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Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005). Methicillinresistant Staphylococcus aureus in community-acquired skin infections. Emerg. Infect. Dis. 11: 928-930.

Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Full Length Research Paper

Comparative study on prevalence and association of some virulence factors with extended spectrum beta-lactamases and AmpC producing *Escherichia coli*

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The aim of this study was to gain further insight into the interplay between both extended spectrum β-lactamase (ESBL) and AmpC enzymes and different virulence factors (VFs) among *Escherichia coli* (*E. coli)* isolated from Mansoura University Hospitals, Egypt. For this purpose 100 *E. coli* isolates were collected from different clinical sources. All isolates were investigated for production of ESBL and AmpC enzymes. The prevalence of VF encoding genes including: *KPsMII* (group2 capsule synthesis), *FyuA* (ferric yersiniabactin uptake), *Afa/dra* (Dr-binding a fimbrial adhesins), *PapA* (P fimbriae), *PapC* (formation of digalactoside-binding Pap pili), *iutA* (aerobactin receptor) and *BssS* (biofilm formation) and associations of these genes with both enzyme types were analyzed by polymerase chain reaction. ESBL was produced by 37% of the isolates while AmpC enzyme was produced by 29%. Virulence genes prevalence among ESBL phenotypes were 43.2% *KPsMII*, 51.35% *FyuA*, 62.1% *Afa/dra*, 43.2% *PapA*, 16.2% *PapC*, 67.56% *iutA* and 78.4% *BssS*. Regarding AmpC phenotypes, the prevalence of virulence genes were: 51.7% *KPsMII*, 86.2% *FyuA*, 68.96% *Afa/dra*, 55.17% *PapA*, 24.1% *PapC*, 82.7% *iutA* and 100% *BssS*. Of the tested virulence factor encoding genes, *BssS*, *FyuA* and *iutA* were significantly more prevalent among AmpC producers. In addition, AmpC producers exhibited a statistically significant higher prevalence of multivirulence (MV) (MV≥4) than ESBL producers. Furthermore, AmpC phenotypes showed very significantly higher expression (P<0.01) of *BssS* gene than ESBLs phenotypes. Our results suggest a correlation between AmpC phenotypes and production of some factors that are reported to be involved in the virulence of *E. coli*.

Key words: extended spectrum β-lactamase, AmpC enzymes, virulence factors, *Escherichia coli.*

INTRODUCTION

Great attention has been paid to antimicrobial resistance due to morbidity and mortality from diseases caused by resistant bacteria. β-lactams are the most predominant antimicrobials for the treatment of infectious diseases. Bacterial resistance to this class of antibiotics is mediated by the production of β-lactamase enzymes (Paterson and Bonomo, 2005), sometimes in association with other mechanisms that might lead to either diminished permeability or active efflux (Poole, 2004).

Numerous β-lactamases are known and more continue

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to be described. Of particular clinical and epidemiological importance are extended-spectrum β-lactamases (ESBLs) and AmpC β-lactamases, which are capable of inactivating the effects of broad –spectrum cephalosporins and penicillins. ESBLs are enzymes that hydrolyze penicillins, cephalosporins, aztreonam and are susceptible to clavulanic acid (Paterson and Bonomo, 2005). Resistance mediated by these enzymes is now observed in all species of Enterobacteriaceae and currently disseminated worldwide (El Naggar et al., 2010; Bourjilat et al., 2011). AmpC are clinically important because they confer resistance to cephamycins such as cefoxitin and cefotetan, in addition to narrow, expanded and broad spectrum cephalosporins and aztereonam, moreover they resist inhibition by clavulanic acid (Jacoby, 2009) but they are inhibited by cloxacillin and 3-aminophenylboronic (Tan et al., 2009). In Gram-negative bacteria, AmpC β-lactamases production is either chromosome or plasmid mediated. Plasmid-mediated AmpC β-lactamases have been detected in some isolates of *Klebsiella* species, *Salmonella* species, *Citrobacter freundii*, *Proteus mirabilis*, *Enterobacter aerogenes* and *Escherichia coli* (*E. coli*) and are usually associated with multidrug resistance. Accurate prevalence data of plasmid-mediated AmpC β-lactamases are limited due to lack of testing, but generally they are less common than ESBLs (Jacoby, 2009).

E. coli is one of the commensal bacteria in human intestinal tract. However, the high plasticity of the genome of this species via gene loss or gain, through lateral gene transfer gives it an enormous capability to evolve, leading to the evolution of pathogenic strains from the commensal cohorts (Kaper et al., 2004).

The virulence factors (VFs) help in the survival of *E. coli* under adverse conditions, which allows *E. coli* to cause extraintestinal infections (Johnson and Russo, 2002). *E. coli* possess a broad range of virulence-associated factors including toxins, adhesions, lipopolysaccharides, polysaccharide capsules, proteases and invasions (Mokady et al., 2005).

VFs comprise mechanisms that allow pathogenic bacteria to cause infections. Genomics offers a good tool for defining virulence factors as it can be used to recognize genes encoding for specific factors that contributes to virulence of pathogens. However, the presence of one factor rarely makes an organism virulent; a combination of different factors will determine if an organism can cause infection (Dobrindt, 2005).

The treatment of infection caused by *E. coli* has been complicated by the emergence of antimicrobial resistance (Da Silva and Mendonça, 2012). β-Lactam antibiotics, especially the third generation cephalosporins, are essential drug class used to treat severe community-onset or hospital-acquired infections caused by *E. coli*. Among *E. coli*, production of β-lactamase enzymes still represents the most important mediator of β-lactam resistance (Livermore and Woodford, 2006). Most importantly is the increasing numbers of *E. coli* isolates producing “newer β-lactamases” which includes the extended- spectrum β-lactamases and plasmid-mediated AmpC β-lactamases (Jacoby and Munoz-Price, 2005). The dissemination of such resistance is associated with genetic mobile elements, such as plasmids, that may also carry virulence determinants and transmit them (Da Silva and Mendonça, 2012). This will necessitate the studying the interplay between antimicrobial resistance and bacterial virulence determinants.

The present study has been undertaken to gain further insight into the interplay between both ESBL and AmpC enzymes and different VFs among *E. coli* isolated from Mansoura University Hospitals, Egypt. For this purpose the prevalence and associations of VFs among ESBLs and AmpC producing *E. coli* was investigated.

MATERIALS AND METHODS

Isolation and identification of bacterial isolates, media and growth requirements

A total of 180 clinical samples were collected from Mansoura University Hospitals Dakhlia governorate, Egypt over a period of 6 months from January 2013 until June 2013. These isolates were obtained from different clinical sources including pus, urine and sputum. Hundred isolates were identified biochemically as *E. coli* based on Gram staining, colony morphology on MacConkey's agar and laboratory biochemical standards (Crichton, 1996). The experimental protocol conducted in the study complies with the ethical guidelines and the principals of care, use and handling of human subjects in medical research adopted by "The research Ethics Committee, Faculty of Pharmacy, Mansoura University, Egypt which is in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki involving use and handling of human subjects).

Phenotypic detection of ESBL enzymes

E. coli isolates were tested for ESBL production using modified double disc synergy test (DDST) by using a disc of amoxicillin-clavulanate along with three cephalosporins discs; cefotaxime (30 µg), ceftazidime (30 µg) and ceftriaxone (30 µg). A disc which contained amoxicillin-clavulanate was placed in the centre of the plate. The discs of cefotaxime, ceftazidime and ceftriaxone were placed around amoxicillin-clavulanate disc keeping the distance of 16-20 mm from it (centre to centre) on Mueller-Hinton agar plate inoculated with suspension equivalent to 0.5 McFarland standards. The plate was incubated at 37ºC for 24 h. When the zone of inhibition around any of these cephalosporins discs showed a clear-cut increase towards the amoxicillin-clavulanate disc, the organism was considered as positive for ESBL production (Paterson and Bonomo, 2005).

Phenotypic detection of AmpC enzymes

The isolates were tested by three dimensional extract method for the production of AmpC enzymes. The surface of the Mueller-Hinton agar plate was inoculated with *E. coli* DH5α. Cefoxitin disc (30 µg) was placed on the plate. Crude enzyme was extracted from nutrient agar slope culture of the tested isolate as described previously (Livermore et al., 1984). Linear slits were cut 3 mm away

Table 1. Oligonucleotide primers used to amplify the tested genes.

Gene name	Type	Sequence
PapA	Fw	ATGGCAGTGGTGTCTTTTGGT
	Rv	GATAAGTCAGGTTGAAATTCGCAA
PapC	Fw	TGGATTGTCAGCCTCAAGGTCTA
	Rv	CACTGACGCCGAAAGACGTA
iutA	Fw	ATCAGAGGGACCAGCACGC
	Rv	TTCAGAGTCAGTTTCATGCCGT
Afa/dra	Fw	ACCCGACGCCGTTTTACATCAACCTG
	Rv	CCCTTCCCGCCACCTTTCAGCA
KpsMTII	Fw	GCGCATTTGCTGATACTGTTG
	Rv	CATCCAGACGATAAGCATGAGC
fyuA	Fw	ATACCACCGCTGAAACGCTG
	Rv	CGCAGTAGGCACGATGTTGTA
BssS	Fw	GATTCAATTTTGGCGATTCCTGC
	Rv	TAATGAAGTCATTCAGACTCATCC
V1to V6 region of 16S-rRNA	Fw	AGAGTTTGATCMTGGCTCAG
	Rv	ACGAGCTGACGACARCCATG

from the edge of cefoxitin disc, small circular wells were made inside the outer edge of the slit. The well was loaded approximately with 30-40 µl of enzyme extract. The plate was kept upright for 5-10 min until the solution dried and was then incubated at 37 ºC for 24 h. The isolates which showed clear distortion of inhibition zone around cefoxitin disc were reported as AmpC β-lactamase producers (Manchanda and Singh, 2003).

Molecular screening of some virulence genes among ESBL and AmpC producers

ESBL and AmpC producers were analyzed by PCR for the presence of seven virulence factors associated genes: *KPsMII* (group2 capsule synthesis), *FyuA* (ferric yersiniabactin uptake), *Afa/dra* (Dr-binding a fimbrial adhesins), *PapA* (P fimbriae), *PapC* (formation of digalactoside-binding Pap pili), *iutA* (aerobactin receptor) and *BssS* (biofilm formation). PCR analysis of tested genes was performed using specific primers listed in Table 1. Genomic DNA was prepared by picking up one – two colonies were grown overnight on nutrient agar medium then they were resuspended in 100µl of nuclease free water and heated to 95 ºC for 10 min. A reaction mixture containing 0.5 µM of each primer, 1.5 Mm $Mgcl_2$, 0.2 Mm dNTPs, 1 U Taq polymerase (Thermoscientific Dream Taq Green DNA polymerase), 5 µl of DNA and nuclease free water was added for a total volume of 25 µl per reaction.

The PCR program consisted of an initial denaturation step at 94 ºC for 3 min, followed by 40 cycles of DNA denaturation at 94 ºC for 30 s, annealing at 68 ºC for *Afa/dra*, at 48 ºC for *BssS* gene and at 60 ºC for other gene, then extension at 72 ºC for 1 min. This is followed by a final extension step at 72 ºC for 7 min.

An aliquot of each reaction was analyzed on a 1% agarose gel stained with ethidium bromide and compared with 100 bp DNA molecular weight ladder to verify the success of the PCR reaction after visualization under UV illumination.

Quantitative real time-PCR

Total RNA was extracted from the tested cultures using Trizol reagent (Sigma, USA) according to the manufacturer's instructions. The concentration and the purity for each RNA sample were determined spectrophotometrically at 260 and 260/280 nm ratio respectively, using NanoDrop (ND-1000 Spectrophotometer, NanoDrop Technologies, Wilmington, Delaware, USA). One microgram of RNA was then reverse transcribed into complementary DNA (cDNA) using QuantiTect Reverse Transcription kit, QIAGEN, USA (according to the manufacturer's instructions).

The expression level of biofilm in both AmpC and ESBL clinical isolates was estimated using the *BssS* primer listed in Table 1. Amplification and expression were performed using 5x FIREPol EvaGreen, qPCR Mix, ROX Dye; Solis BioDyne (according to the manufacturer's instructions). The reaction mixture was prepared as following: (4 µl of FIREPol ® EvaGreen ®qPCR Mix, 100 nM of forward and reverse primers, and 12 µl of RNase-free water). The prepared master mix was distributed as 18 µl in each tube. Control tubes were set as no template control (NTC). To each tube, 2 µl (100 ng) of the template cDNA (sample tubes) or 2 µl of RNase-free water (NTC tube) were added to the master mix and mixed well to give a final volume of 20 µl.

RT-PCR was performed using Rotor Gene Q thermocycler (QIAGEN, Hilden, Germany) programmed as follow: initial denaturation at 95°C for 15 min, followed by denaturation at 95°C for 15 s, annealing at 48°C for 30 s and extension at 72 °C for 1 min for 40 cycles. The expression of the gene in the samples were measured relative to standard sample and were analyzed with NTC sample. Expression of the target genes was normalized to the

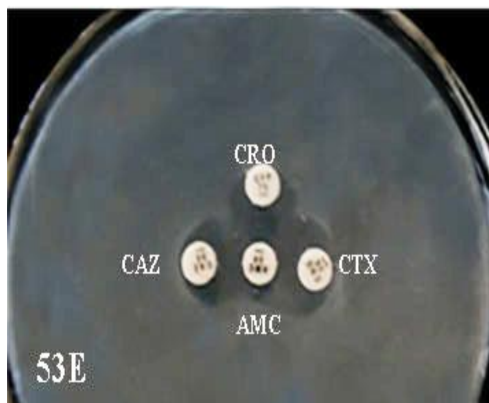

Figure 1. Modified double-disk synergy test on Mueller-Hinton agar plate of isolate 53E. The inhibition zone around all cephalosporins discs showed a clear-cut increase towards the amoxicillin-clavulanate disc (presumptive extended spectrum extended spectrum β-lactamase producer).

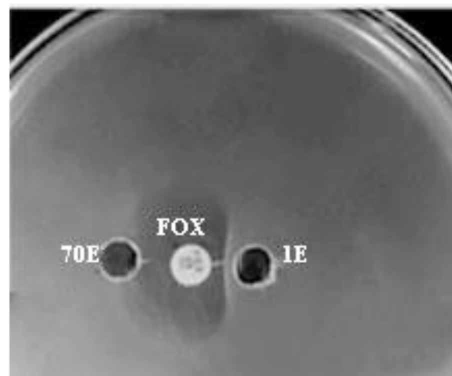

Figure 2. Three-dimensional test on Mueller-Hinton agar plate of isolate 1E and 70 E. Isolate 1E showed clear distortion of inhibition zone around cefoxitin disc (presumptive AmpC producer). No distortion was noted for isolate 70E and it was considered non AmpC producer.

expression of reference gene *16S-rRNA*.

Statistical analysis

Data concerning the presence of various virulence factors associated genes in ESBL and AmpC groups was analyzed using the $\chi 2$ test. Differences were considered significant at $p \leq 0.05$.

Regarding relative expression of *BssS* gene, the Excel data analysis package was used to calculate mean, standard deviation of the mean and standard error. Data was analyzed using t test between ESBL and AmpC groups by the GraphPad Instate software package (version 3.05). Differences were considered significant at p value ≤ 0.05.

RESULTS

A total of 180 clinical isolates were collected from different patients distributed among Mansoura University Hospitals, Dakahlia Governorate, Egypt. One hundred isolates were identified as *E. coli* using standard biotyping methods. Most *E. coli* isolates were obtained from urine (60%) while the remaining isolates were 23 from wound and 17 from sputum.

Phenotypic detection of ESBL and AmpC enzymes

Detection of ESBL activity using modified double disc synergy test method indicated that 37% of the tested isolates were considered positive for ESBL production (Figure 1) and 63 isolates exhibited negative results. On the other hand, detection of AmpC activity using three dimensional extract method have shown that 29% of the tested isolates were considered positive for AmpC production (Figure 2) and 71 isolates were AmpC non producers. None of the tested isolates produced both type of enzymes.

Virulence factors associated genes among ESBLs and AmpC phenotype

The distribution of different virulence factors associated genes among ESBLs and AmpC producing isolates are illustrated in Table 2. PCR detection of *KPsMII* gene reveled that it was harbored by 43.2 and 51.7% of ESBLs and AmpC producing isolates respectively with amplicon size of 269 bp (Figure 3a).

Regarding *FyuA* gene it was detected in 51.35 and 86.2% of ESBLs and AmpC producing isolates respectively with amplicon size of 277bp (Figure 3b).

For *Afa/dra*, it was harbored by 62.1 and 68.96% of ESBLs and AmpC producing isolates respectively with amplicon size of 380 bp (Figure 3c).

Regarding genes for pili it was found that *PapA* is more predominant than *PapC*. For *PapA*, it was harbored by 43.2% and 55.17% of ESBLs and AmpC producing isolates respectively with amplicon size of 519bp (Figure 3d). While, *PapC* was detected only in 16.2% and 24.1% of ESBLs and AmpC producing isolates respectively with amplicon size of 319bp (Figure 3e).

For *iutA*, it was detected in 67.56 and 82.7% of ESBLs and AmpC producing isolates respectively with amplicon size of 253 bp (Figure 3f).

Regarding *BssS* gene for biofilm formation, it is the most predominant among the tested genes. It was harbored by 78.4 and 100% of ESBLs and AmpC producing isolates respectively with amplicon size of 210 bp (Figure 3g).

Among AmpC producing isolates, it was found that four isolates harbored all the tested virulence factors

Table 2. Distribution of different virulence factors associated genes among ESBLs and AmpC producing isolates.

Pattern	MVS	VS	Virulence factor genes							Isolate code	Enzyme type
			BssS	***iutA***	***PapC***	***PapA***	***Afa/dra***	***FyuA***	***KPsMII***		
P1		4	+	+	-	-	+	+	-	1E	
P2		5	+	-	-	+	+	+	+	2E	
P1		4	+	+	-	-	+	+	-	3E	
P3		7	+	+	+	+	+	+	+	4E	
P4		4	+	+	-	+	-	+	-	5E	
P5		6	+	+	+	+	-	+	+	6E	
P1		4	+	+	-	-	+	+	-	7E	
P6		6	+	+	-	+	+	+	+	8E	
P7	4.68	4	+	-	-	-	+	+	+	9E	
P1		4	+	+	-	-	+	+	-	10E	
P8		5	+	+	-	-	+	+	+	11E	
P9		3	+	-	-	+	-	+	-	12E	
P3		7	+	+	+	+	+	+	+	13E	
P3		7	+	+	+	+	+	+	+	14E	
P10		3	+	+	-	-	-	-	+	15E	AmpC
P11		2	+	+	-	-	-	-	-	16E	
P12		5	+	+	-	+	+	+	-	17E	
P8		5	+	+	-	-	+	+	+	18E	
P13		3	+	+	-	-	+	-	-	19E	
P14		4	+	+	-	-	+	-	+	20E	
P15		4	+	+	-	-	-	+	+	21E	
P12		5	+	+	-	+	+	+	-	22E	
P16		3	+	+	-	-	-	+	-	23E	
P17		5	+	-	+	+	-	+	+	24E	
P2		5	+	-	-	+	+	+	+	25E	
P12		5	+	+	-	+	+	+	-	26E	
P3		7	+	+	+	+	+	+	+	27E	
P12		5	+	+	-	+	+	+	-	28E	
P18		5	+	+	+	+	-	+	-	29E	
P19		1	-	-	-	-	+	-	-	30E	
P12		5	+	+	-	+	+	+	-	31E	
P20		3	+	-	-	+	+	-	-	32E	
P21		2	-	-	-	+	-	+	-	33E	
P6		6	+	+	-	+	+	+	+	34E	
P12	3.62	5	+	+	-	+	+	+	-	35E	
P22		5	+	+	-	+	+	-	+	36E	
P23		2	+	-	-	-	+	-	-	37E	
P24		3	-	+	+	-	+	-	-	38E	
P13		3	+	+	-	-	+	-	-	39E	Extended spectrum β-lactamae
P25		5	-	+	+	+	+	-	+	40E	
P26		4	+	+	-	+	+	-	-	41E	
P27		3	+	-	-	+	-	-	+	42E	
P16		3	+	+	-	-	-	+	-	43E	
P28		6	+	+	+	-	+	+	+	44E	
P29		3	+	-	-	-	+	-	+	45E	
P16		3	+	+	-	-	-	+	-	46E	
P18		5	+	+	+	+	-	+	-	47E	
P30		1	+	-	-	-	-	-	-	48E	
P16		3	+	+	-	-	-	+	-	49E	

Table 2. Contd.

Pattern	MVS	VS	Virulence factor genes							Isolate code	Enzyme type
			BssS	*iutA*	*PapC*	*PapA*	*Afa/dra*	*FyuA*	*KPsMII*		
P31		-	-	-	-	-	-	-	-	50E	
P32		1	-	-	-	-	-	-	+	51E	
P16		3	+	+	-	-	-	+	-	52E	
P33		6	+	+	+	+	+	+	-	53E	
P8		5	+	+	-	-	+	+	+	54E	
P6		6	+	+	-	+	+	+	+	55E	
P34		3	+	-	-	-	-	+	+	56E	
P6		6	+	+	-	+	+	+	+	57E	
P6		6	+	+	-	+	+	+	+	58E	
P35		5	-	+	-	+	+	+	+	59E	
P14		4	+	+	-	-	+	-	+	60E	
P36		3	+	-	+	-	+	-	-	61E	
P13		3	+	+	-	-	+	-	-	62E	
P4		4	+	+	-	+	-	+	-	63E	
P11		2	+	+	-	-	-	-	-	64E	
P8		5	+	+	-	-	+	+	+	65E	
P32		1	-	-	-	-	-	-	+	66E	

VS, Virulence score, it was calculated for each isolate as the sum of all virulence-associated genes detected; MVS, Mean virulence score, the sum of all the VS of the isolates was calculated, and this sum was divided by the number of isolates to give the mean virulence score.P, Pattern.

associated genes and 82.7% of the isolates contained four or more virulence factors associated genes. On the other hand, none of ESBLs producers harbored all the tested virulence factors associated genes and there were only 45.92% of the isolates containing four or more virulence factors associated genes.

Statistical analysis revealed that there was extreme significant difference in the presence of *BssS* and *FyuA* gene between AmpC and ESBLs producers as P value was < 0.0001. In addition there was a significant difference in the presence of *iutA* gene between AmpC and ESBLs producers as P value was < 0.05. Regarding other VF associated genes there was no significance difference between AmpC and ESBLs producers.

