier et al. (2002).

TTGE analysis

PCR products obtained fromV3 region amplifications were subjected to TTGE analysis. TTGE was performed by using the Dcode Universal mutation detection system (Bio Rad) that were 16 x 16 cm x 1 mm according to the method described by Ogier et al. (2004).

Statistical analysis

Data reported are the average of three measurements. The SAS statistical analysis software package (SAS, 1999) was used for analysis of variance. Differences were considered significant at $P<0.05$.

RESULTS AND DISCUSSION

Physical and chemical analysis of Karish Cheese

The physico-chemical properties of Karish cheeses are summarized in Table 1. The pH value was ranged from 4.21 to 4.65 in fresh cheeses and it decreased from 3.98 – 4.35 after 15 days of storage, while the average of acidity was 1.56% of fresh cheese and increased to 1.75% after 15 days of storage. The moisture content ranged from 66.8 to 73.4% with an average of 69.5% in fresh cheese and decreased to 66.5% after 15 days of storage, this shows fat and protein content which increased after 15 days of storage, while the salt content was not changed during storage. The fat in Karish cheeses was variable in most of the samples; the minimum fat level was 3.4%, while the maximum fat level was 6.4% with an average of 5.2% in fresh samples. The protein level of cheese ranged from 15.4 to 19.4% in fresh samples with average of 17.8%. The data of physic-chemical analysis of Karish cheese was similar with that reported by Todaro et al. (2013) and Egyptian Standard (1008/4/2000). Various factors affect the chemical composition and physical properties of Karish cheese like, this include the milk type, season of milk production, fat separation method used, micro flora of the milk and/or starter addition, additives in the cheese milk, storage time and temperature. The composition of Karish cheese is in the range of 17.7 to 22.5% protein, 2.2 to 5.8% fat and 64.8 to 71.7% moisture (Todaro et al., 2013).

Texture profile analysis of Karish cheese

The texture profile analysis including hardness, adhesiveness, cohesiveness, springiness, gumminess and chewiness of Karish cheese samples are shown in the Table 2. There was a relationship between chemical composition and texture profile analysis as the hardness increased after 15 days of storage with decrease in the moisture content. The hardness and chewiness are both increased at 15 days of storage due to the increase in protein content and decrease in the moisture content and

Table 1. Physico-chemical analysis of traditional Karish cheese.

Storage/days	pH value		Acidity (%)			Fat (%)			Protein (%)			Moisture			Salt (%)		
	Min	Max	Min	Max	Ave	Min	Max	Ave	Min	Max	Ave	Min	Max	Ave	Min	Max	Ave
1	4.21[a]	4.65[a]	1.25[b]	1.71[b]	1.56[b]	3.4[b]	6.4[b]	5.2[b]	15.4[b]	19.4[b]	17.8[b]	66.8[a]	73.4[a]	69.5[a]	0.2[a]	0.4[a]	0.3[a]
15	3.98[b]	4.35[b]	1.45[a]	1.98[a]	1.75[a]	3.6[a]	6.6[a]	5.5[a]	16.4[a]	20.1[a]	18.9[a]	64.5[b]	71.5[b]	66.5[b]	0.2[a]	0.4[a]	0.3[a]

Each value is the average of three replicates. Min: the minimum value of 15 samples, Max, is the maximum value of 15 samples, Ave: is the average of 15 samples; [a–b] Means within a column with no common superscript differ ($P < 0.05$).

Table 2. Texture profile analysis of traditional Karish cheese.

Days	Hardness (g)	Cohesiveness ratio	Adhesiveness (gs)	Springiness (mm)	Gumminess (gs)	Chewiness (g)
0	128	0.78	5.2	7.4	99.84	738.81
15	174	0.78	5.2	7.4	135.72	1004.32

Each sample has analyzed three times and the present value is the average of 15 samples.

pH value. Many factors influence texture development of Karish cheese. These include those factors that affect the curd moisture content (temperature of coagulation and drain of whey), cheese composition, pH, interactions between casein and serum proteins, Ca content, ionic strength, salt content, and manufacturing protocol, especially rate and extent of acid development. Fat content in the cheese is responsible for its many desirable functional and texture. In addition, decreasing moisture content might result in decrease in the level of free moisture in cheese; this increased the hardness (Awad, 2011).

Microbiological analysis of Karish cheese samples

Enterobacteriaceae and coliform bacterial count

The results of coliform bacterial count of fresh Karish samples on VRB agar ranged from $\log_{10}$ 3.4 to $\log_{10}$ 5.2 cfu/g with an average of $\log_{10}$ 4.6 cfu/g (Table 3). *Enterobacteriaceae* count of fresh Karish samples on Brilliant Green agar ranged from $\log_{10}$ 2.6 to $\log_{10}$ 3.5 cfu/g, there was no bacterial growth in only one sample. The Egyptian Standard for Karish cheese (1008/4/2000) recommended that the coliform bacteria should be not more than 10 cfu/g. The high level of *Enterobacteriaceae* and coliform bacteria in Karish cheese indicated the poor hygienic practice during processing of the cheese. The results are in agreement with Aboul Kheir et al. (1985). They found *Enterobacteriaceae* with average of 1.01×10^6 cfu/g in more than 85% of Karish cheese samples. *E. coli* was in about 81% of examined samples. While El-Kholy (1989) found higher count of *Enterobacteriaceae* in fresh Karish cheese with an average of $1.34 \pm 0.93 \times 10^8$ cfu/g and *E. coli* was detected in 23% of samples. Karish cheese samples collected from different localities in Fayoum city, Egypt, contain high level of coliform and faecal enterococci at average level of 18.9×10^3 and 8×10^6 cfu/g of cheese, respectively (Tawfek et al., 1988). High levels of coliform bacterial group were also reported in Karish cheese by Metwalli (2011).

Escherichia coli β-glucuronidase positive

Tryptone-bile-x-glucuronide (TBX) agar was used for the detection of *E. coli*. After the period of incubation, there were no bacteria growth (blue or blue green colonies) in all tested Karish samples. Svanberg et al. (1992) and Svanberg (1996) reported that lactic acid-fermented gruels inhibited the proliferation of Gram-negative pathogenic bacteria including toxicogenic *E. coli.* The Egyptian Standard for Karish cheese (1008/4/200) recommended that the cheese should be free from *E. coli*.

Table 3. Microbiological analysis of Karish samples.

Number of days	Log_{10} CFU/g on VRA			Log_{10} CFU/g on Brilliant Green			Log_{10} CFU/g on MYP agar			Log_{10} CFU/ g on SF agar			Log_{10} CFU/g on Baired Parker agar			Log_{10} CFU/ g on MRS agar			Log_{10} CFU/g On O.G.Y.E agar		
	Min	Max	Ave	Min	Max	Ave	Min	Max	Ave	Min	Max	Ave	Min	Max	Ave	Min	Max	Ave	Min	Max	Ave
1	3.4	5.2	4.6	2.6	3.5	3.2	4.2	5.6	5.2	3.5	5.8	4.9	1.8	2.7	2.6	6.7	9.8	8.5	2.7	3.5	3.4
15	2.5	4.4	3.2	1.8	2.5	2.2	1.5	2.2	1.8	2.5	4.5	4.0	0	2.6	1.3	5.9	7.8	7.6	3.4	3.8	3.6

Three to six dilutions of each sample were analyzed. Min: the minimum value of 15 samples, Max, is the maximum value of 15 samples, Ave: is the average of 15 samples.

Detection of *Shigella* and *Salmonella* spp.

Desoxycholate and xylose-lysine-desoxycholate (XLD) agars are intermediate selective media and are preferred media to isolate *Shigella and Salmonella* spp. *Shigella* colonies on XLD agar medium are translucent and red (alkaline). The results of this study confirmed the absent of *Shigella* and *Salmonella* in all examined samples.

Bacterial count on MYP agar

The microbial count relating to *Bacillus* ssp. of Kishk samples on MYP agar are variable and the counts on the medium ranged from log_{10} 4.2 to log_{10} 5.6 cfu/g with an average of log_{10} 5.2 cfu/g (Table 3).

It is obvious from the results that all the examined samples contained *Bacillus* ssp. This may be due to the use of raw milk in Karish cheese making and the contamination of the milk from the soil and during the Karish cheese processing. There was significant reduction of bacilli count of Karish cheese samples after 15 days; these is mostly related to the low pH in Karish cheese. Wong and Chin (1988) reported that lactate (0.1 M) completely inactivated multiplication of *B. cereus* at pH 5.6. In general, due to the lower pH value (~4.2- 4.6), fermented milks is not a suitable environments for the majority of spoilage-causing bacteria (Samarzija et al., 2012).

The presumptive colonies on MYP medium were tested for hemolysis on blood agar base No 2. α-Haemolysis was not observed in all Karish samples, while β-hemolysis was represented in 4 out of 15 samples.

***Staphylococci* count on Baird Parker agar and detection of Coagulase-positive *Staphylococci* count**

Results of *Staphylococci* count for kishk samples showed that the *Staphylococci* counts on Baird Parker agar ranged from log_{10} 1.8 to log_{10} 2.7 cfu/g (Table 3). The presumptive colonies were tested for coagulase using dry-spot staphytect, after inoculation in brain heart infusion broth and streaking colonies on Baired Parker agar. Typical colonies on Baird Parker medium are black to gray, brilliant and convex and surrounded by a clear zone, which can be partially opaque and confirmed by coagulase test using dry-spot staphytect. Coagulase-positive *Staphylococci* were detected in five out of fifteen samples. This is also related to poor hygienic practice in cheese manufacturing, these samples are not within Egyptian Standard for Karish cheese (1008/4/2000) as it is recommended that the cheese should be free from Coagulase-positive *Staphylococci. S. aureus* is usually the dominant pathogens in traditional Karish cheese. This microbe was found in about 10% of examined karish cheese samples by Abou-Donia (1984**)**. *S. aureus* was isolated from 66 and 78% of the examined Karish cheese samples by Tawfek et al. (1988) and by Said and Fahmy (1991), respectively. *S. aureus* count was 3.6 $x10^5$ cfu/g and 41% of the isolates were coagulase positive (Said and Fahmy, 1991).

Detection of *C. perfringens*

Sulfite-cycloserine (SC) agar was used for *C. perfringens* count. After incubation, the black were calcaluted. There was no growth in 12 out of 15 tested Kishk samples and presumptive colonies were inoculated into fluid thioglycollate medium then five drops of thioglycollate medium. Cultures were further examined for the production of gas and the presence of a black colour in LS medium. Durham tubes more than one-quarter full of gas and tubes having a black precipitate are considered positive for the occurrence of *C. perfringen*s. From the results, the occurrence of *C. perfringen*s was none in the examined samples.

Lactic acid bacterial count

Enterococci were enumerated on SF agar. Lactobacilli were enumerated on MRS agar and lactococci were enumerated on M17 agar. Enterococci count ranged from $\log_{10}$ 3.5 to $\log_{10}$ 5.8 cfu/g with an average of $\log_{10}$ 4.9 cfu/g. Concerning, Lactobacilli ranged from $\log_{10}$ 6.7 to 9.8 cfu/g with an average of $\log_{10}$ 8.5 cfu/g, while lactococci were in the range of $\log_{10}$ 6.3 to 7.8 cfu/g with an average of $\log_{10}$ 6.9 cfu/g (Table 3). The lactic acid bacterial count is related to the raw milk used in making the Karish cheese.

Detection of L. monocytogenes

The presumptive colonies were streaked onto tryptone soya yeast extract broth. Colonies were confirmed with appropriate morphological test (Gram positive, slim and short rods) and some biochemical tests such as catalase test (3%), the positive catalase reaction indicated *L. monocytogenes*. The obtained results from all Karish samples confirmed all the examined samples were free of *L. monocytogenes.*

Detection of *Campylobacter* spp.

The representative samples were analyzed for the occurrence of *Campylobacter* spp. The results of all the samples confirmed that the detected colonies in the selective medium did not belong to *Campylobacter* spp. Svanberg et al. (1992) and Svanberg (1996) reported that lactic acid-fermented gruels inhibited the proliferation of Gram-negative pathogenic bacteria including *Campylobacter jejuni.*

Yeasts and molds

The results of yeasts and molds count of fresh Karish samples on oxytetracycline glucose yeast extract agar ranged from $\log_{10}$ 2.7 to $\log_{10}$ 3.5 cfu/g with an average of $\log_{10}$ 43.4 cfu/g, the average of yeasts and molds increased to $\log_{10}$ 3.6 cfu/g after 15 days. The Egyptian Standard for Karish cheese (1008/4/200) recommended that the yeasts should not exceed 400 cfu/g while the molds should not exceed 10 cfu/g. The high level of yeasts and molds in Karish cheese indicated the poor hygienic practice during processing of the cheese. Yeasts and molds were also presented at high level: 7.1 $x10^6$ cfu/g in Karish cheese samples (Abou-Donia et al., 1975).

The diversity of lactic acid bacteria using TTGE and rep-PCR

The TTGE database of standard strains was used to identify major bacterial populations present in 5 samples of traditional Karish cheese at 1 day and after 15 of manufacturing as shown in Table 4. The obtained results indicated that the *L. lactis* and *L. delbrueckii* subsp. *lactis* are the predominant species in fresh and 15 days old cheese. Only these both species were detected in all fresh samples by both methods “dependent and independent”. *L. delbrueckii* subsp *lactis* was also detected by TTGE in all five samples after 15 days of storage, while the *L. Lactis was* detected in only four samples out of five after 15 days of storage.

L. garvieae was detected by only TTGE in 3 fresh samples out of 5; it was detected in same samples after 15 days, while *L. garvieae* could not be detected by dependent methods in all the five samples. *Enterococcus feacium* was detected in 4 fresh out of 5 samples by TTGE, while it was detected in only 2 samples by classical methods.

L. plantarum was detected by TTGE in 3 fresh samples (2, 4 and 5) but it was detected in only one sample by rep-PCR (2), while it was detected in 4 samples after 15 days of storage (1, 3, 4, 5). *L. acidophilus* was also detected by TTGE and rep-PCR in 3 samples (1, 3, 5), *L. fermentum* was detected by TTGE and rep-PCR in 3 samples (3, 4, 5). *L. acidophilus* and *L. fermentum* was detected by TTGE in same fresh and 15 days old samples. *S. thermophilus* was detected in 3 fresh samples by TTGE and in only 2 samples by rep-PCR. *Leuconostoc mesenteroides* was detected in 3 samples by TTGE and in one sample by rep-PCR while *Leuconostoc lactis* was detected in 2 samples by TTGE and in one sample by rep-PCR. In comparing between directed and indirect methods in detecting the bacterial culture during storage of traditional Karish cheese, it is clear that more strains could be detected using TTGE than direct method. *L. garvieae* was detected in fresh samples by using TTGE and could not be detected using directed method. *S. thermophilus* was detected in 3 samples by TTGE while it detected in only one sample by rep-PCR. All the strains that were detected by rep-PCR were also detected by TTGE except *L. lactis* and *L. mesenteroides* which were detected in one sample by only rep-PCR. Moreover, *L. garvieae,* which is a dominant population in Karish cheese by TTGE-PCR, has never been detected on lactococcal counting plates, same finding was also found in traditional Egyptian Domiati cheese (El-Baradei et al., 2007) and in traditional, Spanish, blue-veined Cabrales cheese (Flórez and Mayo 2006). TTGE confirmed 10 different lactic acid bacterial species that are present in fresh karish cheese and some strains could be isolated and identified by traditional analyses of microbiota using cultivation on specific or non-specific growth agars. The weakness of phenotypic methods include poor reproducibility and discriminatory power, laboriously investigations, and the ambiguity of some techniques caused by complex growth conditions (Cocolin et al., 2007). It is well documented

Table 4. Lactic acid bacteria biodiversity of Karish cheese during storage.

Cheese age	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5	
	TTGE	Rep-PCR	TTGE	Rep-PCR	TTGE	Rep-PCR	TTGE	Rep-PCR	TTGE	Rep-PCR
Lc .garvieae	+	-	+	-	-	-	+	-	-	-
E .faecium	+	+	-	-	+	+	+	-	+	-
Lb.del. subsp.*lactis*	+	+	+	+	+	+	+	+	+	+
Lb. plantarum	-	-	+	+	-	-	+	-	+	-
Lc. Lactis	+	+	+	+	+	+	+	+	+	+
Lb acidophlus	+	+	-	-	+	+	-	-	+	+
Lb. fermentum	-	-	-	-	+	+	+	+	+	+
Ln. mesenteroides	+	-	+	-	-	-	-	+	+	-
Ln. lactis	+	-	+	-	-	-	-	-	-	+
Str. thermophilus	+	-	-	-	+	+	-	-	+	-
Lc. garvieae	+		+		-		+		-	
E. faecium	+		-		+		+		-	
Lb.del. subsp. *lactis*	+		+		+		+		+	
Lb. plantarum	+		-		+		+		+	
Lc. Lactis	-		+		+		+		+	
Lb. acidophlus	+				+		-		+	
Lb. fermentum	-		-		+		+		+	
Ln. mesenteroides	+		+		-		-		+	
Ln. lactis	+		+		-		-		+	
Str. thermophilus	-		-		+		-		+	

Gels were analyzed using database with 140 reference strain.

that stressed or injured cells could not be recovered in selective media and that cells present in low numbers are very often inhibited by other microbes (Hugenholtz et al., 1998). For these reasons, it is important to have methods that allow monitoring of the microbial populations without cultivation by culture-independent methods (Cocolin et al., 2004; Mangia et al., 2015). The culture-independent methods allowed us to confirm the dominant bacterial community of Karish cheeses that are almost identified in each sample "*L. lactis subsp. lactis, L. delbrueckii* subsp. *lactis*, *L. acidophlus* and *L. lactis"*. These bacteria may play the main role in the fermentation and organoleptic properties of Karish cheeses because of their common presence in the tested samples.

