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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

Detection of extended-spectrum beta-lactamases (ESBLs) in clinical isolates of *Klebsiella pneumoniae* using the ESBL NDP test and flow cytometric assay in comparison to the standard disc diffusion

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This study was undertaken to evaluate the comparison among three different assays: extended-spectrum beta-lactamases (ESBL) Nordmann/ Dortet/ Poirel (NDP) test, flow cytometric assay and disc diffusion method for the detection of ESBL production. Sixty clinical isolates of *Klebsiella pneumoniae* were isolated from patients' clinical samples admitted to Suez-Canal University Hospital, Ismailia Governorate. The percentages of ESBLs producing *Klebsiella pneumoniae* ranged from 70 to 80% by ESBL NDP and flow cytometric assays, respectively in comparison to 76.6% by disc diffusion method. The sensitivity and specificity of the three assays were evaluated and the sensitivity by ESBL NDP and disc diffusion method was 100%, while by the flow cytometric assay, it was 91.3%. The specificity of disc diffusion method in detection of ESBLs was 100%, followed by the ESBL NDP test (85.7%) and flow cytometric assay (77.8%). Kappa testing showed perfect agreement between the ESBL NDP test and disc diffusion method (kappa=0.9), while flow cytometric assay showed substantial agreement (kappa= 0.7). The ESBL NDP test offers an applicable tool for rapid detection of ESBL-production. Although, flow cytometric assay is a promising method that might be used in the clinical microbiology laboratory but there is a need for the experienced personnel along with the device.

Key words: Extended-spectrum beta-lactamases (ESBLs), ESBL NDP test, flow cytometry.

INTRODUCTION

Extended-spectrum beta-lactamases (ESBLs) produced by Gram-negative bacteria are considered one of the largest and rapidly evolving group of plasmid-mediated enzymes that confer resistance to oxyimino-cephalosporins and monobactams (Pitout, 2010). *Escherichia coli* and *Klebsiella pneumoniae*, being the major source of community- and hospital-acquired infections are mostly ESBL producers (Pitout and Laupland, 2008).

ESBL recognition has an important clinical impact as

inappropriate treatment can lead to therapeutic failures and consequently to adverse clinical outcomes (Schwaber and Carmeli, 2007). A variety of ESBLs have been reported in *Enterobacteriaceae*, being mostly of the CTX-M-, TEM- and SHV-types (Bush and Jacoby, 2010; Poirel et al., 2012). ESBL detection is necessary to screen patients, improve hospital infection control practices and to curb inappropriate antibiotic used that prolonged the efficacy of the currently available antibiotics (Schwaber et al., 2006; Zahar et al., 2009).

Current techniques for detecting ESBL producers are based on the determination of susceptibility to expanded-spectrum cephalosporins followed by the inhibition of the ESBL activity, mostly by clavulanic acid or tazobactam (Drieux et al., 2008). Sensitivities and specificities of the double disk test and of the E-test proposed for that purpose are good, ranging from 80 to 95% (Gazin et al., 2012). The automated methods used in the detection of ESBL producing organisms had a much higher sensitivity (80 to 99%) than specificity (50 to 80%). However, those tests require overnight growths consuming 24-48 h before ESBL production is detected with a subsequent delay in the initiation of appropriate antibiotic therapy (Schwaber et al., 2006; Drieux et al., 2008; Gazin et al., 2012).

Molecular detection of ESBL genes (PCR and sequencing) is an interesting alternative but remains costly and requires a certain degree of expertise (Drieux et al., 2008; Gazin et al., 2012) since recently, real time PCR and DNA microarray (Check-Points) are commercially available to detect ESBL gene variants (Cuzon et al., 2012). However, those PCR-based techniques require isolation of bacteria from clinical samples prior to susceptibility testing and phenotypic identifications and hence; those results can be obtained at least 48 h after obtaining the clinical samples. Also, they are usually not performed in a routine laboratory but restricted to epidemiological purposes. Therefore a simple and efficient technique for ESBL producers is required (Nordmann et al., 2012).

The ESBL NDP test is a novel test, based on the hydrolysis of the β-lactam ring of a cephalosporin (cefotaxime), which generates a carboxyl group, by acidifying a culture medium. It uses 96-well microtiter plates or a single tube and the acidity resulted from this hydrolysis is identified by the color change using a pH indicator (red phenol) while, inhibition of ESBL activity is evidenced by adding tazobactam in a complementary well (Cuzon et al., 2012).

A rapid, powerful high-throughput technology allowing analysis of several thousand cells per second and providing quantitative and statistically significant data is the flow cytometry (FC) (Shapiro, 2001). Bacterial cells are incubated with cephalosporins (ceftazidime or cefotaxime) in the presence and absence of clavulanic acid; subsequently, cells are stained with the fluorescent dye Bis-(1, 3-dibutylbarbituric acid) trimethine oxonol [DiBAC4 (3)] which is able to diffuse across depolarized membranes. Susceptible isolates display increased fluorescence after 1 h of incubation; conversely, the increase of the depolarized population was only observed after incubation with clavulanic acid associated with ceftazidime or cefotaxime in ESBL producers (Ramos et al., 2012).

In the present study, we assessed two new methods (a flow cytometric assay and the ESBL NDP test) for detection of ESBLs in clinical isolates of *Klebsiella pneumoniae* in comparison with the standard disc diffusion method.

MATERIALS AND METHODS

Bacterial strains

A total of 60 clinical isolates of *K. pneumoniae* were isolated from patients (24 males and 36 females) with different clinical infections (12 sputum, 26 urine, 12 pus and 10 blood samples) admitted to Suez-Canal University Hospital, Ismailia Governorate from January to August 2014. The samples were collected from various clinical origins. Blood samples were inoculated into blood culture bottles (Egyptian Diagnostic Media, Egypt) then incubated at 37°C for 7-14 days. Subcultures were done every 48 h on blood agar and MacConkey's agar (Oxoid, UK) plates. Other samples were cultured on nutrient agar (Oxoid, UK) blood agar and MacConkey's agar. Gram negative bacilli giving non-lactose fermenting colonies on MacConkey's agar were taken for biochemical tests including mannitol motility, triple sugar iron, indole, citrate, MR, VP and carbohydrate utilization tests for identification (Birgul, 2010). *K. pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 were used as ESBL- positive and negative, respectively (CLSI, 2014). All isolates were kept in soft agar at -20^{0}C till the time for ESBL detection.

Antimicrobial drugs and ESBL phenotypic detection

For the disc diffusion method, antibiotic discs of ceftazidime (CAZ, 30 µg), cefotaxime (CTX, 30 µg), Cefotaxime- clavulanic acid (30/10 µg) (CTC 40 µg) and Ceftazidime- clavulanic acid (30/10 µg) (CZC 40 µg) were purchased from Bioanalyse Chemical Co Ltd, Turkey. Cefotaxime sodium salt, tazobactam (TZB) and clavulonic acid (CLA) were purchased from Sigma-Aldrich, Saint-Quentin-Fallavier, France for the ESBL NDP test. For flow cytometric assay, bis-(1, 3-dibutylbarbituric acid) trimethine oxonol [DiBAC4 (3)], a fluorescent probe that binds to membranes and to intracellular proteins of depolarized cells, was purchased from Invitrogen/Life technologies, Carlsbad, USA; a stock solution (1 mg/ml) was prepared in dimethyl sulphoxide (DMSO).