Based on the distribution of the various VF associated genes, all ESBLs producers exhibit 27 patterns, referred to as P, while AmpC producers exhibit 18 patterns (Table 2). P6 and P16 is the most common pattern among ESBLs producers where each is shared by 4 isolates (10.8%). The majority of patterns (77.7%) were detected by single isolate. Regarding AmpC producers P1, P3 and P12 were the most common patterns (each was exhibited by 4 isolates). The distribution of patterns among all studied isolates revealed that 9 patterns were shared by ESBLs and AmpC producers, 18 patterns were detected only in ESBLs producers and 9 patterns were found only in AmpC producers (Figure 4).

Expression of BssS gene

The relative expression of *BssS* gene in randomly selected 14 ESBL and 15 AmpC producers was performed (Figure 5). Statistical analysis revealed that AmpC producing *E. coli* showed very significantly higher expression (P<0.01) of *BssS* gene than ESBLs producing *E. coli.*

DISCUSSION

E. coli is a serious cause of urinary tract infections, enteric infections and systemic infections in humans (Mandell et al., 2005). Pathogenic *E. coli* clones have acquired specific virulence factors, which confer an increased ability to cause a broad spectrum of diseases (Kaper et al., 2004).

The control of *E. coli* infections is complicated due to the increasing resistance to antibiotics. The greater prevalence of resistance to common antibiotics has been reported by Hassan et al. (2010). The production of β-lactamase remains the most important mediator of β-lactam resistance among *E. coli* as ESBL and AmpC β-latamases are important causes of β-lactam resistance among extraintestinal pathogenic *E. coli* (ExPEC) (Pitout, 2012).

Similar to ESBL-producing bacteria, organisms harbored plasmid- mediated AmpC enzymes have mostly been responsible for nosocomial outbreaks on a worldwide although the risk factors associated with infection are not as well defined as those associated with ESBL- producing bacteria (Philippon et al., 2002). This study was therefore designed to investigate the virulence factors of *E. coli* that produce ESBL and AmpC enzymes.

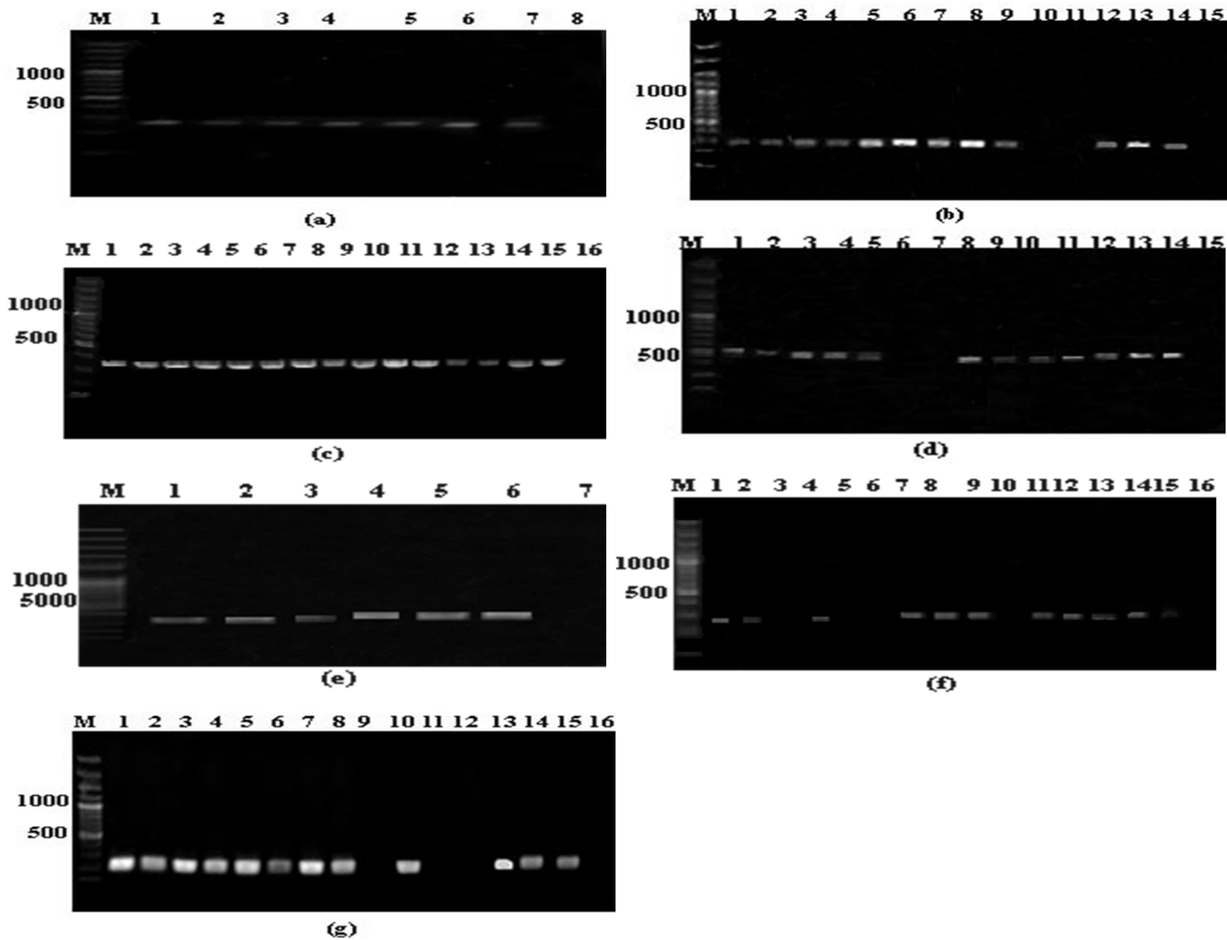

Figure 3. Agarose gel electrophoresis of gene amplicones. a. KpsMT Lane M was 100 bp plus DNA Ladder. Lanes from 1 to 7 were amplicones from *E. coli* isolates No. 18E, 21E, 27E, 40E, 54E, 57E and 60E respectively. Lane 8 was negative control. **b.** fyuA. Lane M was 100 bp plus DNA Ladder. Lanes from 1 to 14 were amplicones from *E. coli* isolates No.1E, 2E, 3E, 4E, 5E, 6E, 7E, 33E, 34E, 41E, 42E, 46E, 47E and 52E respectively. Lane 15 was negative control. **c.** Afa/dra. Lane M was 100 bp plus DNA Ladder. Lanes from 1 to 15 were amplicones from *E. coli* isolates No 8E, 9E, 13E, 14E, 17E, 18E, 19E, 20E, 30E, 31E, 32E, 38E, 39E, 40E and 58E respectively. Lane 16 was negative control. **d.** papA. Lane M was 100 bp plus DNA Ladder. Lanes from 1 to 14 were amplicones from *E. coli* isolates No 4E, 5E, 6E, 14E, 17E, 21E, 23E, 47E, 53E, 55E, 57E, 58E, 59E, and 63E respectively. Lane 15 was negative control. **e.** PapC. Lane M was 100 bp plus DNA Ladder. Lanes from 1 to 6 were amplicones from *E. coli* isolates No.13E, 14E, 29E, 38E, 40E and 61E respectively. Lane 7 was negative control. **f.** iutA. Lane M was 100 bp plus DNA Ladder. Lanes from 1 to 15 were amplicones from *E. coli* isolates No 10E, 11E, 12E, 13E, 24E, 25E, 26E, 27E, 28E, 30E, 49E, 52E, 53E, 54E and 55E respectively. Lane 16 was negative control. **g.** BssS. Lane M was 100 bp plus DNA Ladder. Lanes from 1 to 15 were amplicones from *E. coli* isolates No. 19E, 20E, 21E, 22E, 24E, 25E, 26E, 28E, 30E, 32E, 33E, 50E, 44E, 46E and 53E respectively. Lane 16 was negative control.

Results of our study have shown that ESBL producing *E. coli* was 37% and this result is comparable to those obtained by Chayakulkeeree et al. (2005) who reported that the rate of ESBL producing *E. coli* was 33.3% while a higher level of ESBL producing *E. coli* (60.9%) was reported in Egypt (Al-Agamy et al., 2006). Regarding AmpC enzymes, it was present in lower percentages of isolates (29%) and this result was equivocal with studies carried by El-Hefnawy (2008), Barwa et al. (2012) and Fam et al. (2013) from Egypt where AmpC prevalence was 34, 31.6 and 28.3% respectively while studies from India have shown that 50-75% prevalence of ESBL (Goyal et al., 2009; Chakraborty et al., 2013) and 30-50% prevalence of AmpC production among *E. coli* (Chakraborty et al., 2013). These differences between studies may be assumed to the difference in the study number population, time of collection, types of organisms tested and geographical area (Shah et al., 2004; Thabit et al., 2011).

Extraintestinal pathogenic *E. coli* possess a broad spectrum of specialized VFs responsible for pathogenesis outside the gastrointestinal tract including different adhesions, capsules, toxins, siderophores and invasions. These VFs contribute to fitness, colonization,

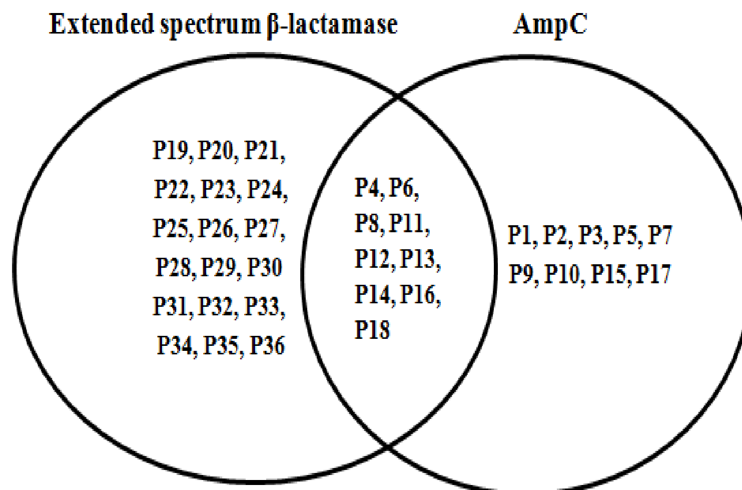

Figure 4. Distribution of virulence gene patterns in relationship with extended spectrum β-lactamase and AmpC phenotypes.

invasion into host tissues, avoidance of immune responses and antimicrobial drugs in addition to acquiring nutrients from the host (Pitout, 2012). Genomics offers an interesting tool for defining virulence factors; it can be used to identify genes encoding for specific factors that are responsible for virulence in pathogens. However, the presence of a single factor rarely makes an organism virulent while combination of factors would determine if a bacterium can cause infection (Dobrindt, 2005). In this study, the detection of several virulence factors associated genes (*KPsMII, FyuA, Afa/dra, PapA, PapC, iutA* and *BssS*) among *E. coli* isolates revealed a higher prevalence in AmpC producers than ESBL producers probably because usually low amounts of AmpC are produced as AmpC is regulated by a weak promoter and strong attenuator (Olsson et al., 1982). Clermont et al. (2008) has suggested that the prevalence of VFs decreased within resistant strains. It was found in this study that biofilm formation gene (*BssS*) and siderophore genes (*fyuA* and *iutA*) were the most prevalent genes in AmpC producers and at the same time Pap pili gene *PapC* was less prevalent as compared to other virulence genes. But among ESBL producers biofilm formation gene (*BssS*) siderophore genes (*iutA*) and adhesion gene (*Afa/dra*) were the most prevalent genes. Similar to AmpC producing isolates Pap pili gene PapC was less prevalent when compared to other virulence genes. In a previous study by Lillo et al. (2014) regarding ESBL producing *E. coli*, *fyuA* and *iuTA* genes were more prevalent, also adhesion gene *afa/dra* was detected in high percentage of strains.

Among the tested VFs in our study, only *BssS*, *fyuA* and *iuTA* genes exhibited a statistically significant higher prevalence among AmpC producers than ESBL producers. Among the investigated isolates, AmpC producers exhibited a statistically significant higher prevalence of multivirulence (MV) (MV≥4) than ESBL producers (Table 2). Our finding suggests that AmpC phenotypes might be more virulent than ESBL phenotypes.

In our study, various combinations of detected genes were designated as virulence patterns. Analysis of virulence patterns distribution in ESBL and AmpC producers did not allow the determination of a clear correlation between a determined genes distribution and the type of β-lactamase enzyme (Figure 4). This is because most of the patterns detected in ESBL and AmpC producers were represented by single isolate. In addition 25% of patterns were shared by both ESBL and AmpC producers.

Lee et al. (2008) had reported that there is a strong relationship between biofilm formation and production of betalactamases. In this study, the relationship between biofilm formation and both ESBLs and AmpC producers was also investigated by detecting the relative expression level of BssS gene in some isolates (Figure 5). It was found that AmpC producers exhibited a statistically very significant higher expression of *BssS* gene than ESBL producers. Swarna et al. (2014) reported that antimicrobial resistance is compounded by their ability to form biofilm that increases their virulence. This could explain our findings where AmpC producers exhibited a statistically significant higher prevalence of multivirulence (MV) (MV≥4) than ESBL producers.

In conclusion, the present results suggest a correlation between AmpC phenotypes and production of some factors that are reported to be involved in the virulence of *E. coli*. In addition *E. coli* isolates producing AmpC are thought to be more virulent than ESBL producers. Investigation of the bacterial pathogenicity associated with ESBL and AmpC phenotypes may contribute to a more clear medical intervention.

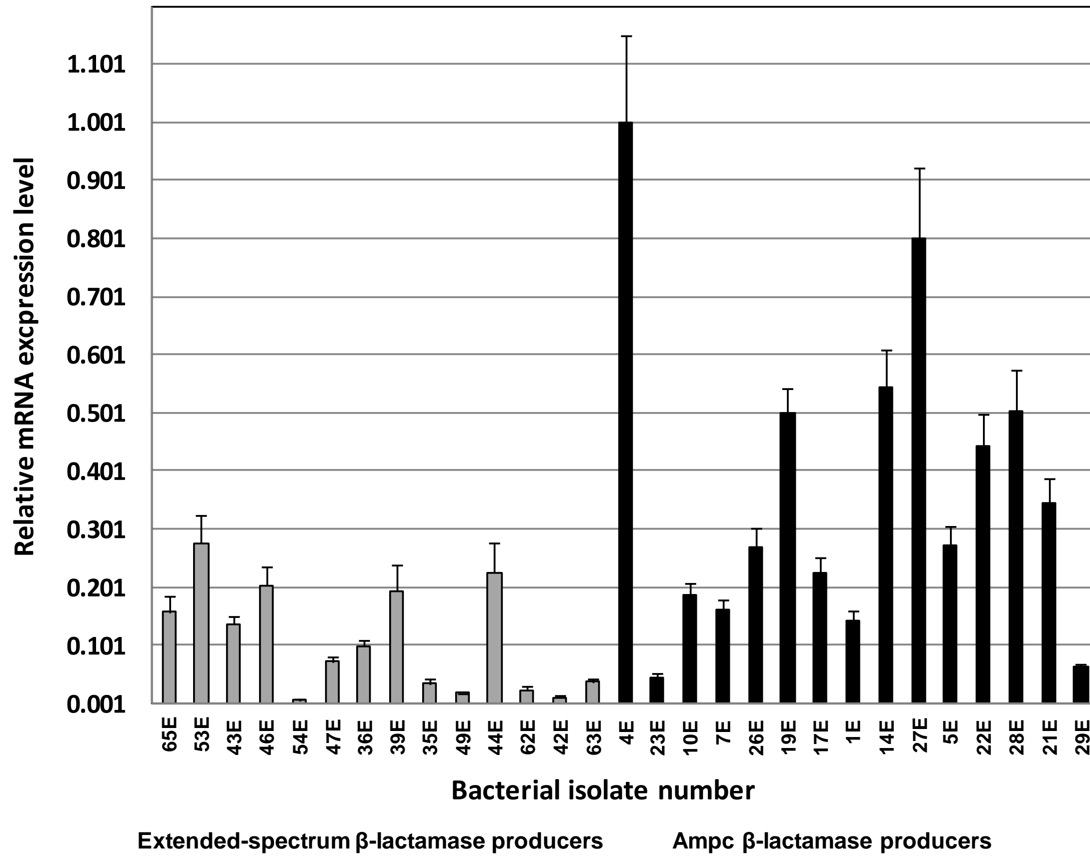

Figure 5. Relative expression of BssS gene in randomly selected 14 extended spectrum β-lactamase and 15 AmpC phenotypes.

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Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Microbial activity in two soils with different clay content contaminated by different diesel/biodiesel mixtures

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Biodiesel is an alternative energy source that has a high biodegradability potential and low toxicity, contributing to ecosystem impact reductions. The aim of this study was to determine, by the natural attenuation technique, the microbial activity of two soils: one clayey (CLA) and the other sandy (SAN), contaminated with different concentrations of biodiesel blended with diesel (B0, B5, B20 and B100) simulating a surface spill. The respirometry, fluorescein diacetate (FDA) hydrolysis and cultivable heterotrophic bacteria and actinobacteria count techniques were used to determine the microbial activity in the different microcosms at up to 48 days of incubation. For the respiration activity, the CLA soil was most active at all mixed fuel concentrations (B0, B5, B20 and B100), as compared to the SAN. Furthermore, the biodiesel addition to the two soil types contributed to the microbial activity increase, and higher CO_2 release values were found in the B20 and B100. For the FDA activity, it was found that the CLA soil showed higher activity at the B5 and B20 concentrations, and heterotrophic count showed a tendency towards a CFU g^{-1} decrease as the incubation time increased. This indicates that the CLA soil, due to a higher amount of nutrients, clay, organic matter and CEC, was associated with the addition of biodiesel and showed higher microbial activity. The results obtained in this study contribute to future studies of surface contamination by different mixtures of diesel/biodiesel in soils with similar physical and chemical characteristics.

Key words: Biodiesel, diesel, biodegradation, natural attenuation, clayey and sandy soils.

INTRODUCTION

The search for alternative energy sources has been encouraged in recent decades due to the disadvantages presented by the use of diesel and other petroleum products. These disadvantages can be exemplified by the high levels of contamination in different environmental compartments and the dependence on non-renewable energy sources, such as the use of petroleum-based fuels. On the other hand, the biodiesel from renewable sources, is produced from vegetable oils or animal fats which are transformed into long-chain esters by various esterification or transesterification processes (Demirbas, 2008; Dias et al., 2014).

The use of biodiesel has been widely stimulated in many countries. Brazil has a regulatory framework that

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authorizes the commercial use and which promotes the growing addition of this biofuel to diesel oil by the fuel distributors (MME, 2014). Depending on the biodiesel addition percentage in the diesel/biodiesel blend, a nomenclature is given to the mixture. For example, B5 has 5% biodiesel; B20, 20%; until reaching B100 corresponding to pure biodiesel (Silva and Freitas, 2008).

The use of biodiesel has many environmental advantages, since this fuel has high biodegradability potential and low toxicity (Lisiecki et al., 2014). This is due to the presence of fatty acids and two oxygen atoms which make up the ester function, which allows greater molecule reactivity (Chao et al., 2010, Bücker et al., 2011). In the absence of sulfur and polycyclic aromatic hydrocarbons in the composition, low CO_2, CO, SO_2 and particulate material atmospheric emission levels are observed during biodiesel combustion (Canakci et al., 2006), biodiesel being considered a non-toxic fuel for ecosystems and the biota that live in them. This proves of great interest because spills, leaks and accidents with fuel used in the country can generate serious contamination events, especially in soils.

One of the most widely used techniques for the decontamination of affected environments is bioremediation. This technology uses the physiological competence of micro-organisms (native or introduced), to completely degrade a contaminant in addition to it being a low cost-effective and environmentally acceptable alternative (Jacques et al., 2007). Among bioremediation strategies, natural attenuation is considered an advantageous process, because the reduction of the contaminant may effectively occur using only the potential of the native microbial population without the addition of nutrients to the soil (Horel and Schiewer, 2011). Thus, a positive point of biodiesel is its accelerated biodegradation when compared with other fuels such as diesel (Sorensen et al., 2011).

Due to the addition of an ever-increasing percentage of biodiesel to diesel, it is essential to assess the behavior of these different mixtures, simulating a surface spill of such fuels in soils. The aim of this study was to determine the whole microbial activity from the soil, by the passive bioremediation technique (natural attenuation), performed by microbial respiration, microbial activity of degradative enzymes and quantification of heterotrophic bacteria in two soil types: 1) Oxisol, basaltic substrate (CLA) and 2) Rhodic Paleudalf (RP), sandy substrate, (SAN) contaminated with different biodiesel/diesel blends (B0, B5, B20 and B100).

MATERIALS AND METHODS

Soils

Surface samples of two types of soil were collected from an area without contamination history at a depth of 0-10 cm (The Horizon), classified by the Brazilian System of Soil Classification (EMBRAPA, 2006 as: 1) Oxisol, basaltic substrate (clayey - CLA) and 2) Rhodic Paleudalf (RP), sandy substrate (sandy - SAN) collected in areas along the Federal Highway BR 386, geographically located in the municipalities of Fazenda Vilanova, RS (29° 34’ 43.6’’ S, 51° 50’33.8’’ W) and Triunfo, in Coxilha Velha, RS (29° 44’ 09’’ S, 51° 37’ 44.7’’ W), respectively. The soil choice was based on differences in the organic matter, clay and cation exchange capacity (CEC) levels. After collection, the material was sieved through 2 mm mesh. Soil moisture was standardized, presenting field capacity around 60%. During the experiment, distilled water was added to respirometric bottles to maintain the same standard of humidity during the incubation process. The physical and chemical properties of the soil were determined in the Soil Science Department Analysis Laboratory of the Faculdade de Agronomia of the Federal University of Rio Grande do Sul and by the Soil Laboratory of the Fundação Estadual de Pesquisa Agropecuária de Porto Alegre, RS, according to the methodologies described by Tedesco et al. (1995) (Table 1).

Table 1. Physical and chemical characterization of two soils evaluated.

Evaluated parameter	Depth (0-10 cm)	
	Sandy (SAN)[a]	**Clayey (CLA)[b]**
Clay (g kg^{-1})	140	480
Organic Matter $^{-1}$)	10	24
CEC ($mmol_c$ dm^{-3})	7.4	10.8
pH (H_2O)	4.5	5.2
SMP Index	5.6	6.0
Available P (mg dm^{-3})	2.8	2.6
Available K (mg dm^{-3})	39	52
Zinc (mg DM^{-3})	0.7	5.8
Copper mg DM^{-3})	0.8	4.5
Manganese (mg dm^{-3})	25	94
Exchangeable aluminum (mmolc dm^{-3})	1.5	0.4

[a]Rhodic Paleudalf; [b]Oxisol.

Fuels

Samples of metropolitan diesel and soy biodiesel were used, provided by the Ipiranga Oil Distributor and BSBios companies, respectively. Sterilization of fuels (diesel and biodiesel) was performed by vacuum filtration through a membrane with 0.22 µm pores, using a sterile *kitassato* flask. After this procedure, the fuels were stored in sterile flasks and sealed. To avoid photo-oxidation, the flasks were protected from light with aluminum foil and stored at room temperature. Different diesel/biodiesel mixtures were prepared: 100% diesel fuel (B0); 95% diesel and 5% biodiesel (B5); 80% diesel and 20% biodiesel (B20) and 100% biodiesel (B100).

