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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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Review

Novel human astroviruses (HAstVs) identified recently

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Human astrovirus (HAstV) plays an important role in human viral gastroenteritis, especially in young children and the elderly. In recent years, some novel astrovirus strains different from HAstV were reported previously and new subtypes or lineages of HAstV were found. These new strains might be responsible for the acute gastroenteritis in hospital or community, thus should be paid more attention in HAstV surveillance. This study reviewed the novel HAstVs identified recently.

Key words: Human astrovirus (HAstV), diarrhea, genotype, lineage.

INTRODUCTION

Human astrovirus (HAstVs), first detected in 1975 by electron microscopy (Madeley and Cosgrove, 1975; Guix et al., 2002), constituted the only *genus* in the family *Astroviridae* (Oh and Schreier, 2001). Its importance was determined as the second most common cause of viral gastroenteritis in young children (Ulloa et al., 2005). The complete genome of HAstV contains three open reading frames (ORFs): ORF1a, ORF1b, ORF2, which encoded the viral non-structural protein, the viral RNA-dependent RNA polymerase, and the capsid protein respectively (Moser et al., 2007). Because the structural protein encoded by ORF2 was associated with the genome packaging and virus particle release (van Hemert et al., 2007), ORF2 was thought to be most important region, and the type-specific reverse transcription-polymerase chain reaction (RT-PCR) and sequence analysis were performed mostly in the ORF2 region (Jakab et al., 2003; Liu et al., 2007, 2008; Wang et al., 2011). Based on the sequence analysis of ORF2, HAstV can be divided into eight genotypes (Oh and Schreier, 2001), and the genotype classification is in agreement with serotypes (Noel et al., 1995). Recently, some novel HAstV strains different from known eight genotypes/serotypes (such as AstV-MLB and HMOAstV/AstV-VA) and subtypes/lineages belong the eight genotypes/serotypes were reported.

AstV-MLB and HMOAstV/AstV-VA

AstV-MLB1 was first reported in 2008 when an epidemiologic study was conducted in North America (Finkbeiner et al., 2008, 2009a, 2009b), and AstV-MLB2 was identified from pediatric patients with diarrhea in two continents (Finkbeiner et al., 2009). Based on the complete genome sequence of AstV-MLB1, it showed high diversity from all previously described animal and human astroviruses (Finkbeiner et al., 2008). However, both AstV-MLB1 and AstV-MLB2 was thought to be globally widespread (Finkbeiner et al., 2009c; Banyai et al., 2010) and may not cause diarrhea (Holtz et al., 2011). The clades of these new distinct strains might be deduced by the selective pressure many years ago.

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HMOAstV/AstV-VA was another novel astrovirus species identified from human stool (Kapoor et al., 2009; Burbelo et al., 2011). HMOAstV consisted of three subgroups (Burbelo et al., 2011), HMOAstV-A, HMOAstV-B, and HMOAstV-C, while HMOAstV-C was also named AstV-VA1. Another two novel astroviruses named AstV-VA2/3 were identified, which showed high homology to AstV-VA1 (Finkbeiner et al., 2009c). Both AstV-MLB and HMOAstV/AstV-VA were most closely related to ovine and mink astroviruses (Finkbeiner et al., 2009a; Kapoor et al., 2009), Xiao et al. (2011) also found AstV-VA1/2/3 were detected from bat species. Because of their closest phylogenetic relatives in animals, AstV-MLB and HMOAstV/AstV-VA may suggest the possibility of cross-species transmissions (Kapoor et al., 2009). Interestingly, further studies of human humoral responses showed the novel astrovirus HMOAstV-C was a highly prevalent human infectious agent (Burbelo et al., 2011).