The disc diffusion method

Stored isolates were subcultured on MacConkey's agar and the

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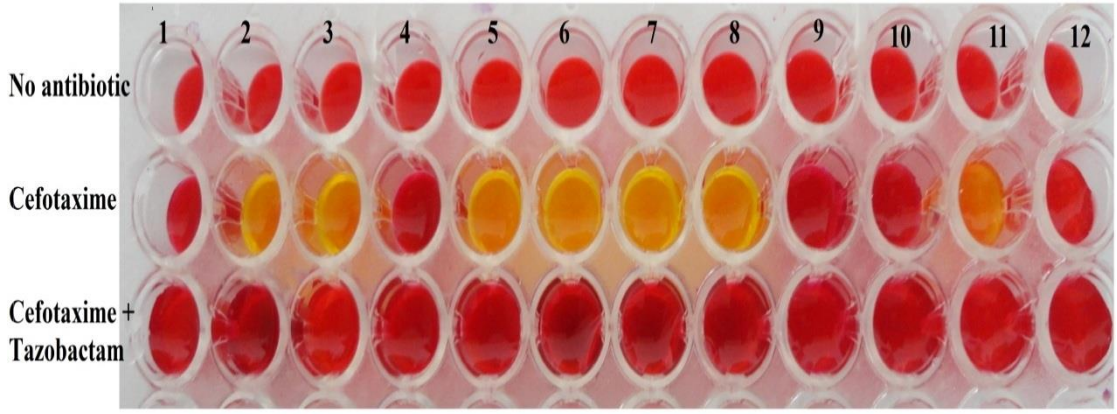

Figure 1. Representative results of the ESBL NDP test. Strains 1 and 2 are negative and positive controls, respectively; strains 3, 5,6,7,8 and 11 are ESBL producers; strains 4,9,10 and 12 are non-ESBL producers.

pure isolated colonies of identified bacteria was adjusted to 0.5 McFarland turbidity standards in 0.85% saline and lawn culture was spread using sterile swabs on Muller Hinton Agar media (Hi-media). All the strains were screened for ESBL production using CTX (30 µg) and CAZ (30 µg). Strains showing zone of inhibition of ≤ 27 mm for CTX and ≤ 22 mm for CAZ were selected for ESBL combined disc conformation test. Combined discs of CTC (40 µg) and CZC (40 µg) were used in the confirmation test according to the CLSI M2-A10 protocol (CLSI, 2009).

The ESBL NDP (Nordmann/ Dortet/ Poirel) test

Strains were isolated on MacConkey's agar and incubated at 37°C for 24 h before performing the NDP rapid ESBL test as described by Nordmann et al. (2012). Briefly, one calibrated loop inoculum (10 µl) of the tested strain was resuspended in 150 µl of 20 mM Tris-HCl lysis buffer in eppendorf tubes containing microbeads. Then, microbead tubes were vortexed for 30 min at room temperature for the mechanical lysis of bacteria. After centrifugation, 30 µl of the supernatant was mixed in a well of a 96-well tray with 100 µl of a 1 ml solution made of 3 mg of purified cefotaxime sodium salt in a pH 7.8 phenol red solution. The pH value was then adjusted to a 7.8 value by the addition of drops from 1 N NaOH solution. Mixture of the phenol red solution and the enzymatic suspension being tested was incubated at 37°C for 30 min. Similarly, culture extracts were analyzed in wells containing cefotaxime and tazobactam (4 mg/ml). A test was considered as positive when the well containing cefotaxime alone turned from red to yellow/orange and the well containing cefotaxime suplemented with tazobactam remained red (ESBL producer).

Flow cytometric analysis

Bacterial isolates from fresh agar plates were inoculated in trypticase soy broth and incubated at 37°C with shaking until the log phase was reached (about 1 h and 15 min). Subsequently, a suspension containing 5 x 10^6 cells/ml in fresh medium was prepared and the bacterial cells were exposed either to 4 mg/L of CTX, or 16 mg/L of CAZ, alone or with 4 mg/L of CLA, for 60 and 120 min. In parallel, after incubation, the cells were centrifuged and washed in PBS. The dye DiBAC4 (3) was added in a concentration of 1 µg/ml for 30 min, at room temperature and protected from light. The flow cytometric assay was used according to Ramos et al. (2012). It was performed on a FACSCalibur flow cytometer (BD, Sparks, USA). Nearly, 10,000–30,000 events of each sample were measured with the Software Cell Quest. The acquisition settings were defined using non-treated, non-stained cells (autofluorescence) and after adjusting the photomultiplier tubes' voltage to the first logarithmic (log) decade. The fluorescence intensity at 530/30 nm (FL1) was registered after incubation with antimicrobials and staining with 1 µg/ml DiBAC4 (3).

Statistical analysis

Sensitivity, specificity, positive and negative predictive values were assessed for the ESBL NDP test and the flow cytometric assay considering the standard disc diffusion method as a gold standard. The kappa values were calculated to evaluate the agreement between each of the ESBL NDP test and the flow cytometric assay and the disc diffusion method (Viera and Garrett, 2005).

RESULTS

The disc diffusion method had classified the 60 tested strains into 46 (76.6%) ESBL producers and 14 (23.3%) non- ESBL producers. Using the disc diffusion method, an ESBL producer isolates showed resistance to CTX and CAZ then the susceptibility increased (≥5 mm increase in zone diameter) to combined discs CTC and CZC while non- ESBL producer isolates were resistant to CTX and CAZ with no increase in the susceptibility to combined discs CTC and CZC.

Using the ESBL NDP test, 80% (n= 48) of the tested isolates produced ESBLs as the color of the wells turned from red to yellow in presence of cefotaxime and remained red when tazobactam was added (Figure 1) and 20% (n=12) tested negative for ESBL production. The sensitivity and specificity of the test were 100 and 85.7%, respectively in comparison with the standard disc diffusion method whereas the positive and negative

Table 1. Results of the disc diffusion method, the ESBL NDP test and the flow cytometric assay for detection of ESBLs in clinical isolates of *Klebsiella pneumoniae*.

Test result	The disc diffusion method	The ESBL NDP test	Flow cytometric assay
ESBL producers	46	48	42
Non- ESBL producers	14	12	18
Total	60	60	60
Sensitivity	100%	100%	91.3%
Specificity	100%	85.7%	77.8%
Positive predictive value		95.8%	91.3%
Negative predictive value		100%	77.8%

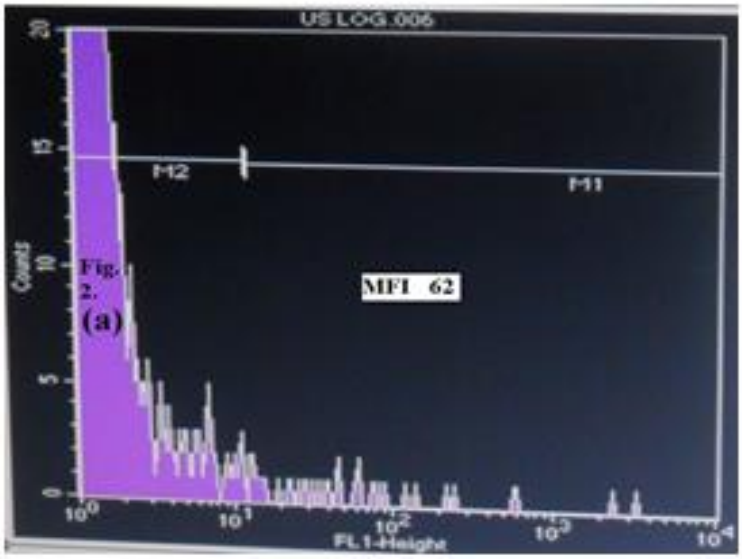

Figure 2a. Flow cytometric histogram representing the emitted fluorescence at FL1 (green- 530 nm) of non-treated and non-stained cells (autofluorescence). The mean fluorescence intensity (MFI) was 62.

predictive values of this test were 95.8 and 100%, respectively. Kappa testing showed an almost perfect agreement between the ESBL NDP test and disc diffusion method in detecting ESBLs (kappa =0.9).