Respirometric analysis

The methodology used was described by Bartha and Pramer (1965), which quantifies the carbon dioxide released via microbial respiration in the soil. Soil samples (300 g) were added in 1000 ml glass jars and were then infected with 15 ml from fuel (B0, B5, B20 or B100) per kg^{-1} dry soil. The flasks were equipped with a CO_2 capture apparatus, containing plastic cups with 20 ml from a 0.75 M

NaOH solution and were hermetically sealed. The flasks were slightly open every 12 days, removing the container with the NaOH solution and mixing 3 ml of $BaCl_2.2H_2O$ 30% in order to precipitate the CO_2 in solution. Afterwards, 200 µL from phenolphthalein indicator were added, and finally the solution was titrated with 0.5 M HCl until the solution changes the color. A soil sample was removed with moisture equal to that present in the experiments to determine the mass of dry soil in kg, expressing the production of C-CO_2 in mg.Kg^{-1} dry soil. The experiments were conducted in triplicate and incubated at 28°C for 48 days. The negative control corresponded to the soil samples (CLA and SAN) in which there was no fuel addition (NC). The production of C-CO_2 was determined according to Stotzky (1965), the values in mg CO_2 determined by the formula below:

$$\text{mg C-CO}_2 = \frac{[(B - T) \times eq \times M\ HCl \times CF]}{Mc}$$

Where: B = volume (ml) of HCl solution used to titrate the negative control (without soil); T = volume (ml) of HCl solution used to titrate the treatment; eq = gram-equivalent of C (= 6); M = molarity of standardized HCl solution; CF = correction factor for acid/base normality = M HCl/M NaOH; Mc = soil dry mass (kg).

Microbial activity by degradative enzymes in the soil

The microbial activity of the contaminated soil sample was evaluated according to the fluorescein diacetate (FDA) hydrolysis technique, wherein said substrate is mostly cleaved by esterases, lipases and dehydrogenases (Schnürer and Rosswall, 1982). Samples of 1 g of each soil type (sandy and clay) were distributed into centrifuge tubes in which 20 ml from a 60 mM sodium phosphate buffer was added. These tubes were incubated under agitation at 180 rpm and 28°C for 15 min. Afterwards, 100 µL from stock FDA solution was added into all tubes. The same procedure was used for negative controls (no added FDA) to subsequently subtract the turbidity values of the soil samples. All samples were incubated again at 28°C for 15 min under agitation at 180 rpm, promoting the stoppage of the enzymatic reaction by the addition of 20 ml of acetone p.a. The samples were centrifuged for 5 min at 6000 rpm, and the supernatant was filtered in Whatman No. 4 filter paper. The solutions were evaluated in a spectrophotometer (λ = 490 nm). To determine the standard curve, the following fluorescein concentrations were used: 0.00; 0.25; 0.75; 2.00; 3.74 and 7.48 µg.ml^{-1}. Microbial activity was evaluated at 0, 13 and 48 days and the experiment was conducted in triplicate.

Quantification of heterotrophics in the soil

To estimate the count of cultivable heterotrophic bacteria present in the soil samples, the plate count technique was used (APHA, 1998). The plate count agar (standard agar for counting) (PCA HiMedia) and starch casein agar (SCA) were used in order to determine the total heterotrophic bacteria and actinobacteria cell counts, respectively (CFU.ml^{-1}). The experiment was conducted in triplicate, and 1 g from soil samples (SAN and CLA) underwent serial dilutions in saline solution (0.85%), using an aliquot of 0.1 ml for spreading on the plates. For counting of bacteria and actinobacteria, the Petri dishes were incubated, respectively, for 24 h and 5 days at 28°C. The experiments were performed at 0, 13 and 48 days.

RESULTS

Soil physical and chemical characterization

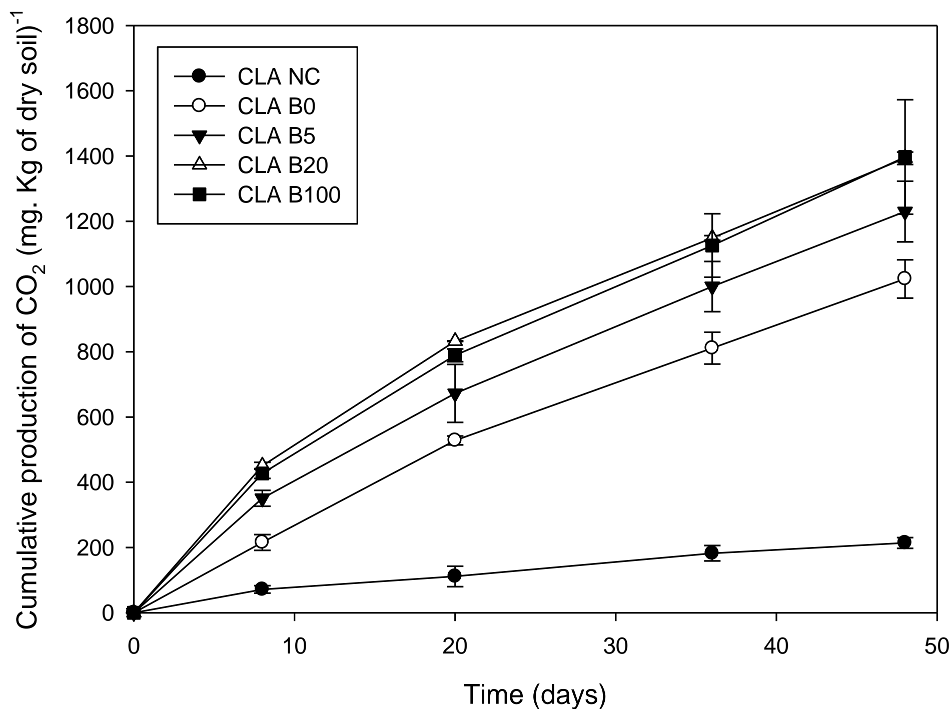
Table 1 shows the physical and chemical characterization of the soils. For all physico-chemical parameters evaluated, it can be seen that the clay soil (CLA) presents the highest values, such as those for clay, organic matter and cation exchange capacity, than the sandy soil (SAN). The clay content ranged from 480 g kg^{-1} in the clayey to 140 g kg^{-1} in the sandy, and the organic matter values ranged from 24 g kg^{-1} in the clayey to 10 g kg^{-1} in the sandy. The cation exchange capacity (CEC) was also higher in the clayey soil, with values ranging from 10.8 mmol dm^{-3} in clayey soil to 7.4 mmol dm^{-3} in the sandy soil. The clayey soil also showed higher amounts of available K (52 mg dm^{-3}), zinc (5.8 mg dm^{-3}), copper (4.5 mg dm^{-3}) and manganese (94 mg dm^{-3}), and the sandy soil presented a more acidic pH than the clayey soil.

Respiratory activity

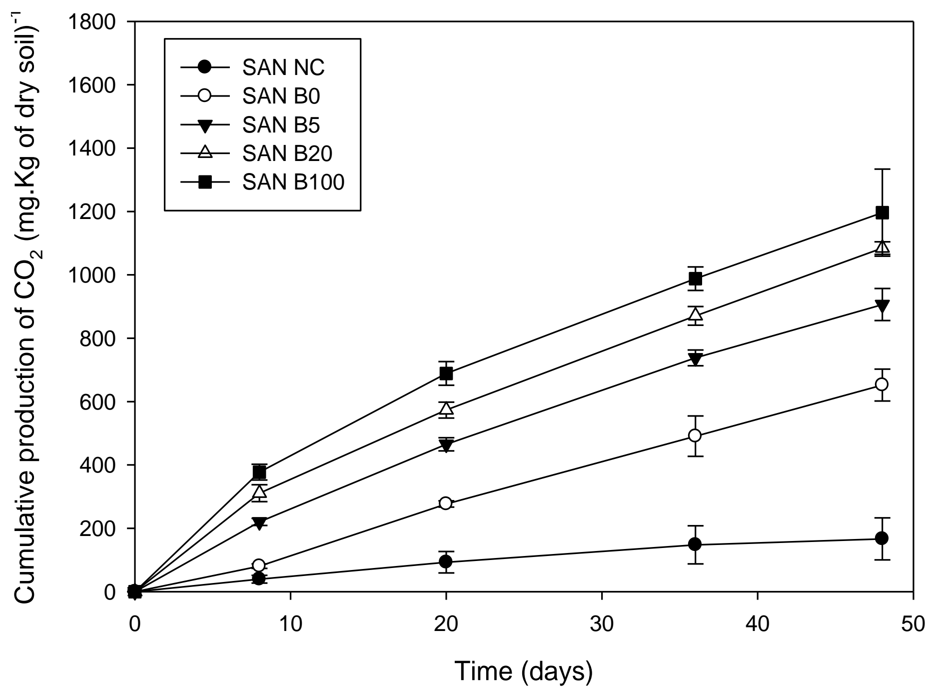
Figure 1A, B and Table 2 show the cumulative CO_2 release in Oxisol (CLA) and Rhodic Paleudalf (SAN), contaminated by different mixtures of diesel/biodiesel. By comparing the respirometric analysis of both soil samples, it can be seen that at all concentrations (B0, B5, B20 and B100), the clayey soil showed higher cumulative CO_2 release values than the sandy soil ($p < 0.05$). The highest cumulative CO_2 release values were observed in the clayey soil in the CLA concentration of B20 and CLA B100 at 48 days, the values ranging, on average, from 1393.14 to 1396.9 mg kg^{-1} dry soil, respectively. The sandy soil had the lowest average cumulative CO_2 release values under the same concentrations and the same incubation time (1084.46 and 1196.43 mg kg^{-1} dry soil, respectively). The lowest average values of cumulative CO_2 release were observed in sandy soil in the control treatment (NC SAN) and SAN B5 after 48 days of incubation (1023.36 and 166.63 mg kg^{-1} dry soil, respectively).

Fluorescein diacetate (FDA)

Figure 1A, B and Table 2 show the sum of the average fluorescein activity in samples of the two soil types in the presence of different diesel/biodiesel mixtures. It can be seen that the highest total fluorescein activity averages were found in treatments CLA B5 and CLA B20 (104.1 and 101.2 mg of F g^{-1} dry soil h^{-1}, respectively). The same treatments in sandy soil had lower values than the clay soil (55.7 and 64.5 mg of F g^{-1} dry soil h^{-1}, respectively). The CLA, NC and CLA B0 control treatments of the clayey soil had the lowest average total fluorescein



(A)



(B)

Figure 1. (A) Cumulative CO_2 release in a Oxisol soil (CLA). ANOVA, tukey < 0.05 for the respective incubation times: 8 days: NCd (Negative Control), B0c, B5b, B20a, B100a; 20 days: NCd, B0c, B5b, B20a, B100a; 36 days: NCc, B0b, B5a, B20a, B100a; 48 days: NCc, B0b, B5ab, B20a, B100a. (B) Cumulative CO_2 release, in a rhodic paleudalf soil (SAN), contaminated by different mixtures of diesel/biodiesel. ANOVA, Tukey < 0.05 for the respective incubation times: 8 days: NC^d (Negative Control), $B0^d$, $B5^c$, $B20^b$, $B100^a$; 20 days:NC^e, $B0^d$, $B5^c$, $B20^b$, $B100^a$; 36 days: NC^d, $B0^c$, $B5^b$, $B20^a$, $B100^a$; 48 days: NC^d, $B0^c$, $B5^b$, $B20^{ab}$, $B100^a$

Table 2. Microbial activity in soils contaminated with different mixtures of diesel/biodiesel.

Fuel concentration	SAN		CLA	
	CO_2[1] Release	FDA[2] Activity	CO_2 Release	FDA Activity
NC	166.63^{Ad}	25.38^{A}	213.98^{Ac}	29.26^{A}
B0	652.11^{Bc}	24.56^{A}	1023.36^{Ab}	29.55^{A}
B5	906.31^{Bb}	18.57^{B}	1229.56^{Aa}	34.71^{A}
B20	1084.46^{Ba}	21.52^{B}	1393.14^{Aa}	33.73^{A}
B100	1196.43^{Ba}	16.41^{A}	1396.9^{Aa}	17.18^{A}

[1]Cumulative CO_2 production during the 48 days of incubation (mg of CO_2. Kg^{-1} dry soil.); [2]average FDA values for the three monitoring points (at times 0, 13 and 48 days) (mg of fluorescein. g^{-1} dry soil. h^{-1}). Uppercase letters: comparison of fuel concentrations between soils (Tukey <0.05); lowercase letters: comparison of fuel concentrations within each soil type (Tukey<0.05). NC: negative control.

activity, however, still higher than that in the sandy soil (87.8 and 88.6 mg of F g^{-1} dry soil h^{-1}, respectively). Thus, it can be seen that most of the treatments incubated in clayey soil showed higher microbial activity relative to the sandy soil, as compared to the same diesel/biodiesel mixtures. These results confirm the responses of the CO_2 release between the two soils, reaffirming that, in clayey soil, due to its physicochemical properties, the indigenous microbiota showed higher metabolic activity. Thus, it was expected that B100 treatment, in the clayey soil, as well as in the sandy, would present the highest average values and hence higher average total for fluorescein activity. However, this behavior did not occur (Figure 2).

Cultivable heterotrophic bacteria count

The cultivable heterotrophic bacteria and actinobacteria count in the diesel/biodiesel contaminated treatments in both CLA (clay) and SAN (sandy) soils are presented in Table 3. The results demonstrate a score reduction trend, with passing experiment incubation time (Table 3). Bacteria had higher CFU counts at baseline (0 days). Treatments B5 SAN and B100 CLA correspond to the samples with the highest counts (4870.7 g^{-1} and 512.14 CFU, respectively). At the 13th day of the experiment, a microbial population significant reduction can be observed, particularly in the sandy soil (SAN), with an average of 7.36 CFU.g^{-1} among treatments SAN B0, B5, B20, B100 and NC. This trend was not observed in clayey soil (CLA) for treatments CLA B0, B5 and B20, it was only observed in the control treatment (NC CLA). However, at 48 days, there was a drastic reduction of the microbial population in all treatments (B0, B5, B20, B100 and control), with average values of 13.79 a 3.50 CFU.g^{-1}.

The count of actinobacteria colonies showed lower count values in relation to the total bacteria, and presented a count number in a progressive reduction. However, the CLA B5 treatment was an exception, as there was an increase of populations during the experiments, observing count values of 12.6 CFU.g^{-1} at 13 days and 24.72 CFU.g^{-1} at 48 days. At the end of the experiment, CLA B5 was the treatment with the highest actinobacteria count.

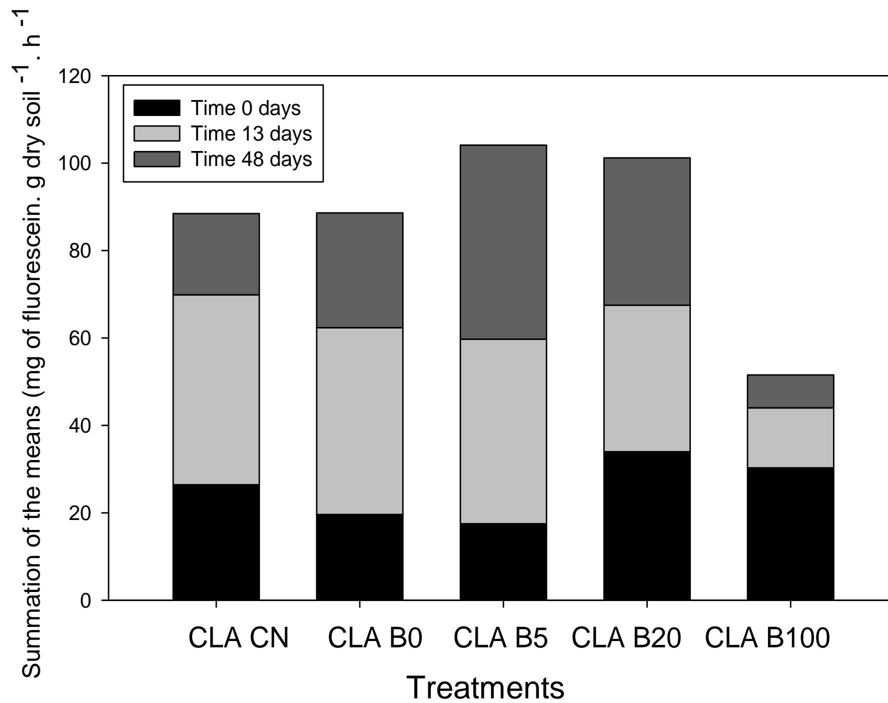
DISCUSSION

Physicochemical characterization of studied soils

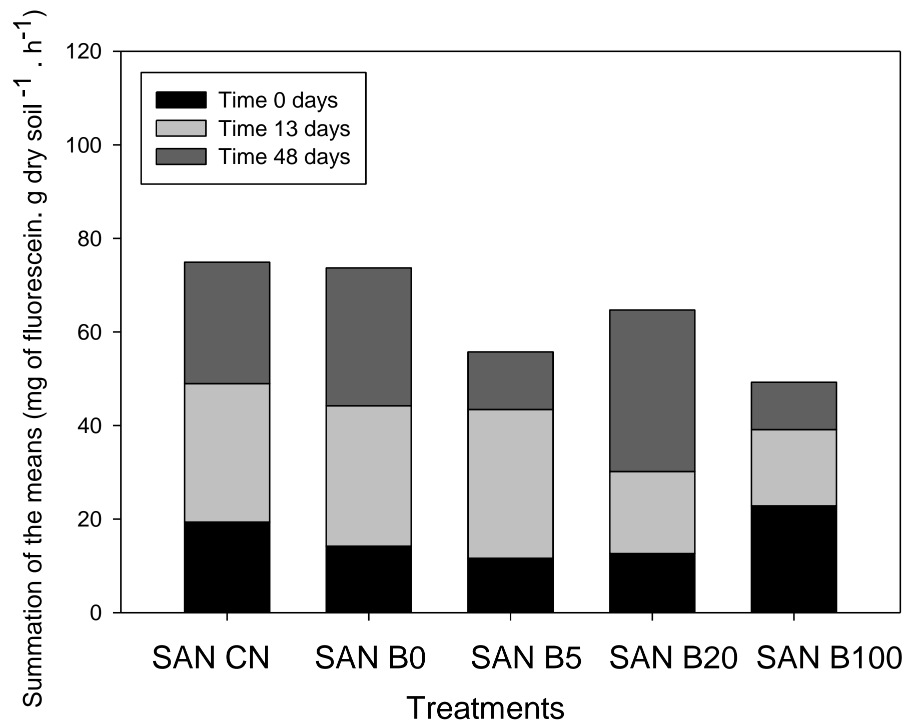
When evaluating the results of the cumulative CO_2 release and physicochemical characterization of the two soils, we can infer that some characteristics of the clayey soil (CLA) such as pH, CEC and organic matter content, may have contributed to the higher cumulative CO_2 release values in this soil. The pH of the clayey soil (CLA) was monitored with a value above 5.0, closer to neutrality in relation to the sandy soil (SAN). Soil pH is an important factor because it is responsible for controlling and maintaining the biological processes, and the pH range required for such activity is between 5.5 and 8.8. In addition, the soil CEC values and the organic matter content were higher in the clayey soil (CLA). These characteristics may have contributed to the growth and maintenance of the native microbiota present in this soil, aiding survival in the presence of the contaminant. Furthermore, this soil also had the highest K concentrations, and this macronutrient is considered essential for microbial growth. Melo et al. (2010), studying the covering soil attenuation process in a municipal solid waste landfill, observed that the samples containing higher values of MO, P and K were those that also showed the highest cumulative CO_2 values throughout the experiment. Thus, the physicochemical characteristics of impacted soils can positively or negatively influence the passive attenuation process.

Respiratory activity

The release of CO_2 is an indirect measure used to evaluate microbial activity in the soil through which the respiration of existing microbiota is evaluated. The contribution of the presence of biofuel in the diesel/-biodiesel blend was confirmed in this study by the highest cumulative CO_2 release values in treatments B100 (100% biodiesel) and B20 in both soils. These results corroborate the results found by Mariano et al. (2008), although they found high cumulative CO_2 release values in the B5 and B20 blends as compared to diesel separately (B0). The highest values were also found in the B100 mixture. The biofuel presence in the diesel/biodiesel blend in soil may facilitate microbial



(A)



(B)

Figure 2. Total of the average fluorescein activity in Oxisol soil (CLA) (A) The sum of average fluorescein activity in a rhodic paleudalf soil (SAN); (B) contaminated by different mixtures of diesel/biodiesel.

Table 3. Count of cultivable heterotrophic bacteria and actinobacteria from various treatments contaminated with diesel/biodiesel mixtures in two types of soil.

Treatment	Actinobacteria Count (10^3 UFC. g^{-1} dry soil)			Bacteria Count (10^3 UFC. g^{-1} dry soil)		
	0 days	13 days	48 days	0 days	13 days	48 days
CLA NC	0.00	4.85	2.56	105.96	24.72	2.20
CLA B0	8.83	16.77	0.79	105.96	176.60	4.24
CLA B5	0.00	12.36	24.72	97.13	194.26	3.97
CLA B20	17.66	5.33	0.26	79.47	211.92	53.00
CLA B100	0	2.65	2.65	512.14	256.00	5.56
SAN NC	9.19	0.92	1.38	91.90	8.64	1.47
SAN B0	9.19	1.28	0.73	91.90	8.27	1.93
SAN B5	18.38	0.78	0.46	4870.70	6.98	0.32
SAN B20	1838	0.37	0.46	275.70	5.51	6.89
SAN B100	0.00	0.55	0.92	321.65	7.44	6.89

degradation of the fuel (Soares Junior et al., 2009). Biodiesel is more readily metabolized than diesel, first, because it is a labile compound consisting of fatty acids with oxygen atoms attached at one end. Therefore, when recognized, they are immediately attacked by enzymes such as acyl-CoA dehydrogenase (Balat, 2011; Yusuf et al., 2011). The highest CO_2 rate release estimated in the clayey soil treatments can be explained by probably having had variations in environmental factors that influence the bioavailability of the contaminant and the structure and density of the biodegrading community in the soil. Furthermore, it is important to note that clayey soils have a relatively higher CEC, among other physical and chemical factors, which helps in the breakdown of covalent bonds of a xenobiotic (abiotic degradation), thus allowing an increase in biotic degradation due to the hydrolysis process (Fay et al., 2008).