Conclusion

Traditional cheeses like Karish are widely consumed and also contribute to the Egyptian culture. Microbial diversity is considered essential to the sensory richness and safety of Karish cheeses. Although, traditional Karish cheese is made from raw milk without the addition of any starters, most of the bacteria identified in this study as dominant are lactic acid bacteria. However, some members of these complex communities were identified as responsible for cheese flavor defects (coliform group, *Enterobacteriaceae*) and some other constitute a health risk (coagulase-positive *Staphylococci*). The results of this study confirmed the improvement in Karish cheese manufacturing which is necessary to obtain a safe

and homogenous product.

Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Systemic elicitation of defense related enzymes suppressing *Fusarium* wilt in mulberry (*Morus* spp.)

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Antagonist and organic amendments based bioconsortia (seri bed waste+Pf1+Bs4+Th1+neem cake) was found to lead the restriction of *Fusarium solani* pathogen in mulberry roots mainly by inducing inherent defense enzymes. Induction of defense enzymes such as peroxidase, polyphenoloxidase, phenylalanine ammonia lyase, phenols, catalase and superoxide dismutase, was studied in mulberry plants pretreated with bioconsortia and comparative fungicides challenged inoculation with *Fusarium solani* in glasshouse condition. There was increased expression of defense enzymes in mulberry plants treated with bioconsortia, when compared to control. The bio-chemical reaction of the above defense enzymes started to increase at the 3rd day, reached maximum on the 5th day and thereafter declined gradually. The native polyacrylamide gel electrophoresis (PAGE) experiment showed that one to four isoforms of the defense enzymes each with greater intensity were expressed in these treatments. This is indicating that the restriction of *F. solani* in mulberry plants was mainly due to application of microbial bio-consortia.

Key words: Bio-consortia, defense enzymes, *Fusarium* wilt, isoforms, mulberry.

INTRODUCTION

Mulberry (*Morus* spp.) is a fast growing deciduous woody perennial plant belonging to the genus *Morus* and grows under various climatic conditions ranging from temperate to tropical regions. Mulberry foliage is the best food for the silkworm (*Bombyx mori L.*). The total acreage of mulberry in India is around 282,244 ha. Cultivation of mulberry is one of the most important aspects in sericulture industry. The diseases have become more alarming because of its epidemic nature and propensity to kill the plant completely. Among the various diseases, *Fusarium* wilt of mulberry is considered as severe disease leading to death of entire plant. In India, the wilt is caused by soil borne fungal pathogen *Fusarium solani* (Siddaramaiah and Hegde, 1990).

Although fungicides are effective in the management of the diseases, they leave harmful residues in the soil, causing environmental pollution and other deleterious effects on the ecosystem (Misato and Yamayuchi, 1977). The biological control method has been considered as a promising approach

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for the management of soil borne diseases by enhancing the inherent systemic resistance. However the most of the approaches mainly biological control of plant disease have used a single organism. The application of a single antagonist is not likely to be better in all environmental conditions where it is applied. Thus more emphasis is laid on the combined used of two or more antagonist with preferred substrates, which has turned out to be more successful than either of them alone, as reported by several researchers (Thilagavathi et al., 2007; Sundaramoorthy et al., 2013). Antagonistic microorganisms *viz., Trichoderma harzianum, Pseudomonas fluorescens* and *Bacillus subtilis* were evaluated in host plants individually and in combinations for their resistance inducing ability against *F. solani* pathogen in mulberry crop. Plant has endogenous defense mechanisms that can be induced in response to attack by insects or pathogens. Defense reaction occurs due to the accumulation of PR-proteins, phytoalexins, chalconesynthase, phenylalanine ammonia lyase (PAL), peroxidase (PO), catalase (CAT) polyphenoloxidase (PPO), superoxide dismutase (SOD) and phenolics (Manikandan and Raguchader, 2014). Considering the importance of mulberry, destructive nature of the disease, the present studies were undertaken to study the induction of defense-associated enzymes against *F. solani* of mulberry using potential biocontrol agents (Choudhari et al., 2012).

MATERIALS AND METHODS

Collection and maintenance of biocontrol agents

Ten (10) isolates of *Bacillus* spp., 7 isolates of *Pseudomonas* spp*.,* and 10 isolates of *Trichoderma* spp., were isolated from the rhizosphere soil samples collected from mulberry fields by serial dilution (Pramer and Schmidt, 1956) using *Trichoderma* selective medium for *Trichoderma* spp., King's B (KB) medium for *Pseudomonas* spp. and nutrient agar (NA) for *Bacillus* spp. One isolate of *Pseudomonas fluorescens* (Pf1) and one isolate of *Trichoderma harzianum* (Th1) were obtained from the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore. Each of agents were further purified and sub-cultured in the selective medium. The efficient strains of *T. harzianum* and with plant growth-promoting rhizobacteria (PGPR) bacteria biocontrol agents, *P. fluorescens* and *B. subtilis* were evaluated for their antagonistic activity against *F. solani* by dual culture technique (Dennis and Webster, 1971).

Pot culture experiment

A pot culture experiment was laid out in completely randomized design to test the efficacy of fungicides (carbendazim, pre mixture fungicide carbendazim + mancozeb, tebuconazole) and selected compatible biocontrol agents (Pf1, Bs4 and Th1) along with seri-bed waste and neem cake. Potting medium (red soil: cow dung: manure at 1:1:1 w/w/w) was autoclaved twice and filled in pots. The culture of *F. solani* was mass multiplied separately in sand maize medium and inoculated separately in different pots grown with mulberry plants at 5% (w/w) around collar region. The pathogen alone inoculated served as control. Three replications (three pots per replication) were maintained and the pots were arranged in a randomized manner. The incidence of *F. solani* wilt disease was recorded on 60 days post inoculation (dpi). The treatments details were as follows.

Treatments

T_1: Soil drenching of consortia (seribed waste at 100 g+ Pf_1 at 10 g + Bs_4 at 10 g+ Th_1 at 50 g + neem cake at 30 g/plant) at 200 g/plant
T_2: Soil drenching of *Pseudomonas fluorescens* (Pf_1) at 10 g/plant.
T_3: Soil drenching of *Bacillus subtilis* (Bs_4) at 10 g/plant.
T_4: Soil drenching of *Trichoderma harzianum* (Th_1) at 50 g/plant
T_5: Soil drenching of tebuconazole at 0.1%
T_6: Soil drenching of carbendazim at 0.1%
T_7: Soil drenching of carbendazim + mancozeb at 0.1%
T_8: Control (untreated)

Collection of samples and enzyme extraction

Leaf samples were collected from bottom portion of the plant at two days interval up to 9 days. Two leaves from each replication of the treatment were collected for biochemical analysis. The leaf tissues were homogenized with liquid nitrogen. One gram of powdered sample was extracted with 2 ml of 0.1 M sodium phosphate buffer (pH 7.0). The homogenate was centrifuged for 20 min at 10,000 rpm at 4°C. The supernatant was used as enzyme extracts and used for the assay of phenylalanine ammonia lyase, peroxidase, polyphenoloxidase, superoxide dismutase and catalase enzymes.

Assay of defense related enzymes

Assay of phenylalanine ammonia lyase (PAL)

PAL activity was determined as the rate of conversion of L-phenylalanine to *trans*-cinnamic acid at 290 nm. 0.4 ml of enzyme extract was incubated with 0.5 ml of 0.1 M borate buffer, pH 8.8 and 0.5 ml of 12 mM L-phenylalanine in the same buffer for 30 min at 30°C. Enzyme activity was expressed in fresh weight basis as nmol *trans*-cinnamic acid $min^{-1}mg^{-1}$ of sample (Dickerson et al., 1984).

Assay of peroxidise (PO)

Fresh mulberry leaves (1 g) were homogenized in 3 ml of 0.1 M sodium phosphate buffer (pH 7.0) with pre-chilled mortar and pestle. The homogenate was centrifuged at 18,000 rpm at 4°C for 15 min. The supernate served as an enzyme source and was used within 2 to 4 h. To a spectrophotometric sample cuvette, 3 ml of sodium phosphate buffer solution, 0.05 ml guaiacol solution, 0.1 ml enzyme extract and 0.03 ml H_2O_2 solution were added and mixed well. The absorbance was recorded at 420 nm using spectrophotometer. The enzyme activity was expressed as changes in absorbance min^{-1} g^{-1} of fresh tissue (Hammerschmidt and Kuc, 1982).

Assay of polyphenoloxidase (PPO)

The polyphenoloxidase activity was determined as the procedure given by Mayer et al. (1965). The reaction mixture consisted of 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5) and 200 µl of the enzyme extract. To start the reaction, 200 µl of 0.01 M catechol was added and the activity was expressed as change in absorbance min^{-1} g^{-1} of protein.

Assay of phenol

One gram of the mulberry leaves was ground in 10 ml of 80% methanol. The homogenate was centrifuged at 10,000 rpm for 20 min. The supernate was evaporated to dry and the residue was dissolved in 5 ml of

distilled water. 0.2 ml of solution was taken and was made up to 3 ml with distilled water and then 0.25 ml of (1 N) Folin-Ciocalteau reagent was added. After 3 min, 1 ml of 20% sodium carbonate was added and mixed thoroughly. Thus the tubes were placed in boiling water for one minute and cooled. The absorbance was measured at 725 nm against a reagent as blank. The phenol activity was expressed as µg of catechol g^{-1} of plant tissue (Zieslin and Ben-Zaken, 1993).

Assay of catalase (CAT)

CAT activity was assayed spectrophotometrically as described by Chaparro-Giraldo et al. (2000) using 3 ml assay mixture containing 100 mM potassium phosphate buffer (pH 7.5), 2.5 mM H_2O_2 prepared immediately before use and 100 µl enzyme extract. The activity was measured by monitoring the degradation of H_2O_2 using UV-visible spectrophotometer (Varian Cary 50) at 240 nm over 1min, against a plant extract-free blank. The decrease in H_2O_2 was followed as the decline in optical density at 240 nm, activity was calculated using the extinction coefficient ($\varepsilon_{240\ nm}$ = 40 mM^{-1} cm^{-1}) for H_2O_2 and expressed in µmol min^{-1} mg^{-1} of sample.

Assay of superoxide dismutase (SOD)

The enzyme extracts were prepared by homogenizing 1 g of the mulberry leaf in 2 ml of 0.2 M citrate phosphate buffer (pH6.5) at 4°C. The homogenate was centrifuged at 15,000 rpm at 4°C for 30 min. The supernate served as enzyme source and SOD activity was determined as its ability to inhibit the photochemical reduction of NBT (Giannospolitis and Ries, 1977). The assay mixture (3 ml) contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 2 µM riboflavin, 0.1 mM EDTA, 100 µl of the enzyme extract and the riboflavin which was added at the end. Tubes were shaken and placed under a 40-W fluorescent at 25°C. The reaction was initiated and terminated by turning the light on and off respectively. The absorbance at 560 nm was measured against identical non illuminated in parallel to the sample tubes for blank. Each extract was subtracted from the blank and multiplied by 100 to obtain the percentage inhibition of NBT-photoreaction. The SOD activity was expressed in SOD units g^{-1}tissue (50% NBT inhibition=1 unit)

Native anionic polyacrylamide gel electrophoresis

Peroxidase (PO)

To study the expression pattern of different isoforms of peroxidases in different treatments, native anionic polyacrylamide gel electrophoresis was carried out. One gram of mulberry leaf tissue was homogenized in 2 ml of 0.01 M potassium phosphate buffer (pH 7.0), centrifuged at 10,000 rpm for 15 min at 4°C and the supernatant was used as enzyme source. For native anionic polyacrylamide gel electrophoresis (PAGE) resolving gel of 8% acrylamide concentration and stacking gel of 4% acrylamide concentration were prepared. After electrophoresis the gels were incubated in a solution containing 0.15% benzidine in 6% NH_4Cl for 30 min in darkness. Then a few drops of 30% H_2O_2 were added with constant shaking until the appearance of bands. After staining the gel was washed with distilled water and photographed (Sindhu et al., 1984).

Polyphenoloxidase (PPO)

One gram of mulberry leaf tissue was homogenized in 2 ml of 0.01 M potassium phosphate buffer (pH 7.0), centrifuged at 10000 rpm for 15 min at 48°C and the supernatant was used as an enzyme source. After native electrophoresis, the gel was equilibrated for 30 min in 0.1% *p*-phenylenediamine in 0.1 M potassium phosphate buffer (pH 7.0) followed by 10 mM catechol in the same buffer. The addition of catechol was followed by a gentle shaking which resulted in the appearance of dark brown discrete enzyme bands. After staining, the gel was washed with distilled water and photographed (Jayaraman et al., 1984).

Superoxide dismutase (SOD)

Electrophoresis was carried out under native condition in 8% polyacrylamide gels for SOD activity staining. Electrophoresis running conditions were as described by Vitoria et al. (2001) and buffers and gels were prepared as described by Laemmli (1970) lacking SDS. Equal amounts of enzyme (40µg) were loaded on to each lane. SOD activity was determined on native PAGE gels as described by Beauchamp and Fridovich (1971) and modified by Azevedo et al. (1998). The gels were rinsed in deionized water and incubated in the dark for 30 min at room temperature in an assay mixture containing 50 mM potassium phosphate buffer (pH 7.8), 1 mM EDTA, 0.05 mM riboflavin, 0.1 mM nitroblue tetrazolium and 0.3% (v/v) N,N,Ń,Ń-tetramethylethylenediamine (TEMED). At the end of this period, the gels were rinsed with deionized water and placed in deionized water and exposed on a light box for 5 to 10 min at room temperature until the development of colourless bands of SOD activity in a purple-stained gel was visible. The reaction was stopped by transferring the gels to 6% (v/v) acetic acid.

Statistical analysis

The data were statistically analyzed. The treatment means were compared by Duncan's multiple range test (DMRT). The software package used for analysis was IRRISTAT version 92 developed by the International Rice Research Institute Biometrics unit, the Philippines.

RESULTS AND DISCUSSION

Induction of defense related enzymes in mulberry plants

Results of colorimetric assay revealed that bio-consortia (seri bed waste+Pf1+Bs4+Th1+neem cake) significantly increased activities of PO (Figure 1), PPO (Figure 2), PAL (Figure 3), total phenol (Figure 4), CAT (Figure 5) and SOD (Figure 6) enzymes in mulberry plant against wilt disease. The increased activity was observed up to five days in all the treatments and thereafter declined. The mulberry treated with bio-consortia (seri bed waste+Pf1+Bs4+Th1+neem cake) gave the highest anti-oxidative and defense related enzymatic activity. The mulberry treated with Pf1 gave the second highest enzymatic activity. The mulberry treated with pathogen alone recorded (changes in absorbance $min^{-1}g^{-1}$ of fresh tissue) only slightly increase in enzymes activity. The healthy mulberry without any treatments showed lesser activity. Accumulation of PO has been correlated with ISR in several crops (Ramamoorthy et al., 2001). Isolates of *Pseudomonas* systemically induced resistance against *Fusarium* wilt of chickpea and suppressed the disease by 34.45% when compared to control (Saikia et al., 2005). Ramamoorthy and Samiyappan (2001) observed accelerated PPO activity in chilli plants treated with *P. fluorescens* when co-inoculated with *Colletotrichum capsici*. Induction of PAL by

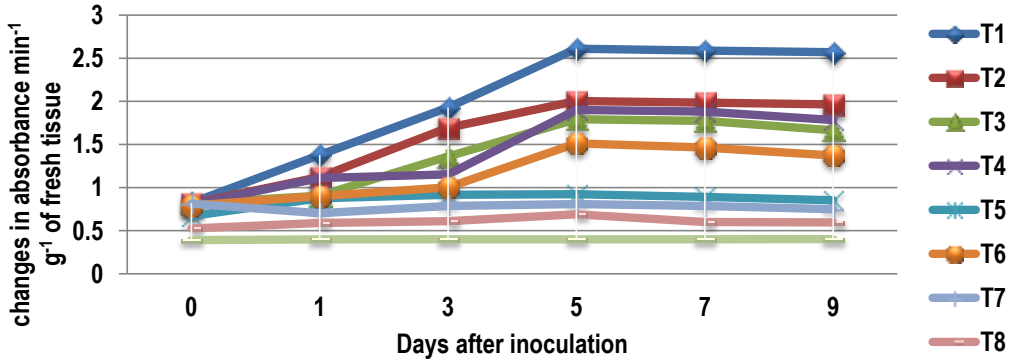

Figure 1. Induction of peroxidase (PO).

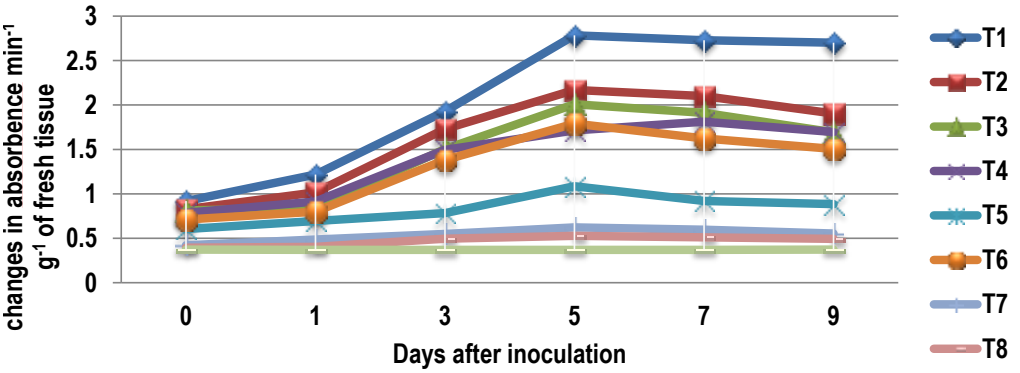

Figure 2. Induction of polyphenoloxidase (PPO).