Out of the 60 tested isolates, 42 (70%) and 18 (30%) were ESBL and non- ESBL producers, respectively with the flow cytometric assay method. The sensitivity and specificity of the test were 91.3 and 77.8% whereas the positive and negative predictive values of this test were 91.3 and 77.8%, respectively in comparison with the standard disc diffusion method (Table 1). The intrinsic autofluorescence signal of bacterial cells was detected at the first decade of the logarithmic scale [the mean fluorescence intensity (MFI) was 62]. This corresponds to very low fluorescence intensity without interference with the assessment of membrane depolarization using DIBAC4 (3) as a voltage sensor probe (Figure 2a). Higher intensity of green fluorescence (530/30 nm - FL1) was obtained with dead cells compared with viable cells; consequently, two distinct regions were defined, respectively, for depolarized and polarized cells after staining with DIBAC4 (3). Considering the ESBL-positive clinical isolates, the MFI was 293 after treatment with CTX for 60 min, and then drastically increased to 1541 following simultaneous incubation with both CTX and CLA for 60 min (Figure 2b, c). For non- ESBL producer isolates, the MFI was 72 after treatment with CTX (4 mg/L) for 60 min and remained around value 73 after treatment with both CTX and CLA for 60 min (Figure 3a,b).

For evaluation of agreement between the flow cytometric assay and disc diffusion method, Kappa testing showed substantial agreement between both tests (kappa= 0.7).

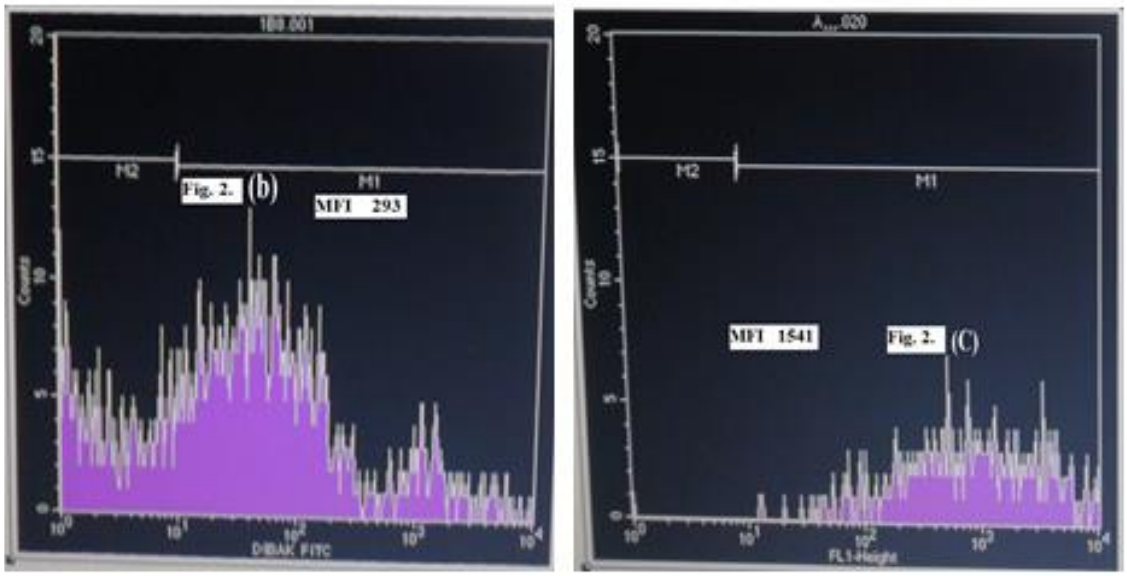

Figure 2b, c. Flow cytometric histogram representing the emitted fluorescence at FL1 (green- 530 nm) of an example of ESBL producer isolate. (B) After treatment with CTX (4 mg/L) for 60 min; the MFI was 293. c) After treatment with CTX (4 mg/L) and CLA (4 mg/L) for 60 min: the MFI was 1541.

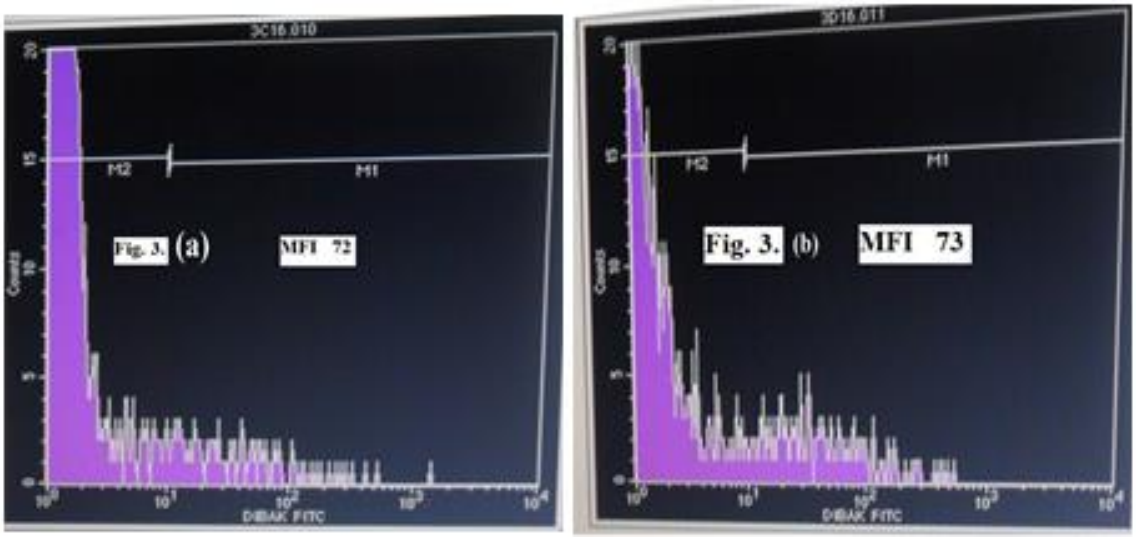

Figure 3a,b. Flow cytometric histogram representing the emitted fluorescence at FL1 (green- 530 nm) of an example of a non-ESBL producer isolate. (a) After treatment with CTX (4 mg/L) for 60 min; the MFI was 72. b) After treatment with CTX (4 mg/L) and CLA (4 mg/L) for 60 min: the MFI was 73.

DISCUSSION

ESBLs are the main cause of resistance to beta-lactam antibiotics which are among the safest and most frequently prescribed antimicrobial agents all over the world. As their occurrence has been increasing, it becomes essential to evaluate their occurrence in *E. coli* and *K. pneumoniae* which are mostly ESBL producers (Pitout and Laupland, 2008; Sahu et al., 2011).