Fluorescein diacetate (FDA)

The diacetate fluorescein (FDA) hydrolysis method is considered a simple and quick procedure to evaluate microbial activity since the FDA is hydrolyzed by various enzymes (lipases, proteases and esterases) produced by the microorganisms. Biodegradation of biodiesel begins with methyl or ethyl ester hydrolysis from lipase or esterase action (Boczar et al., 2001). However, the results obtained by analysis of the fluorescein diacetate hydrolysis in the B100 treatment for both soils were not consistent, pointing to probable improper use of this indicator in this study. Orantas (2013) also detected a similar problem when studying the effect of oily sludge in isolation in the soil. The authors reported that the results indicated that the hydrolysis of FDA would not be a good microbial activity indicator related to hydrocarbon degradation because probably the lipases, proteases and esterases hydrolyzed were less related to hydrocarbon degradation process than dehydrogenases. Furthermore, the presence of sludge interfered with the detection of this enzyme, thus there was no good standard of evaluation. A similar problem was detected in 100% biodiesel in the present study: possibly the presence of this compound may have caused an interference in the detection of fluorescein activity in both soils. It can be inferred that in the first 13 days of treatment, there were significant changes in enzyme production (lipases and esterases). However, as the biodiesel concentration in the diesel/biodiesel mixture increased, the enzyme activity decreased. These results were not expected since biodiesel contains long chain fatty acids in its composition and thus can be considered a good stimulator for the synthesis of lipase and esterase. However, it can be seen that at lower concentrations, the addition of diesel fuel in the soil will probably have stressed the native microbiota, stimulating the enzyme production in an attempt to degrade the xenobiotics. Some studies have argued that biodiesel, when added to diesel/biodiesel blends, acts as a co-metabolizing agent, enhancing the degradation rate of the most recalcitrant compound by leading to an increase in the microbial population and stimulating the production of degradative enzymes (Pasqualino et al., 2006; Schleicher et al., 2009; Horel and Schiewer, 2011).

Cultivable heterotrophic bacteria count

Regarding the cultivable heterotrophic bacteria, it should be pointed that the two soil samples used in this study did not have a contamination history. Thus, the tested bacteria were subjected to an intense adaptation process and probably top an intensive reorganization of the structure and composition of the microbial population. It is

still important to note that, due to the addition of the diesel/biodiesel blend, the bacterial community may have undergone physiological stress and led through an intense rehabilitation process. In turn, due to the fact that the native heterotrophic bacteria belong to a soil without a contamination history, a large part may have been selected, with potentially degrading microbiota, from the added xenobiotic, surviving for the most part.

In the CLA B100 treatment, microbial reduction was observed whose control had more emphatic difference, and that progressively maintained itself until the end of the 3rd processing, with the possibility of depletion of readily available carbon sources. Similar results were found by Silva et al. 2012. The same was not observed in CLA treatments B0, B5 and B20, which showed an increase of the bacterial population until of the 2nd processing, probably by selection/physiological adaptation for the biodegradation of the contaminant, either by expression of specific enzymes, adjustments at a genic level, or by selection of organisms suitable for specific degradation activity (Leahy and Colwell, 1990). However, there was a sudden reduction in the count of bacteria and actinobacteria on the SAN treatments, as compared to count of CLA treatment. Consistent with other studies (Margesin and Schinner, 2001), we observed a decrease in heterotrophic count during the incubation time, probably due to increased degradation of the different treatments, including the negative control, exhausting the sources of carbon and other nutrients of an inorganic nature. In other hand, another study of biodegradation that presented the same concentrations of diesel and biodiesel from the brazilian tropical forest soil showed that the whole heterotrophic bacteria populations had a tendency for increasing until the half incubation time and afterwards the populations kept in highest count after the end of the experiment, totaling 60 days (Silva et al., 2012). This fact shows that several factors are responsible for those different results. It is clear that the structural and nutritional composition of the soil has some fundamental role, as observed in the CLA treatments, whose soil presents the highest clay and organic matter content. In general, the soils that show this profile tend to adsorb both nutrients as contaminants for more time, turning them available bit by bit to the microbiota from the site (Labud et al., 2007). Presumably, these slow and gradual processes may contribute to the high adaptability of the micro-organisms that act on the natural attenuation, as compared to those of the SAN treatment. Although natural attenuation is a very slow bioremediation strategy as compared to other treatments, it becomes efficient, considering it for a long time period, besides its low-cost and its *in situ* facilities.

Conclusion

In a general way, the biodiesel and the clay soil contributed to stimulation of the native microbiota activity. Therefore, the biodiesel can present as a potential replacement regarding diesel fuel. Microbial activity was observed for the natural attenuation along the incubation time. However, the reduction of the native population in both soils along the experiment was not an unexpected factor, since this strategy was not favorable for the establishment of the population for a long period of time (nutrients lack), especially to the sandy soil. The results obtained in this paper contribute to future studies of surface contamination by different mixtures of diesel/biodiesel in soils with similar physical and chemical characteristics.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Cyanide influence on the growth of mycotoxigenic fungi from cassava flour *in vitro*

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The hypothesis that residual cyanide present in cassava flour influences the growth of mycotoxigenic *Aspergillus* was established. Therefore, cyanide concentrations were measured in three types of flour: mixed, dry and watery (*n=30*), as the basis for the establishment of doses to be used. Fungi sowing were performed in solid Saboraud medium with a central well (500 µL), in which 5, 10, 15 and 20 mg CN kg^{-1} concentrations were applied, and control group was established using distilled water (0 mg CN kg^{-1}). Plates were incubated at 25°C for 24, 48 and 120 h and evaluated on direct observation of fungal growth. Results showed that fungi grown unevenly according to the applied concentration. The initial development at all concentrations, in both genera, was by the edge of plates, at a slower rate in larger doses. After 120 h only the 20 mg kg^{-1} concentration did not grow near the center circle. Thus, results show that cyanide slows fungi growth of mycotoxigenic *Aspergillus flavus* assessed, with dose-dependent effect. It is important to apply the good practices in cassava flour production in order to control cyanide levels, as well as the reduction of toxigenic fungi, to promote the food safety.

Key words: Mycotoxin, linamarin, *Manihot*, *Aspergillus.*

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is part of the food base and cuisine of Latin America and Africa. In Brazil, where the plant is domesticated, its importance is not only economic but also social and cultural. Among its main derivatives, the flour is of great prominence due to the heterogeneity of manufacturing processes, resulting in typical products of each region. In Brazil, a classify-cation criterion was established dividing the flour into

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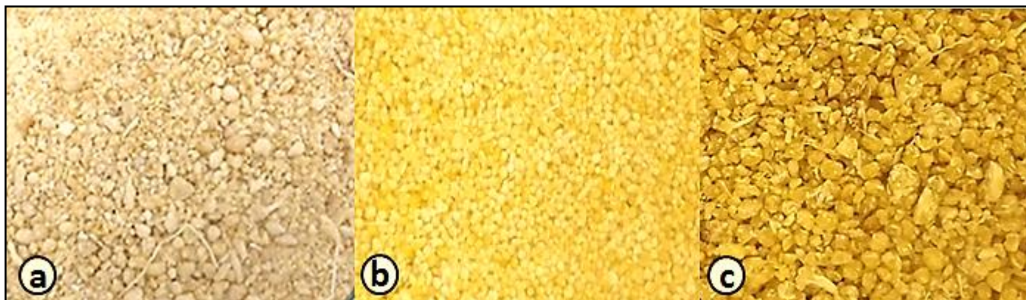

Figure 1. Cassava flour. a) Mixed; b) dry and c) watery.

groups (dry, watery and “Bijusada”), classes (fine, medium or semi-coarse and coarse) and types (1, 2 and 3) (Brazil, 2011). In the Amazon region (northern Brazil), the main type of flour used is the watery. This type of flour is produced through an empirical process of roots fermentation, followed by pressing, grating and roasting (Cereda and Vilpoux, 2010; Chisté and Cohen, 2011). This product is available heterogeneously in the market due to a large physicochemical and microbiological variation.

Microbiological evaluations of cassava flours show the incidence of mycotoxigenic fungi, especially from genera *Aspergillus* and *Penicillium* (Gomes et al., 2007; Santos et al., 2012), which mainly produce Aflatoxins (AFLs), Ochratoxin A (OTA), Citrinin and Patulin (Santos et al., 2012; Morales et al., 2007; Singh et al., 2007; EFSA, 2006). Due to a high toxicity (hepatotoxicity, nephro-toxicity, neurotoxicity, blood toxicity and even death), human exposure to food consumption is a matter of public health concern in the world (Caldas et al., 2002). The culinary use of cassava could be more valued, even for export, if not for the concern for indigenous cyanide content and its possible toxicity. However, during the flour processing steps, cyanogenic compounds (linamarin and lotaustralin) present in the roots decrease substantially (Unung et al., 2006; Chisté and Cohen, 2008), decreasing the possibility of human poisoning (Cereda, 2003; Brito et al., 2013). For example, during processing stages, the cyanide levels decrease in flours (Cardoso et al., 2005) and fermentation of tubers (Chikezie and Ojiako, 2013).

On the other hand, as the exposure to cyanide is inevitable due to natural cianoglycosides, many organisms have developed mechanisms to perform the detoxification of this compound. Only when the amount of cyanide is higher than the amount that natural detoxification mechanisms are able to eliminate, the possibility of cyanide poisoning is considered (Ramalho et al., 2010). In this sense, the presence of residual cyanide in cassava flour can potentially provide an inhibitory effect on the development of mycotoxigenic fungi. The aim of this study was to evaluate the influence of cyanide on the growth of mycotoxigenic *Aspergillus flavus in vitro*.

MATERIALS AND METHODS

Samples of cassava flour were selected, according to the manufacturing process, corresponding to groups mixed (*n=16*), dry (*n=3*) and watery (*n=11*) (Figure 1).

Samples were collected in the local market of Coari-Amazonas (Brazil), in different batches sampled randomly. They were placed in sterile plastic bags (300 g), identified and stored under controlled temperature and light conditions until analysis. The flowchart of cassava flours production steps is in Figure 2.

Methods

Cyanide content

Cyanide analysis was performed according to that described by Brito et al. (2009) using potassium cyanide (KCN Vetec®, 96% purity). Standard curve was established from free cyanide increasing doses, ranging from 0.00104 to 0.0520 mg. The color reaction with alkaline picrate developed a color gradient according to CN concentration variation, measured through a spectrocolorimeter (Bel Photonics® spectrophotometer SP 1105) at 535 nm. Absorbance values obtained in analyzes were computed, obtaining the equation $Y = 0.6635.X - 0.0215$ with $r^2 = 0.996$. Samples were suspended in 0.01 M sulfuric acid solution, homogenized for 10 min, followed by filtering and collection of the supernatant. Colorimetric reaction with alkaline picrate was subsequently performed (15 myths at 37°C), followed by spectrophotometry (triplicate). Absorbance values were calculated according to the calibration curve. Results were expressed as milligrams of total cyanide per kilogram of dry weight ($mgHCNkg^{-1}$ DW).

Fungi

The isolation was obtained by using 10 g of each sample, diluted in 90 ml of 0.1% peptone water and submitted to consecutive decimal dilutions up to 10^{-4}. Sowing was performed in triplicate, using 0.1 mL from the extract on the surface in 18% Dichloran Glycerol Agar (DG18), at 28°C for 07 days (Pitt and Hocking, 2009). Then, colonies were purified in test tubes containing Malt Extract Agar (MEA). Purified fungi were identified to genus level by microculture technique: fungi were subcultured on plates containing MEA at three equidistant points. From the same plate, a cube of approximately 1 cm^3 was cut and the sample was seeded in its surface. The cube was then covered with a coverslip and the plate was incubated at 28°C/07 days. After, the coverslip was removed to prepare a slide, to identify the fungus genus under a microscope (Kern and Blevins, 1999).

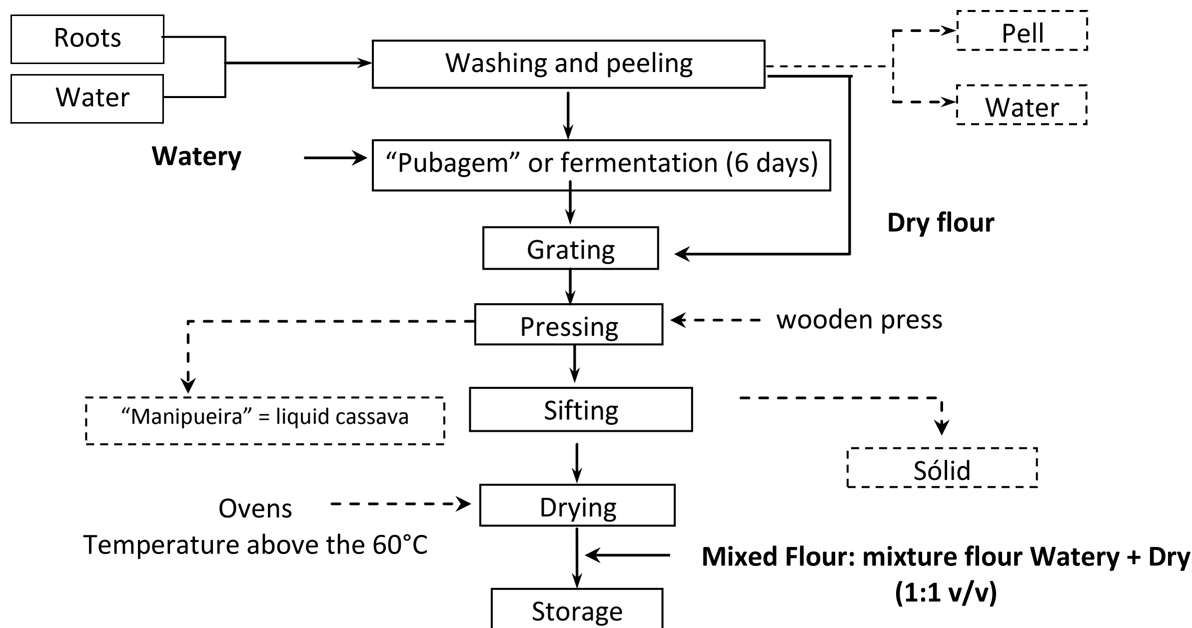

Figure 2. Flowchart of the basic steps of processing of watery, dry and mixed cassava flour. Adapted from Cereda and Vilpoux (2010) and Cardoso (2005).

Table 1. Cyanide concentrations in cassava flour from Coari-Amazonas (Brazil), according to the group (dry, mixed, watery).

Flour samples	Cyanide[a] (range) $mgHCN.kg^{-1}$
Dry	2.55±0.5 (0.50-4.20)
Mixed	2.91±0.8 (0.99-4.70)
Watery	5.46±1.2 (1.12-15.60)

[a]Results in DW±SD.

Cyanide versus fungi (in vitro tests)

Doses were established from the concentrations of cyanide determined in cassava flours. For growth tests, solid Saboraud medium was used into Petri dishes (Ø 10 cm), with wells established in the center of each plate (500 µL capacity) for the application of cyanide solution. A solution of potassium cyanide (KCN Vetec®, 96% purity) was applied in spaced concentrations of 5, 10, 15 and 20 $mgHCN.kg^{-1}$. Control group was established using 0 $mgHCN.kg^{-1}$ of distilled water. The experiment was performed in triplicate. Plates were incubated at 25°C for 24, 48 and 120 h. The evaluations consisted of direct observation of fungal growth.

RESULTS AND DISCUSSION

Cyanide content

As expected (due to processing), mixed, dry and watery cassava flour evaluated had low concentrations of cyanide as compared to other authors (Table 1). Chisté et al. (2007) showed that the concentration of cyanide in cassava products could be different according to the cassava cultivar. Charles et al. (2005) found mean of 17.1 (8.3-28.8) $mgHCN.kg^{-1}$ in different cassava genotypes. Cumbana et al. (2007) studied cassava flour samples from Mozambique, which the authors considered as typical for a year of average rainfall, and found an average of total cyanide of 4.3 (0.8-8.85) $mgHCN.kg^{-1}$. Chisté and Cohen (2008) studied cassava flour of another city from Northern Brazil (Belem), and their results ranged from 7.68-20.57 $mgHCN.kg^{-1}$ (dry) and 3.57-12.36 $mgHCN.kg^{-1}$ (watery). Comparing our findings with those authors' even in *watery* cassava flour with 5.46 (1.12-15.60) $mgHCN.kg^{-1}$, the results were lower.

FAO/WHO (1991) establishes a LD_{50} value of 10 mg HCN kg^{-1} of body weight, a dose determined from HCN administered to experimental models via inhalation. The LD_{50} was established using extracted linamarin, orally applied, which is consistent with its usual absorption by the body, and the lethal dose was 35.35 $mgHCN.kg^{-1}$ of body weight (Chisté at al., 2010). Thus, cyanide values found in these cassava flours from Brazil, suggest low risk to human health. It is important to emphasize the samples have passed through different processing steps, including heating, when some linamarin could be hydrolyzed to acetone cyanohydrin (catalyzed by endogenous linamarase) which decomposes to HCN gas, thus reducing the cyanogen content.

Cyanide versus fungi (*in vitro* tests)

Depending on the established concentration of cyanide in cassava flour, *in vitro* tests were performed to evaluate

Figure 3. Growth of *Aspergillus* after 24 h of incubation with cyanide (0.00; 5.00; 10.00; 15.00 and 20.00 mg kg^{-1}).

Figure 4. Growth of *Aspergillus* after 48 h of incubation with cyanide (0.00; 5.00; 10.00; 15.00 and 20.00 mg kg^{-1}).

Figure 5. Growth of *Aspergillus* after 120 h of incubation with cyanide (0.00; 5.00; 10.00; 15.00 and 20.00 mg kg^{-1}).

the effect of this compound on the growth of mycotoxigenic *A. flavus*. Qualitative assessments indicated that the initial development of fungi at all concentrations in both genera was by the edge of plates, at a slower rate in tests with higher doses, as shown in Figures 3 to 5. After 120 h of incubation, only the 20 mg kg^{-1} concentration had no growth of fungi near the center circle (Figure 5). However, it was observed that the total colonization of plates was only a matter of time. In the literature, there are some fungal pathogens of cyanogenic plants (*Stemphylium*, *Gloeocercospora* and *Fusarium* genera) that have detoxification capacity due to its colonization habits (Nazly et al., 1983).

Cyanide presented a delaying action on the growth of fungi genera studied, with dose-dependent effect, only though for a short period, indicating a possible inhibition of these types of fungi just after the flour preparation, thereby precluding the production of mycotoxins. In addition to the observations of fungi growth and cyanide influence, the possibility of AFL production by toxigenic strains, in cassava could be considered. On the other hand, previous work did not detect AFL in cassava flour samples (Muzanila et al., 2000). Adjovi et al. (2014) studied the ability of cassava to block AFB1 production by a toxigenic strain of *A. flavus*. The fungi was inhibited by heat treatment, sun drying or freezing of cassava samples. When each of these processes was applied, the growth of a toxigenic strain of *A. flavus* on treated cassava was associated with the production of AFB1. The assays demonstrated that the molecule responsible for the inhibition of toxin production is quite sensitive and could correspond to a peptide or small protein. Many fungi display natural linamarase activity and are therefore able to break down cyanogenic glucosides present in cassava. They conclude that cassava is a substrate non-permissive for secondary metabolism of fungi and aflatoxin production.

Despite the intrinsic mechanisms of cassava, it is important to promote the good practices in the cassava flour production to avoid the contamination of toxigenic fungi. Products intended for the most demanding markets must comply with strict standards of contamination control. Among several parameters that determine food quality, the most important are those that define their

microbiological characteristics and safety. Therefore, it is likely that in flours packed immediately after processing, there is a reduction in the risk of mycotoxigenic fungi growth. Nevertheless, hygienic conditions by manufacture, packaging and good storage practices remain as the main forms of product quality assurance and safety.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Molecular screening for the presence of *Streptococcus agalactiae*, *Escherichia coli* and *Listeria monocytogenes* in samples of milk from dairy herds

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This study aims to verify the predominance of contamination with pathogenic microorganisms in dairy herds. In order to validate the initially used methodology, an artificial contamination was conducted in commercially acquired whole UHT milk, with strains of *Listeria monocytogenes*, *Streptococcus agalactiae* and *Escherichia coli*, in final concentrations from 2.10^{-7} to 2.10^{0} CFU/mL, which were submitted to a DNA extraction protocol and to a later amplification using the polymerase chain reaction (PCR) technique. The 702 bp fragments were identified, 884 and 524 bp corresponding, respectively, to *L. monocytogenes*, *E. coli*, *S. agalactiae*. In order to verify the presence of these pathogens in *in natura* milk, the samples were obtained directly from the teats of 125 cows from the dairy herds of four producers, and from the cooling tanks of eight producers, being submitted to DNA extraction, and posterior PCR analysis. The data were analyzed with the Chi-squared test (χ^2) and different sensibility and specificity values were obtained for each microorganism. In cooling tanks, a prevalence of 37.5% of contamination with *S. agalactiae* and of 31.25% by *E. coli* was found. Regarding samples obtained from cow teats, we observed the presence of *S. agalactiae* and *E. coli* in 16.2 and 47.5% of the samples. No sample tested positive for *L. monocytogenes.* The results obtained indicate that the isolation protocol of bacterial DNA directly from the milk, and the PCR technique were efficient to detect the analyzed microorganisms, and may be incorporated as part of routine tests. Moreover, PCR may be an important mechanism to evaluate the quality of milk to be consumed.

Key words: Milk, microorganism, predominance, polymerase chain reaction (PCR).

INTRODUCTION

Milk is one of the most complete and nutritive foods, and is necessary to the human diet. Its high nutritional value is due to the great amount of proteins and dietary elements, characteristics that make it susceptible to contamination by microorganisms.

Milk contamination may occur inside the mammary gland

of the cows with clinical and subclinical mastitis; and during milking and storage, due to ineffective cleaning of udder, teats and milking equipment (Huck et al., 2007). These contamination risks are all associated with improper herd and milk management; therefore, the Brazilian Agriculture Ministry launched the National Program for Milk Quality Improvement. Aiming to increase milk and dairy products safety, this program set higher standards for physicochemical and microbiological parameters of raw milk. Brazil is currently the fourth largest milk producer globally, producing approximately 32.3 billions of liters annually (FAO, 2014), thus improving milk quality is important both for public health and for the country's economy.

Contamination of milk with pathogenic microorga-nisms is a fact in Brazil. Among them, are prominent: *Streptococcus agalactiae*, *Escherichia coli* and *Listeria monocytogenes*. *S. agalactiae,* one of the pathogens associated with cattle and human infections and could be identified through a small quantity of tests as it presents specific biochemical characteristics (Santos et al., 2007). *E. coli* is part of the intestinal microbiome of several animals, also associated with mastitis, which, depending on the virulence factors, can be classified as enterohemorrhagic, enteropathogenic, among others (Bavaro, 2012). *L. monocytogenes*, is a psychotropic bacterium that reproduces at temperatures between -0.4 and 50°C (Donnelly, 2001) and may also be associated with mastitis (Dias, 2007).