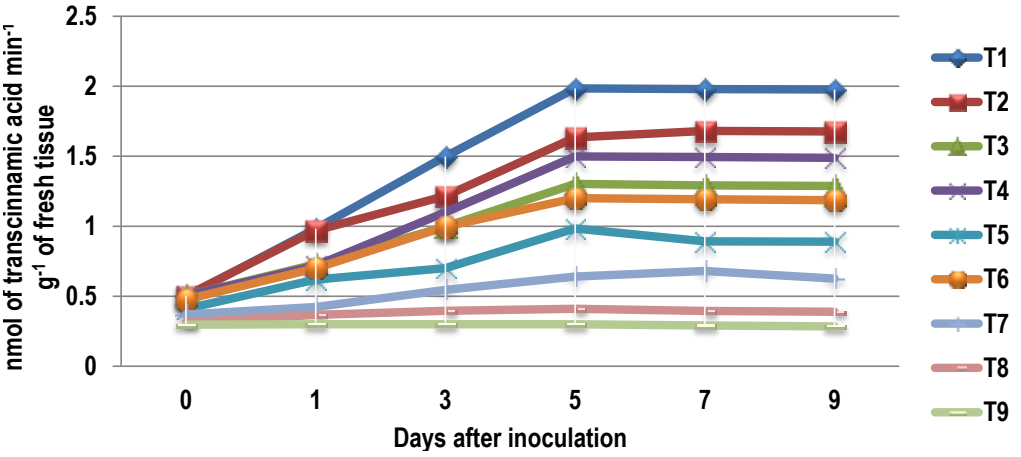

Figure 3. Induction of phenylalanine ammonia lyase (PAL).

fluorescent pseudomonads was reported in cucumber against *Pythium aphanidermatum* (Chen et al., 2000). Accumulation of phenolics by prior application of *P. fluorescens* in pea has been reported against *Pythium ultimum* and *F. oxysporum* f. sp. *pisi* (Benhamou et al., 1997). Superoxide dismutase and catalase (which scavenges O^{-}_{2}) suppresses the oxidative burst during the pathogen infection (Vera-Estrell et al., 1993).

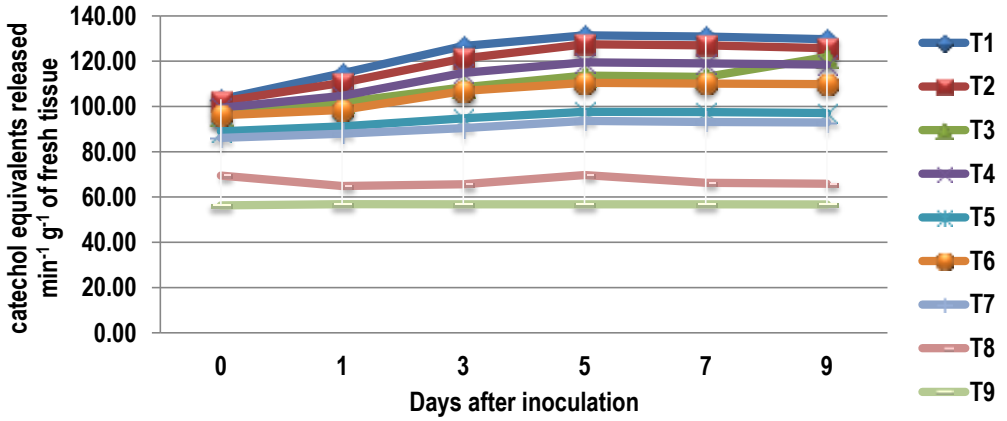

Figure 4. Induction of total phenol.

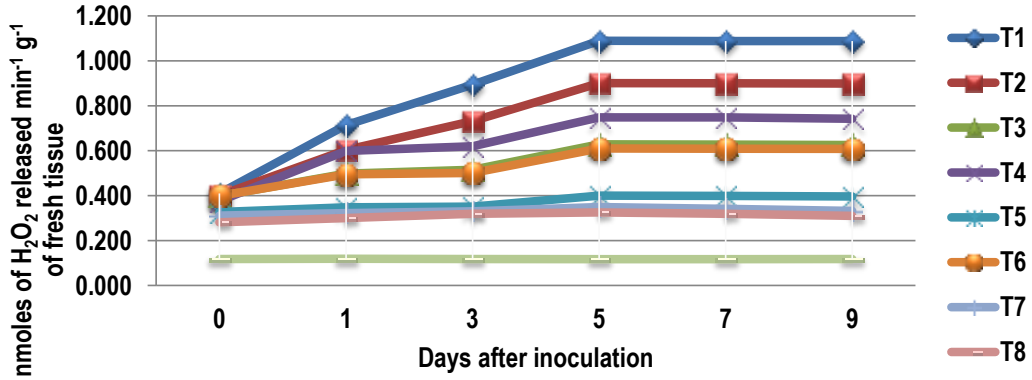

Figure 5. Induction of catalase (CAT).

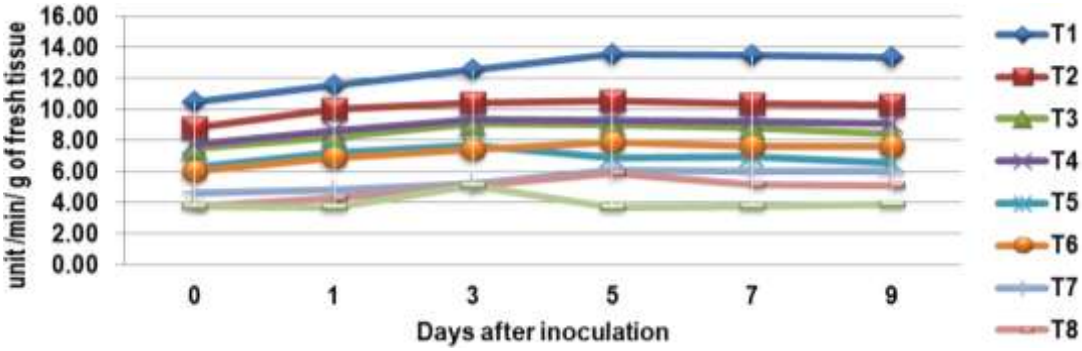

Figure 6. Induction of superoxide dismutase (SOD).

Detection of different isozymes by native gel

Isoforms pattern of peroxidase (PO)

In the present investigation, native polyacrylamide gel electrophoretic separation of enzyme extracts from biocontrol agents and fungicides treated plants were tested for the induction of PO and PPO isoforms separately. The results showed that plants were treated with bio-consortia (seri bed waste+Pf1+Bs4+Th1+neem cake) and Pf1 showed four isoforms *viz*., PO1, PO2 PO3 and PO4 (Plate 1). The other treatments *viz.,* Th1, tebuconazole, carbendazim showed three isoforms *viz.,* PO1, PO2, and PO3. PO is a useful marker of plant development, physiology, infection and

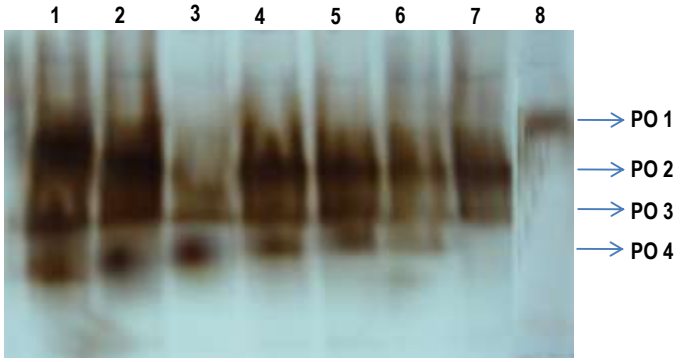

Plate 1. Native page profile of peroxidase (PO).

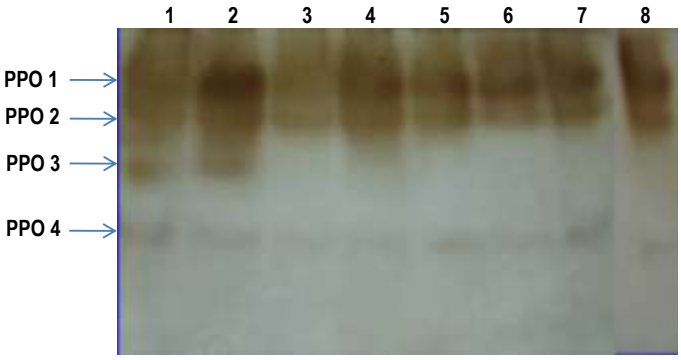

Plate 2. Native page profile of polyphenoloxidase (PPO)

stress (Welinder, 1992). Interestingly, increased peroxidase activity in leaf extracts of black gram infested with pathogens might be due to its utilization in cell wall lignifications (Parthasarathy et al., 2015). Therefore, induction of PO by bio-consortia in mulberry during wilt can be considered as one of the marker of disease resistance. Plants are equipped with well-organized and coordinated defense network of biochemical reactions, which are inducible in response to appropriate signals (Jones and Dangl, 2006). Inducing innate biochemical defense mechanisms in plants by treating them with biocontrol agents are thought to be novel plant protection strategies (Van Loon et al., 2008; Kashyap and Dhiman, 2009).

Isoforms pattern of polyphenoloxidase (PPO)

Similarly, the bio-consortia and Pf1 induced four isoforms of PPO *viz*., PPO1, PPO2, PPO3 and PPO4. The other treatments *viz.,* Pf1, Bs4, Th1, tebuconazole, carbendazim, and sprint showed three isoforms *viz.,* PPO1, PPO2, and PPO3 (Plate 2). The result indicates that the expression of PO4 and PPO4 in mulberry plants may be due to the treatment with bio-consortia, which expressed additional enzymatic activity.

The other treatments Bs4 and sprint revealed two isoforms and the control shown only one isoforms with less intensity. Increased PPO activity contributed to disease resistance due to its property to oxidize phenolic compounds to more toxic quinines which invade pathogenic micro-organisms (Vinale et al., 2008). It substantiated the role of PPO in disease resistance during pathogenesis. Our results are in affirmation with the study that reported a gradual increase in polyphenol content in red pepper (Sriram et al., 2009) and tomato (Nawrocka et al., 2011) treated with biocontrol agents.

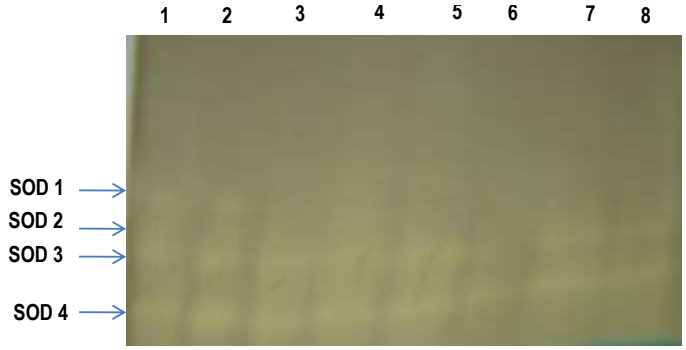

Plate 3. Native page profile of superoxide dismutase (SOD).

Isoform pattern of super oxide dismutase (SOD)

The results showed that plants treated with consortia (seri bed waste+Pf1+Bs4+Th1+neem cake) and Pf1 showed four isoform *viz*., SOD1, SOD2 SOD3 and SOD4 (Plate 3). The other treatments *viz.,* Bs4, Th1, tebuconazole, carbendazim, and sprint showed two isoforms *viz.,* SOD and SOD2. The result indicates that the expression of SOD3 and SOD4 super oxide dismutase in mulberry plants may be due to the treatment with bio-consortia. Super oxide dismutase suppresses the oxidative burst (Vera-Estrell et al., 1993) and inhibits tissue necrosis.

Conclusion

The present study supports that the bio-consortium (seri bed waste+Pf1+Bs4+Th1+neem cake) effectively reduced the *Fusarium* wilt disease in mulberry plant. Antagonistically, at the same time bioconsortia triggers the activity of defense enzymes in mulberry roots during the infection with *Fusarium solani.* Hence, simultaneous and inherent induction of defense metabolites revealed that slowdown of the pathogen might be due to activation of phenylpropanoid metabolism. In this regard, it is recommended to use consortia based biocontrol agents as a promising alternate to chemical fungicides to minimize the adverse impact on the

environment and ensuring plant disease management.

Conflicts of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Anti-HSV type-1 activity of olive leaves extract crude form acting as a microemulsion dosage form

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***Olea europeae* (L.) has been reported to have antibacterial, antifungal and antiviral activities. The aim of the present study is to evaluate antiviral activity of olive leaves extract (OLE) against herpes simplex viruses (HSV) type-1 virus. Screening of antiviral activity was assessed by measuring inhibition of viral-induced cytopathic effect of *in vero* cells of different OLE fractions that have been successively extracted using solvents of increasing polarities, against HSV type-1 virus. Negligible antiviral activity has been shown of different fractions, except for ethyl acetate and n-butanol fractions, showing strong and moderate anti-HSV type -1 activity, respectively. High performance liquid chromatography (HPLC) chromatographic analysis of both fractions revealed high oleuropein content in ethyl acetate fraction in addition to other phenolic and flavonoid contents, whereas n-butanol fraction showed only high content of other phenolic and flavonoid compounds. Cytotoxicity of ethyl acetate fraction was assessed in vero cell line, the mean cytotoxic concentration CC50, was reported to be 610 µg/ml. On the other hand, the 50% inhibitory concentration (IC50), against HSV-1, was of value as low as 40 µg/ml (SI = 15.2). This concentration could be more reduced to 33 µg/ml (SI = 16.9); that is, 17% reduction in dose, by formulating a microemulsion dosage form, with particle size of 13 to 19 nm, being assessed by Malvern Zetasizer Ver. 6.2 and electron microscopy. Acyclovir, a recommended anti-HSV agent, was used as a positive control. Oleuropein pure standard and the main phenolic component of OLE, was also assessed for its anti-HSV type-1 virus. As conclusion, microemulsion formulation enhanced antiviral activity of crude OLE.**

Key words: Olive leaves extract, OLE, acyclovir, anti-HSV activity, microemulsion, oleuropein.

INTRODUCTION

Medicinal plants, including the olive tree (*Olea europaea* L.) have been used for treatment of different types of diseases (Vijayan et al., 2004; Salah et al., 2012). Olive tree leaves have been widely used in European and Mediterranean countries as a traditional remedy in the form of extracts, herbal teas and powder (EI and Karakaya, 2009).

Olive leaves extract has been recommended as a potential nutraceutical; its phytochemical content as polyphenols and flavonoids (Micol et al., 2005) were

known to have diverse pharmacological activities (Lee et al., 2009). Olive leaves extract showed antibacterial and antifungal actions at low concentrations (Markin et al., 2003) as well as antiviral activity such as flu and colds. Olive leaves extract (OLE) is also effective against various diseases such as coronary artery disease, hypertension, high cholesterol level, arrhythmia, cancer, diabetes, overweight and osteoporosis (Erdohan and Turhan, 2011).

Oleuropein was reported as the most abundant biophenol in olive leaves (Ilias et al., 2011), and it has been claimed in a U.S. patent to have potent antiviral activities against herpes mononucleosis, hepatitis virus, rotavirus, bovine rhinovirus, canine parvovirus and feline leukemia virus (Fredrickson, WR, F & S group, Inc., 2000).

Oleuropein and the phenolic compounds of OLE have been reported to show antimicrobial activities against viruses, retroviruses, bacteria, yeasts, fungi and other parasites (Korukluoglu et al., 2010). Other clinical effects of oleuropein and hydroxytyrosol are the potentiation of cellular and organismal protection through the macrophage-mediated response, and the inhibition of platelet aggregation and eicosanoid production, respectively (Petroni et al., 1995; Benavente-Garcia et al., 2000).

The aim of the present study is to investigate the *in vitro* antiviral activity of olive leaves extract against HSV type-1 virus, in its crude form and after incorporation in a microemulsion formulation.

MATERIALS AND METHODS

Plant material, reagents and standard

1. Olive leaves were kindly provided from the Experimental station of Medicinal and Aromatic Plants, Faculty of Pharmacy, Cairo University, Giza, Egypt.
2. N-hexane, ethyl acetate, n-butanol and chloroform were purchased from Adwic, El-Nasr pharmaceutical chemicals company.
3. Pure oleuropein standard (HPLC grade), acyclovir, isopropyl myristate (IPM), tween 80, and transcutol were purchased from Sigma-Aldrich Chemical Company, USA. All chemicals were of analytical grade.

Cell line and virus

African Green Monkey kidney cells (Vero cell line) were obtained from the Egyptian Organization for Biological Products and Vaccines (VACSERA). Hepatitis A virus (HAV), Coxsackievirus group B (Cox B virus), and Herpes simplex virus type 1 (HSV-1) were kindly provided from the antimicrobial activity unit in The Regional Center for Mycology and Biotechnology, Al Azhar University.