The incidence of ESBL-producing *K. pneumoniae* varies from country to another depending upon various factors, like antibiotic policy, the carriage rate among hospital personnel, and the type of disinfection used especially in the ICU (Sarojamma and Ramakrishna, 2011). It is recognized that Egypt has an extremely high rate of ESBL producers, with up to 70% of isolates

producing the enzyme (Borg et al., 2006). In the present study, 76.6% (n= 46) of the 60 tested strains were ESBL producers and 23.3% (n=14) were non- ESBL producers. This could be attributed to the empirical usage of 3rd generation cephalosporins in treatment of nosocomial infections in our hospitals.

Although molecular methods brought speed and accuracy, they are costy and not suitable for low income developing countries (Gazin et al., 2012). In this work, we assessed two phenotypic methods; the ESBL NDP test and the flow cytometric assay for detection of ESBLs in *K. pneumoniae* clinical isolates in comparison with the standard disc diffusion method. The ESBL NDP test was able to detect all ESBL-producing isolates that hydrolyze cefotaxime (color change from red to yellow in the first well), while the second well that contained tazobactam remained red (inhibition of hydrolysis), thus corresponding to a positive test. The sensitivity and positive predicative value of the test were 100 and 95.8%, respectively. This result was higher than that of Nordmann et al. (2012) who evaluated the ESBL NDP test retrospectively on a collection of 255 strains (from various clinical and geographical origins and previously characterized at the molecular level). In their published study, the sensitivity of the test was 92.6%. Also, our results are higher than those of Dortet et al. (2014) who applied the ESBL NDP test on 500 ESBL producing Enterobacteriaceae recovered from urine samples. They reported that the sensitivity of the ESBL NDP test was 98% and the positive predicative value was 98% which is higher than ours. The discrepancy of the results may be attributed to the different geographical origins and the large number of tested isolates in comparison with our study. Two false positive isolates were detected by the ESBL NDP test as some isolates could contain combined ESBL and AmpC-overproducing enzymes giving a positive result, if the corresponding AmpC hydrolyses cefotaxime at high level.

The specificity and the negative predictive value of the ESBL NDP test in our study were 85.7% and 100%, respectively. These results are lower than those of Nordmann et al. (2012) and Dortet et al. (2014) whereas, it was 100% in the first study and 99.8% in the second one. This could be explained by the inability of the test in detecting non-CTX-M ESBL producers and strains which had MIC values of cefotaxime lower that the resistance breakpoint for that molecule (>8 µg/ml).

Our results show an almost perfect agreement between the ESBL NDP test and disc diffusion method in detecting ESBLs (kappa =0.9) which agrees with those of Dortet et al. (2014) who observed a perfect correlation between cefotaxime resistance and positivity of the ESBL NDP test.

Compared to the standard disc diffusion method, flow cytometric assay yielded a sensitivity of (91.3%) while the specificity was 77.8%. It correctly detected 42 isolates out of the 46 ESBL positive isolates previously catalogued by the standard disc diffusion method. Only 4 strains tested false negative result which might be obtained whenever complex mutant or rare ESBL types are present as isolates expressing these enzymes confer resistance to cephalosporins but are partially inhibited or not inhibited by CLA acid, respectively (Canton et al., 2008; Drawz and Bonomo, 2010).

Our results are in concordance with those of Ramos et al. (2012) who tested 20 ESBL-negative and 41 ESBL-positive isolates phenotypically catalogued by the standard disc diffusion method and molecular typing. In their study flow cytometric analysis correctly detected all the 41 ESBL-positive isolates. It showed an excellent correlation either with phenotypic analysis or molecular typing however, in our study flow cytometric analysis showed substantial agreement with the standard disc diffusion method (kappa= 0.7).

The ESBL NDP test offers a simple and rapid test with an almost perfect agreement with the standard disc diffusion method in detecting ESBLs which could significantly help in guiding first-line antibiotic therapy and improve the outcome of infected patients. Flow cytometric assay is a promising method that might be used in the clinical microbiology laboratory provided that the availability of the device and a trained personnel. Although, the standard method remains the best one because of its low price for the lab and the patient.

Conflict of interests

The author(s) did not declare any conflict of interest.

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Full Length Research Paper

Effects of lithium compounds on the growth of white-rot fungi

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Identifying the most suitable lithium compounds for fungal growth is important for the lithium (Li) enrichment of mushrooms. However, enrichment thus far has been carried out using LiCl. In an effort to identify an alternative source of lithium, we investigated the effect of five lithium compounds on lag phase, growth rate and biomass of ten species of white-rot fungi. The objective of this work was to make a screening of promising lithium compounds for further studies of Li enrichment of mushrooms. The fungal growth was affected depending on the species and lithium form tested. Lithium sulfate and lithium chloride were found to be the most promising candidate compounds for the Li enrichment of mushrooms.

Key words: Li-enriched fungi, mycelial growth, Lithium compounds.

INTRODUCTION

Lithium is not an essential mineral for humans (Schrauzer, 2002), but studies have suggested that its uptake can influence human behavior (Dawson et al., 1970; Schrauzer and Shrestha, 1990; Severus et al., 2009). Indeed, lithium salts are commonly used to treat bipolar disorder, but the side effects caused by ingestion of these salts are numerous and severe (Kjølholt et al., 2003; Aral and Vecchio-Sadus, 2008; Ghaemi, 2010), so much so that their sale in the USA was prohibited in 1949 by the Food and Drug Administration and not resumed until 1970.

The main sources of lithium for human consumption are vegetables and grains (Schrauzer, 2002). Lithium level in mushrooms varies depending lithium availability and ability of fungi to accumulate lithium. In the environmental, this element has been found at average concentration of 0.189 ppm in mushrooms (Vetter, 2005). According to Vetter (2005) due to the low levels of lithium, the mushrooms are not suitable source of lithium for humans. However, mushrooms can be enriched with lithium (de Assunção et al., 2012).

Li-enriched mushrooms have been shown by de Assunção et al. (2012) to be a promising alternative source of lithium due to the higher solubility in water of the lithium found in the mushrooms than lithium carbonate, which is interesting as solubility in water is one of the factors that affect the absorption of compounds in the intestine. Therefore, the lithium found in the mushrooms can be more bioavailable to humans than lithium carbonate. As the control of lithium

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Table 1. Concentration of lithium in culture medium according to fungi specie.

Fungi	Lithium level in Li-medium (mg L^{-1}) [a]
Hericium erinaceum (HE 01)	50
Lyophillum shimeji (LY 01)	50
Lentinula edodes (UFV 73)	50
Ganoderma subamboinense (GR 117)	100
Grifolla frondosa (GF)	100
Pleurotus ostreatus (PLO 06)	150
Pleurotus ostreatus (P.98)	150
Pleurotus eryngii (PE 04)	150
Pholiota nameko (PHOLI)	200
Pleurotus djamor (PLO 13)	270

[a] The above lithium levels were used for all lithium compounds tested and based on a previous experiment (Nunes et al., 2014).

consumed by direct consumption of Li-enriched mushrooms is difficult, development of medicines based on the lithium found in the mushrooms seems to be a good approach to provide new lithium compounds for humans and perhaps, decrease the side-effects currently observed.

Only the mushroom *Pleurotus ostreatus* was shown to be capable of Li enrichment. Nunes et al. (2014) screened 12 white-rot fungi with the goal of identifying other mushrooms suitable for Li-enrichment and found that *Pleurotus djamor* and *Pholiota nameko* were more resistant to LiCl than *P. ostreatus*, making these species promising candidates.