Studies have shown the presence of several types of pathogenic microorganisms (*Staphylococcus aureus, Streptococcus* spp, *Corynebacterium bovis, Streptococcus agalactiae* and *Staphylococcus* spp.) in samples of milk in Brazil and other countries (Nornberg et al., 2010; Ribeiro et al., 2009; Arcuri et al., 2006; Bennedsgaard et al., 2006).

Due to the importance of these pathogens and the difficulty of obtaining a quick and precise diagnostic, some studies use molecular techniques to detect these microorganisms. However, they do so indirectly by sowing and enriching the milk with specific growth mediums (Borela et al., 1999; Perez et al., 2002; Zocche et al., 2009).

Among the techniques used, the polymerase chain reaction (PCR) technique has been the most used, since it does not need viable microorganisms in the samples, which enables analysis of samples submitted to improper conservation processes. This technique is also able to detect non-cultivable microorganisms and does not suffer interference by the presence of antibiotic in milk, providing quick, selective and specific results (Meiri-bendek et al., 2002; Ahmadi et al., 2010; Amagliani et al., 2012).

Thus, the aim of the present study was to validate a PCR protocol to detect DNA from *S. agalactiae*, *E. coli* and *L. monocytogenes* directly from milk samples and to evaluate the predominance of raw milk contamination for these microorganisms in dairy herds in Brazil.

MATERIALS AND METHODS

Sample preparation

Artificial contamination was conducted in commercially acquired UHT milk, with *S. agalactiae* (ATCC 13813), *E. coli* (ATCC 25922) and *L. monocytogenes* (ATCC 19114) strains. The dilutions were made from the stationary phase of each microorganism, cultivated in 18-24 h brain heart infusion (BHI) in a heater at 36°C. Quantification in this phase was made by plating suspensions of the microorganism in depth in plate count agar (PCA), and incubation at 36°C for 48 h. For the artificial contamination we used 1.8 mL of UHT milk, and 200 uL of dilutions, with final concentrations from 2.10^{7} to 2.10° CFU/mL. 12-16 curves were built, with each point in triplicate.

Samples of raw milk (15 mL) were collected from the cooling tanks of eight properties in July and December, 2011. A sample from each producer per month was collected, with a total of 56 samples. In addition, we performed an analysis of samples of milk (15 mL) collected directly from the teats of 125 cows from dairy herds of three producers in September and October, 2012. All samples were submitted to the same DNA extraction protocol and PCR reactions described below.

DNA isolation

For DNA extraction, 200 µL of raw milk sample were used. Briefly, 20 µL of Tween-20 were added to the samples, followed by centrifugation (12000 xg, 15 min). Pellets were suspended in 60 µL extraction buffer (100 mM Tris-HCl, 100 mM EDTA, 250 mM NaCl, pH 8.0) (Invitrogen, Carlsbad, CA, USA), 30 µL 10% SDS (Sigma-Aldrich, St Louis, MO, USA), 15 µL proteinase K (Ambion®, Austin, TX, USA) (20 mg/mL), and 195 µL ultrapure water, and they were incubated for 1 h at 37°C. Subsequently, 100 µL of buffered phenol (ANRESCO, Solon, OH, USA) were added, and the samples were centrifuged (12000 xg, 5 min); the supernatant was collected and mixed with 100 µL of phenol-chloroform-isoamyl alcohol (25:24:1) (Sigma-Aldrich, St Louis, MO, USA) and centrifuged (12000 xg, 5 min).

A total volume of 26.5 µL of 2 M sodium acetate (Sigma-Aldrich,St Louis, MO, USA) was added to the supernatant, followed by addition of 400 µL of absolute ethanol (Merck, Darmstadt, GER) and overnight incubation at 4°C. The samples were centrifuged (12000 xg, 20 min) and the pellets with DNA were resuspended in TE buffer (10 mM Tris-HCL, 1 mM EDTA, pH 8.0), and stored at -20°C until use. The optical density ratio (260/280 nm) of DNA preparations was considered suitable of downstream applications when greater than 1.6.

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Figure 1. Detection curve for *S. agalactiae*. Samples in triplicate and in CFU/mL: 2 x 10^6 (lanes 1 to 3), 2 x 10^5 (lanes 4 to 6), 2 x 10^4 (lanes 7 to 9), 2 x 10^3 (lanes 10 to 12), 2 x 10^2 (lanes 13 to 15), 2 x 10^1 (lanes 16 to 18), 2 (lanes 19 to 21). C - negative control. M - Molecular weight marker 100 bp (New England Biolabs®, UK). Agarose gel stained with ethidium bromide.

Figure 2. Detection curve for *E. coli*. Samples in triplicate and in CFU /mL: 2 x 10^5 (lanes 1 to 3), 2 x10^4 (lanes 4 to 6), 2 x10^3 (lanes 7 to 9), 2 x 10^2 (lanes 10 to 12), 2 x 10^1 (lanes 13 to 15), 2 (lanes 16 to 18). C - negative control. M - Molecular weight marker 100 bp (New England Biolabs®, UK). Agarose gel stained with 1.5% ethidium bromide.

PCR analysis

PCR was conducted using the primers for the 16S rRNA of *S. agalactiae* (GI: 386081764): Forward 5'CGGGTGAGTAACGCGTAGGTAA3' and reverse 5'GGTTAAGCCACTGCCTTTAACTTC3'. The conditions of the reaction were: 94°C for 30 s, 58°C for 45 s and 72°C for 45 s, during 30 cycles, providing a fragment of 524 bp corresponding to *S. agalactiae*. For *E. coli*, the primers: up 5´CCGATACGCTGCCAATCAGT3´ and down: 5´ACGCAGACCGTAGGCCAGAT3´ were used for the *uspA* gene (Chen and Griffiths, 1998). Reaction conditions were: 94°C for 2 min, 58°C for 1 min, and 72°C for 1 min, during 35 cycles producing a 884 bp fragment. For *L. monocytogenes*, primers for listeriolysin gene were used, generating an amplicon of 702 pb: LM1 5'CCTAAGACGCAATCGAA3' and LM2 5'AAGCGCTTGCAACTGCTC3' (Lawrence and Gilmour, 1994). Reaction conditions were: 94°C for 80 s, 50°C for 90 s and 72°C for 2 min during 30 cycles.

PCR reactions were performed on the thermal cycler (Techne® Barloworld Scientific, Stone, Staffordshire, UK) with a final volume of 50 µL using a PCR Buffer (20 mM of Tris HCL pH 8.4 and 50 mM of KCL), 1.5 mM of $MgCl_2$, 1.25 U of Taq polymerase, 0.4 µM of sense and antisense primers, and 0.2 mM of dNTP mix. The fragments generated were analyzed in agarose gel colored with ethidium bromide. The analysis of the gels was made with the image capture and photodocumentation system Gel Logic 200 (KODAK®RaytestGmbH, Straubenhardt, Germany).

Statistical analysis

All statistical analyses were performed using the Prism 5 (Graphpad®, California, EUA). The prevalence and frequency of contamination and sensitivity and specificity determination were accessed with exact 95% confidence interval (CI), Fisher's exact test and Chi-square; The gold method used was microbiological analysis. A $p < 0.05$ was considered statistically significant.

RESULTS

Sensibility and specificity of the PCR protocols tested were calculated based on the analysis of 16 curves for each of the microorganisms (Figures 1 to 3), and the PCR technique proved itself more sensible and specific for *S. agalactiae,* with a sensibility of 89.5% for a detection limit of 2 CFU/mL ($P < 0.001$, Table 1). For *E. coli*, the sensibility was 79.1% for a limit of 2 CFU/mL ($P < 0.001$, Table 2). The *L. monocytogenes* PCR protocol presented the lowest sensibility (16%) with a detection limit of 2 x 10^4 CFU/mL ($P = 0.0057$, Table 3).

The present study also evaluated the predominance of contamination in milk samples from cooling tanks, and obtained directly from the teats of the cows by PCR

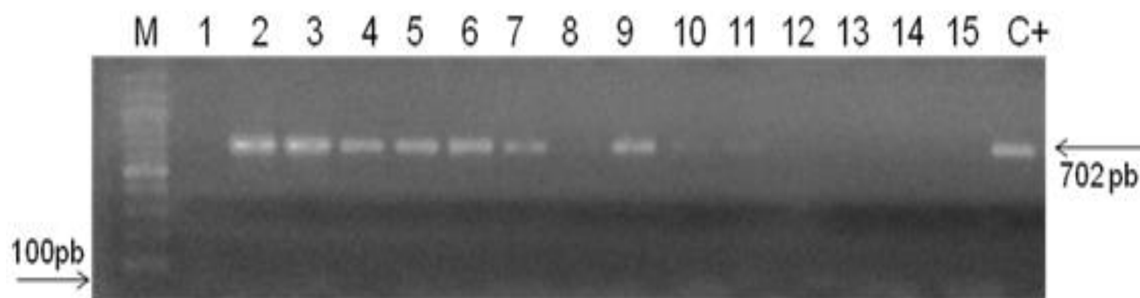

Figure 3. Detection curve for *L. monocytogenesi*. Samples in triplicate and in CFU /mL: 2 x 10^7 (lanes 1 to 3), 2 x 10^6 (lanes 4 to 6), 2 x 10^5 (lanes 7 to 9), 2 x 10^4 (lanes 10 to 12), 2 x 10^3 (lanes 13 to 15). C - negative control. C+ - positive control. M - Molecular weight marker 100 bp (New England Biolabs®, UK). Agarose gel stained with ethidium bromide.

Table 1. PCR sensitivity and specificity for *S. agalactiae* detection in raw milk. Each concentration was tested in 48 samples.

CFU/mL	2 x 10^6	2 x 10^5	2 x 10^4	2 x 10^3	2 x 10^2	2 x 10^1	2
Sensibility	100%	95.8%	100%	93.7%	97.9%	97.2%	89.5%
Especificity	100%	100%	100%	100%	100%	100%	100%
P-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Table 2. PCR sensitivity and specificity for *E. coli.* detection in raw milk. Each concentration was tested in 36 samples.

CFU/mL	2 x 10^6	2 x 10^5	2 x 10^4	2 x 10^3	2 x 10^2	2 x 10^1	2
Sensibility	100%	83.3%	95.8%	83.3%	95.8%	83.3%	79.1%
Especificity	100%	100%	100%	100%	100%	100%	100%
P-value	<0.0001	<0.0001	<0.0001	<0.0001	<00001	<0.0001	<0.0001*

Table 3. PCR sensitivity and specificity for *L.monocytogenes*.detection in raw milk. Each concentration was tested in 36 samples.

CFU/mL	2 x 10^7	2 x 10^6	2 x 10^5	2 x 10^4
Sensibility	83.3%	79,1%	50%	16.6%
Especificity	100%	100%	100%	100%
P-value	<0.0001	<0.0001.	<0.0001.	0.0057

technique. We verified that none of the 125 samples collected from the teats, as well as the 56 samples collected from the tanks, tested positive for *L. monocytogenes*. Regarding the samples collected from the tanks, 37.5% were positive for *S. agalactiae,* and 31.25% were positive for *E. coli*, and the analysis of the milk collected in the teats revealed that 47.5% of the samples tested positive for *E. coli,* and only 16.2% for *S. agalactiae.*

DISCUSSION

Raw milk is known as a carrier of pathogens, contributing to the acquisition of infectious diseases. In the USA, between 1998 and 2011, 2.384 diseases were associated with the consumption of raw milk and byproducts (CDC, 2013). The identification of the presence of microorganisms in the food is the gold standard method to determine food poisoning sources. Routinely, milk bacterial contamination is determined by means of microbiological culture of milk and biochemical characterization of isolated microorganisms. Despite being the gold standard method to determine food poisoning sources, the period necessary to obtain the

results by the current method is quite long. In average, it takes at least five days to confirm a negative result, and more than 10 days to confirm a positive result (Gasanov et al., 2005). Also, there are disadvantages associated with microbiological culture, such as: a milk culture may yield no bacteria from truly contaminated milk due to the presence of very low numbers of bacteria in the samples; and a result of negative culture may also occur due to the presence of residual therapeutic antibiotics that may inhibit *in vitro* bacterial growth.

Molecular techniques have been used as an alternative to microbiological methods for the detection of pathogens in food; however, regarding milk, most molecular studies isolate the microorganisms after sowing them in growth mediums, and not directly from the milk (Borela et al., 1999; Perez et al., 2002; Zocche et al., 2009). This probably happens due to the presence of inhibitory substances of the PCR in milk composition or because milk fat could cover bacterial surface and make lysis more difficult, thus lowering sensibility to PCR (Kim et al., 2001; Aslan et al., 2003).

The present study demonstrated a high sensibility and specificity for the PCR technique when detecting *S. agalactiae* and *E. coli*. A study obtained inferior results, with 85% sensibility and 82% specificity when detecting *S. agalactiae* in milk samples obtained from expansion tanks (Elias et al., 2012). The specificity and sensibility value achieved by this study is quite relevant since they demonstrate the capacity of the test to correctly identify samples that were really infected, and the samples that were really not infected, showing 100% reliability. Similarly, the detection limits achieved in this study were superior to those of Martinez et al. (2001) who achieved a sensibility limit of 100 CFU/mL for the same pathogen. Moreover, a detection limit of 2 CFU/mL was verified, being similar to the one of Kumar et al. (2013) that detected *E. coli* producers of the Shiga toxin (STEC) in samples of milk without pre-enriching. Meiri-Bendek et al. (2002) conducted a contamination of milk with different concentrations of *S. agalactiae* for later detection by PCR. Without enrichment, detection capacity varied from 10^4 to 10^5 CFU/mL, and its detection capacity was lower than the one presented in the present study. It is noteworthy to mention that enrichment of milk for microorganism detection increases the sample analysis time from 6 to 12 h. This contrasts with the need for fast and reliable methods aimed to be used in industrial routine.

L. monocytogenes has been a great concern for the food industry, since it is responsible for a significant percentage of food-transmitted diseases around the world (Gandhi and Chikindas, 2007). The detection of *L. monocytogenes* in food through traditional microbiological methods is time consuming, requiring the use of specific cultivation mediums for isolation (Vanegas et al., 2009). Therefore, the development of new ways for screening milk contamination by *L. monocytogenes* is of great interest. In the conditions tested, we observed a high sensibility only in contamination higher than 2 x 10^7 CFU/mL. Aznar and Alarcón (2003) also detected, through the PCR technique, *L. monocytogenes* artificially inoculated in different foods obtaining a sensibility from 1 to 10 CFU/mL. However, differently from the present study, the bacterial DNA was isolated directly from the cultivation plate after enrichment in a specific liquid for Listeria (LEB). Another study compared the PCR technique with conventional microbiology for the detection of *E. coli* O157, *Salmonella* spp. and *L. monocytogenes* in milk obtained from cooling tanks. Results revealed that the real time PCR was more sensitive in the detection of *E. coli* O157 than the culture method; while both methods were equally efficient in detecting *L. monocytogenes* (Amagliani et al., 2012). The diversity of results found in studies may occur due to the variation in quality and quantity of the DNA obtained with the use of different protocols. Furthermore, DNA extraction usually is performed using culture plates, and not directly from the milk or its byproducts.

The present study also evaluates the prevalence of contamination in samples of milk. Such scenario differs from others recently published in developed and developing countries. A study evaluated a total of 446 samples of raw milk, obtained from tanks of Iranian producers, in order to verify the presence of Listeria species. *Listeria* spp. was isolated in 18.6% of the samples, and the most found species was *Listeria innocua* (57.8%); followed by *L. monocytogenes* (21.7%); *Listeria welshimeri* (12%) and *Listeria seeligeri* (8.4%) (Jamali et al., 2013). In contrast, in the USA, a low prevalence of *L. monocytogenes* was observed, present in only three (2.3%) of the 113 samples collected from 11 dairy farms (D'amico et al., 2008).

Conclusion

The PCR methods here proposed for detection of milk contamination by *S. agalactiae* and *E. coli* have high sensibility and specificity. Providing quick and reliable results, the use of molecular methods can optimize and increase milk quality control in the dairy industry. Also, an important occurrence of contamination of milk prior industrial processing indicates milking and herd management problems. Industrial milk processing eliminates viable bacterial contamination; however in developing countries, the consumption of raw milk and dairy products is common, putting part of the population at risk of milk-borne infectious diseases.

Conflict of interest

The authors did not declare any conflict of interest

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Full Length Research Paper

Mycobiota from the eggs, nests and stillbirths of *Eretmochelys imbricata* Linneus 1766 (Testudines: Cheloniidae) in Pernambuco State, Brazil

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***Eretmochelys imbricata* Linneus 1766 was the subject of trade due to egg collection and consumption of the flesh of the females, being the fishery one of the main impacts towards the coastal area. The pathogens are also worrying factors of mortality of sea turtles especially those caused by fungi; these can cause the death of embryos and cutaneous mycoses. This study aimed to investigate the mycoflora isolated from soil, eggshells and stillbirths from *E. imbricata* in three beaches of Ipojuca (Brazil). We recorded data on the reproductive biology of the species after incubation of nests. Soil samples and fragments of eggshells were collected at the end of the nesting season for fungi identification. A total of eight species of fungi were identified by their morphological characteristics: *Aspergillus terreus*, *Aspergillus niger*, *Aspergillus flavus*, *Cladosporium cladosporioides*, *Nigrospora grisea*, *Fusarium solani*, *Fusarium lateritium* and *F. oxysporum* in the soil samples, eggshells and stillbirths. *Fusarium* was recorded in other studies interfering with the development of turtles embryos. The data from this study will provide information to support the management and conservation of sea turtles.**

Key words: Eggshell, fungi, *Fusarium*, Hawksbill, testudines.

INTRODUCTION

Eretmochelys imbricata Linnaeus 1766, commonly known as the hawksbill turtle, uses the beaches in the Brazilian states of Bahia, Rio Grande do Norte and Sergipe as its main nesting site (Marcovaldi et al., 2011); the breeding also occurring in the states of Pernambuco and Paraíba (Mascarenhas et al., 2003; Moura et al., 2012). In spite of its wide distribution, this chelonian is classified as critically endangered in the red list of the International

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Union for Conservation of Nature (Marcovaldi et al., 2011).

According to Gallo et al. (2006), one of the main anthropogenic impacts on chelonians is coastal fishing, which is considered the main cause of mortality of sea turtle. Other negative factors include the disorderly growth of coastal regions (artificial lighting, erosion, beach occupation by houses and hotels, vehicular traffic, the construction of ports and other marine structures) that degrade the marine environment and impact chelonian populations (Tuxbury and Salmon, 2005; Deem et al.,2007). Several studies also have reported finding plastic residues in the digestive tracts of sea turtles, resulting in their mortality (Thomas et al., 2002; Wabnitz and Nichols, 2010; Marcovaldi et al., 2011).

In addition to anthropogenic impacts, pathogenic agents have been factors causative of the mortality of sea turtles, including ectoparasites (leeches) and endoparasites (nematodes and trematodes) (Greiner, 2013), viral diseases (such as fibropapilloma, a debilitating or deadly disease for these animals, and now the focus of research investigations) (Baptistote et al 2005; Higgins, 2003; Rodenbush et al., 2014), respiratory diseases, and fungal infections (Cabañes et al., 1997; Phillott and Parmenter, 2001; Higgins, 2003; Coelho, 2009; Sarmiento-Ramírez et al., 2010).

Studies carried out to evaluate the influence of fungi on turtle eggs and hatchlings of *Caretta caretta* (Linneaus, 1758) have reported neonate mortality through mycoses caused by the fungal species *Fusarium solani* (Mart.) Sacc. (Phillott et al., 2004; Sarmiento-Ramírez et al., 2010) on Boa Vista Island (Cape Verde). These infections were considered responsible for the mortality of *C. caretta* embryos inside the nest (Sarmiento-Ramírez et al., 2010). Phillott et al. (2006) reported that these fungi degrade the calcium in sea turtle eggshells, facilitating the penetration of hyphae and consequently affecting the embryo. Cabañes et al. (1997) noted that *F. solani* isolated from chelonian rehabilitation tanks has caused cutaneous mycoses and weakened those turtles.

Fusarium species are actually a monophyletic “species complex”, that is *F. solani* species complex, including up to 60 species (O’Donnell, 2000; Short et al., 2013). This complex has a huge range of distribution and includes borne saprobes from soil and plant debris (O’Donnell et al., 2008; Short et al., 2011). Although, studies focusing on *F. solani* isolated from nests of sea turtles are still scarce (Sarmiento-Ramirez et al., 2014). *Fusarium* infections were identified in eggs of sea turtles in different stage of development, since the second week of incubation presenting initial infection, until advanced stage of incubation with evident mycelia inside the egg (Sarmiento-Ramirez et al., 2014).

According to Marcovaldi and Marcovaldi (1999), the turtle population on the Brazilian coast is considered one of the largest in the world, but additional studies investigating biotic and abiotic aspects that can influence the reproductive success of these animals are still needed, including the potential role of the mycobiota in the development of *E. imbricata*. In view of the impact caused in the reproductive success of this endangered species by fungi infections, we sought to isolate and identify fungi from nests of *E. imbricata* on the coast of Ipojuca (Pernambuco State, Brazil), due to provide data to the nesting conservation of this taxon.

MATERIALS AND METHODS

Study area

The study area was located on the beaches of Muro Alto, Cupe and Merepe (08°24'06"S and 35°03'45"W) in the municipality of Ipojuca, Pernambuco State, Brazil. The coastline there features areas of mangrove vegetation, Atlantic Forest remnants, flooded areas, vegetation shins, and restinga vegetation (sandy substrate, near-shore vegetation) (Santos, 2005). The Muro Alto Beach is characterized by sandstone reefs, with native vegetation behind the tidal zone. Cupe Beach has rock formations parallel to the coast that is breached in a number of locations, facilitating the entry of the waves that shape the coastline through sand deposition. Merepe Beach is 3.47 km long, without sandstone or coral reef barriers, and only sparse vegetation behind the tidal zone (Moura et al., 2012).

Sampling

Soil samples, eggshells, and stillbirths were collected from seven monitored nests of *E. imbricata* on the beaches at Merepe (3 nests), Cupe (3), and Muro Alto (1). Data of the seven nests were collected regarding the reproductive biology of the species determined after opening the nests, including: the numbers of eggs per nest, the numbers of live offspring, numbers of stillbirths (estimated from the number of offspring that had not completed their development), the numbers total of eggs originally laid (including those that had not completed their development), and the hatching success of each nest (Table 1); the numbers of nests per beach were also determined. These evaluations were carried out *in situ* during low tides during the rainy season in June/2011 on three beaches (Muro Alto, Cupe, and Merepe). The samples were collect from seven nests, which were opened to depths of approximately 50 cm, and soil samples, the shells of eggs and stillbirths were collected. The soil samples and egg fragments were placed in sterile bags, while stillbirths were sampled using sterile swabs rubbed on the skins of the animals (the swabs were subsequently held in sterile distilled water). All of the samples were transferred to the laboratory for proper processing.