Model virus selection

Screening of OLE antiviral activity was performed against Hepatitis A virus (HAV), Coxsackievirus group B (Cox B virus), and Herpes simplex virus type 1 (HSV-1). Significant antiviral activity was observed only against HSV-1 virus, which was selected as a model virus in the present study. It was propagated in Vero cells and stored at -80°C until use. Virus titre was determined by a plaque assay on Vero cell monolayers.

Preparation of olive leaves extract

The olive leaves were air dried in shade, powdered, and kept in tightly closed dark glass container till extraction. The powdered leaves were extracted with 70% ethanol at room temperature, then the extract was concentrated under vacuum in a rotary evaporator, further dissolved in distilled water and subjected to liquid-liquid fractionation in a glass separating funnel by successive extraction with solvents of increasing polarities (n-hexane, chloroform, ethyl acetate and n-butanol). The obtained four extracted fractions were then examined for their antiviral activity against HSV type-1 virus.

High performance liquid chromatography (HPLC) analysis and identification of olive leaves extract components

HPLC analysis of the fractions that showed anti-HSV type-1 activity was performed using HPLC Hewlett Packard (series 1050) equipped with UV detector to identify their components. Phenolic compounds of the active extract fractions, ethyl acetate and n-butanol extractives, were determined at λ280 nm by reverse phase HPLC (RP-HPLC) using Alltima C18 (5 µm, 150 mm×1.6 mm id) packed column. Gradient separation was carried out with methanol and acetonitrile as a mobile phase at a flow rate of 1 ml/min. Retention time and peak area were used to calculate the concentration of phenolic compounds by the data analysis of Hewlett Packard software (Goupy et al., 1999) and (Mattila et al., 2000).

Preparation of microemulsion formulation

The most active fraction (ethyl acetate) of olive leaves extract was formulated into a topical microemulsion dosage form. Pseudoternary phase diagrams were constructed using the water titration method. Three phase diagrams were prepared, consisting of the surfactant/co-surfactant (S-Cos) mixture Tween80/Transcutol with mass ratios 3:1, 1:1 and 1:3, mixed with the oil phase (isopropyl myristate) at the weight ratios of 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1. These mixtures were diluted dropwise with distilled water mixed with DMSO (10%), under moderate agitation. Clear transparent liquid was classified as microemulsion. The chosen microemulsion is illustrated in Table 3. The ethyl acetate extract was dissolved in the distilled water with 10% DMSO, then the aqueous phase was added dropwise to the mixture of oil/S-Cos and the microemulsion with concentration of 1 mg/ml was obtained by stirring the mixtures at ambient temperature by vortex (Zheng et al., 2010; Moghimipour et al., 2013).

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Particle size measurement and electron microscopy scanning of microemulsion

The mean particle size and zeta potential of OLE microemulsion were measured using Malvern Zetasizer Ver. 6.2 (Malvern Instruments Ltd., UK). Samples were placed in clear disposable zeta cells and results were recorded. The microstructure of formulated microemulsion was investigated by electron microscope (JOEL, JEM-1400 TEM) at a voltage of 80 kV (Munin and Edwards-Levy, 2011).

Evaluation of cytotoxicity of olive leaves crude extract, formulated microemulsion, pure oleuropein standard and acyclovir

Cell toxicity was monitored by observing the change in cell morphology and viability. Vero cells were grown and propagated in growth medium (Dulbecco's modified Eagle medium, DMEM; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.22% sodium bicarbonate (Sigma) and 50 µg/ml gentamicin (Gibco). The cells were maintained at 37°C in a humidified incubator with 5% CO_2 for 24 h. Serial twofold dilutions of the prepared extracts and formulated microemulsion (31 - 1000 µg/ml) were added to confluent Vero cell monolayers in a 96-well microtitre plate (Falcon, NJ, USA), and incubated at 37°C in a humidified incubator with 5% CO_2 for 48 h. Control cells were incubated without the test samples. At the end of incubation period, media was aspirated and crystal violet staining solution (0.1%) was added to each well for at least 15 min. Afterwards, the stain was aspirated and the plate was rinsed using distilled water, then the absorbance of the plate was measured with a microplate reader (Bio-Rad 550) at wavelength of 490 nm. Absorbance values of treated cell samples were compared to the control cells cultured without the test sample (Lee-Huang et al., 2003; Shoeib et al., 2011). The concentration reducing the cell viability by 50% (CC50), was calculated by regression analysis using the dose-response curve (Figure 3), generated from the experimental data.

Evaluation and comparison of antiviral activity of olive leaves extracted fractions, formulated microemulsion, pure oleuropein standard and acyclovir using cytopathic effect inhibition assay

Different non-toxic concentrations of the extracted fractions of OLE and formulated microemulsion (1.5 to 200 µg/ml) were checked for their antiviral activity, using cytopathic effect (CPE) inhibition assay, against HSV type-1 virus. In brief, confluent monolayers of Vero cells were challenged with 10^4 PFU/ml herpes simplex type-1 virus doses, and simultaneously the cultures were treated with equal volumes of two-fold serial dilutions of the crude extracts, microemulsion, acyclovir as a positive control and pure oleuropein standard; then incubated at 37°C in a humidified incubator with 5% CO_2 for 48 h. Also, an infection control as well as untreated Vero cells growth control, without the tested compounds, was made. Every 24 h, observation under the inverted microscope was made until the virus in the control wells showed complete viral-induced cytopathic effect (CPE). The cell monolayer sheet was stained with 0.1% crystal violet staining solution for at least 15 min, then removed by aspiration and rinsing with distilled water, then the absorbance was measured with microplate reader (Bio-Rad 550) at wavelength of 490 nm. The viral inhibition rate was calculated according to the study of Bag et al. (2012) and Dargan (1998) as follows:

(ODtv-ODcv) **/** (ODcd-ODcv) x 100%

Where, ODtv indicates the absorbance of the test sample with virus infected cells. ODcv indicates the absorbance of the virus infection control. ODcd indicates the absorbance of the cell growth control.

The concentration reducing the viral-induced CPE by 50% (IC50), was calculated by regression analysis using the dose-response curve (Figure 4), generated from the experimental data. A selectivity index (SI) was calculated for each test sample by dividing its CC50 by the corresponding IC50 value.

Statistics

Data were analyzed using GraphPad Prism for Windows version 6. Reduction of viral-induced CPE for each compound was expressed as mean value $\pm$ standard deviation. Comparison between different compounds within each concentration was done using analysis of variance (ANOVA), followed by multiple comparison tests (post-Hoc tests), that were used for pair-wise comparison with multiplicity adjusted (exact) p values. All tests were two-tailed. A p-value <0.05 was considered significant.

RESULTS

HPLC analysis and identification of olive leaves extract components

The phenolic compounds of the ethyl acetate extract that showed strong anti-HSV type-1 virus activity were determined by reverse phase HPLC (RP-HPLC) at λ280 nm. As shown in Figure 1, oleuropein content was the major component of phenolic compounds (1.8% w/w) of ethyl acetate extract, compared to pure oleuropein standard; whereas, the content percent in butanol extract was only 0.06% w/w (Figure 2).

Evaluation of cytotoxicity of olive leaves extract and its microemulsion form relative to acyclovir and pure oleuropein standard on vero cells

The cytotoxicity of crude olive leaves extract and its microemulsion form was assessed, compared to Acyclovir and pure Oleuropein standard; the results are presented in Table 1. As shown in Table 1 and Figure 3, the cytotoxicity of the tested samples on Vero cell line was concentration-dependent. The concentration reducing cell viability by 50% (mean cytotoxic concentration, CC_{50}) of the tested samples was calculated by GraphPad prism for Windows Version 6 statistical program. Pure Oleuropein standard showed highest cytotoxic effect on vero cells followed by formulated microemulsion. The crude olive leaves extract showed lower cytotoxic effects. Acyclovir had no significant cytotoxicity on Vero cells.

Comparison of anti-HSV type-1 activity of olive leaves extract and its microemulsion form with acyclovir and pure oleuropein standard using cytopathic effect inhibition assay

The antiviral activity of the tested samples was assessed

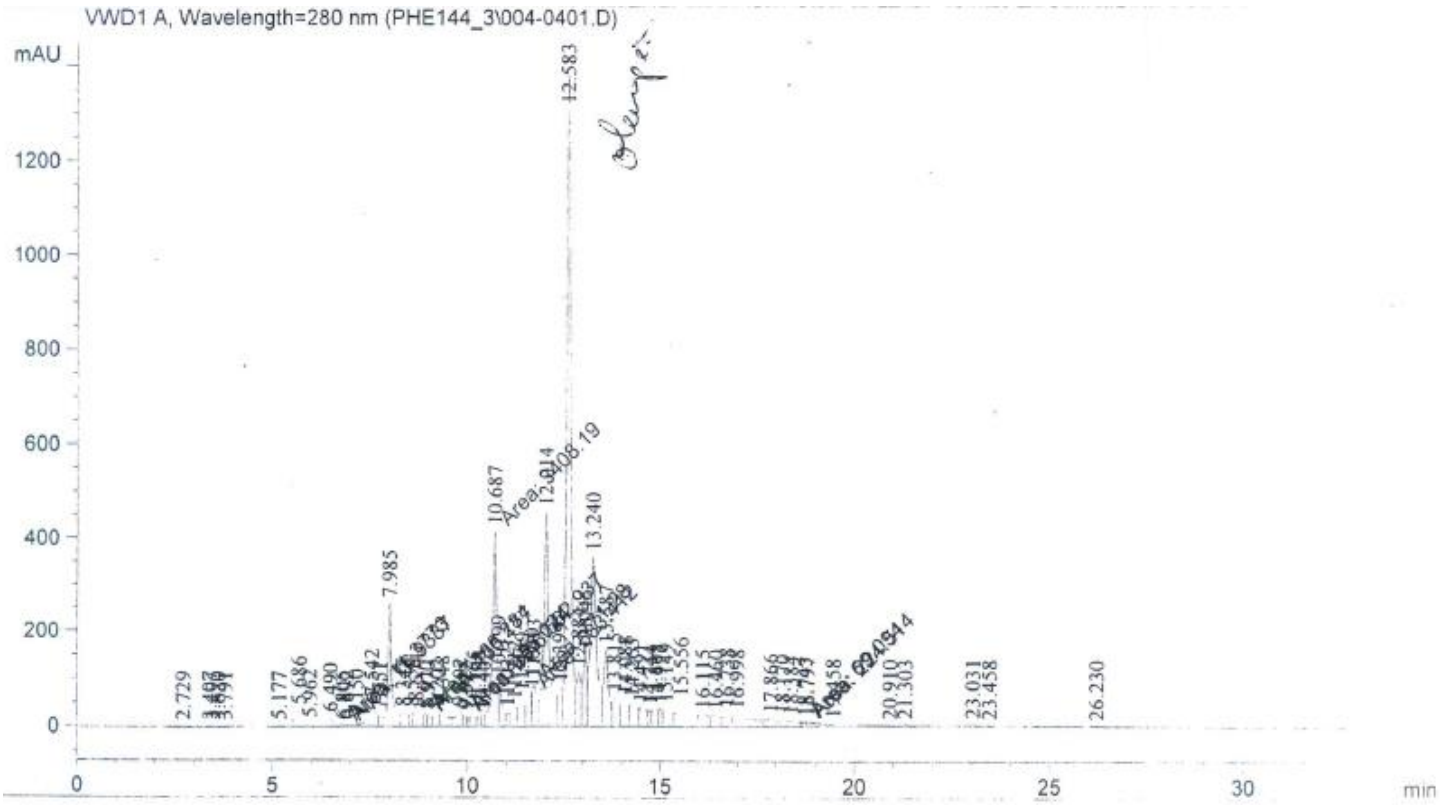

Figure 1. Chromatogram showing the phenolic compounds of ethyl acetate extract with high percent of oleuropein at λ_{max} 280 nm.

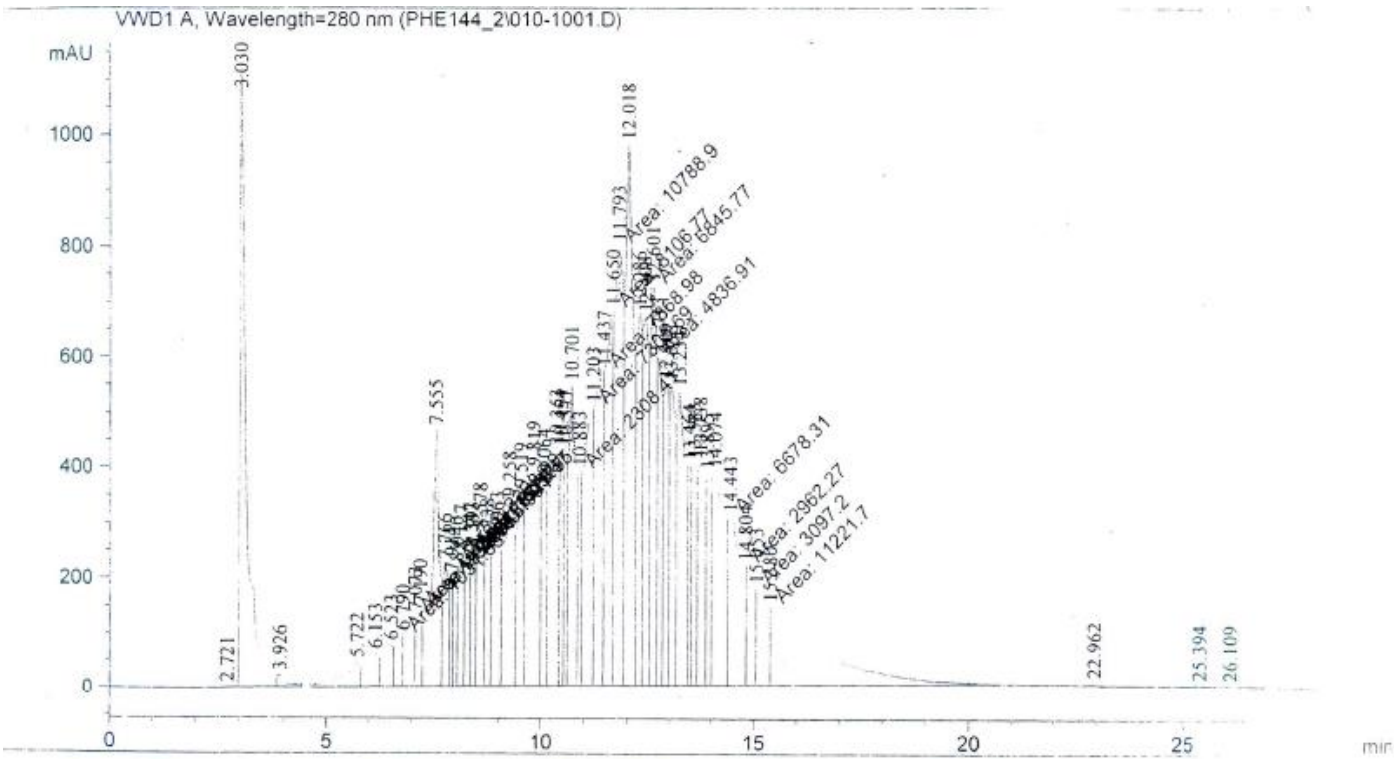

Figure 2. Chromatogram showing the phenolic compounds of butanol extract with negligible amount of oleuropein at λ_{max}280 nm.

Table 1. Comparison of cytotoxicity effect of formulated microemulsion, olive leaves extract, pure oleuropein standard and acyclovir on vero cells.

Sample conc. (µg/ml)	Cytotoxicity % of Microemulsion	Cytotoxicity % of crude olive leaves extract	Cytotoxicity % of pure Oleuropein standard	Cytotoxicity % of Acyclovir
0	0	0	0	0
31.25	17.02	3.78	21.72	0
62.5	25.77	5.32	30.29	3.44
125	31.88	11.41	36.32	6.86
250	40.33	30.38	44.58	13.52
500	47.99	48.92	58.73	21.74
1000	63.83	64.56	72.11	33.41
*CC_{50} (µg/ml)	563	610	346	>1000

*CC_{50}: cytotoxic mean concentration that reduces cell viability by 50%.

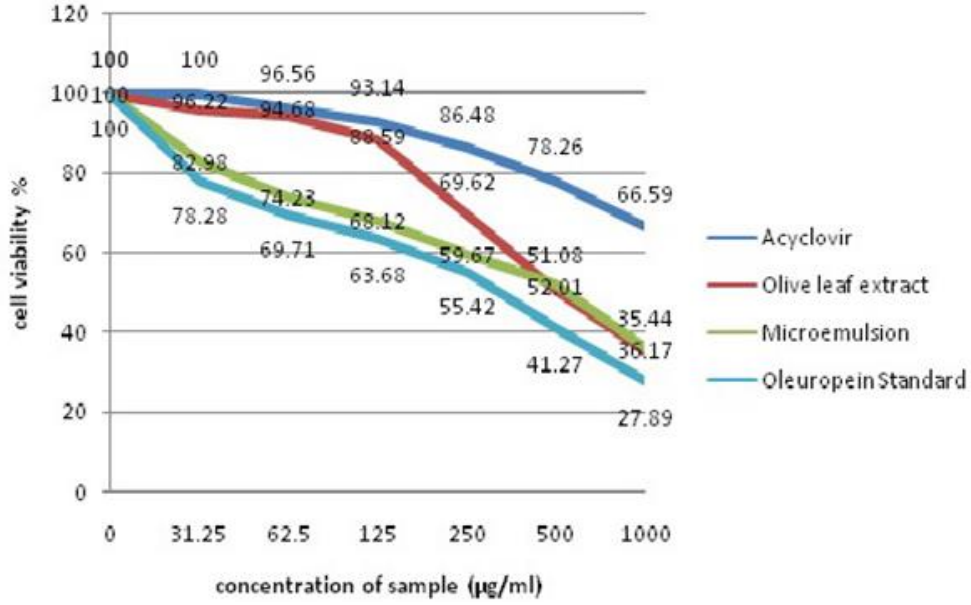

Figure 3. Curve showing percentage of Vero cell viability inhibited by formulated microemulsion, Olive leaves extract, pure oleuropein standard and acyclovir.

by measuring their protective effects on infected vero cells (Cann, 1999; Del Barrio and Parra, 2000).