However, both studies use LiCl for enrichment (de Assunção et al., 2012; Nunes et al., 2014). Chlorine is known to have antimicrobial properties (Wilson et al., 2005). Therefore, other lithium compounds may be more suitable for use in Li enrichment. Furthermore, the accessibility and price of different lithium compounds vary between regions. Therefore, knowing which lithium compounds can be used effectively is useful information.

To make a screening of promising lithium compounds for further studies of Li enrichment of mushrooms, we tested the effect of five lithium compounds on development of nine white-rot species that produce commercially available edible mushrooms.

MATERIALS AND METHODS

Microorganism

The fungi tested were all currently available commercial species able to produce mushrooms on non-composted substrate, which is easier and less expensive to use than composted substrates. The fungi used were *Ganoderma subamboinense* var. *laevisporum* Bazzalo and Wright (GR 117), *Grifola frondosa*. (Dicks.) Gray (GF), *Hericium erinaceus* (Bulls.) Pers. (HE), *Lentinula edodes* (Berk.) Pegler (UFV 73), *Lyophyllum shimeji* (Kawam.) Hongo (LY), *Pleurotus eryngii* (DC.) Quél. (PLE 04), *Pleurotus ostreatus* (Jacq.: Fr.) Kummer (PLO 06), *P. ostreatus* (Jacq.: Fr.) Kummer (P.98), *Pleurotus djamor* (Rumph. ex Fr.) Boedijn (PLO 13) and *Pholiota nameko* (T. Itô) Ito and Imai (PHOLI). These fungi belong to the collection of the Laboratório de Associações Micorrízicas / Departamento de Microbiologia / BIOAGRO / UFV. The fungi were grown in Potato Dextrose Agar (PDA, Fluka Analytical, St. Louis, Missouri, USA) at 22 ± 1 ºC for seven days. Two different isolates of *P. ostreatus* that presented different commercially important characteristics were included.

Culture media and cultivation conditions

Fungi were grown on PDA containing one of the following lithium compounds: lithium acetate, lithium chloride, lithium hydroxide, lithium sulfate, or lithium carbonate. The pH of all media was adjusted to 5.5. The concentrations chosen for enrichment of the PDA medium were determined by the lithium content of each compound (Table 1) and the concentration of LiCl that allowed mycelial growth for each fungus in previous experiments (Nunes et al., 2014). It should be noted that lithium concentrations used were different for each fungus, but lithium molar concentrations were equal among different compounds. The culture medium was then autoclaved at 121°C for 20 min. PDA plugs of inoculum 5 mm in diameter containing active mycelium were cut from the board of the colony. Inoculum plugs were firmly placed with the mycelium side down in the centers of Petri dishes. Four replicate plates were prepared for each lithium salt and fungus and were incubated at 22 ± 1ºC with room moisture.

Lag phase and growth rate

After incubation, colonies were observed daily to determine the start of mycelial growth. The fungal growth rate was determined by measuring each colony's diameter in two orthogonal directions. Measurements were made for 45 d or until maximum Petri dish colonization. Measurements were taken every 48 h.

Biomass

To determine the mycelial dry mass, the entire contents of the Petri dish (mycelium + culture media) were placed in a bottle with approximately 200 mL of distilled water and heated in a water bath for 1—5 min to dissolve all culture medium (da Silva et al., 2013). The solution was then filtered, and retained mycelium was dried in an oven at 80 ºC until a constant weight was reached.

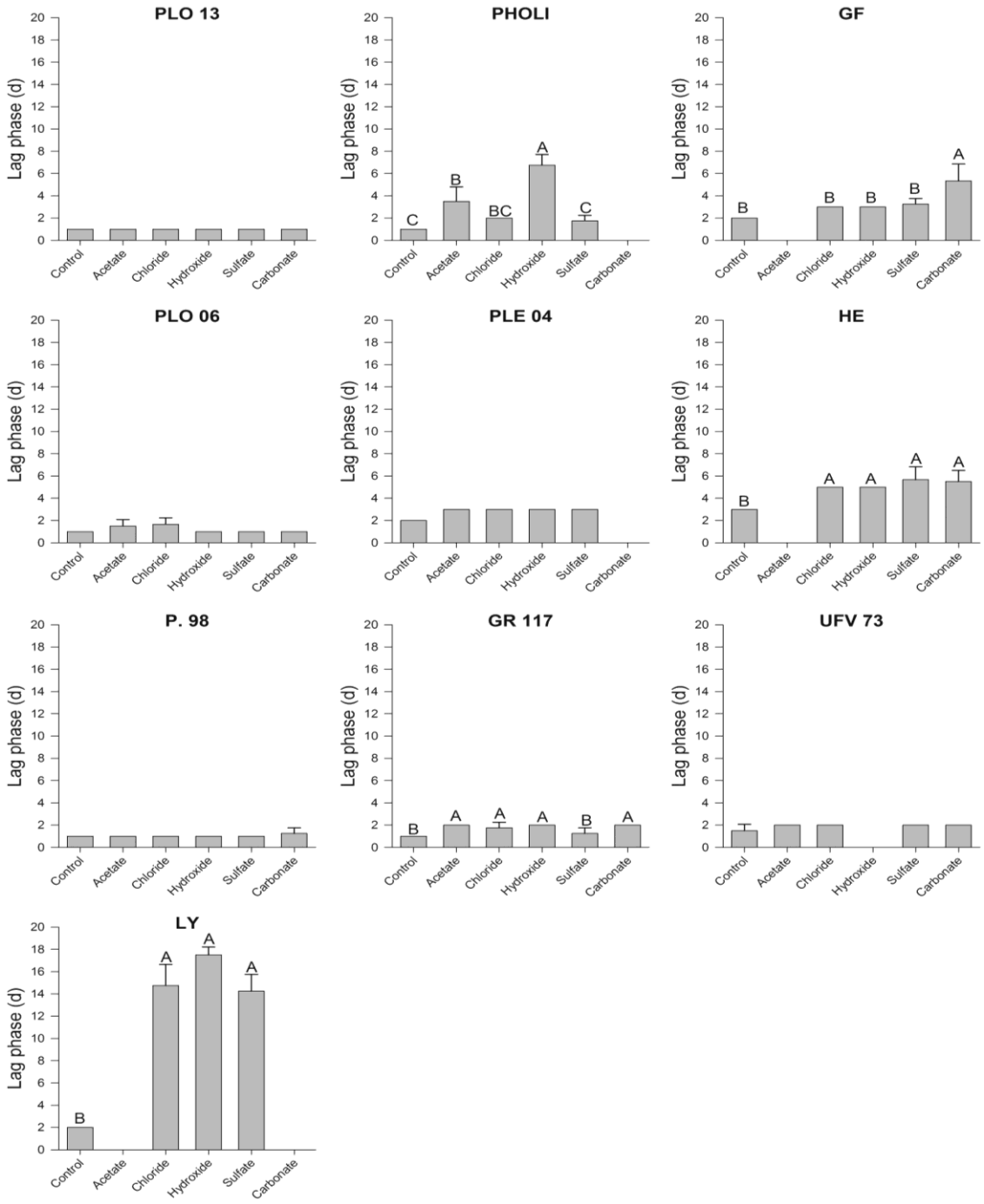

Figure 1. Lag phase of white rot fungi in culture media supplemented with different lithium compounds. Mean growth of 4 replicates. Missing bars indicates fungal growth inhibition. Letters above columns compare individual fungi by lithium compounds level (Tukey´s test; $p \leq 0.05$).