Fungi isolation and identification

The soil samples were processed using the successive serial dilution technique proposed by Clark (1965), with modifications. Dilutions of the soil samples were made up to 1:1000 in Petri dishes containing Potato Dextrose Agar (PDA) with added chloramphenicol (100 mg/l). After dilution, the samples were sown into Petri dishes (as triplicates) containing PDA culture medium. The eggshell fragments were disinfected with sodium hypochlorite (3 min), rinsed 3 times with distilled water, and subsequently sown into Petri dishes containing PDA medium with 100 mg/l chloramphenicol (as triplicates). The swab samples from the stillbirths (1 ml aliquots) were sown into Petri dishes containing PDA with chloramphenicol (100 mg/l) (as triplicates). All the plates were

Table 1. Total of nests, number of living, stillborn and unhatching, and percentage of living.

Beaches	Nests	Living	Stillborn	Unhatching	% living
Merepe	1	2	0	111	1.76
Merepe	2	90	6	45	63.82
Merepe	3	26	0	85	23.42
Cupe	4	56	11	63	43.75
Cupe	5	0	0	97	0
Cupe	6	46	9	72	36.22
Muro Alto	7	100	4	11	86.96

Table 2. Fungi isolated from soil, stillbirths and unhatched eggs on the beaches Merepe, Cupe and Muro Alto (08°24'06'' S e 35°03'45'' W), in 2011.

Species/Beaches	Merepe			Cupe			Muro alto		
	Soil	Eggs	Stillbirths	Soil	Eggs	Stillbirths	Soil	Eggs	Stillbirths
Aspergillus flavus Link	X								
A. niger (Tiegh.)	X								
*A. terreus (*Thom)	X								
Cladosporium cladosporioides (Fresen.) G.A. de Vries						X			
Fusarium lateritium Nees					X				
F. oxysporum Schlecht.		X	X						
F. solani (Mart.) Sacc.	X	X	X		X	X			
Nigrospora grisea (Herbert) Barr							X		X

incubated in BOD incubator (26°C) during 72 h. To identify the fungal species, samples were transferred to specific media and their macroscopic and microscopic characteristics were subsequently evaluated using the specific literature.

RESULTS AND DISCUSSION

Eight fungal species were isolated and subsequently identified based on macrostructure and microstructure characteristics: *Aspergillus flavus, A. niger, A. terreus*, *Cladosporium cladosporioides, F. solani*, *F. lateritium*, *F. oxysporum*, and *N. grisea* (Table 2). A total of 58 x 10^3 g/ml of colony forming units were observed at Merepe Beach, followed by Cupe Beach (26 x 10^3 g/ml) and Muro Alto Beach (23 x 10^3 g/ml). Colonies of *F. solani* were more abundant on eggs, in the soil, and associated with stillbirths. The species identified from Merepe Beach were *A. flavus, A. niger, A. terreus* and *F. solani* isolated from soil; *F. oxysporum* and *F. solani* were also identified on eggs and Stillbirths. Cupe Beach had colonies from *F. solani*, *F. lateritium* and *Cladosporium cladosporioides* obtained from eggs and stillbirths. In samples from Muro Alto Beach only the species *N. grisea* was isolated from soil samples and on stillbirths. The genus Aspergillus occur commonly in soil in warmer climates, in compost, decaying plant matter, stored grain, and could survive in many environments, which are abundant in tropical and subtropical regions (Domsch et al., 2007; Rosa et al., 2002).

The amount of fungi found was proportional to the birth index of the nests. The reproductive success of Muro Alto (86. 96%) was the higher between the beaches, presenting lower colony forming units and the least fungi diversity. Besides that, only the species *N. grisea* was found in the samples collected in this beach. However, Moura et al. (2012) recorded Merepe beach with significant occurrence of *E. imbricata* nests and higher reproductive success in comparison with the other beaches (Cupe and Merepe), therefore we suggest increasing the number of samples for future analysis due to correlate the incidence of fungi contamination and the birth index.

According to Mader (2006), some species of opportunistic fungi cause infections in marine turtles. These microorganisms are usually saprobes but can invade living tissue under favorable conditions, and studies have reported that some of these fungi species infects sea turtle eggs and cause embryo mortality. Patino-Martinez et al. (2012) reported similar results in the eggs of the sea turtle *Dermochelys coreacea* in Colombia, where *F. solani* and *F. oxysporum* were

identified by phylogenetic analyzes; these fungi were possibly affecting the phenotype of the hatchling (body size). The first way of contamination of the eggs by *Fusarium* species probably occur through the secretion present in the oviduct, as suggested by Phillot et al. (2002), likewise it might be the reason for fungi contamination in nests of other species that belong to Cheloniidae family, since several species of fungi have been isolated from the cloaca of females during breeding. The most possible via of infection of the microorganisms come from spores present in the nesting substrate into the coacla during the copula with males (Phillott and Parmenter, 2001; Phillott et al., 2002).

Several biological and abiotical parameters influence the reproductive success of sea turtles, simlarly it might create conditions more farovables to the development of fungi infections in the nests (Phillott et al., 2002). In the present study, *F. solani* and *F. oxysporum* were observed in infected eggs and probably interfering in the development of embryo and increasing the mortality of sea turtles, as reported by Phillott and Parmenter (2004). Phillot and Parmenter (2004) identified and analyzed the mycobiota in nests of *E. imbricata*, *Chelonia mydas, Natator depressus* and *Caretta caretta* in Australia and noted that *F. solani* and *F. oxysporum* were present on the eggshells. Sarmiento-Ramírez et al. (2010) also observed *F. solani* on the eggshells and embryos of *C. caretta* in Cape Verde, and determined that this specie was responsible for mass nest mortality. Phillott et al. (2006) likewise reported that calcium losses in eggs could be attributed to the presence of fungi and would interfere with embryonic development. Cabañes et al. (1997) reported skin fungal infections in weakened marine turtles in recovery tanks mainly caused by *F. solani*. According to Wiles and Rand (1987), skin mycoses are more frequent among sea turtles in captivity than in their natural environment.

According to Marcovaldi et al. (2011) these monitoring and nesting biology studies are of significant importance, because the data will help in improving the conservation plans for *E. imbricata* - classified as critically endangered in Brazil based on data available until the year 2009 and being considered for similar conservation status in other countries (IUCN, 2008). The hawksbill is among the most endangered species of sea turtles among the seven existing species in the world (Wallace et al., 2011).

Species of the genus *Fusarium* are not part of the normal microbiota of marine animals, as they are saprobic fungi that normally live alone or as plant pathogen (Frasca et al., 1996), but studies have shown that opportunistic infections caused by these fungi occur relatively frequently in humans and animals (such as sea turtles) (Rebell, 1981). *Colletotrichum acutatum* was identified as the causative agent in the death of a young specimen of *Lepidochelys kempii* in Florida in 2000 (Manire et al., 2002), emphasizing that the immune statuses of animals will determine their pathogen resistance. Elshafie et al. (2007) reported 14 species of fungi isolated from soil and eggs in *C. mydas* nests, with high incidence of *Aspergillus* species, especially: *A. flavus*, *A. niger*, *A. terreus*, *A. nidulas*, *A. fumigatus*, and *A. ochraceus*. Among these, *A. flavus*, *A. niger* and *A. terreus* were likewise isolated from the soil on the beaches at Merepe and Cupe (Ipojuca- PE), and it appears that fungal growth on eggs and on the sea turtles themselves, and the production of mycotoxins, will affect embryonic development and contribute to chelonian mortality.

Our research has shown the presence of *F. solani*; it could be considered another threat to sea turtle, especially on beaches with anthropic impacts, as the occupation of reproductive and consequently the decrease of spawning areas. Beaches that are more exposed to environmental pressures such as erosion, sediment movement, and anthropic pressure as pollution might been more susceptible to the colonization of these pathogenic fungi. The nests sampled in this study were located in beaches with high exploitation of tourism and under anthropic pressure.

We characterized the mycobiota present in the nest envionment of *E. imbricate*. Finding the pathogenic fungi infections, and understanding the environmental conditions that favor colonization by *Fusarium* and other pathogenic species of fungi within the nests of sea turtles still need to be clarified as well as their influence in the development of sea turtles.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Prevalence and characterization of carbapenemase producing isolates of *Enterobacteriaceae* obtained from clinical and environmental samples: Efflux pump inhibitor study

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The present study was undertaken to screen for carbapenem resistance and the ability of efflux pump inhibitors to inhibit this resistance in enterobacteriaceae strains isolated from clinical specimens from patients attending Jawaharlal Institute of Post Graduate Medical Education and Research (JIPMER) hospital and water samples in and around Puducherry. A total of 425 carbapenem resistant isolates from clinical samples were studied by both phenotypic and genotypic methods. Two hundred and forty eight (248) strains were positive for metallo beta lactamase in the double disk synergy test (DDST) and 264 strains were positive for modified Hodge test (MHT). Multiplex PCR assays revealed that 262 of the 425 strains harboured *blaNDM-1* gene. Efflux pump inhibitory activity of Phenylalanine arginine beta-naphthylamide (PAβN) was detected for these strains. This study demonstrates that, 30 strains out of 163 blaNDM-1 negative strains were found to exhibit efflux pump activity. This study brings out the fact that such carbapenem resistant strains are limited only to clinical samples and not found in water samples in and around Puducherry.

Key words: *Enterobacteriaceae, bla*NDM-1, Efflux pump, carbapenem resistance.

INTRODUCTION

Antibacterial resistance continues to be a global public health concern, threatening the effectiveness of therapy, and challenging the efforts for developing novel antibacterial (Li and Nikaido, 2009). The introduction of carbapenem into clinical practice represented a great advancement in the treatment of serious bacterial infections caused by beta-lactam-resistant bacteria (Li and Nikaido, 2009; Walsh et al., 2002; Bush, 1998). Due to their broad spectrum of activity and stability to hydrolysis by most beta-lactamases, the carbapenem have been the drugs of choice for the treatment of infections caused by cephalosporin-resistant Gram

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negative bacilli, especially ESBL-producing Gram-negative infections (Li and Nikaido, 2009; Walsh et al., 2002; Bush, 1998; Kahan et al., 1983; Bradley et al., 1999).

Resistance to carbapenem is mediated by decreased outer membrane permeability (Walsh et al., 2005), efflux systems (Coyne et al., 2011), alteration of penicillin-binding proteins and carbapenem hydrolyzing enzymes-carbapenemases (Fernandez-Cuenca et al., 2003). Carbapenemases are class B metallo-β-lactamases or class D oxacillinases or class A clavulanic acid inhibitory enzymes. Metallo-β-lactamases which belong to class B, require divalent cations of zinc as cofactors for enzyme activity (Urban et al., 2003). They have potent hydrolyzing activity not only against carbapenem but also against other β- lactam antibiotics (Walsh et al., 2005; Bush, 1999).

The increase in resistance among bacteria, most notably *Klebsiella* spp. and *Escherichia coli*, by the production of extended-spectrum β-lactamases (ESBLs) has led to the increased use of carbapenem antibiotics (Paterson and Bonomo, 2005). Resistance to carbapenem among these bacteria remains remarkably rare in most countries. However, *Klebsiella* spp. that produce serine-based carbapenemase enzymes, referred to as *Klebsiella pneumoniae* carbapenemases (KPCs), have been identified in recent years (Bulik et al., 2010)

The emergence of carbapenem resistance constitutes an alarming development in the field of infectious diseases, with major public health implications. More intensive efforts are urgently required to elucidate the epidemiological and infection control issues related to these organisms and to improve the management of patients with infections. In this study, attempts have been made (i) to characterize the carbapenem resistant enterobacteriaceae species in clinical specimens and water samples and (ii) to detect the role of efflux pumps in mediating carbapenem resistance in *Enterobacteriaceae*.

MATERIALS AND METHODS

Clinical isolates and susceptibility tests

This study was carried out in the Department of Microbiology, JIPMER, for a period of 2011 and 2012. A total of 425 carbapenem resistant bacterial isolates belonging to the family *Enterobacteriaceae* were collected from different clinical specimens such as wound swab, tracheal aspirate, blood, pus, peritoneal fluid, CSF and sputum during the study period. The isolates were stocked in 0.2% semi-solid agar until analyzed. Patients' demographic data, clinical diagnoses and specimen types were recorded. Only one positive culture per patient was included.

Antimicrobial susceptibility test for all isolates were performed on Mueller Hinton agar (MHA) plates by the standard Kirby Bauer disk diffusion method. A panel of 9 antibiotics of different classes (in-house disks of amikacin (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), ceftazidime (30 µg), ceftriaxone (30 µg) and meropenem (30 µg) from Hi-Media, Mumbai, India) were encountered. The diameter of the zones of inhibition of growth was interpreted as per CLSI guidelines 2011. *E. coli* ATCC 25922 was used as a control organism. Strains found to be meropenem resistant by disc diffusion test were tested by both broth micro dilution and commercial E-strips (Biomerieux, France), in order to determine the MIC (µg/ml) level.

Phenotypic test for carbapenemase detection

Metallo β-lactamase (MBL) detection

In the present study, imipenem-EDTA (10 µg/750 µg) and Imipenem (10 µg) commercial disks (Hi-Media, Mumbai) were used. The test was performed on Muller-Hinton agar plate by disk diffusion method. A 0.5 McFarland adjusted suspension of the test organism was inoculated on MHA. A 10 µg imipenem disk was placed on the plate and the I-EDTA disk was placed at a distance of 20 mm, centre to centre, from the imipenem disk and the plate were incubated at 37°C overnight (Lee et al., 2001). An increase in the zone diameter of imipenem-EDTA disk of ≥5 mm as compared to imipenem disk alone indicates a positive test.

Modified Hodge's test (MHT)

This is a phenotypic test which can be used to determine if reduced susceptibility to carbapenems is mediated by a carbapenemase production. Mueller-Hinton agar plate was inoculated with a 0.5 McFarland suspension of *E. coli* ATCC 25922 and streaked for confluent growth using a swab. A 10 µg imipenem disk was placed in the center, and each test isolate was streaked from the disk to the edge of the plate and the plates were incubated at 37°C overnight. After incubation, the plates were examined for a clover leaf-type indentation at the intersection of the test organism and the *E. coli* ATCC 25922, within the zone of inhibition of the carbapenem susceptibility disk (Lee et al., 2001).

Water sample strains and susceptibility tests

A study was conducted to determine the prevalence of carbapenemase producing strains among enterobacteriaceae species from water samples in and around Puducherry. Five hundred milliliters of water samples were collected from lakes (4), ponds (2) and taps (9) in screw capped wide mouthed sterile bottle and were filtered through sterile membrane filters (0.2 µM, Millipore, India) and the membrane was placed upside down on the surface of sheep blood and MacConkey agar plates and allowed for few minutes before being taken off. Then, the surface of MacConkey agar and sheep blood agar were streaked for isolated colonies. Further, the membrane was introduced into McCartney bottles containing brain heart infusion broth using sterile forceps and the broth was sub-cultured on MacConkey agar and blood agar after overnight incubation at 37°C. The isolates were identified by standard methods and antimicrobial susceptibility testing was carried out by disk diffusion method as per CLSI guidelines 2011.

Multiplex PCR analysis

Genomic DNA was extracted from all the strains by boiling lysis method. Multiplex PCR was carried out in order to detect metallo-β-lactamase genes such as blaNDM-1, blaVIM, blaKPC and blaIMP. Primer sequences used are given in Table 1. Thermal cycler gradient, Eppendorf was used for multiplex PCR. An initial denaturation step of 15 min at 95°C was followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 59°C for 1 min and extension at 72°C for 90 s. This was followed by a step of final extension at 72°C for 10 min. The amplified products were analyzed

Table 1. Primers used to identify *bla*NDM-1, *blaIMP, blaVIM* and *blaKPC* genes by multiplex PCR analysis (Michael et al., 2011).

Name	Forward	Reverse	Size (bp)
NDM-1	GGTGCATGCCCGGTGAAATC	ATGCTGGCCTTGGGGAACGS	660
VIM	GTTTGGTCGCATATCGCAAC	AATGCGCAGCACCAGGATAGAA	382
IMP	CCWAATITAAAAATYGAGAAGCTTG	TGGCCAHGCTTCWAHATTTGCRTC	522
KPC	ATGTCACTGTATCGCCGTC	AATCCCTCGAGCGCGAGT	863

Table 2. Clinical specimens from which meropenem resistant organisms were isolated.

Specimen	Number of organisms isolated	Percentage of organisms isolated
Wound swab	162	38
Blood	85	20
Tracheal aspirate	55	13
Pus	51	12
CSF	17	4
Sputum	17	4
Catheter tip	13	3
Pleural pus	9	2
Peretonial fluid	8	2
Synovial fluid	8	2

Table 3. Results of tests showing positivity with respect to different organisms isolated.

Organism	Number of organismsisolated	MBL positive (%)	MHT positive (%)	blaNDM-1 positive (%)	Efflux positive (number)
Klebsiella sp.	229	58	62	65	14
Enterobacter sp.	89	60	63	53	8
Escherichia coli	85	58	62	66	6
Citrobacter sp.	14	57	64	57	1
Providencia sp.	5	60	60	60	1
Proteus sp.	3	67	0	0	0
Total	425	58	62	62	30

in 1.5% agarose gel stained with ethidium bromide and visualized under UV.

Efflux pump inhibitors

The presence of efflux pump as the mechanism of carbapenem resistance was analyzed using MIC assay. The strains which were negative for carbapenemase genes by PCR were used for efflux pump studies. Meropenem trihydrate pure powder (Orchid, Chennai) was used for MIC assay. Broth micro dilution was performed using Muller Hinton broth in the presence and absence of 25 µg/ml of phenylalanine arginine beta-naphthylamide (PAβN) (Sigma Aldrich, US). A threefold or more decrease in the MICs of the strains in the presence of PAβN suggests efflux pump inhibition by PaβN, indicating the presence of an active efflux mechanism.

RESULTS

Among 425 meropenem resistant clinical isolates, 229 were *Klebsiella* sp., 89 were *Enterobacter* sp., 85 were *E. coli*, 14 were *Citrobacter* sp., 5 were *Providencia* sp. and 3 were *Proteus* sp. Table 2 shows the different clinical specimens from which meropenem resistant organisms were isolated. Two hundred and forty eight (58%) strains were positive for metallo beta lactamase double disk synergy test (MBL) and 264 (62%) strains were positive for modified Hodge test (MHT). Among 425 strains, 262 (62%) strains had blaNDM-1(660 bp) gene. Table 3 presents the positive percentage of these tests.

The efflux pump inhibitory studies showed that out of

Table 4. Organism isolated from water samples.

Organism	Number of isolates
Escherichia coli	5
Klebsiella sp.	3
Enterobacter sp.	2
Proteus mirabilis	1
Total	11

163 *blaNDM-1* gene negative strains, 30 (23%) isolates had shown three fold decrease in MIC values in the presence of PAβN, which suggests the presence of efflux pump-mediated carbapenem resistance.

The prevalence rate of carbapenem resistant enterobacteriaceae from water samples in 15 areas of Puducherry was studied. It was observed that the *Enterobacteriaceae* species isolated from lakes (4) and ponds (2) were all susceptible to meropenem, amikacin, ciprofloxacin, ceftazidime, ceftriaxone and gentamicin by disc diffusion method. The most common isolates were *E. coli* (5), *Klebsiella* sp. (3), *Enterobacter* sp. (2) and *Proteus mirabilis* (1) (Table 4). However, all the tap water samples (9) did not yield any Gram negative bacteria.

DISCUSSION

Carbapenemase producing organisms display higher levels of resistance to almost all antibiotics (Li and Nikaido, 2009; Lee et al., 2001). Meropenem resistance is used in practice as an indicator of the presence of metallo beta lactamases. The present prospective study was carried out to reveal the prevalence rate of MBL-producing enterobacteriaceae from clinical and environmental isolates. It has been mostly reported that resistance to beta lactam antibiotics is on the rise among clinical isolates in different Indian hospitals, indicating the need for exhaustive research. Several studies have reported the risk role of MBL and ESBL-producing *Pseudomonas aeruginosa, Acinetobacter* species or enterobacteriaceae among hospitalized patients. The occurrence of an MBL-positive isolate in a hospital environment not only poses a therapeutic problem, but also is a serious concern for infection control management (Behera et al., 2008).

In the present study, MHT was positive in 62% of the isolates. This indicates that carbapenemase mediated mechanism of resistance is more frequent than the non-carbapenemase mediated mechanism of resistance. 62% of the isolates showed the presence of blaNDM-1 gene and the predominant is *K. pneumoniae.* NDM-1 which is a recent addition to the carbapenemase list, was the only carbapenemase identified in our isolates. In a recent study (Rahman et al., 2014) which evaluated the detection and molecular characterization of NDM variants in Enterobacteriaceae at a tertiary care hospital in India, all carbapenem-resistant isolates were bla_{NDM} positive by PCR.

Efflux pump inhibitor studies revealed that out of 163 clinical isolates negative for carbapenemase genes by PCR, 30 isolates showed efflux pump activity. Previous studies (Baroud et al., 2013) reported that efflux pump inhibition has significantly decreased MICs to carbapenems in *E. coli*. Carbapenem resistance in ESBL-producing *K. pneumoniae* and *E. coli* is due to the combined effect of β-lactamases with porin impermeability and/or efflux pump activity observed in these organisms, and in a number of isolates is due to the production of the carbapenemase-encoding genes and the newly emerging blaNDM-1 (Cunningham et al., 2013).

The prevalence of carbapenem resistant strains of *Enterobacteriaceae* in water samples from 15 places in Puducherry was studied. It was observed that the *Enterobacteriaceae* species isolated from lakes and ponds were susceptible to meropenem. Tap water samples were found to be negative for *Enterobacteriaceae* species.

Findings of this study emphasize the role of carbapenem resistance among hospital isolates and leads to consideration of empirical treatment for infection caused by these organisms especially in patients compromised by underlying disease or immunological status.

Conclusion

In this study, it was observed that efflux pumps play an important role in carbapenem resistant *Enterobacteriaceae* species apart from other resistance mechanisms such as metallo beta lactamases and outer membrane proteins. The results of environmental studies indicate the absence of carbapenem resistant enterobacteriaceae species in pond, lake and tap water from the area under study. This study provides reliable data for researchers working in the area of carbapenem resistant bacteria in clinical and environmental samples.

Conflict of interest

The authors did not declare any conflict of interest.