Anti-HSV type-1 activity of crude olive leaves extract and its formulated microemulsion dosage form was determined by the inhibition of virus-induced cytopathic effect, compared to positive (Acyclovir) and negative controls (Table 2). As shown in Figure 4, formulated microemulsion exhibited significant higher virus inhibitory activity than the crude form of the olive leaves extract within the concentration limit range from 1.56 to 12.5 µg/ml. Pure oleuropein standard exhibited significant higher antiviral activity relative to the tested samples. Whereas, Acyclovir (positive control) showed the highest anti-HSV type-1 activity compared to all samples. All samples exhibited concentration-dependent *in-vitro* antiviral activity against HSV type-1 virus.

Also the selective index of olive leaves crude extract has increased after incorporation in the microemulsion dosage form, indicating increased selectivity against HSV type-1 virus. Oleuropein pure standard has higher selective index value relative to the tested samples. Acyclovir has the highest selective index value. All the tested samples have higher selective index values than 10, showing potential promising and selective anti-HSV type-1 virus candidates (De Clercq, 1993; Dargan, 1998).

Phase studies of formulated microemulsion

The aim of construction of pseudoternary phase diagrams was to find out the existence region of micro- emulsions.

Table 2. Comparison of the reduction of viral-induced cytopathic effect by 50% (inhibitory mean concentration, IC_{50}) of formulated microemulsion, crude olive leaves extract, pure Oleuropein standard and Acyclovir as a positive control against HSV-1 virus.

Inhibition percentage (%) ± SD*									IC_{50}(µg/ml)	SI (CC_{50}/ IC_{50})
Conc.(µg/ml)/Test sample	**1.56 (µg/ml)**	**3.12 (µg/ml)**	**6.25 (µg/ml)**	**12.5 (µg/ml)**	**25 (µg/ml)**	**50 (µg/ml)**	**100 (µg/ml)**	**200 (µg/ml)**	-	-
Crude olive leaves extract	5% ±2	7% ± 3	9% ±2	23% ±5	41%±6	56% ±5	62%±6	76%±7	40.1	15.2
Formulated microemulsion	12%±4^{ss}	19%±3^{ss}	32%±5^{ss}	40%±4 ^{ss}	47%±5	56% ±7	65%±6	71%±3	33.3	16.9
Pure Oleuropein standard ^{s*}	31%±4	34%±6	41%±5	47%±7	66%±6	75%±4	83%±6	89%±6	14.5	23.9
Acyclovir ^{S}	45%±6	68%±4	76%±5	87%±4	91%±6	93%±4	94%±5	98%±2	1.9	526.3

Results represent inhibition percentage of cytopathic effect of HSV type-1virus against Vero cells of 3 test samples ± standard deviation; S = All results of inhibition percentage of Acyclovir are significantly higher than those of other tested samples; S= Pure Oleuropein standard showed significant anti-HSV type-1 activity compared to formulated microemulsion; SS = Formulated microemulsion showed significant anti-HSV type-1 activity, compared to crude extract, within the concentration limit range from 1.56 – 12.5 µg/ml; IC_{50} = inhibitory mean concentration that reduces virus-induced cytopathic effect by 50% calculated by GraphPad prism version 6; SI = Selective index (CC_{50}/ IC_{50}).

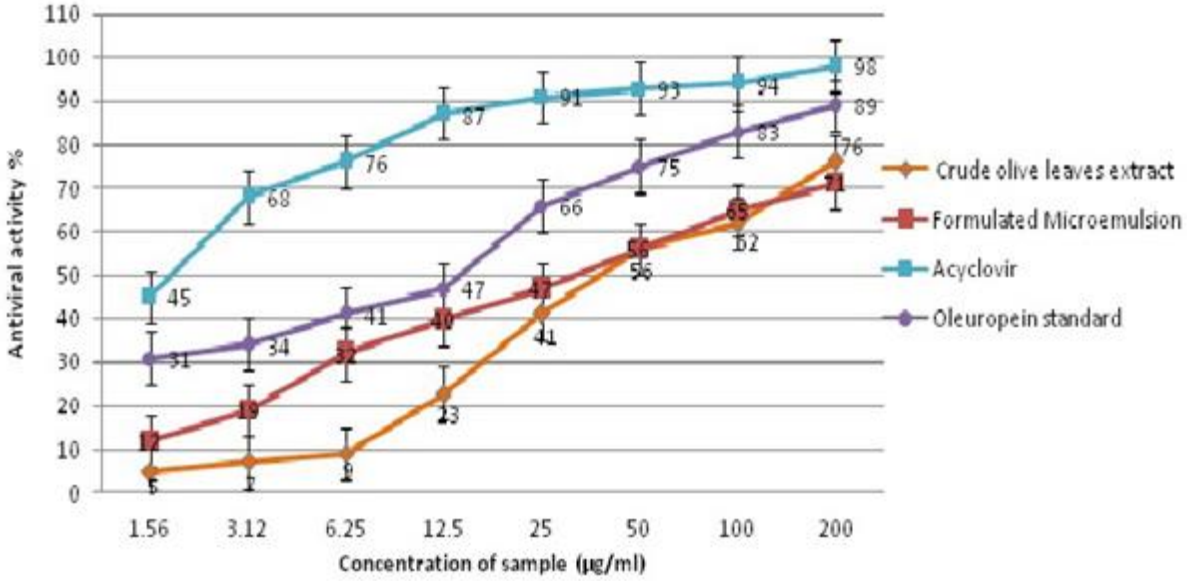

Figure 4. Antiviral activity curve of olive leaves extract, formulated microemulsion, pure oleuropein standard & Acyclovir against HSV type-1 virus.

The pseudoternary phase diagrams of isopropyl myristate / Tween80: Transcutol (3:1) / Water system is presented in Figure 5 (a to c). It was found that o/w microemulsion created with the three systems (Figure 5 a, b and c) was thermodynamically stable, optically transparent

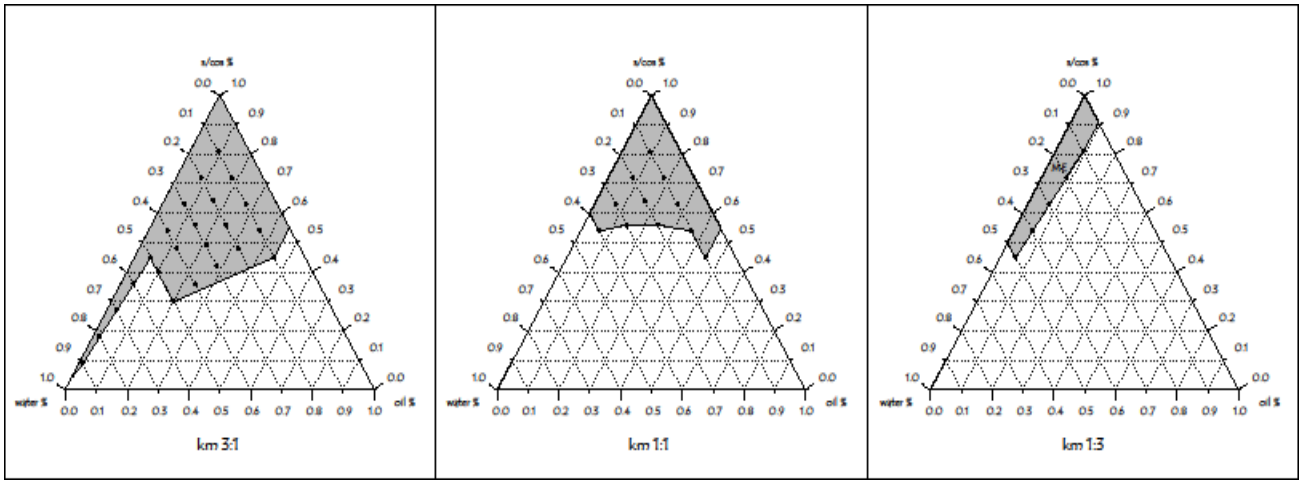

Figure 5. The pseudoternary phase diagrams of oil/surfactant: co-surfactant/water system at the 3:1 (a), 1:1 (b), 1:3 (c) weight ratio of Tween80: Transcutol, respectively, at ambient temperature; Dark areas show microemulsion zone.

Table 3. The selected formulation of Km mass ratio 3:1 of tween 80: transcutol, respectively.

Sample	Content of isopropyl myristate (%)	Content of Tween80/Transcutol (S/Cos) in ratio 3:1(%)	Water content with 10% DMSO (%)
Microemulsion	0.5	4.5	95

*S/Cos: surfactant/co-surfactant mixture

Table 4. Mean particle size, Polydispersity index and zeta potential of medicated microemulsion.

Sample	Mean particle size (nm) ± SD	Polydispersity index (Pdl) ± SD	Zeta potential (mV) ± SD
Microemulsion	15.71±2.92	0.429 ± 0.09	-2.58 ± 1.75

The values are the mean value of three samples ± standard deviation.

and single phase of liquid solution. The effect of the weight ratio of surfactant and co-surfactant (Km) on the area of o/w microemulsion region was compared. It can be seen from the figures that the area of o/w microemulsion increased with increasing the ratio of Km to be 3:1 (tween 80: transcutol), respectively. The maximum area of o/w microemulsion region was observed at Km 3:1.

Characterization of the selected formulation

According to the constructed pseudoternary phase diagrams, one formulation of the Km ratio 3:1 (tween 80: transcutol) was selected and crude olive leaves extract was incorporated in it as shown in Table 3.

Measurement of particle size and zeta potential

Polydispersity is a measure of particle homogeneity and it varies from zero to 1. The nearer the value of polydispersity to zero, the higher is the homology between the particles. The polydispersity index of microemulsion was 0.429 ± 0.09 (Table 4), indicating wide particle size distributions (Jia et al., 2010). These results suggest that the mixture favoured to form nanoparticles with more homogenous size distributions due to size reduction. The zeta potential evaluation is an indication of long-term stability due to electrostatic repulsion that prevents particle aggregation; the higher the value, the more stability.

Scanning of medicated microemulsion by electron microscopy

Scanning electron micrograph demonstrated spherical droplets in the nanometer range in a multi-disperse system (Figure 6) which is in agreement with the particle size data determined by Zetasizer.

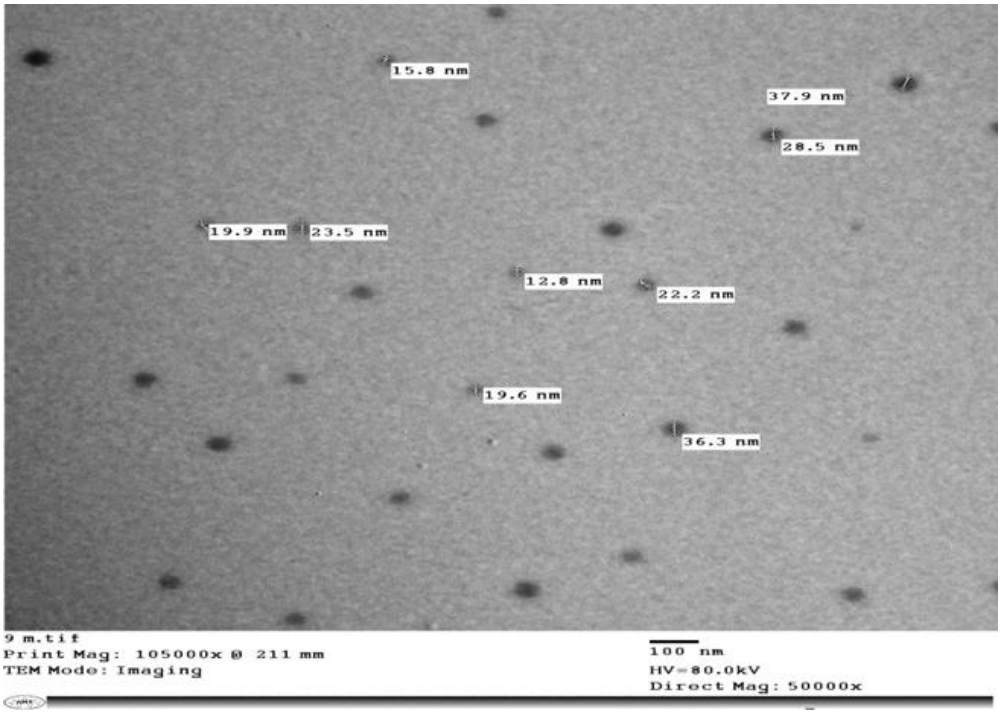

Figure 6. Microemulsion under transmission electron microscope JEOL (JEM-1400 TEM).

DISCUSSION

The earlier mentioned results show that incorporation of the olive leaves crude extract in a microemulsion formulation improved its therapeutic index as an anti-HSV type-1 virus, where the inhibitory mean concentration (IC50) of the crude extract was reduced from 40 to 33 µg/ml, with increased selective index (SI) from 15.2 to 16.9.

Accordingly, the antiviral activity and selective index values of the extract was enhanced by micronization. It has also been reported by Kulkarni (2011) and Soliman et al. (2010) that a number of plant constituents have shown enhanced therapeutic effect at similar or lower doses when being incorporated into novel drug delivery systems, as a result of the micronized particle size of the extract relative to its conventional crude form, which has led to better bioavailability and improved drug uptake due to better adherence to membranes and transport of bioactive molecules in a more controlled pattern (Soliman et al., 2010; Kogan and Garti (2006). Also, it was reported that the lower the viscosity of the vehicle, the faster is the release (Kogan and Garti, 2006).

Moreover, the higher extract solubility in the external water phase of o/w microemulsion has caused enhanced release of the solubilized extract and consequent improvement in the antiviral activity than the crude form itself at the concentration limit of 1.56 to 12.5 µg/ml as shown in Figure 4, followed by slower release of the extract from the internal oil phase of microemulsion. It has been also reported by Kogan and Garti (2006) that incorporation of the drug in the external phase of o/w microemulsion increases the diffusion rate, leading to release of larger amounts of the drug.

Also, the antiviral activity of pure oleuropein standard, which is the main biophenol constituent of olive leaf extract, is relatively high (IC50 of 14.5 µg/ml) with selective index (SI= 23.9), compared to the formulated ME and the crude extract. This can indicate that this active constituent could be responsible for the antiviral activity of olive leaves extract (Ritchason, 1999).

In previous study, it has been reported that the antiviral activity of OLE might be attributed to the prevention of attachment and adsorption of virus particles to the cell; thereby blocking their entry into the cells. Oleuropein interacts with the viral envelope by interacting with the surface of phospholipid bilayer present in the virus envelope, promoting drastic changes on the membrane surface to interfere with the binding of proteins to phospholipid domains, enriched in negatively charged phospholipids, such as phosphatidylglycerol or phosphatidylserine, leading to reduced membrane-fusion capacity. Anionic phospholipids have been shown to play a crucial role on the viral entry process (Micol et al., 2005).

Also micronized topical dosage forms of herbal extracts increase their solubilization and bioavailability at deeper epidermal layers, leading to more specifity and efficiency with decreased risk of toxicity due to reduction in the effective dose and improved absorption of active

ingredient at site of action, with reduced systemic side effects (Kesarwani and Gupta, 2013; Gupta et al., 2013).

Conclusion

Consequently, it can be observed that microemulsion formulation of OLE can be a promising technique of enhancing their pharmacological action and reducing their side effects.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Virulence factors and antibiotic susceptibility patterns of multidrug resistance *Klebsiella pneumoniae* isolated from different clinical infections

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Many strains of *Klebsiella pneumoniae* can produce several virulence factors, such as siderophores, capsules, fimbrial adhesins and antibiotic resistance enzymes, which are important in the adherence, colonization, invasion and development of the infection. Therefore the aim of this study was to investigate ten virulence gens in 32 *K. pneumoniae* isolated from different clinical infections: urine (13), burns (12), sputum (4) and blood (3). Phenotypic methods were used to detect capsule, hypermucoviscosity, ability to form biofilm, produce siderophores, primary and confirmatory test of extended spectrum beta lactamase (ESBL). Polymerase chain reaction (PCR) technique was used to detect ten virulence genes encoding attachments (*fimH*, *ycfM*), siderophores (*kfu*: iron uptake system, *entB*: enterobactin, *irp-2*: yersiniabactin), capsule synthesis or invasions (*rmpA*, *uge, wabG*) and beta lactamase enzymes (*SHV*, *TEM*). Antimicrobial susceptibility was tested by disk diffusion method. According to phenotypic methods, the results demonstrated that, the capsule and siderophores production and biofilm formation were observed in 100% of isolates, while hypermucoviscosity phenomenon was observed in 62.5%. The most common virulence genes were *fimH-1*, *ycfM* and *entB* (100%), *uge* and *TEM* (93.75%), *wabG* and *SHV* (87.5%). *Kfu* and *rpmA* genes were found at medium rates of 65.62 and 62.5%, respectively and at lower prevalence was gene *Irp-2* (37.5%). A rate of 84.375% of isolates showed a multidrug resistance (MDR) pattern, 12.5% extensive drug resistance (XDR), 3.12% pandrug resistance (PDR) and the rate of extended-spectrum β- lactamases (ESBL) producing *K. pneumoniae* was 62.5%.The study concluded that *fimH*-1, *ycfM* and *entB* genes were commonly found in all isolates, they seem to be at the basis of classic pathogenicity of *K. pneumoniae*. The most common virulence genes were observed in isolates from burns and blood samples. *K. pneumoniae* became highly resistant to antibiotics especially to 3rd generation cephalosporins and there was positive relationship between presence of virulence genes and ability of the bacterium to antibiotics resistance.