Statistical analysis

Experiments used a randomized design. The data were subjected to analysis of variance (ANOVA), and the averages were compared by Tukey's test ($P < 0.05$) using Minitab statistical software (Version 16.0).

RESULTS AND DISCUSSION

The lag phase of five fungi increased due to the addition of lithium compounds to the culture medium (Figure 1). One-way ANOVA revealed significant effects of lithium

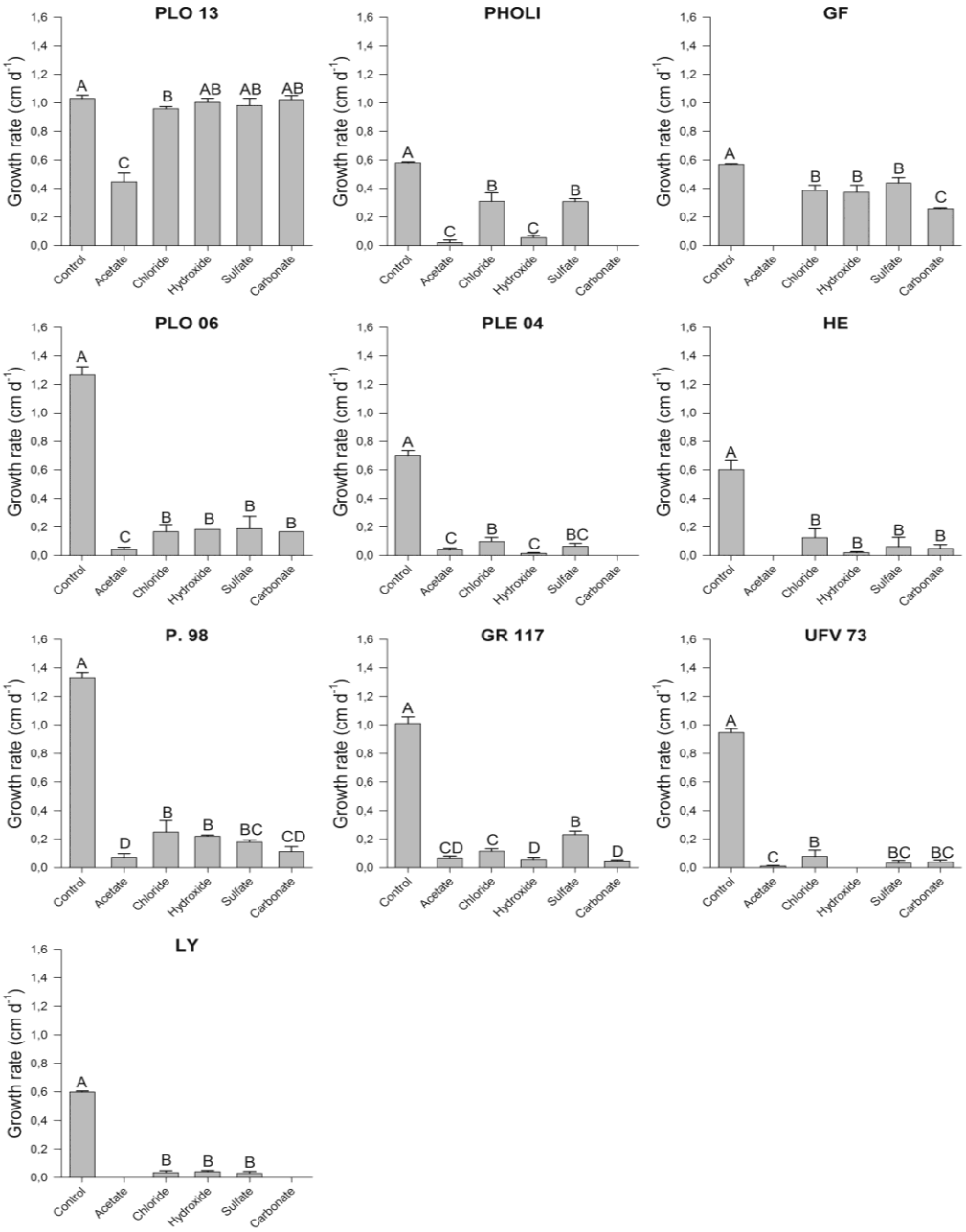

Figure 2. Mycelial growth rate of white rot fungi in culture media supplemented with different lithium compounds. Mean growth of 4 replicates Missing bars indicates fungal growth inhibition. Letters above columns compare individual fungi by lithium compounds level (Tukey´s test; p ≤ 0.05).

compound addition on the lag phase of *H. erinaceus* (HE 01; $F_{(4;15)}$ = 10.80; $p < 0.001$), *G. frondosa*. (GF; $F_{(4;15)}$= 12.76; $p < 0.001$), *G. subamboinense* var. *laevisporum* (GR 117; $F_{(5;18)}$ = 18.68; $p < 0.001$), *P. nameko* (PHOLI; $F_{(4;15)}$ = 32.65, $p < 0.001$) and *L. shimeji* (LY 01; $F_{(3;12)}$ = 93.56; $p < 0.001$), showing a increases in lag phase. *L. shimeji* showed the highest increase in lag phase (Figure 1). Moreover, we observed that different lithium compounds had a similar negative influence on the lag phase (Figure 1), which suggest that the lithium is the main factor influencing this fungal growth phase.

We observed a significant effect ($P < 0.05$) of lithium compounds on the growth rate and biomass of all fungi tested (Figures 2 and 3). Richter et al. (2008) noted that the radial growth rate may not represent fungal biomass reduction. Indeed, biomass evaluation has been shown to