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Full Length Research Paper

Candida albicans ssp. *dubliniensis stat.et comb. nov*., a new combination for *Candida dubliniensis* based on genetic criteria

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One accredited species, *Candida albicans* subspecies *dubliniensis*, has been proposed to replace the existing designations of *Candida dubliniensis*. The study of the genetic diversity among the clinical isolates of *C. albicans* and *C. dubliniensis* was performed based on the amplified transposable intron region in the 25S rRNA gene. This study attempts to verify the unequivocal understanding of the genetic relationship between *C. albicans* and *C. dubliniensis*. Twenty (20) isolates of *C. albicans* and *C. dubliniensis* were studied using the method of typing by rDNA, restriction fragment length polymorphism (RFLP), random amplification polymorphism DNA-polymerase chain reaction (RAPD-PCR) and intron sequencing in the 25S gene. The results reveals that the specific primer pair CABF59F and CADBR125R was the successfully amplified target for all the *C. albicans* isolates and three isolate of *C. dubliniensis*. The *Candida* isolates revealed a genetic pattern based on the analysis of the RAPD-DNA fingerprinting pattern. The RFLPs generated by HhaI and Hae 111 enzymes elucidated similar recognition sites for both the *C. albicans* and *C. dubliniensis* isolates. Analysis of the intron sequence in the 25S gene region of the genotype *C. albicans* and *C. dubliniensis* showed identical with only a few differences in the base substitution. The sequence variations appear among the same isolates in each species. In all the cases, the clinical isolates of both species showed a percentage sequence similarity of >99.5%. This result emphasizes a high indication of similarity between *C. dubliniensis* and *C. albicans*. It was concluded that the taxonomic position of *C. dubliniensis* was puzzled due to insufficient genetic and phenotypic characters to warrant species status. Variations were occasionally observed to occur among the same isolates, within the same species; however, this indication is applied to other taxonomic criteria between them, with no credibility for the great differences observed between *C. dubliniensis* and *C. albicans*. This is the final taxonomic decision for *C. dubliniensis* to merit an amendment in order to be included as *C. albicans* subspecies *dubliniensis* stat. et comb. nov. C. *dubliniensis* with a revised synonymy for *C. albicans*.

Key words: Amendment, *Candida albicans* ssp. *dubliniensis stat. et comb. nov*, restriction fragment length polymorphism (RFLP), random amplification polymorphism DNA (RAPD), polymerase chain reaction (PCR), sequence, phylogenetic tree.

INTRODUCTION

Over the past 19 years, several studies have been done to evaluate the relationship between *Candida dubliniensis* and *Candida albicans,* a typical *Candida* strain difficult to identify up to species level, because of the heterogeneous morphological, biochemical and genetic characteristics they exhibit (Sullivan et al., 1995; Pujol et al., 1997; Sullivan and Coleman, 1997).

C. dubliniensis had been described as a separate

species in 1995 by Sullivan et al. (1995). Retrospective studies revealed that it had been earlier commonly identified as *C. albicans*, to which *C. dubliniensis* is closely related, and with which it shares several characteristics including those of growth conditions, germ tube formation, chlamydospore formation and color interaction on CHROMagar (Tamura et al., 2001). Systematic studies of *Candida* spp. based on phenotypic criteria alone have been revealed to be unreliable markers, although they enabled us to elucidate some taxonomic complications between *C. albicans* and *C. dubliniensis,* such as similarities in the phenotypic characters, especially in their color on chromogenic agar, as well as following other conventional criteria, although not the perfect solution for the differences between them (Ahmed et al., 2002; Marot-Leblond et al., 2006; Imran and Al Asadi, 2014). In fact, misidentifications between some *Candida* spp., particularly between *C. dubliniensis* and *C. albicans* have frequently been observed (Tamura et al., 2001; Abaci et al., 2008). Coronado-Castellote and Jiménez-Soriano (2013) referred to *C. dubliniensis* as exhibiting similarity with *C. albicans* in their germ tubes and chlamydoconidia, as well as the high probability for mating between them and the similarity in some of their sequences at different loci (Pujol et al., 2004). Most of these reasons were critical in the identification of the taxonomic position of *C. dubliniensis*. Ribosomal DNA, considered an essential marker in *Candida* and other fungi, is ideally suited for the development of molecular studies. The high discriminatory power of the molecular tools like polymerase chain reaction (PCR) PCR, PCR-RFLP, RAPD-PCR, as well as sequencing, have provided change barter fast, relatively simple to perform, precise and reliable methods for the diagnosis of the *Candida* spp. (Mirhendi et al., 2005; Santos et al., 2010; Mijiti et al., 2010; Shokohi et al., 2010).

McCullough et al. (1999) utilized CABF59F and CADBR125R primers designed to span the region that includes the site of the transposable intron of the 25S rRNA gene (rDNA). The molecular target, the transposable intron and the design CA-INT primer were the highly reproducible markers for typing the *C. albicans* subgenotypes and differentiated the *C. dubliniensis* from the closely related isolates of the *C. albicans* compartment with a selection of the ITS or other regions (Tamura et al., 2001). The simple PCR attached to the inserted intron in 25S rDNA classified the strains of *C. albicans* into three or four subgroups as the given genotype A, genotype B, genotype C and sometimes even genotype D (McCullough et al., 1999). A special genotype was also assigned to *C. dubliniensis* (Tamura et al., 2001). However, Tamura et al. (2001) showed that no group I intron was observed in the other *Candida* species tested, including those of *Candida glabrata*, *Candida krusei*, *Candida parapsilosis* and *Candida tropicalis*. Imran and Al. Asadi (2014) revealed the presence of introns in most non albicans species. In contrast, if reproducible markers like the restriction enzyme are utilized, they could facilitate solving the difficult cases. By analyzing the restriction fragment length polymorphisms (RFLPs), the unique polymorphism in the monomorphic PCR bands can be identified (Mirhendi et al., 2005). From this perspective, the intron inserted in the region of the 25S rRNA gene sequences offers several advantages to the *Candida* spp. genotypes (McCullough et al., 1999). The intron in the 25S rRNA gene has been shown to have a high heterogeneity within the *Candida* species (Hanafy and Morsy, 2012). The contribution of the intron inserted in the region of the 25S rRNA in clinical diagnosis remains to be determined, due to the lack of a complete molecular database that could enable the systematic comparison of the inter- and intra-species variations in the different isolates among the *Candida* spp.

Most of the regions of the large ribosomal subunit genes (LRSg) of yeasts are reproducible markers, which provided very useful information concerning the phylogenetic relationships among the various marine yeasts (Fell and Kurtzman, 1990).

It is not surprising that some irregularities are seen in the taxonomy of *Candida*. Several recent studies have described the *Candida* isolates, whose properties do not correspond precisely with the descriptions of the classical species, leading to further confusion (Mahrous et al., 1990; McCullough et al., 1994, 1995; Boerlin et al., 1995). It is, therefore, the right time to assess the potential contribution that other techniques could make towards the identification of the relationships between *C. albicans* and C. *dubliniensis*. Sullivan and Coleman (1997) indicated the necessity of further confirmation, which can be obtained by conducting any of the several DNA fingerprint techniques available, as well as by RFLP and RAPD analysis. These are also effective, as well as quicker and easier to perform in order to discriminate between *C. albicans* and *C. dubliniensis*.

Indeed, the comparative nucleotide sequence analysis of the rDNA has been used extensively to study the evolutionary relationships among a wide variety of fungi. Most of these studies have been performed on small ribosomal subunit gene sequences (Hendriks et al., 1991; Fleischmann et al., 2004). A search conducted in the Gene Bank nucleotide sequence databases over the past few decades revealed that the sequence data on the rDNA genes have been reported only for a large number of *Candida* spp. However, these studies indicate that the

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25S gene sequences can be used to confirm the natural relationships within the genus, such as the close evolutionary relationship between *C. albicans* and C. *dubliniensis,* based on biochemical and phenotypic criteria (Kumar et al., 2006; Nawrot et al., 2010).

The aim of this study was to achieve a detailed and unequivocal understanding of the evolutionary relationships between *C. albicans* and C. *dubliniensis*, by performing rapid genotyping based on simple PCR, RFLP-PCR, RAPD-PCR and emphasizing the identification of genotype patterns for both *C. dubliniensis* and *C. albicans* by using the sequencing tools.

MATERIALS AND METHODS

Yeast collection and cultural characters

A total of 60 clinical vaginal swabs were collected from the clinics in the province of Babylon, Iraq, during the study conducted in 2013-2014. Clinical samples using a sterile cotton swab were taken from the vagina of patients exhibiting clinical signs of the vaginal candidiasis based on Imran and Al. shukry (2014). They were transferred to the biotechnical laboratory where they were directly streaked on Sabouraud agar medium (SDA) supplemented with chloramphenicol and streptomycin (50:50 µg/ml). After inoculation, the Petri plates were incubated for 24-48 h at 37°C (Sivakumar et al., 2009; Baveja, 2010). All the sixty isolates in this study were subjected to preliminary identification was done based on CHROMagar Candida (Ghelardi et al., 2008; Marsh and Martin, 2009; Nadeem et al., 2010).

Extraction of genomic DNA

Twenty isolates out of 60 isolates of *Candida* spp. were subjected to DNA extraction and PCR assays .A loop full of *Candida* colony was suspended in the lysis buffer (200 mM Tris-HCl, 20 mM EDTA, 150 mM NaCl and 0.5% SDS) and heated in water bath at 95°C for 2 min. The suspension was centrifuged at 5000 rpm for 2 min and the supernatants were decanted into new sterile tubes, and precipitated with an absolute alcohol and then, washed DNA pellet by 70% ethyl alcohol, dried pellet of DNA dissolved in elution buffer and preserved at -20°C until use (Fredricks et al., 2005).

PCR assays

The phenotypic results were confirmed by simple PCR by specific primer pair for *C. albicans*: CABF59F: 5'-TTGAACATCTCCAGTTTCAAAGGT-3' and CADBR125R: 5 '-AGCTAAATTCATAGCAGAAAGC-3'. amplified target 665 bp (Kanbe et al., 2002). Genotypes and subgenotypes of *Candida* isolates determination by PCR based on method of Tamura et al. (2001). The primer pairs whose sequences span the site of the transposable intron in the 25S rDNA were those described by McCullough et al. (1999). The PCR primer pairs used were CA-INT-L (5′-ATAAGGGAAGTCGGCAAAATAGATCCGTAA-3′) and CA-INT-R (5′-CCTTGGCTGTGGTTTCGCTAGATAGTAGAT-3′).

1 µL of DNA (20 µg/ml) from each of 20 *Candida* isolates were mixed with PCR mixture (final reaction volume 25 µL) consisted of 12 µL of 2x Master Mix (Promega), 2 µL of primers (10 pmole) and rest molecular-grade water. The PCR conditions for CA-INT-L and CA-INT-R primers were 95°C for 3 min followed by 30 cycles 94°C for 1 min, annealing temperature 65°C for 1 min. Extensions temperature 72°C for 2.5 min followed by final extension temperature 72°C for 7 min. The PCR conditions for primer pairs CABF59F and CADBR125R was similar to previous cycle except annealing temperature which was 55° C in place of 65° C. The PCR mixture was amplified by thermal cycler PCR System (Labnet, USA).

The PCR products for each target region were run on 1.2% agarose gel (Bio Basic Canada Inc.). Electrophoresis was performed at 100 V. in TBE buffer. The gel was pre-stained with 0.05% ethidium bromide. The DNA bands were detected by Desktop Gel imager scope 21 ultraviolet transilluminator (Korea Com.).

PCR-RFLP assay

The PCR-RFLP assay was performed as described earlier by Mirhendi et al. (2006). In brief, the incubation of a 10 µL aliquot of the PCR products consisted of 12 µL of 2x Master Mix (Promega), 2 µL of primers (10 pmole) and rest molecular-grade water of amplified intron region of 25S rRNA gene with 10 µL of Hae111 and Hhal cocktail restriction enzymes (Promega, USA) was performed in single reaction, at 37°C for 3 h, using both enzymes. Next, 8 µL of the RFLP-PCR products were run on 1.5% agarose gel at 70 V for 60 min. The gel was pre-stained using 0.05% ethidium bromide and visualized under UV light and photographed by the Desk Gel imager scope 21 ultraviolet transilluminator (Korea Com).

RAPD-PCR assay

RAPD-PCR was accomplished by utilizing a total volume of 34 µL consisting of 0.7 µL (20 µg/ml) genomic DNA, 18 µL of 2x master mix (Promega USA) 12 µL molecular-grade water and 1.5 µL (50 pmole) of random primer GGTGTAGTGT. The mixture was amplified under the following conditions: 95°C for 4 min; 38 cycles at 94°C for 1 min; 36°C for 1.5 min; 72°C for 1.30 min and 72°C for 8 min (Labnet PCR System). Further, 8 µL of the PCR products were run on 1.5% agarose gel at 70 V for 60 min. The gel was pre-stained with 0.05% ethidium bromide.

Sequencing assay

To study the relationship and similarity at morphological and molecular level that are sometimes exhibited between the *C. albicans* and *C. dubliniensis* isolates particularly because *C. albicans* and *C. dubliniensis* also possess the transposable intron in the 25S rDNA, the genomic DNA of the representative isolates for both *C. albicans* and *C. dubliniensis* were amplified with the CA-INT primers. After PCR amplification, the purified products for 8 isolates were sequenced. The PCR primers CA-INT-L was used for DNA sequencing of transposable intron in the 25S rDNA of *Candida* isolates. Sequence analysis was performed using the Macro Gene Company, USA. The sequence alignment of *C. albicans* and *C. dubliniensis* was compared with the BLAST database and were aligned with sequences from the BLAST database derived from the following reference strains: (*C. dubliniensis* sequence ID: embl FM992695.1 United Kingdom isolate; *C. albicans* sequence ID: gbl DQ465844.1 New Zealand isolate).

Phylogenetic analysis

The phylogenetic tree dendrograms of RFLP and RAPD-PCR products for isolates of *C. albicans* and *C. dubliniensis* was created by clustering methods applied on distance matrix unweighted pair

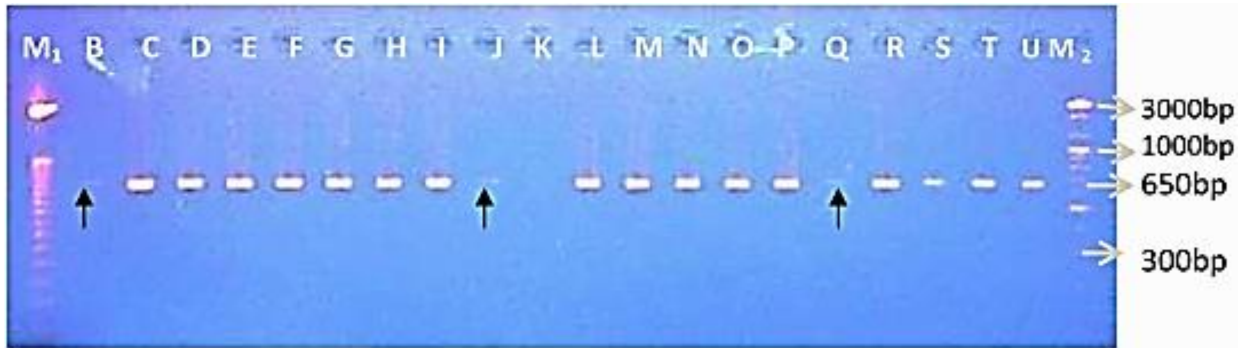


Figure 1. PCR products of 20 *Candida* isolates were amplified by the primer pair CABF59F and CADBR125R. (M_1 = molecular size marker one step 50 bp ladder; M_2 = molecular size marker 100 bp ladder); all positive lanes are *C. albicans* and faint or negative bands are *C. dubliniensis* (labeled by arrows).

group method with arithmetic mean (UPGM) which offers automatic lane/band detection, band matching and molecular weight computation. The phylogeny tree computation was analyzed based on UVI band software GD/45230 for gel image analysis. The software is able to analyze gel image patterns of bands for different isolates or species and generates phylogenetic tree based on the information available in gel image. It also evaluated the similarity coefficient factor according to Mackenstedt et al. (1994) and Ute et al. (1994). The phylogenetic tree based on sequencing and sequence table were constructed employing the Mega 6 software.

RESULTS

Phenotypic and molecular diagnosis for *Candida* spp.

All the sixty isolates in this study showed up in green color on the CHROMagar Candida. The results of molecular assay showed that 20 isolates of *Candida* were identified as *C. albicans*. The amplification of the targeted region produced an amplicon of size 665 bp (Figure 1). The target regions of three isolates for *Candida* showed faint bands as seen in Figure 1, lanes B, J and Q.

Genotyping of *Candida* spp. by CA-INT primer pair

The specific primer CA-INT was designed to flank the transposable intron region of the 25S rRNA gene. PCR was successfully amplified the target region of the genomic DNA of the 20 isolates. The amplification result designated five isolates as *C. dubliniensis,* which had a high PCR product (1080 bp). Thus, 16 isolates of *C. albicans*, with low PCR products could be classified and three genotypes could be designated viz., (i) genotype C, (ii) (450 and ~840 bp), (iii) A genotype of (~840 bp) of *C. albicans* (450 bp) (Figure 2).

RFLP-PCR assay

Both restriction enzymes (HhaI and Hae 111 enzyme) have an equal chance of making a cut anywhere in the PCR product. However, the restriction banding patterns by using the HhaI enzyme showed large fragments (500 bp) of *C. dubliniensis,* as in Figure 3a (lanes B, H, J, K and Q).

This enzyme also revealed a similar basal band with the PCR fragments of average length approximately 380 bp in all the isolates for *C. albicans* and *C. dubliniensis*. However, the use of the HhaI enzyme cut PCR products into many short PCR fragments (<100 bp), as seen in Figure 3a. The restriction banding patterns by using the Hae 111 enzyme showed characteristic cleavage profile (350, 300, 180 and 60 bp fragments) for *C. dubliniensis*.

However, the PCR products of *C. albicans* also showed variation in their RFLP patterns. The first pattern revealed two fragments such as C, E-G, I and M, whereas the second pattern was composed of four fragments such as D, L, N, O, PT and U (Figure 3a). The isolates of *C. dubliniensis* B, H, J, K and Q showed variation in their RFLP-PCR patterns, in which the H, J and K appeared closely related, while the B and Q showed differences (Figure 3b). The Hae 111 enzyme resulted in a large fragment of the PCR, of *C. dubliniensis,* of about 350 bp as in the B, H, J, K and Q lanes. The PCR product of the albicans isolates showed fragments of about 300 bp, as in C, E-G, I, M, R and R-S, as well as a fragment of 270 bp in D, L, N-P, T-U; This enzyme also revealed a similar basal band of average length of the PCR fragments, approximately 100 bp, in all the isolates for *C. albicans* and *C. dubliniensis* (Figure 4a).

RAPD-PCR assay

The results show that the oligo primer GGTGTAGTGT successfully genotyped the 20 isolates of *C. albicans* and *C. dubliniensis* into 7 genotypes: *C. dubliniensis* revealed three genotypes, while *C. albicans* showed four genotypes (Figure 5). RAPD-PCR produced multiple bands, the main band of which was consistently present in all the

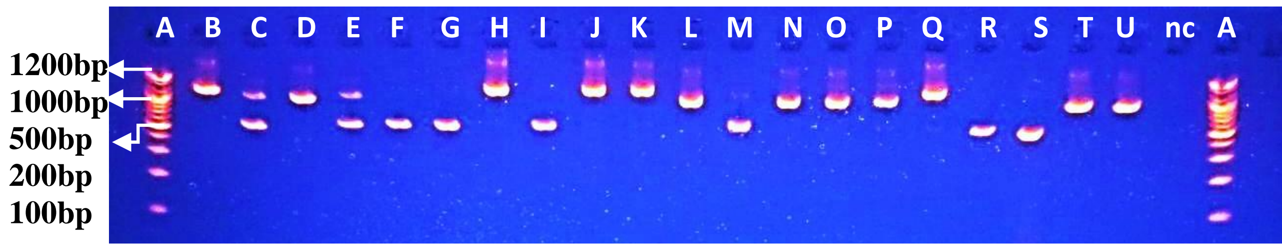
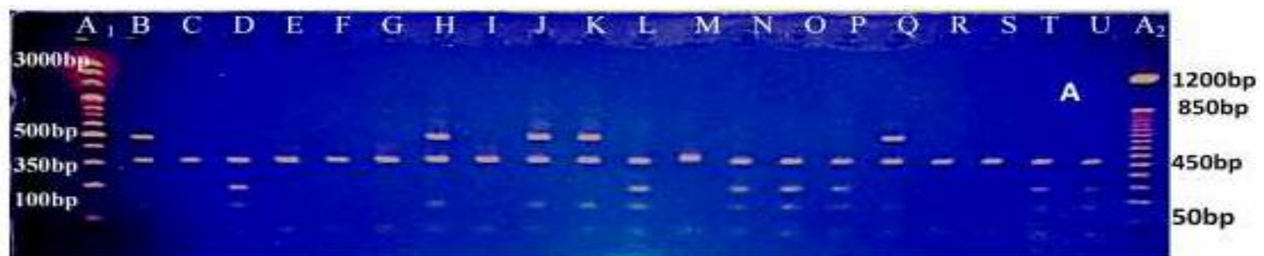


Figure 2. Gel electrophoresis of the PCR product amplified by the primer pair CA-INT. Bands in lanes B, H, J-K and Q: *C. dubliniensis* (1080 bp), lanes, C and E = genotype C (450 and 840 bp), lanes D, L, N-P and T-U. A genotype of *C. albicans* (~840bp), lanes F-G, I, M and R-S = B genotype of *C. albicans* (450bp) A= molecular marker 100 bp.



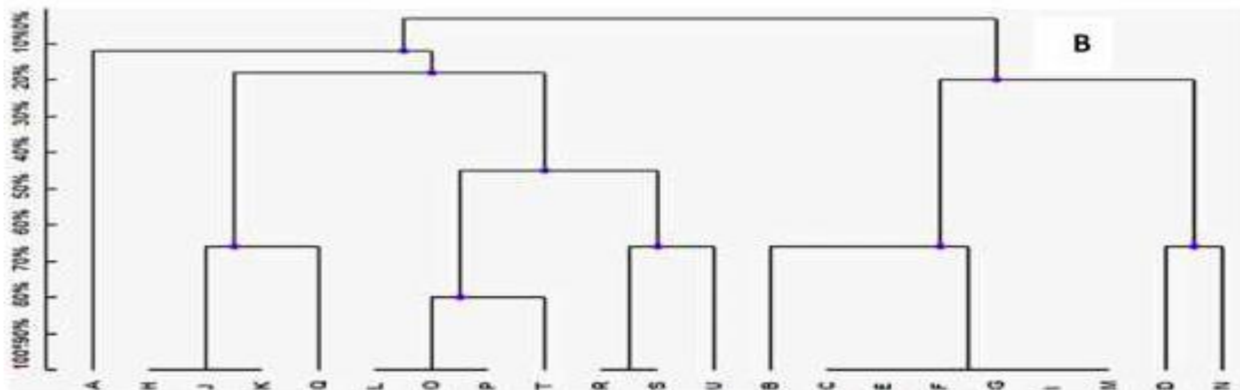


Figure 3. A- Restriction digestion patterns of the transposable intron region of the 25S rRNA gene PCR products with the HhaI enzyme from 20 randomly selected representative isolates of the *Candida* species. Lane A: DNA molecular size marker (A_1 =One step 100 bp ladder; A_2 = one step 50 bp), lanes B, H, J-K and Q: *C. dubliniensis*, lanes C and E = genotype C lanes: D, L, N-P and T-U. A genotype of *C. albicans*, lanes F-G, I, M and R-S = B genotype of *C. albicans*. B- Phylogenetic relationships (homologous coefficient %) between different isolates of *Candida.* The consensus tree was based on data of RFLP-PCR by HhaI enzyme generated via UPGMA cluster analysis. A = molecular size marker 100 bp; B-T, clinical isolates of *Candida*.

isolates (constant basal band 450-550 bp); the greatest variation occurred among the upper bands of the constant single bands at 600-800 bp (Figure 5). *C. dubliniensis* revealed three genotypes: (B-Q 70%, K-H 45% and J) .and *C. albicans* showed 10 genotypes (M-N, O-U, P-S, F-G, R-T, J, C, L, I and E).