Key words: *Klebsiella pneumoniae*, drug resistance, virulence genes, Iraq.

INTRODUCTION

Klebsiella pneumoniae is one of the most important pathogenic bacteria. It is gram negative, bacilli, non-motile and causative agent of many diseases, such as pneumonia, urinary tract infections, bacteremia, burns

and wounds infections and pyogenic liver abscesses (Rahamathulla et al., 2016). Pathogenicity of *K. pneumoniae* is due to the presence of many virulence genes which encode virulence factors that allow it to attack the immune system of mammalians and cause many kind of diseases. Some of these virulence factors are: biofilm formation, hypermucoviscosity, capsule synthesis, adhesions, iron uptake and lipopolysaccharides formation (Fertas-Aissani et al., 2013; Chung et al., 2015). *K. pneumoniae* has been found capable to resist many antibiotics especially third generation cephalosporins like cefotaxime, ceftriaxone and ceftazidime (Yeh et al., 2007). Many clinical features of *K. pneumoniae* infections are related with virulence gens according to number and mode of action of these genes (Wiskur et al., 2008). Recently *K. pneumoniae* is found causing acute liver abscess as reported in many Asian countries like China, Kuwait and Iraq (Christopher et al., 2014; Chung et al., 2015). *K. pneumoniae* mostly contains extended spectrum beta-lactamase genes (*SHV, TEM* and *C-TXM*) that encoded by plasmid. These genes have shown resistance to many types of antibiotics (more than three classes) which is considered as multidrug resistant bacteria (Ahmed et al., 2014). In spite of *K. pneumoniae* is considered as one of the most important opportunistic bacteria, knowledge of the mechanism by which this bacterium causes many diseases is still not fully understood. The aim of this study was to investigate the presence of virulence genes in 32 *K. pneumoniae* clinical isolates from different clinical sources.

MATERIALS AND METHODS

Bacterial isolates

This study was carried out in Al-Sadder Medical City in AL-Najaf Governorate-Iraq. A total of 464 specimens from different clinical sources were collected from patients with (urinary tract infections, respiratory tract infections, burns infections and blood infections) during the period of January to October 2015. All urine and sputum samples were collected in sterile containers, a loopful of the urine and sputum samples were streaked onto the surface of MacConkey agar and blood agar (Oxoid,UK) and incubated at 37°C for 24 h. Burns samples were collected by sterile swabs and streaked onto the surface of MacConkey agar and blood agar (Oxoid, UK) and incubated at 37°C for 24 h. Five milliliter of blood samples were collected by a sterile syringes and mixed with 45 ml of brain heart infusion broth (Oxoid, UK) and incubated at 37°C for 7 days , and streaked (by sterile loop) onto the surface of blood and MacConkey agar (Oxoid, UK) and incubated at 37°C for 24 h (Collee et al.,1996). All isolates were identified according to morphological and biochemical tests (Gram stain, Growth on MacConkey Agar, Capsule stain, Catalase and oxidase test, Urease production test, Motility test, Indole production test, Methyl Red test, Voges-Proskauer test, Simmons Citrate test and Triple Sugar Iron test) (MacFaddin, 2000) and then by cultivation on Chrome agar medium (Orientation company – France). Finally all suspected isolates of *K. pneumoniae* were identified by using Vitek2® system (BioMerieux® -France).

Phenotypic determination of virulence factors

Capsule detection

The presence of capsule was investigated by staining with nigrosin, a loopful of overnight bacterial colony was transferred on a dry and clean slide, then gently mixed with nigrosin and allowed to dry in air, then rinsed with water , the slide was stained with methylen blue for 2 min and allowed to air dry, then the slide was gently washed with water, under light microscope the nigrosin stain provides a dark background to unstained capsule and metylene blue stain provides blue color to the cells (Soensen, 1995).

Hypermucoviscosity testing

Single colonies after culturing on Brain Heart Infusion agar plates (Oxoid, UK) were obtained and tested for their ability to form viscous strings. When a standard inoculation loop was touched onto their surface and slowly raised. The formation of string greater than 5 mm in length is indicative of hypermucoviscosity positive phenotype (Grange, 1988).

Siderophores production assay

Nutrient agar supplemented with 200 mM of 2.2'-dipyridyl was used as iron-restricted agar medium. All bacterial isolates were streaked on agar plates, and then incubated at 37°C for 24 h. Any bacterial growth was considered as positive results for ability of bacteria to siderophores production (Schwyn and Neilands, 1987).

Biofilm formation testing

Briefly, *K. pneumoniae* isolates were sub cultured three times in LB broth for 18 h at 37°C. The optical density was adjusted to 0.55 to 0.65 (2.106 to 8.109 CFU/mL) at 540 nm. Two hundred milliliter of the adjusted bacterial cultures were transferred to 96-well polystyrene microtiter plates and incubated for 24 h at 25°C. After addition of 25 mL of 1% crystal violet to each well, the plate was shaken and incubated for 15 min at room temperature. After removal of medium and three washes with phosphate-buffered saline, crystal violet was dissolved by the addition of ethanol, and the absorbance (Izquierdo et al., 2003).

DNA extraction

DNA was extracted by a boiling method as follows: Three to five pure and fresh colonies were suspended in 300 µl of distilled water, then cells were lysed by heating at 100°C for 20 min (in water bath), immediately the cells were placed in ice for 30 mi and the other cellular components was removed by centrifugation at 8500

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Table 1. Primers used in the current study.

Target genes	Primers'sequences (5′ to 3′)	Product size (bp)	References
entB	F-ATTTCCTCAACTTCTGGGGC R-AGCATCGGTGGCGGTGGTCA	371	(Fertas et al*.,* 2013)
irp-2	F-TCCCTCAATAAAGCCCACGCT R- TCGTCGGGCAGCGTTTCTTCT	287	(Schubert et al.,2000)
kfu	F-GAA GTG ACG CTG TTT CTG GC R-TTT CGT GTG GCC AGT GAC TC	797	(Ma et al., 2005)
rpmA	F-ACTGGGCTACCTCTGCTTCA R-CTTGCATGAGCCATCTTTCA	535	(Yeh et al.,2007)
wabG	F-CGGACTGGCAGATCCATATC R-ACCATCGGCCATTTGATAGA	683	(Brisse et al., 2009)
uge	F-TCT TCA CGC CTT CCT TCA CT R-GAT CAT CCG GTC TCC CTG TA	534	(Regue et al., 2004)
Fimh1	F-ATGAACGCCTGGTCCTTTGC R-GCTGAACGCCTATCCCCTGC	688	(Fertas et al*.,* 2013)
ycfm	F-ATCAGCAGTCGGGTCAGC R-CTTCTCCAGCATTCAGCG	160	
SHV	F- GGCCGCGTAGGCATGATAGA R- CCCGGCGATTTGCTGATTTC	714	(Ensor et al., 2009)
TEM	F- CAGCGGTAAGATCCTTGAGA R- ACTCCCCGTCGTGTAGATAA	643	

rpm for 10 min. Finally the supernatant was used as the DNA template (Yang et al., 2008).

PCR detection of virulence-associated genes

PCR was used to detect genes encoding type 1 adhesins (*fimH*-1), outer membrane lipoprotein (*ycfM*), enterobactin biosynthesis (*entB*), yersiniabactin biosynthesis (*irp-2*), iron uptake system (*kfu*), regulator of mucoid phenotype A (*rmpA*), invasions (*uge, wabG*) and beta lactamase enzymes (*SHV* , *TEM*). All primers used are listed in Table 1. And polymerase chain reaction (PCR) thermo cycling conditions are listed in Table 2.

Antimicrobial susceptibility testing

Antibiotic susceptibility testing was performed by the Kirby-Bauer method according to the Clinical Laboratory Standards Institute (CLSI, 2014). A colony from each *K. pneumoniae* isolate was grown overnight in Mueller Hinton broth (Oxoid,UK) at 37°C. Bacterial cultures were adjusted to 0.5 on the MacFarland nephelometer scale (1.5×10^8 CFU/ml) and plated on Mueller Hinton agar (Oxoid, UK) by the streaking method using a sterile swab. Antimicrobial susceptibility and resistance was determined by isolate growth zone diameter according to CLSI guidelines (2014). E. *coli* ATCC 25922 strain was used as controls. Antibiotics disks were purchased from Bioanalyse, Turkey. All types of antibiotics are listed in Table 3.

Primary test for production of ESBL

Antibiotic susceptibility testing was done to three types of 3rd generation cephalosporins antibiotics: ceftazidime, cefotaxime, and ceftriaxone. If inhibition zone for bacterial isolates were: ≤27 mm for cefotaxime, ≤22 mm for ceftazidime and ≤25 mm for ceftriaxone, this result considered as positive result for production of extended spectrum beta lactamase (CLSI, 2006).

Confirmatory test for ESBL

Augmentin disc 30 µg was placed in the center of Mueller Hinton agar plate (Oxoid, UK). Around of three sides of augmentin disc (30 µg), a disc of ceftazidime (30 µg), ceftriaxone (30 µg) and cefotaxime (30 µg) were placed with distance of 15mm from center to center of augmentin disc. Then the plate was incubated at 37°C for 24 h.

If inhibition zone was increased towards the augmentin disc that considered as positive results for production of ESBL (Sarojamma and Ramakrishna, 2011).

Table 2. PCR thermo cycling conditions.

Gene	Initial Denaturation °C / Time	Denaturation °C / Time	Annealing °C / Time	Extension °C / Time	Number of Cycles	Final extension °C / Time	References
entB	94°C /4 min	94°C /30 s	57°C /40 s	72°C /60 s	30	72°C /10 min	(Fertas et al., 2013)
irp-2	94°C /4 min	94°C /30 s	57°C /40 s	72°C /60 s	30	72 °C /10 min	(Schubert et al.,2000)
kfu	94°C /5 min	94°C /60 s	54°C /45 s	72°C /60 s	35	72 °C /7 min	(Ma et al., 2005)
rpmA	94°C /4 min	94°C /30 s	50 °C /40 s	72°C /60 s	30	72 °C /10 min	(Yeh et al.,2007)
uge	94°C /5 min	94°C /60 s	54°C /45 s	72°C /60 s	35	72 °C /7 min	(Brisse et al., 2009)
wabG	95°C /3 min	94°C /30 s	53°C /30 s	72°C /60 s	30	72 °C /5 min	(Regue et al., 2004)
fimH-1	94°C /4 min	94°C /30 s	55 °C /40 s	72°C /60 s	30	72 °C /10 min	(Fertas et al., 2013)
ycfM	94°C /4 min	94°C /30 s	55 °C /40 s	72°C /60 s	30	72°C /10 min	
TEM	95°C /5 min	94°C /30 s	52°C /45 s	72°C /45 s	30	72 °C /7 min	(Ensor et al., 2009)
SHV	95°C /5 min	94°C /30 s	55°C /60 s	72°C /45 s	30	72 °C /7 min	

Table 3. Antibiotic discs used in the current study.

Antibiotic	Symbol	Concentration (µg)
Amoxicillin	AX	25
Ticarcillin	TIC	75
Amoxicillin + Clavulanic acid	AMC	30
Cefotaxime	CTX	30
Ceftriaxone	CRO	30
Ceftazidime	CAZ	30
Imipenem	IMP	10
Meropenem	MEM	10
Gentamicin	CN	10
Amikacin	AK	30
Tobramycin	TM	10
Tetracycline	TE	30
Doxycycline	DO	30
Ciprofloxacin	CIP	5
Chloramphenicol	C	30
Nitrofurantoin	F	300

Statistical analysis

Statistical analysis was performed with GraphPad Prism version 5 software, percentages was used for the comparison between samples of the study.

RESULTS

K.pneumoniae isolates

Out of the 464 total specimens there were 243 specimens (52.370%) it has been diagnosed as gram negative bacteria, while there were 196 specimens (42.241%) were gram positive bacteria and 25 specimens (5.389%) with no any growth (Table 4). A total of 243 g negative bacterial isolates were collected from different clinical sources (106 from urine, 90 from burns, 25 from sputum and 22 from blood) there were 13 isolate of *K. pneumoniae* (12.264%) from urine, 12 isolates (13.333%) from burns, 4 isolates (16%) from sputum and only 3 isolates (13.636%) from blood (Table 5).

Virulence factors

In the present study, phenotypic methods were used to detect virulence factors, the capsule, biofilm formation and siderophores productions were observed in 32 isolates (100%), hypermucoviscosity phenomenon was observed in 20 isolates (62.5%) (Figure 1). Out of total 32

Table 4. Numbers and percentage of total samples (N=464).

Bacterial growth	No.	Percentage
Gram negative	243	52.370
Gram positive	196	42.241
No growth	25	5.389
Total	464	100

Table 5. Numbers and percentage of *K. pneumoniae* according to total gram negative bacterial isolates and sites of infection (N= 243).

Site of infection	Numbers of Gram negative bacteria	*K.pneumoniae*	Percentage (100%)
Urine	106	13	12.264
Burns	90	12	13.333
Sputum	25	4	16
Blood	22	3	13.636
Total	243	32	13.168

Figure 1. Stretching of *K. pneumoniae* colonies isolated from patient with blood infection resulted in the formation of a string >5 mm in length, demonstrating the hypermucoviscosity phenotype.

isolates, 32 isolates (100%) were positive for *fimh1*, *ycfm* and *entB* genes (Figures 2, 3 and 4, respectively), 30 isolates (93.75%) were positive for *uge* and *TEM* genes (Figures 5 and 6 , respectively), 28 isolates (87.5%) were positive for *wabG* and *SHV* genes (Figures 7 and 8, respectively), 21 isolates (65.62%) were positive for *kfu* gene (Figure 9), 20 isolates (62.5%) were positive for *rpmA* gene (Figure 10) and 12 isolates (37.5%) were positive for *Irp-2* gene (Figure 11). The prevalence and distribution of virulence factors were given in Tables 6 and 7.

Antimicrobial susceptibility

All isolates of *K. pneumoniae* showed resistance to AX, TIC and F (100%, 32/32), AMC and CTX (97.75%, 30/32), CRO (87.5%, 28/32), CAZ (71.875% 23/32), CIP

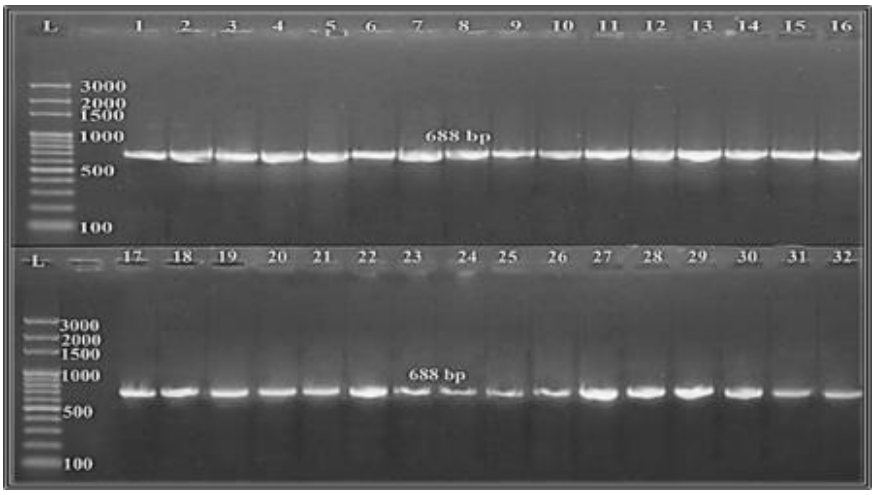

Figure 2. Ethidium bromide-stained agarose gel electrophoresis of monoplex PCR amplified products from extracted total DNA of *klebsiella pneumoniae* . lane: (1 to 32 isolates) amplified with diagnostic *fimH-1* gene, show positive results at 688 bp. The electrophoresis was performed at 80 volt for 95 min.

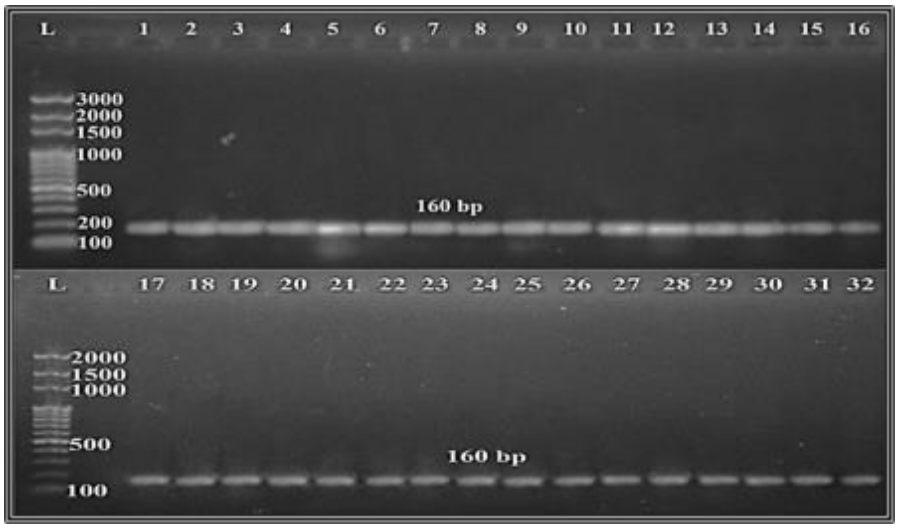

Figure 3. Ethidium bromide-stained agarose gel electrophoresis of monoplex PCR amplified products from extracted total DNA of *klebsiella pneumoniae*. Lane: (1 to 32 isolates) amplified with diagnostic *ycfM* gene, show positive results at 160 bp . The electrophoresis was performed at 80 volt for 95 min.