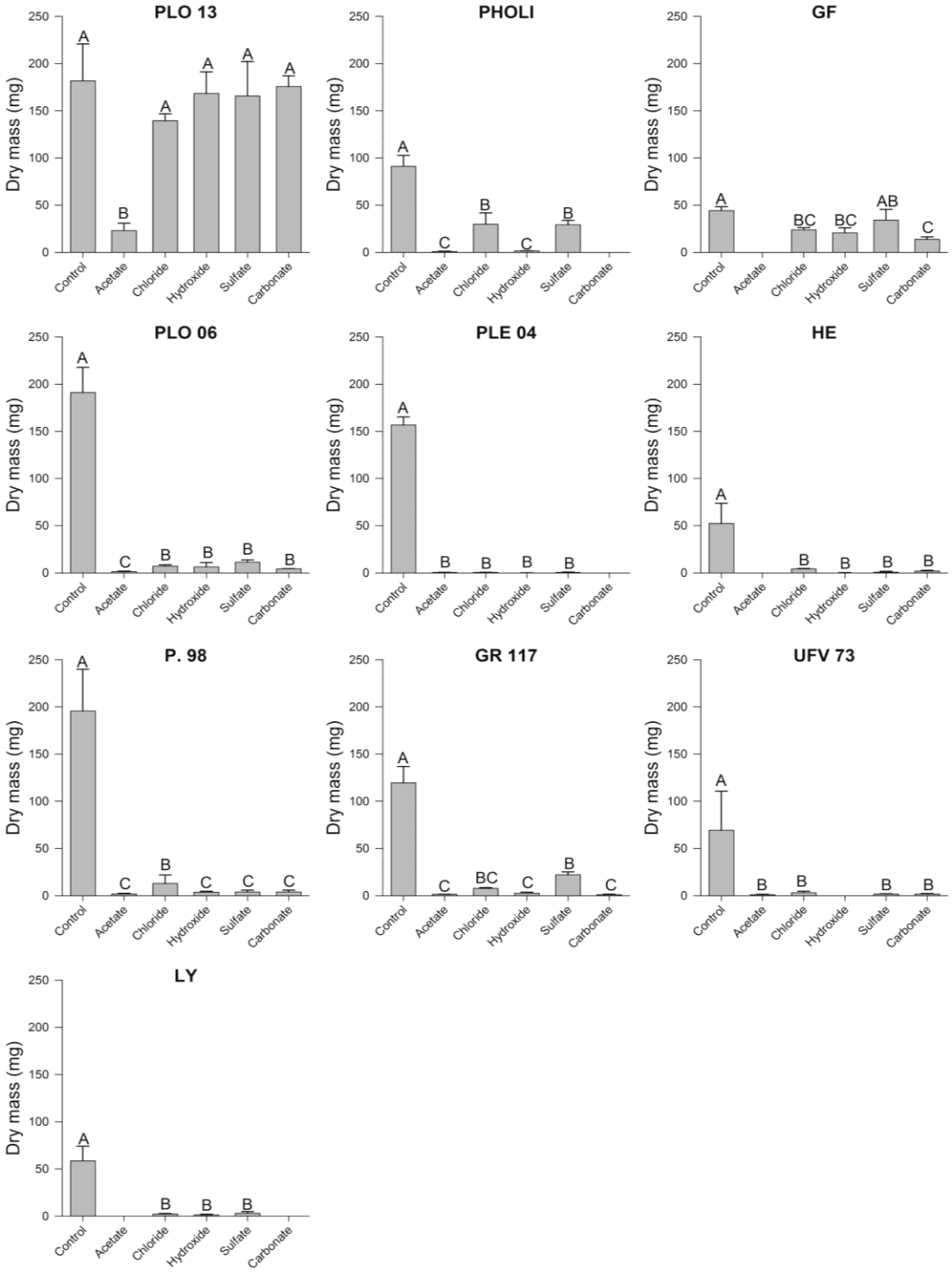

Figure 3. Mycelial dry mass of white rot fungi in culture media supplemented with different lithium compounds. Mean growth of 4 replicates. Missing bars indicates fungal growth inhibition. Letters above columns compare individual fungi by lithium compounds level (Tukey´s test; $p \leq 0.05$).

be a more sensitive method (Nunes et al., 2014). The profiles of biomass and growth rate data observed clearly show that lithium acetate and lithium carbonate were the most toxic compounds (Figures 2 and 3). Acetate is an organic acid that is able to cross the plasmatic membrane and affect cytoplasmic pH, negatively affecting many

metabolic pathways (Cheung et al., 2010). Carbonate has been shown to strongly inhibit Nce103, an enzyme that participates in many physiological processes in eukaryotes (Innocenti et al., 2008). The fungal growth inhibition observed (Figure 2) suggests that these phenomena may be occurring in the fungal cell. Thus, these compounds are not recommended for use in fungal Li enrichment.

The effect of lithium hydroxide on growth rate and biomass varies among the fungal strains tested (Figures 2 and 3). For some fungi, lithium hydroxide results were similar ($P > 0.05$) to those obtained for lithium sulfate and lithium chloride (Figures 1 and 3). In contrast, the addition of lithium hydroxide decreased ($P < 0.05$) the growth rate and biomass of *P. nameko* (PHOLI), *G. subamboinense* var. *laevisporum* (GR 117), *P. eryngii* (PLE 04) and *L. edodes* (UFV 73), clearly showing that this lithium compound was more toxic for these fungi. Furthermore, lithium hydroxide increased ($P < 0.05$) the lag phase of *P. nameko* (PHOLI). In addition, Xu (1997) showed that the activity of laccase, an important factor for white-rot fungus growth, was affected by hydroxide. Furthermore, it is not known how the effects of lithium hydroxide may vary with variation among fungal strains. Therefore, we do not recommend using lithium hydroxide for the Li enrichment of fungi.

The similar profile for lag phase, growth rate and dry mass observed in this study shows that lithium sulfate and lithium chloride were the less toxic compounds to fungi (Figures 1 and 3). As observed by de Assunção et al. (2012), Li is incorporated in the fungus when LiCl is added to the substrate used for mycelial growth. Thus, we can assume that lithium sulfate and chloride are the most promising for the Li enrichment of mushrooms. However, the effect of these lithium compounds on important parameters of mushroom cultivation and bioaccumulation of Li should be investigated.

Conflict of interests

The author(s) did not declare any conflict of interest.

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Full Length Research Paper

Microbiological load of yoghurt sold in Omoku schools, Rivers State, Nigeria

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Sachets of various brands of yoghurt were randomly purchased from different retail outlets within Omoku schools and its pH and microbiological quality were determined using standard method. Total bacterial count (TBC) and Coliform count were done using standard plate count method after making serial dilutions of yoghurt samples. Nutrient agar (NA) was used for enumeration of TBC. NA plates were incubated at 37°C for 48 h. Coliform count was carried out using MacConkey agar (MCA) incubated at 37°C for 48 h. Total fungi were determined by using potato dextrose agar (PDA) and the plates were incubated at room temperature for 5-7 days. The pH of yoghurt samples ranged from 2.38±0.81 to 3.2±0.08, TBC ranged from 3.1 x 10^5 to 5.1x 10^5 cfu/mL. Total fungi count ranged from 3.2 x 10^3 to 4.9 x 10^3 cfu/mL. Total coliform count ranged from 0 to 1.0 cfu/mL. There was no significant difference in TBC and total coliform counts ($p>0.05$), but there was significant differences in total fungi counts ($p<0.05$). Results indicate that yoghurt sold in Omoku schools is of poor microbiological quality and thus their production and sale should be closely monitored in order to protect students and pupils and the general public from food-borne infection.

Key words: Contaminated, total bacteria count, total coliform, total fungi, bacteria, pathogens, yoghurt.

INTRODUCTION

Yoghurt is a sour milk beverage made by blending fermented milk with various ingredients that provide flavour and colour. Although, it is a traditional beverage in the Balkans and Middle East (Ghandge et al., 2008), yoghurt is consumed by all people of all nations. Yoghurt is produced by symbiotic actions of two lactic acid bacteria, namely *Streptococcus thermophilus* and *Lactobacillus bulgaricus* which ferment lactose to lactic acid, which gives it its sour taste (Steinkraus, 1997; Tamine and Robinson, 2004; Kumar and Mishra, 2004; WDC, 2014). Yoghurt can serve as food and plays an important role in human nutrition, health maintaining, therapeutic and dietetic functions (Younus et al., 2002; Khan et al., 2008).

The nutritional quality of yoghurt has been reported and is known to contain high-quality protein, calcium and phosphorous. Its carbohydrate can be utilized easily by those intolerant to lactose (Younus et al., 2002; Alakali et

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al., 2008; Ghandge et al., 2008). It is also believed that yoghurt has valuable therapeutic properties and helps in curing gastrointestinal disorders (Athar, 1986; Wolinsky, 2000; Younus et al., 2002; Vasiljevic and Shah, 2008).