Sequence analysis

The results of sequence analysis for eight isolates of *Candida* spp. showed a similarity with the entry in the percentage sequence of >99.5% with the intron region of the 25S rRNA gene. Genotypes A and C of *C. albicans* isolates (D, T and E) showed high similarity of about 99.98% in their sequence at the same time, the genotype of *C. dubliniensis* isolates (B and Q) showed high similarity of about 99.97% with the A and C genotypes of *C. albicans* isolates (D, T and E), genotype B of *C. albicans* isolate (R) showed similarity of 99.96 when compare with the genotypes C and A of *C. albicans* isolates (C and L) (Figure 6).

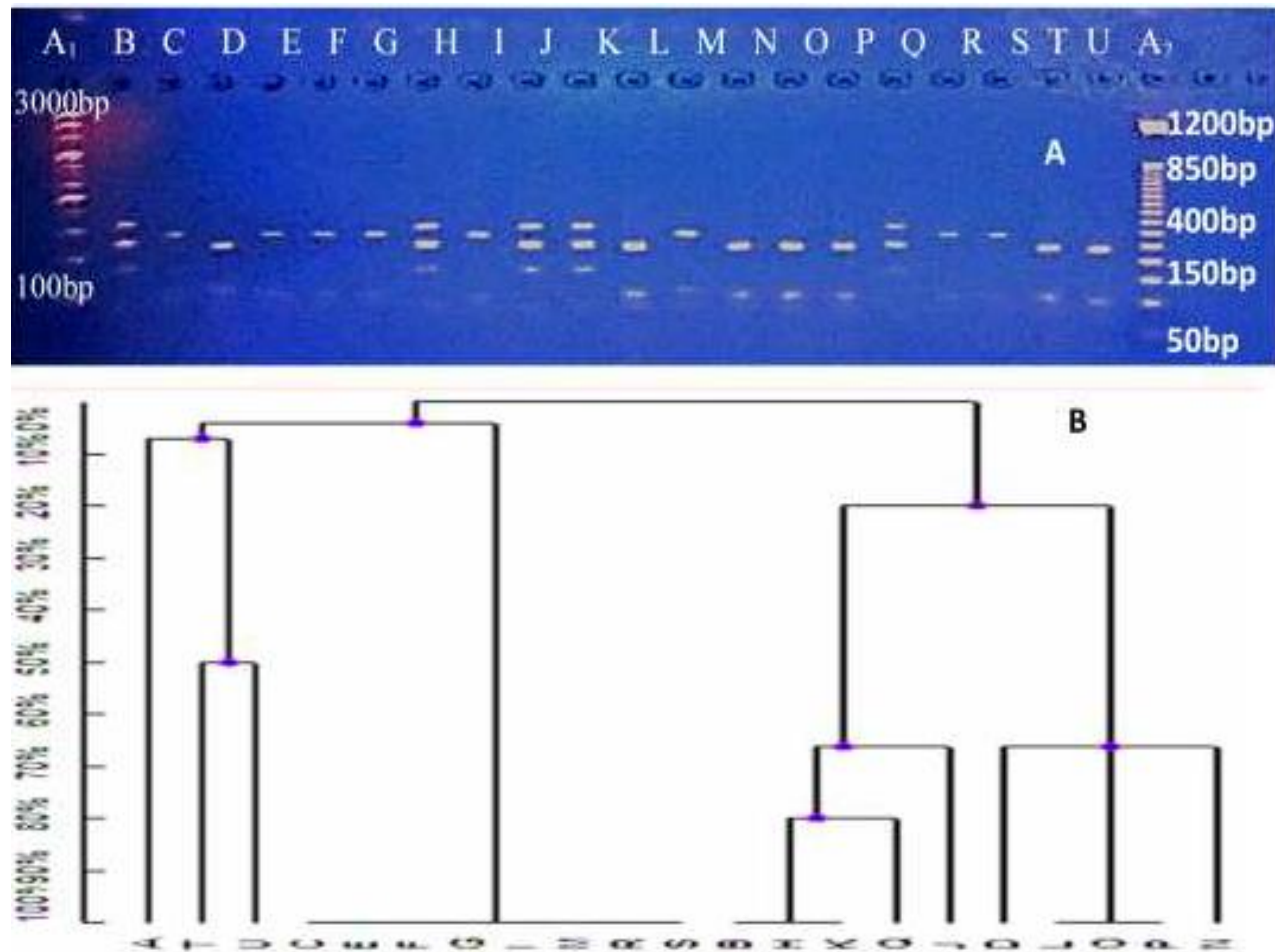


Figure 4. A-Restriction digestion patterns of the transposable intron region of the 25S rRNA gene PCR products with Hae 111 enzyme from 20 randomly selected representative isolates of the *Candida* species. Lane M: DNA molecular size marker (One step 100 bp ladder), lanes B, H, J-K and Q: *C. dubliniensis*, lanes: C and E=genotype C, lanes: D, L, N-P and 19-20) A genotype of *C. albicans*, lanes F-G, I, M and R-S= B genotype of *C. albicans*. B- Phylogenetic relationships (homologous coefficient (%)) between different isolates of *Candida.* The consensus tree was based on data of RFLP-PCR by Hae 111 enzyme generated via UPGMA cluster analysis. A, Molecular size marker 100 bp; B-T, clinical isolates of *Candida*.

Figure 7 showed sequence analysis for eight isolates of *Candida* spp. *C. albicans* isolates D, T and E showed high similarity in their sequence with *C. dubliniensis* isolates B and Q except difference in two adenine nucleotides at the same time, the *C. albicans* isolates C, L and R showed high difference in their sequence with *C. albicans* isolates D, T and E *and C. dubliniensis* isolates B and Q.

DISCUSSION

Our results concurred with several recent studies and demonstrated a wide degree of genetic homogeneity between *C. dubliniensis* and *C. albicans* (Jackson et al., 2009). The results of Boucher et al. (1996) found that both the intron and ribosomal RNA nucleotide sequences were almost perfectly identical between the different *C. albicans* strains, as well as between the *C. albicans* and *C. dubliniensis*. Although it is difficult to distinguish between the *C. albicans* and *C. dubliniensis* colonies formed on CHROMagar which are green in color, the CHROMagar medium can be unstable following subculture or storage (Schoofs et al., 1997; Sullivan and Coleman, 1998). This result indicated that the specific primer pair (CABF59F and CADBR125R) for *C. albicans* amplified its target in C. *dubliniensis* as well as in 16 isolates of *C. albicans.* Despite the concurrence in the results for *C. albicans* with those of Costa et al. (2010), the result with C. *dubliniensis* indicated the similarity of the sequence target region for *C. dubliniensis* and *C. albicans*. This result concurred with the report of Pujol

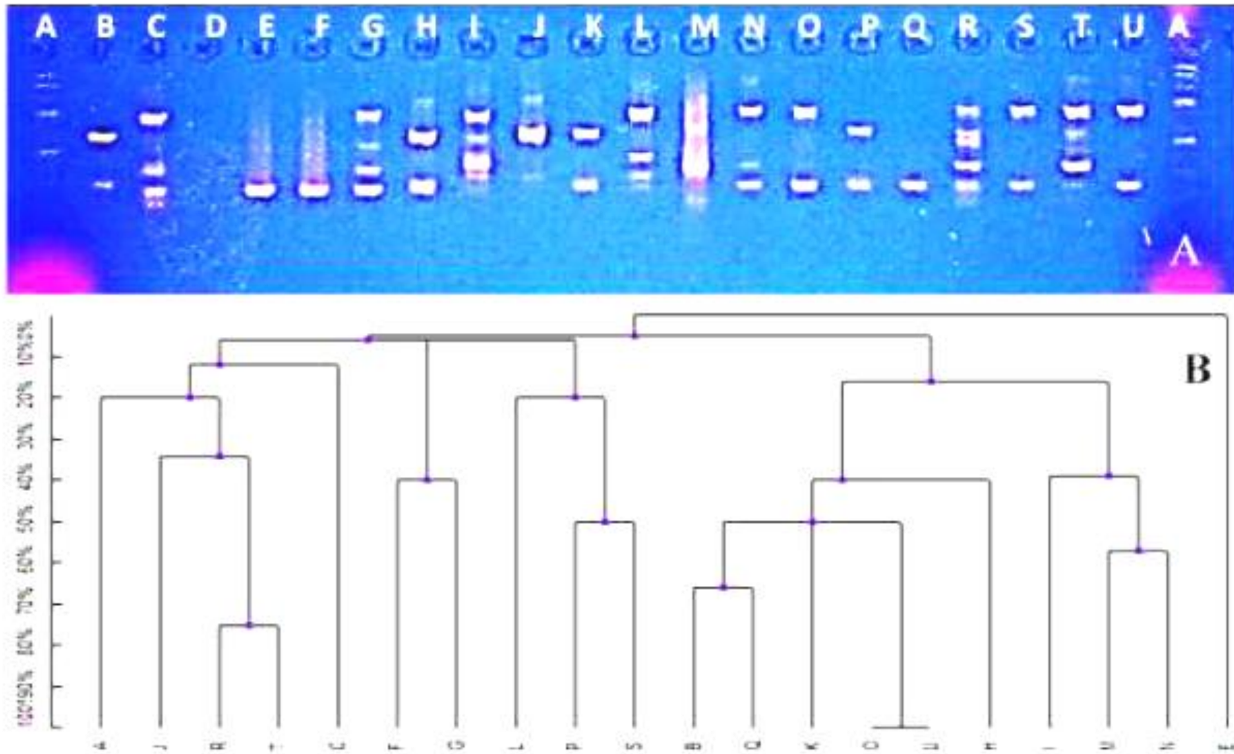


Figure 5. A- Agarose gel electrophoresis of the amplified RAPD-PCR products for *Candida* spp. Detection polymorphism of 20 clinical isolates of *Candida* spp. using the oligo primer GGTGTAGTGT. B- Phylogenetic relationships (homologous coefficient %) between different isolates of *Candida.* The consensus tree was based on data of RAPD-PCR generated via UPGMA cluster analysis. A = Molecular size marker 100 bp; B-T, clinical isolates of *Candida.*

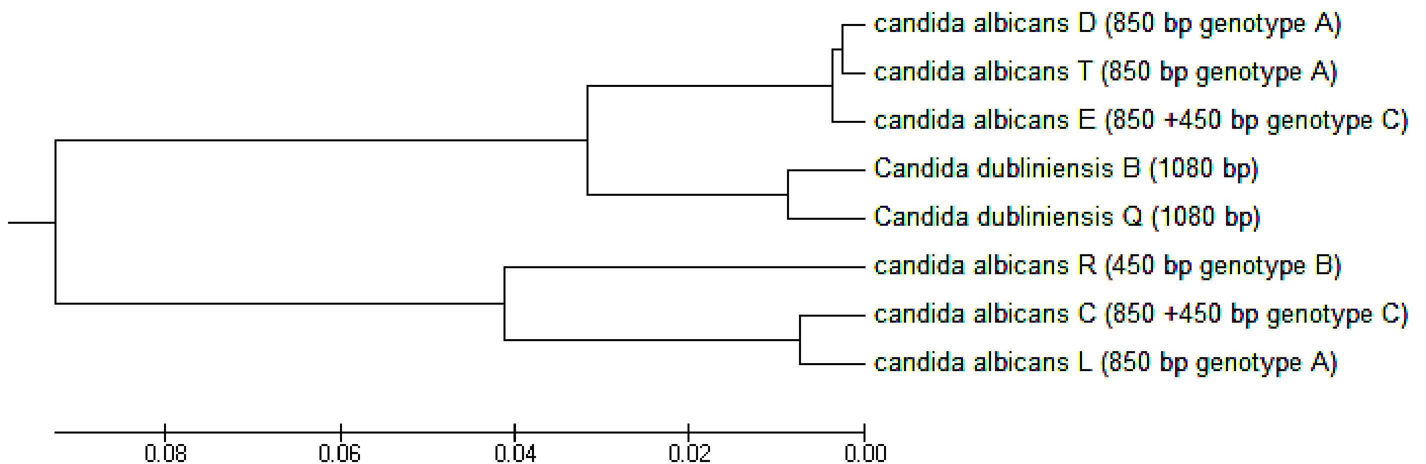


Figure 6. Phylogenetic relationships (homologous coefficient %) between different isolates of *Candida* based on sequence data were constructed employing the Mega 6 software.

et al. (2004) as far as the similarity in some of the loci sequences between the two species. The specific primer CA-INT designed to flank the transposable intron region of the 25S rRNA gene successfully typed 5 isolates out of the 20 isolates designated as *C. dubliniensis* (1080 bp), while the other isolates were designated as three genotypes of *C. albicans* (genotype C = 450 and ~840 bp; genotype A = ~840 bp and genotype B= 450 bp). This result concurred with the earlier studies of Tamura et al. (2001), Kumar et al. (2006) and Nawrot et al. (2010).

DNA Sequences | Translated Protein Sequences

Species/Abbrv	Grou	Sequence
1. candida albicans D (850 bp genotype A)		TATGACTCTCAACCTATAAGGGAGGCAAAAGTAGGGACGCC------ATGGTTTCCAGAAATGGGCCGCGGTGTTTTTGACC-T
2. candida albicans E (850 +450 bp genotype C)		TATGACTCTCAACCTATAAGGGAGGCAAAAGTAGGGACGCC------ATGGTTTCCAGAAATGGGCCGCGGTGTTTTTGACC-T
3. candida albicans T (850 bp genotype A)		TATGACTCTCAACCTATAAGGGAGGCAAAAGTAGGGACGCC------ATGGTTTCCAGAAATGGGCCGCGGTGTTTTTGACC-T
4. Candida dubliniensis Q (1080 bp)		TATGACTCTCAACCTATAAGGGAGGCAAAAGTAGGGACGCT------ATGGTTTCCAGAAATGGGCCGAGGTGTTTTTGACC-T
5. Candida dubliniensis B (1080 bp)		TATGACTCTCAACCTATAAGGGAGGCAAAAGTAGGGACGCT------ATGGTTTCCAGAAATGGGCCGAGGTGTTTTTGACC-T
6. candida albicans C (850 +450 bp genotype C)		TATGACTCTCT-----TAAGGTAGCCAAATGCCTCGTCATCTAATTAGTGACGCGCATGAATGGATTAACGAGATTCCCACTGT
7. candida albicans L (850 bp genotype A)		TATGACTCTCT-----TAAGGTAGCCAAATGCCTCGTCATCTAATTAGTGACGCGCATGAATGGATTAACGAGATTCCCACTGT
8. candida albicans R (450 bp genotype B)		TATGACTCTCT-----TAAGGTAGCCAAATGCCTCGTCATCTAATTAGTGACGCGCATGAATGGATTAACGAGATTCCCACTGT

Figure 7. Sequence data were constructed employing the Mega 6 software.

Both the restriction enzymes, HhaI and Hae 111, revealed a high degree of polymorphisms with respect to RFLP-fingerprinting in both *C. albicans* and *C. dubliniensis*. In total, 16 isolates of *C. albicans* showed polymorphic RFLP-patterns. However, the use of the HhaI enzyme revealed a similar basal band with an average length of the PCR fragments at approximately 380 bp in all the isolates for *C. albicans* and *C. dubliniensis*, the phylogenetic tree highlighting the degree of homogeneity in the sequence of recognition site of enzyme between *C. albicans* and *C. dubliniensis.*
The Hae111 enzyme elucidated polymorphic RFLP-patterns in both *C. albicans* and *C. dubliniensis* and yielded different fragment sizes with *C. dubliniensis* having other short PCR fragments. On the other hand, the PCR products of *C. albicans* also showed variation in their RFLP-patterns. This enzyme also revealed a similar basal band of average length of the PCR fragments, approximately 100 bp in all the isolates for *C. albicans* and *C. dubliniensis.* This implied a similarity in the recognition site and the same sequence in this site for both species.

The phylogenetic tree supported the natural variation such as mating, mutations and recombination which may occurred in many isolates related to the same species. Our result coincidence with many studies (Mirhendi et al., 2005; Mirhendi et al., 2006; Shokohi et al., 2010).

The sequence introns of 25S from eight isolates of *C. albicans* and *C. dubliniensis* were retrieved through BLAST analysis, the sequences in *C. albicans* and *C. dubliniensis* were found to differ by approximately 0.02%. This difference is not similar to the differences in the sequences found between *C. tropicalis* and *C. maltosa* (2.8%) and between *C. parapsilosis* and the ascosporic species, *Lodderomyces elongisporus* (3.2%). Based on this, results were contrary to those of Peterson and Kurtzman (1991), in which they earlier suggested that strong evidence for a separate species exists when this region contains a substitution of greater than 1% of the nucleotide between the two organisms. Most of the phenotypic characters of *C. dubliniensis* did not serve to confirm the taxonomic status of a distinct species.

Amendment of *C. dubliniensis:* subspecies *novus*

In the light of these results, despite the *C. dubliniensis* had been described as a separate species over the past decade (Sullivan et al., 1995; Coleman et al., 1997; Tamura et al., 2001), the understanding that prevailed was that a few variations occasionally occurred in many strains due to natural selection, which were, however, insufficient to justify the emergence of a new species. besides strong confirmation from many studies including the work of Coleman et al. (1997) who revealed the very close similarity among the isolates of *C. dubliniensis* to those of *C. albicans.* Particularly, because *C. dubliniensis* and *C. albicans* are phenotypically very similar, it is highly likely that the isolates of *C. dubliniensis* had been misidentified as *C. albicans* or *C. stellatoidea* in the past (Anthony et al., 1995; Boerlin et al., 1995; Coleman et al., 1997). On the other hand, it is shown that it is impossible to consider any variation in the phenotypic and genetic properties, which were not contingent upon their definitive identification as *C. albicans,* based on the views of Coleman et al. (1997) to be the emergence of a new species.

In spite of the *C. albicans* and *C. dubliniensis,* isolates produced chlamydospores on the TOC agar medium based on Tamura et al. (2001). Our results showed the density of the chlamydospores produced by both species were not a good taxonomic character from which to draw any conclusion.

Our results demonstrate that C. *dubliniensis* shares a very close relationship with C. *albicans* based on the results of both the RFLP, RAPD-PCR patterns and the sequence marker shown in Figures 6 and 7, this result coincides with prior studies that suggested that C. *dubliniensis* isolates were not merely mutant derivatives

of *C. albicans;* in the same trend, we do not observe sufficient differences to separate C. *dubliniensis* from *C. albicans* to warrant a species status. Therefore, we provided further support for its designation and confirmed that C. *dubliniensis* should be considered as a subspecies of *C. albicans.* This judgment, based on the molecular RFLP patterns, such as the HhaI and Hae 111 enzymes revealed a similar basal band, this indicated the presence of the same sequence and recognized the region in both species. Sequence also confirmed part of this truth based on sequence analysis. These results are in agreement with those of Jackson et al. (2009) and Sullivan Coleman (1997) where they refer to the requirement for further confirmation, which can be obtained by performing any of the several DNA fingerprint techniques available. The phylogenetic tree, based on the sequencing of the introns on the 25S gene, showed close similarity (99.5%) between *C. dubliniensis* and *C. albicans* as shown in Figure 7, with only subtle differences in sequence between the two species.

The amendment of *C. dubliniensis* taxonomic state agrees with early and recent studies that are closely related *C. dubliniensis* to *C. albicans* which was routinely misidentified as *C. albicans* (Sullivan et al., 1995; 2004; Moran et al., 2004; Jones et al., 2004). Based on the results of Tamura et al. (2001), the genotype 1080 bp was elucidated only as *C. dubliniensis* on typing a transposable intron region in the 25S rRNA gene from other four genotype strains viz.: genotypes of *C. albicans* (genotype C = 450 and 840 bp, genotype A = ~ 840bp, genotype B = 450 bp and genotype E= 1400 bp), these genotypes continued to remain as different strains of *C. albicans.* Tamura et al. (2001) referred to the genotype E strain which showed a high degree of similarity to *C. dubliniensis* when compared with the degree of similarity of the strains of the other *C. albicans* genotypes, in which the similarity was determined based on the group I intron sequence; however, from his results, he neglected to include this genotype within *C. dubliniensis*, as was expected. From our view, with his erroneous taxonomic judgment along with his temperament and individuality, based on the trend of Tamura et al. (2001), each one of the all the genotypes of *Candida* (450, ~840, 450+850 and 1400 bp) was merited to be included as a new species at the same time. We think it is insufficient to justify the emergence and support genotype 1080 bp of a new species by Tamura et al. (2001). Therefore, the differentiation of the two taxa was based on the color of the colony on CHROMagar. *Candida,* thus, may not be as reliable as was considered earlier, to utilize CHROMagar to differentiate between *C. albicans* and *C. dubliniensis.* Tamura et al. (2001) revealed a dark blue color of the colonies on CHROMagar, which could not confirm the differentiation of four of the *C. dubliniensis* isolates out of five. They also used the growth at 45°C as a criterion for the differentiation between the two species. This was confuted by Tamura et al. (2001) when he referred to all the *C. albicans* genotypes, including the five *C. dubliniensis* strains, which grew well at 45°C on a culture media such as PDA and Sabouraud dextrose agar.

We concluded that, it is impossible to consider any few variations in the phenotypic and genetic properties of the *Candida* strains, and showed that a few variations occasionally occurred in many strains due to natural selection, which were however, insufficient to justify the emergence of a new species. Besides strong confirmation from previous studies which revealed the very close similarity among the isolates of *C. dubliniensis* to those of *C. albicans, C. dubliniensis* is not an emerging new species. We provided further support for its designation and confirmed that C. *dubliniensis* do not merit being included as a new species and should be considered as *C. albicans* subspecies *dubliniensis* stat. et.comb. nov.

Ethical approval

Author hereby declare that all actions have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Conflict of interest

The authors did not declare any conflict of interest.

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