(43.75%, 14/32), DO (40.62%, 13/32), TM and TE (34.37, 11), CN and C (31.25%, 10/32), AK (28.12% , 9/32), MEM(25% , 8/32) and IMP (18.75% 6/32). Also in this study, 84.37% (27/32) of *K. pneumoniae* isolates were MDR, 12.5% (4/32) were XDR and 12% (1/32) were PDR. Both primary and confirmatory test for ESBL production were positive for 62.5% (20/32) of the isolates resistant to third-generation cephalosporins (Figures 12 and 13). Resistance test to 16 antibiotic of *K. pneumoniae* isolates were given in Tables 8, 9 and 10.

DISCUSSION

The genes *fimH*-1 and *ycfm* were found in all of our isolates. This result is in agreement with the ubiquitous nature of this structure in *K. pneumoniae* as reported in Fertas-Aissani et al. (2013). Microorganisms with biofilm formation are associated with many human infections. The biofilm formation role and development by bacteria has been documented to be a crucial step in the pathogenesis of *Klebsiella*. The flushing action of sterile

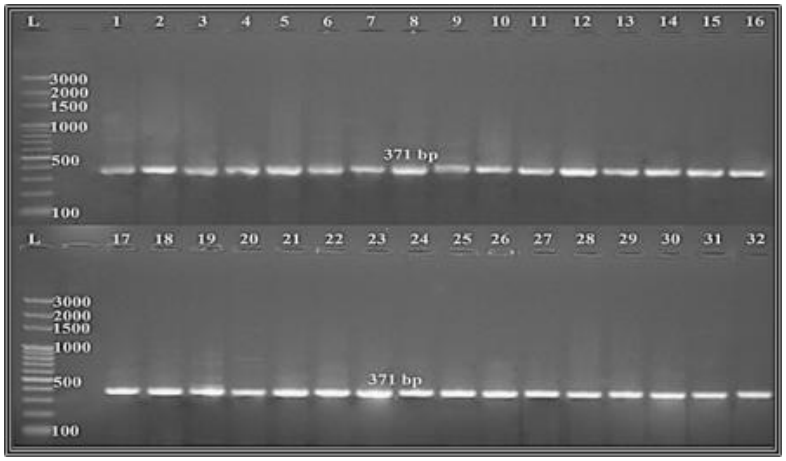

Figure 4. Ethidium bromide-stained agarose gel electrophoresis of monoplex PCR amplified products from extracted total DNA of *klebsiella pneumoniae*. Lane: (1 to 32 isolates) amplified with diagnostic *entB* gene, show positive results at 371 bp . The electrophoresis was performed at 80 volt for 95 min.

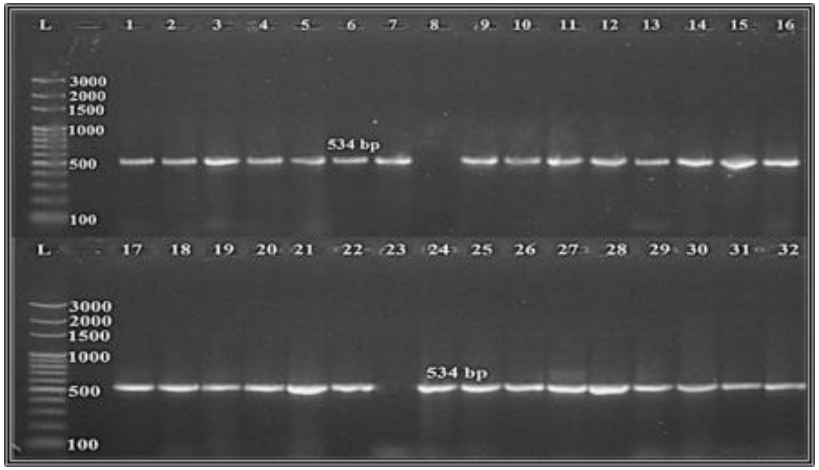

Figure 5. Ethidium bromide-stained agarose gel electrophoresis of monoplex PCR amplified products from extracted total DNA of *klebsiella pneumoniae*.Lane: (1 to 32 isolates) amplified with diagnostic *uge* gene, show positive results at 534 bp .The electrophoresis was performed at 80 volt for 95 min.

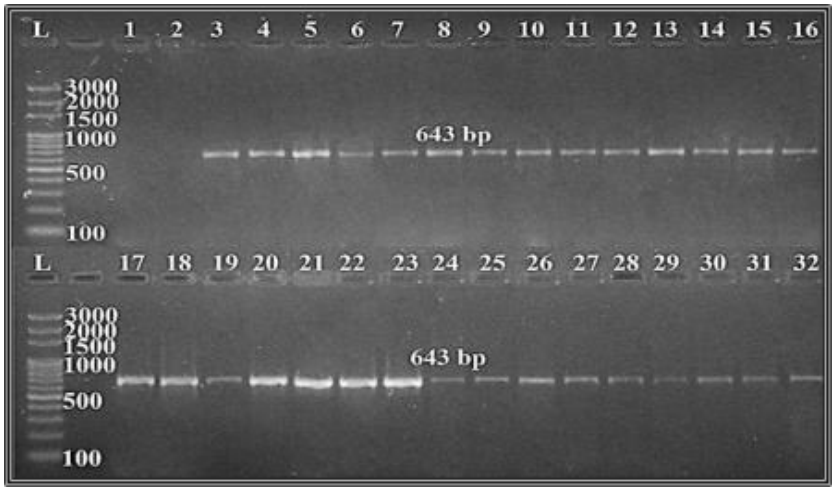

Figure 6. Ethidium bromide-stained agarose gel electrophoresis of monoplex PCR amplified products from extracted total DNA of *klebsiella pneumoniae* . Lane: (1 to 32 isolates) amplified with diagnostic *TEM* gene, show positive results at 643 bp . The electrophoresiswas performed at 80 volt for 95 min.

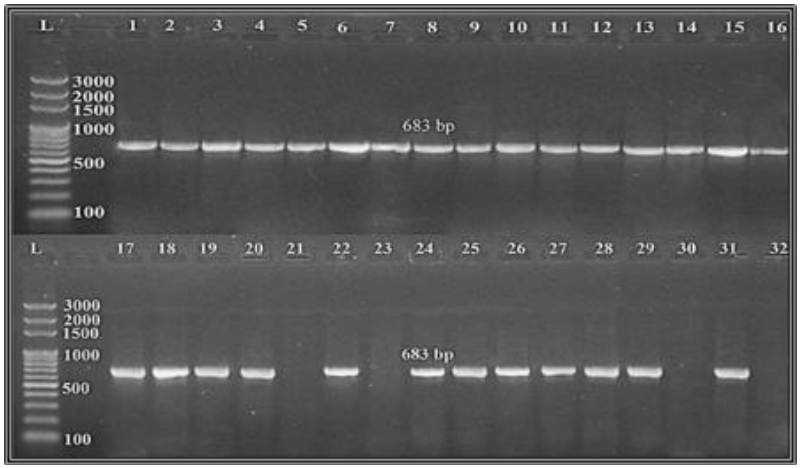

Figure 7. Ethidium bromide-stained agarose gel electrophoresis of monoplex PCR amplified products from extracted total DNA of *klebsiella pneumoniae*. lane: (1 to 32 isolates) amplified with diagnostic *wabG* gene, show positive results at 683 bp . The electrophoresis was performed at 80 volt for 95 min.

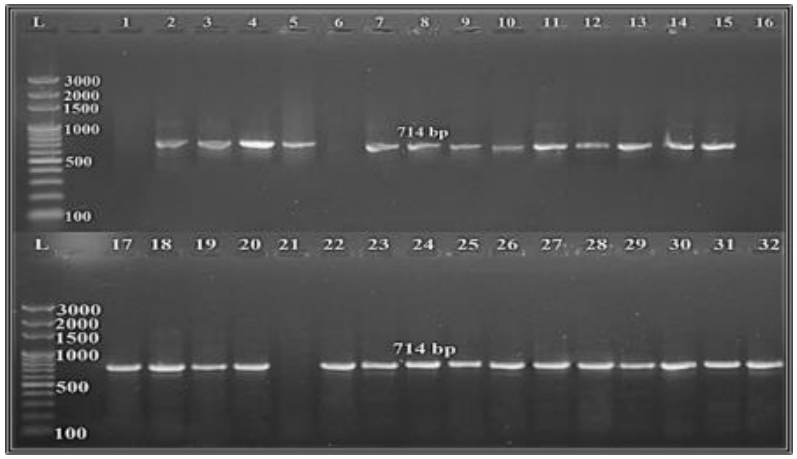

Figure 8. Ethidium bromide-stained agarose gel electrophoresis of monoplex PCR amplified products from extracted total DNA of *klebsiella pneumoniae*. Lane: (1 to 32 isolates) amplified with diagnostic *SHV* gene, show positive results at 714 bp. The electrophoresis was performed at 80 volt for 95 min.

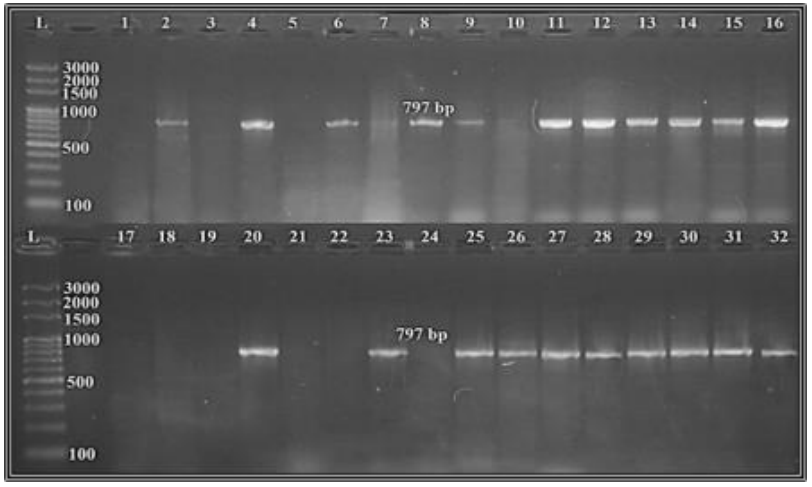

Figure 9. Ethidium bromide-stained agarose gel electrophoresis of monoplex PCR amplified products from extracted total DNA of *klebsiella pneumoniae*. Lane: (1 to 32 isolates) amplified with diagnostic *kfu* gene, show positive results at 797 bp. The electrophoresis was performed at 80 volt for 95 min.

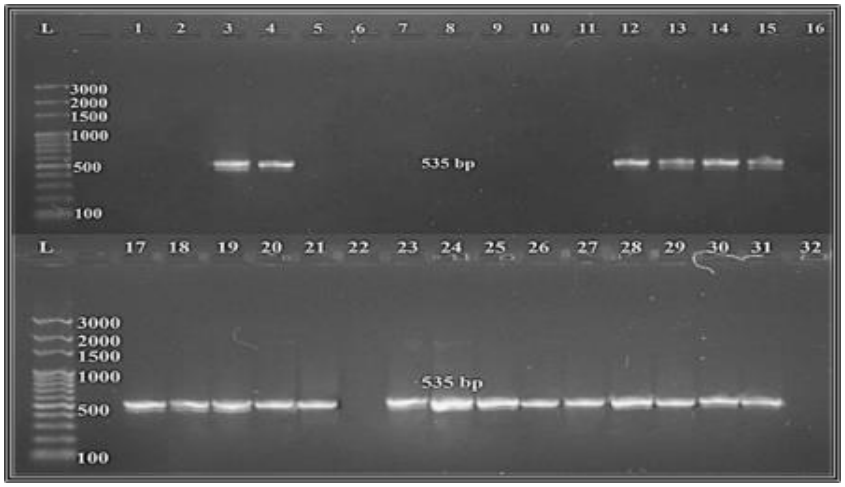

Figure 10. Ethidium bromide-stained agarose gel electrophoresis of monoplex PCR amplified products from extracted total DNA of *klebsiella pneumoniae*. Lane: (1 to 32 isolates) amplified with diagnostic *rpmA* gene, show positive results at 535 bp. The electrophoresis was performed at 80 volt for 95 min.

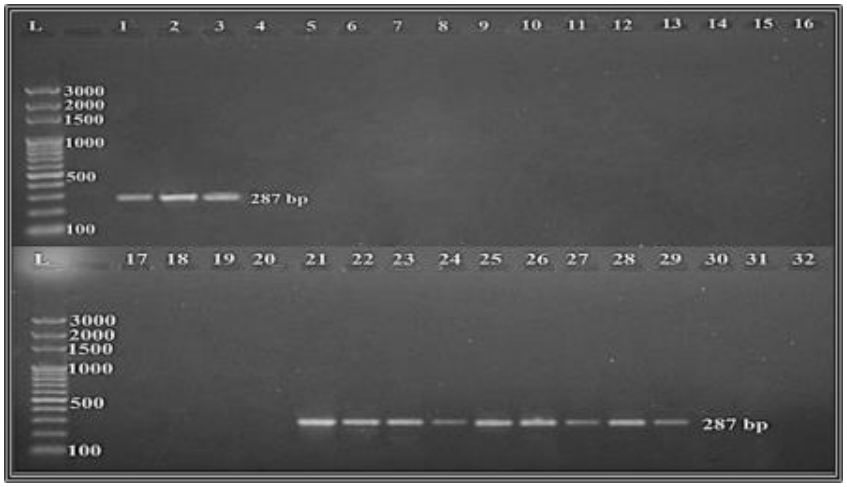

Figure 11. Ethidium bromide-stained agarose gel electrophoresis of monoplex PCR amplified products from extracted total DNA of *klebsiella pneumoniae*. Lane: (1 to 32 isolates) amplified with diagnostic *irp-2* gene, show positive results at 535 bp. The electrophoresis was performed at 80 volt for 95 min.

Figure 12. Confirmatory test for ESBL of *Klebsiella pneumoniae* by Double Disc Synergy Test. Ceftazidime (CAZ 30 µg) ; Ceftriaxone (CRO 30 µg); Amoxi/Clavulanic acid (AMC 30 µg); Cefotaxime (CX 30 µg)

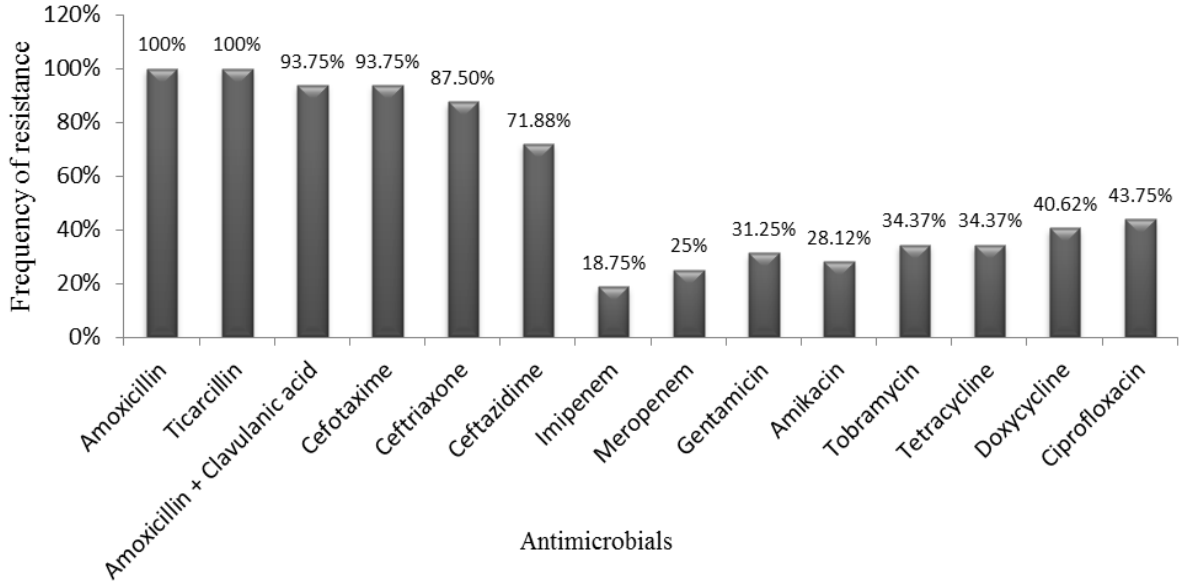

Figure 13. Frequency of antimicrobial resistance to *Klebsiella pneumonia.*

urine in urinary tract is protected from colonization by pathogen, but formation of biofilms by bacteria in urinary tract was lead to infect by chronic urinary tract infections. Biofilms and outer membrane lipoproteins can play an important role in protecting bacteria from drugs exposure when compared with other bacteria do not have these virulence factors (Bellifa et al.,2013). Therefore, there was strong relationship between resistance of antibiotics and prevalence of these virulence factors in bacteria. Bacteria with biofilm forming are generally more resistant

Table 6. Prevalence and distribution of virulence factors according to clinical samples (n = 32).