Yoghurt also serves as a medium for the growth of microorganisms due to its high nutritional content hence it is liable to contamination. Moulds and yeast are the primary contaminants in yoghurt. Fungi growing in yoghurt utilize some of the acid, which will invariably reduce the acidity and hence favour the growth of putrefactive bacteria (Oyeleke, 2009) or other pathogenic organisms such as *Staphylococcus aureus* (Ifeanyi et al., 2013; De et al., 2014; Makut et al., 2014). Evaluation of the bacterial quality of yoghurt is necessary due to the high risk associated with consuming sub-standard or unhygienic yoghurt containing pathogenic organisms. Although, there are reports of qualities of yoghurt sold in some parts of Nigeria, no research has been done for yoghurts sold in the oil producing community of Ogba/Egbema/Ndoni local government area (ONELGA). Analyzing yoghurt for its quality is therefore required in order to create awareness among ONELGA people about the existing situation and hence protect the consumers' health from food-borne epidemic.

MATERIALS AND METHODS

Sample collection and preparation

Eight sachets of four different brands of yoghurt samples (names withheld) were purchased randomly from retail outlets within schools in Omoku and transported to the Integrated Science Laboratory of Federal College of Education (Technical) Omoku for analyses.

pH analysis

The pH values of the samples were analyzed using a Jenway 3505 pH meter (Camlab, UK).

Total bacterial count

Total bacterial count was carried out by pour plate technique as described by Kawo et al. (2006). Briefly, one mL of 10^{-3} dilution series of yoghurt samples was plated on Nutrient agar (Oxoid) and incubated aerobically at 37°C for 24-48 h. Colonies that developed were counted and expressed in colony forming units per milliliter (cfu/mL).

Total coliform count

Coliform count was carried out using MacConkey agar (Oxoid). One millimeter of the diluted yoghurt sample was transferred into well labeled Petri-dishes. Fifteen millimeter of the sterile melted agar at 45°C was poured and then swirled to mix with the agar thoroughly. This was allowed to solidify before incubation at 37°C for 48 h. Colonies obtained were counted.

Total fungi count

Total fungi were determined by plating the diluted yoghurt samples in potato dextrose agar (PDA) (Oxoid) to which 20 µg chloramphenicol was added. The plates were incubated at room temperature for 5-7 days.

Table 1. pH of yoghurt

Sample	Mean ±SD*
Y1	3.1 ± 0.08
Y2	3.20± 0.30
Y3	2.94± 0.01
Y4	2.38± 0.81

*SD, Standard deviation.

Table 2. Microbial load of yoghurt in Omoku Schools (cfu/mL).

Sample	Total bacteria	Total coliform	Total fungi
Y1	$3.1 \times 10^5 \pm 0.45$	1.0 ± 0.50	$3.2 \times 10^3 \pm 0.26$
Y2	$4.1 \times 10^5 \pm 0.71$	1.0 ± 0.50	$3.4 \times 10^3 \pm 0.59$
Y3	$4.9 \times 10^5 \pm 0.10$	0.0 ± 0.0	$4.0 \times 10^3 \pm 1.25$
Y4	$5.1 \times 10^5 \pm 1.20$	1.0 ± 0.0	$4.9 \times 10^3 \pm 1.24$

Values are presented as mean and standard deviation.

RESULTS AND DISCUSSION

The pH of yoghurt samples is shown in Table 1 and ranged from 2.38± 0.81 to 3.2± 0.08. The pH recorded is within the range of 2.35±3.18 reported by Makut et al. (2014) but lower than the ranges reported by Ifeanyi et al. (2013) which is from 3.93±4.50 and Digbabul et al. (2014) which is from 4.73±5.11. This low acidity tended to inhibit coliform and favour the growth of acidophilic yoghurt bacteria as well as yeast and moulds hence their presence in the product.

Total bacteria count in yoghurt sold in public schools in Omoku is shown in Table 2 and ranged from 3.1 x 105±0.45 to 5.1 x 105 ±1.20cfu/mL. High bacteria count is expected because of the presence of starter cultures, which are mainly lactic acid bacteria. The count obtained is comparable to the values obtained in yoghurts from Abuja (Okpalugo et al., 2008), Onitsha (Ifeanyi et al., 2013) and Makurdi (Digbabul et al., 2014). The standard count is 106-107cfu/mL (Codex *Alimentarius*, 2003; Rodrigues et al., 2010). Very high count however is used as an indication of post-pasteurization contamination (Tamine and Robinson, 2004).

Total fungi count ranged from $3.2 \times 10^3 \pm 0.26$ to $4.9 \times 10^3 \pm 1.24$ (Table 2). These values are above the limits stipulated (Codex *Alimentarius*, 2003; Tamine and Robinson, 2004). High counts of yeast and mould have also been reported in yoghurts (Okpalugo et al., 2008; Ifeanyi et al., 2013; De et al., 2014; Digbabul et al., 2014). The presence of yeast and mould is attributed to

Table 3. One-way ANOVA statistics.

Parameter	F -Statistic	p value	Remark
Total Bacteria Count	F=4.47	p=0.102	NS
Total Fungi	F=0.50	p=0.518	NS
Total yeast and mould	F=8.72	P=0.042	S
pH	F=2.16	P= 0.172	NS

NS, not significant; S, significant at p=0.05.

poor handling and production (Kawo et al., 2006; Oyeleke, 2009; Amakoromo et al., 2012; Ifeanyi et al., 2013).

Total coliform count ranged from 0.0 to 1.0 x 10^5 ±0.50 cfu/mL (Table 2). The low level is attributed to acidity of yoghurt and/or heat treatment (Jay, 1992). Coliform was reported in some yoghurts produced locally at Kampala (Mukisa and Kyoshabire, 2010) and Keffi (Makut et al., 2014) but not in yoghurt sold at Ibadan (Alli et al., 2010) and Makurdi (Digbabul et al., 2014), although they contained yeast. The presence of coliform bacteria indicates unhygienic practices during handling of the product (Montagana et al., 1998). *Escherichia coli* in particular indicates failure in general manufacturing practices (FAO, 1998; Tamine and Robinson, 2004). Singh and Prakash (2008) also noted that the presence of *E. coli* in a milk product indicates presence of other enteropathogenic microorganisms which constitute a public health hazard.

One-way analysis of variance indicated that there was no significant difference in pH, total bacteria and total coliform counts (p>0.05), but there was significant differences in total fungi counts (p<0.05) (Table 3). Similar results have been obtained for yoghurt samples sold in Onitsha in which no significant difference in coliform but significant difference was observed in total bacteria count (Ifeanyi et al., 2013).

Conclusion

Although no attempt was made to isolate any organisms in this study, related researches have shown that most yoghurt sold in Nigerian markets are contaminated with pathogenic bacteria such as *E. coli*, *Staphylococcus aureus*, *Bacillus* sp. and moulds, such as *Rhizopus* sp., *Aspergillus* sp. etc. The presence of coliform in the yoghurt samples in this study confirms the unhygienic standards under which it is produced as also observed by other researchers. The implication of the findings is that consumption of contaminated yoghurt may contribute to high prevalence of gastroenteritis in the area. The high fungi count is equally disturbing because some moulds have the potential to produce aflatoxins, which are known to cause food intoxication and some type of cancer.

Therefore, sanitary inspection of production premises as well as consumer protection is hereby advocated.

Conflict of interests

The authors did not declare any conflict of interest.

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