Virulence factors n (%)										
PCR amplification										
	Adhesins		Siderophores			Biosynthesis of capsule / invasins			ESLB	
Samples	***Fimh-1***	***ycfm***	***Entb***	***irp-2***	***kfu***	***RmpA***	***wabG***	***uge***	***TEM***	***SHV***
Urine (n=13)	13	13	13	3	7	5	9	11	12	11
Burns (n=12)	12	12	12	4	8	11	12	12	12	11
Sputum (n=4)	4	4	4	2	3	1	4	4	3	3
Blood (n=3)	3	3	3	3	3	3	3	3	3	3
Total / 32	32	32	32	12	21	20	28	30	30	28
	(100)	(100)	(100)	(37.5)	(65.6)	(62.5)	(87.5)	(93.7)	(93.7)	(87.5)

Virulence factors n (%)									
Phenotypic determination									
	BF	CPS	HMV	Siderophores	Antibiotics resistance pattern			ESBL	
Samples					**MDR**	**XDR**	**PDR**	**Pri.t**	**Conf.t**
Urine (n=13)	13	13	5	13	10	0	0	8	8
Burns (n=12)	12	12	8	12	10	1	0	8	8
Sputum (n=4)	4	4	4	4	4	0	0	1	1
Blood (n=3)	3	3	3	3	3	3	1	3	3
Total / 32	32	32	20	32	27	4	1	20	20
	(100)	(100)	(62.5)	(100)	(84.3)	(12.5)	(12)	(62.5)	(62.5)

ESLB: extended spectrum beta lactamase; *fimh-1*: FimH-like adhesin ; *ycfM*: outer membrane lipoprotein; *Entb*: enterobactin; *irp-2*: yersiniabactin; *kfu*: iron uptake system; *rmpA*: regulator of mucoid phenotype; *wabG*: lipopolysaccharides- core gene; *uge*: uridine diphosphate galacturonate 4-epimerase; *TEM*: β-lactamase named after first patient isolated from Temarian; *SHV*: Sulfhydryl variable β-lactamas; BF: biofilm formation; CPS: capsule; MHV: hypermucoviscosity ; MDR: Multidrug resistance; XDR:Extensive drug resistance; PDR: Pandrug resistance; Pri.t: primary test; conf.t: onfirmatory test.

Table 7. Numbers and percentage of virulence genes among 32 *K. pneumoniae* isolates according to site of infection.

Gene	Urine Total isolates: 13	Burns Total isolates: 12	Sputum Total isolates: 4	Blood Total isolates: 3	Total (32)
Entb	13(100%)	12(100%)	4(100%)	3(100%)	32(100%)
Irp-2	3(23.07%)	4(33.33%)	2(50%)	3(100%)	12(37.5%)
Kfu	7(53.84%)	8(66.66%)	3(75%)	3(100%)	21(65.62%)
Fimh1	13(100%)	12(100%)	4(100%)	3(100%)	32(100%)
Ycfm	13(100%)	12(100%)	4(100%)	3(100%)	32(100%)
wabG	9(76.92%)	12(100%)	4(100%)	3(100%)	28(87.5%)
rpmA	5(38.46%)	11(91.66%)	1(25%)	3(100%)	20(62.5%)
Uge	11(84.61%)	12(100%)	4(100%)	3(100%)	30(93.75%)
TEM	12(92.30%)	12(100%)	3(75%)	3(100%)	30(93.75%)
SHV	11(84.61%)	11(91.66%)	3(75%)	3(100%)	28(87.5%)

to many antibiotics. Biofilms and outer membrane lipoproteins act as biodegradable effect on beta-lactamases antibiotics. Beta-lactamase enzymes are secreted and maintain their activity inside of biofilm matrix, and decompose beta-lactam antibiotics before these antibiotics reach the bacterial cells (Høiby et al., 2010). Antibiotics penetration inside of biofilms could be blocked by other factors, such as the presence of surfaces with negatively-charged, particularly for large polar molecules such as aminoglycosides antibiotics with positively charged, Also, trace amount of metabolic rates and limited oxygen are maybe important factors contributing to increasing resistance to fluoroquinolones, cephalosporins, aminoglycosides and beta-lactamase

Table 8. Antibiotics sensitivity test for 32 clinical isolate of *K. pneumonia.*

Antibiotics	Concentration	Sensitive	Intermediate	Resistant	Percentage of resistance (%)
Amoxicillin	25 µg	0	0	32	100
Ticarcillin	75 µg	0	0	32	100
Amoxicillin + Clavulanic acid	20+10 µg	2	0	30	93.75
Cefotaxime	30 µg	2	0	30	93.75
Ceftriaxone	30 µg	4	0	28	87.5
Ceftazidime	30 µg	9	0	23	71.875
Imipenem	10 µg	23	3	6	18.75
Meropenem	10 µg	23	1	8	25
Gentamicin	15 µg	22	0	10	31.25
Amikacin	30 µg	20	3	9	28.12
Tobramycin	10 µg	19	2	11	34.37
Tetracycline	30 UI	20	1	11	34.37
Doxycycline	30 µg	19	0	13	40.62
Ciprofloxacin	5 µg	16	2	14	43.75
Chloramphenicol	30 µg	22	0	10	31.25
Nitrofurantoin	30 µg	0	0	32	100

antibiotics (Kwon et al., 2008; Lewis, 2001).

Siderophores are bio-synthetically compounds produced and secreted by many microorganisms such as, fungi and bacteria for iron (Fe^{+3}) uptake, and they are selective chelators. These ions are weakly soluble and almost found in all oxygenated environments. In this study, *Entb* gene was found in all our isolates (100%, 32/32), while *irp-2* gene was found in some of our isolates (37.5%, 12/32). These results were in agreement with some previous studies showed that almost all *K. pneumoniae* clinical isolates were having these chelators (Podschun et al., 1993; Koczura and Kaznowski, 2003; Fertas-Aissani et al., 2013) also in this study, *kfu* gene was found in 21 isolates (65.62%). Enterobactin and yersiniabactin they are siderophore compounds produced by bacterial cells to uptake iron (Fe^{+3}) from iron-binding proteins of the host, and they are have strong-affinity extracellular ferric chelators, which; they have an important role in pathogenesis and virulence of bacteria, virulence of these compounds are remain unclear; however, the expression of enterobactin induced biofilm formation (May and Okabe, 2011). But Lai et al. (2001) suggested that the expression of gene for iron-enterobactin that responsible for outer membrane receptor appears to be activated during bacterial infection.

The *kfu* gene is a putative pathogenic gene which codes for an iron uptake system. *kfu* gene associated with the purulent tissue infections ,capsule formation and virulent hypermucoviscosity phenotype. Therefore, this gene is considered as a very important gene in iron up take from host cell (Aher et al., 2012).

In this study, all our isolates had a capsule, while only 62.5% (20/32) had a hypermucoviscosity phenotype and *rpmA* gene. Almost *K. pneumoniae* produce large amounts of muco-polysaccharide mass and extra-capsular polysaccharides to produce strain with more virulent (Wiskur et al., 2008). In line with this Victor et al. (2007) reported that mucoid phenotype was seen in all isolates of *K. pneumoniae* that caused the invasive syndrome and in more than 90% of isolates in human with community- acquired pneumonia and in South Africa and Taiwan. Also he found that mortality of laboratory animals injected with mucoid strains was higher than that occurring in the same laboratory animals injected with non-mucoid strains.

The *rmpA* gene is a main factor that has an important role in virulence of *K. pneumoniae* strains, is controlled by plasmid with strongly mucoviscous phenotype promoted and synthesis regulator of the capsular polysaccharide (Rivero et al., 2010). The results demonstrated that out of total 32 isolates, 30 isolates (93.75%) were positive for *uge* gene and 28 isolates (87.5%) were positive for *wabG* gene.

wabG gene is less virulence factor studied, although it has been observed that many of *wabG* gene positive strains are from the patients with invasive and serious infections. But the mechanism of mode of action is still unclear (Turton et al., 2010). Izquierdo et al. (2003) proved that strains of *K. pneumoniae* with mutant *wab G* gene were non capsulated and less virulent in murine pneumonia model (rats). This fact proves the important role of *wab G* gene in pathogenicity of *K. pneumoniae*. Because the defect in synthesis of core lipopoly-saccharides in strains of *K. pneumoniae* with mutant *wab-G* gene were unable to induce urinary tract infection in experimental rats. Since they were unable to colonize in urinary tract in comparison with that of the normal

Table 9. Profile of virulence genes and resistance pattern among 32 clinical isolates of *K. pneumoniae* according to site of infection

No. of isolate	samples	*Entb*	*irp2*	*Kfu*	*FimH1*	*Ycfm*	*wabG*	*rpmA*	*Uge*	*TEM*	*SHV*	Phenotypic detection
1	Urine	+	+	-	+	+	+	-	+	-	-	
2	Sputum	+	+	+	+	+	+	-	+	-	+	
3	Burns	+	+	-	+	+	+	+	+	+	+	
4	*Burns	+	-	+	+	+	+	+	+	+	+	
5	*Urine	+	-	-	+	+	+	-	+	+	+	
6	Sputum	+	-	+	+	+	+	-	+	+	-	
7	*Urine	+	-	-	+	+	+	-	+	+	+	
8	*Urine	+	-	+	+	+	-	-	-	+	+	
9	*Urine	+	-	+	+	+	+	-	+	+	+	
10	*Urine	+	-	-	+	+	+	-	+	+	+	
11	*Burns	+	-	+	+	+	+	-	+	+	+	
12	*Burns	+	-	+	+	+	+	+	+	+	+	
13	*Burns	+	-	+	+	+	+	+	+	+	+	
14	*Sputum	+	-	+	+	+	+	+	+	+	+	MDR
15	*Burns	+	-	+	+	+	+	+	+	+	+	
16	Urine	+	-	+	+	+	+	-	+	+	-	
17	Burns	+	-	-	+	+	+	+	+	+	+	
18	Burns	+	-	-	+	+	+	+	+	+	+	
19	Urine	+	-	-	+	+	+	+	+	+	+	
20	Burns	+	-	+	+	+	+	+	+	+	+	
21	Urine	+	+	-	+	+	-	+	+	+	-	
22	*Sputum	+	+	-	+	+	+	-	+	+	+	
23	*Urine	+	+	+	+	+	+	+	-	+	+	
24	*Burns	+	+	-	+	+	+	+	+	+	+	
25	*Blood	+	+	+	+	+	+	+	+	+	+	PDR
26	*Burns	+	+	+	+	+	+	+	+	+	+	
27	*Blood	+	+	+	+	+	+	+	+	+	+	
28	*Blood	+	+	+	+	+	+	+	+	+	+	XDR
29	*Burns	+	+	+	+	+	+	+	+	+	+	
30	*Urine	+	-	+	+	+	-	+	+	+	+	
31	Urine	+	-	+	+	+	+	+	+	+	+	MDR
32	Urine	+	-	+	+	+	-	-	+	+	+	

MDR: Multidrug resistance, XDR: Extensive drug resistance, PDR: Pandrug resistance, *: Extended spectrum β-lactamase. +: present, -: absent.

strains. But the mutant bacterial strains were still able to perform some colonization. Regué et al. (2004) proved that the mutant strains of *K. pneumoniae* (without *uge* gene) were non-virulent in laboratory animals; this fact proves the important role of *uge* gene in pathogenicity of *K. pneumoniae*.

The antibiotic susceptibility analysis of the isolates showed that 100% (32/32) were resistance to amoxicillin, ticarcillin and nitrofurantoin. On the other hand, 84.37% (27/32) of isolates were MDR, 12.5% (4/32) were XDR and 12% (1/32) were PDR. The presumptive test for ESBL production was positive for 65.5% (20/32). The genes *TEM* and *SHV* were 93.75% (30/32) and 87.5% (28/32). These resistance patterns can be due to the expression of different enzymes such as extended-spectrum beta lactamases. Multi-drug resistances are often associated with extended spectrum beta-lactamase producing bacteria, that is, resistance to other classes of drugs like aminoglycosides and quinolones (Roshan et al., 2011). Isolates of *K. pneumoniae* may be have natural resistance to amoxicillin, ampicillin and ticarcillin but not to ESBL antibiotics. The resistance to Extended Spectrum Beta-Lactamase could happen class A chromosome beta-lactamase *SHV* and *TEM* genes are expressed (Mendonca and Ferreira, 2009). *K. pneumoniae* plasmids contain many B-lactamase genes including those encoding AmpC beta-lactamases, extended-spectrum B-lactamases, inhibitor resistant, metallo enzymes, *SHV* and *TEM* beta-lactamases. These enzymes make bacteria enable to resistant to different

Table 10. Sensitivity test and type of resistant among 32 clinical isolates of *K. pneumoniae* to 16 types of antibiotics according to site of infection.

No. of isolate	Sours of samples	Antibiotics																Phenotypic detection
		AX	TIC	AMC	CTX	CRO	CAZ	IMP	MEM	CN	AK	TM	TE	DO	CIP	C	F	
1	Urine	R	R	S	S	S	S	S	S	S	S	S	R	S	S	R	R	
2	Sputum	R	R	R	R	S	R	I	R	R	R	R	S	R	R	R	R	
3	Burns	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S	R	
4	*Burns	R	R	R	R	R	R	S	S	S	S	S	S	R	S	S	R	
5	*Urine	R	R	R	R	R	S	S	S	S	S	R	R	S	S	S	R	
6	Sputum	R	R	R	R	R	R	S	S	S	S	S	S	R	R	S	R	
7	*Urine	R	R	R	R	R	S	S	S	S	S	S	R	S	S	R	R	
8	*Urine	R	R	R	R	R	S	S	S	S	S	S	R	R	S	R	R	
9	*Urine	R	R	R	R	R	S	S	S	S	S	S	S	R	S	S	R	
10	*Urine	R	R	R	R	R	S	S	S	S	S	S	S	S	S	S	R	
11	*Burns	R	R	R	R	R	R	S	S	S	S	S	R	S	S	S	R	
12	*Burns	R	R	R	R	R	R	I	R	R	R	R	S	R	R	S	R	
13	*Burns	R	R	R	R	R	R	S	S	S	S	S	S	R	S	S	R	MDR
14	*Sputum	R	R	R	R	R	R	I	S	S	S	S	R	S	S	S	R	
15	*Burns	R	R	R	R	R	R	S	S	S	I	I	S	R	R	S	R	
16	Urine	R	R	R	R	R	S	S	S	S	S	I	S	S	S	S	R	
17	Burns	R	R	R	R	R	R	S	S	S	I	S	S	R	R	S	R	
18	Burns	R	R	R	R	R	S	S	S	S	I	S	R	S	R	S	R	
19	Urine	R	R	R	R	R	S	S	S	S	S	S	S	S	S	S	R	
20	Burns	R	R	R	R	R	R	S	I	R	R	R	S	S	S	S	R	
21	Urine	R	R	S	S	R	R	S	S	S	S	S	S	S	S	S	R	
22	*Sputum	R	R	R	R	R	R	S	S	S	S	S	S	S	R	S	R	
23	*Urine	R	R	R	R	S	R	S	S	R	S	R	S	S	S	S	R	
24	*Burns	R	R	R	R	R	R	S	S	S	S	S	S	S	R	S	R	
25	*Blood	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	PDR
26	*Burns	R	R	R	R	R	R	R	R	R	R	R	R	S	R	S	R	
27	*Blood	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	
28	*Blood	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	XDR
29	*Burns	R	R	R	R	R	R	R	R	R	R	R	S	S	R	R	R	
30	*Urine	R	R	R	R	S	R	S	S	S	S	S	S	S	I	R	R	
31	Urine	R	R	R	R	R	R	S	S	S	S	S	S	R	I	R	R	MDR
32	Urine	R	R	R	R	R	R	S	S	S	S	S	I	S	R	S	R	

AX: Amoxicillin 25 µg, TIC: Ticarcillin 75 µg , AMC: Amoxi/Clavulanic acid 30µg,CTX: Cefotaxime 30 µg, CRO: Ceftriaxone 30 µg, CAZ: Ceftazidime 30 µg, IMP: Imipenem 10 µg, MEM: Meropenem 10 µg, CN: Gentamicin: 10 µg, AK: Amikacin 30 µg, TM:Tobramicine 10 µg , TE: Tetracycline 30 µg, DO: Doxycycline 30 µg CIP: Ciprofloxacin 5 µg, , C: Chloramphenicol 30 µg, F: Nitrofurantoin 300 µg. R: Resistances , I: Intermediate , S: Sensitive, MDR: Multidrug resistance, XDR :Extensive drug resistance, PDR: Pandrug resistance, ESBL: Extended spectrum β-lactamase. *: ESBL.

antimicrobial agents including Imipenem, Meropenem, 3rd generation cephalosporins and others (Moland et al., 2003; Poirel et al., 2004; Essack et al., 2009). Padilla et al. (2010) reported that resistance mechanisms such as efflux pump can contribute to virulence of *Klebsiella*, and there was positive relationship between production of ESBL with virulence factors (Wiskur et al., 2008). As conclusions *fimH*-1, *ycfM* and *entB* genes were commonly found in all isolates, they seem to be at the basis of classic pathogenicity of *K. pneumoniae*. The most common virulence genes were observed in clinical sources of burns and blood infections. *K. pneumoniae* became highly resistant to antibiotics especially to 3rd generation cephalosporins. There was positive relationship between resistance to antibiotics and prevalence of virulence factors in *K. pneumoniae.*

RECOMMENDATIONS

Study the prevalence of another virulence gene in *k. pneumoniae* such as *kpn, traT and iroN*. And study the prevalence of virulence genes in another bacterium like *serratia* and *streptococcus pneumoniae.*

Conflict of Interests

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