HAstV SUBTYPES/LINEAGES

As suggested by Jakab et al. (2003) and Walter et al. (2001), a strain with an identity of less than 95% and a distance of >0.05 can be considered as a new subtype. Thus, based on the sequence analysis of the complete ORF2 region, a new HAstV-3 subtype was identified from an infant with diarrhea in China (Liu et al., 2008). However, the identification of a new HAstV subtype would be reliable only when the sequence analysis was performed on a long genome, because the evolutionary relationships among HAstV genotypes based on a long genome was found to be different from the results from analyses of short genetic regions (Lukashov and Goudsmit, 2002; Liu et al., 2008).

Nowadays, lineage is more popularly used for the genetic diversity analysis within the HAstV genotypes. A new lineage can be defined when a strain has the sequence diversity of at least 7% (Medina et al., 2000; Guix et al., 2002; Gabbay et al., 2007). Thus, based on the 348-bp sequence analysis in HAstV ORF2, there were six lineages for HAstV-1 (Gabbay et al., 2007; Wang et al., 2011), four lineages for HAstV-2 (De Grazia et al., 2011; Malasao et al., 2012), and two lineages for HAstV-4 (Gabbay et al., 2007), respectively. Importantly, some lineages were responsible for the outbreak of HAstV infection among the children (Li et al., 2010). Although, there were no new lineages reported for other HAstV genotypes, more and more lineages would be found, as well as the recent-reported lineages of HAstV-1 (Gabbay et al., 2007) and HAstV-2 (De Grazia et al., 2011). Interestingly, lineages can be further divided into numerous groups (Gabbay et al., 2007; Wang et al., 2011) and the emergence of new groups may be related to selection and spread of specific HAstV variants for the several years interval (Wang et al., 2011).

Complete genomic sequence analysis indicated that HAstV has high evolutionary rate of $3.7 \times 10(-3)$ nucleotide substitutions per site per year, and $2.8 \times 10(-3)$ nucleotide substitutions per site per year for the synonymous changes (Babkin et al., 2012), which is similar to those of other RNA viruses, but significantly higher than that of rotavirus (Yang et al., 2004), a leading cause of viral gastroenteritis in humans. Thus, based on high genetic evolutionary and cross-species recombinant, more and more novel HAstV strains will be found in the future, which may be responsible for the outbreaks of acute gastroenteritis and become the dominant epidemic strains.

Conflict of Interests

The author has not declared any conflict of interest.

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Full Length Research Paper

Characterization of oregano (*Origanum vulgare*) essential oil and definition of its antimicrobial activity against *Listeria monocytogenes* and *Escherichia coli in vitro* system and on foodstuff surfaces

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First aim of this research was to characterize oregano (*Origanum vulgare*) essential oil and the characterization of its minimum inhibitory concentration against the pathogenic species, *Listeria monocytogenes* and *Escherichia coli*. Moreover, the oregano essential oil antimicrobial activity was tested against these pathogenic species, inoculated onto wood and stainless steel surface. The GC/MS profile of oregano essential oil revealed the presence of 34 compounds, principally terpinolene, carvacrol and p-cymene accounting for about 70% of the total area of the identified molecules. Oregano essential oil showed higher antimicrobial activity against *L. monocytogenes* in comparison with *E. coli*. In fact, the *L. monocytogenes* minimum inhibitory concentration ranged between 125 and 200 mg/L while those for *E. coli* ranged between 250 and 350 mg/L. Regarding the decontamination efficacy, the washing of the two surfaces with oregano fastened the viability decrease of both the inoculated microorganisms over time. This phenomenon was more pronounced for wood as compared to steel. The data obtained suggests the great potential of this essential oil to be employed, as alternative to traditional chemicals, and as sanitizing strategy for surfaces.

Key words: Oregano essential oil, GC/MS, surface decontamination, minimum inhibitory concentration.

INTRODUCTION

The adhesion and persistence of microorganisms in equipment surfaces have the potential to spread pathogens and spoilage microorganisms to foods, influencing their shelf-life and safety (Bae et al., 2012). This is particularly significant in the food processing industry (Giaouris and Nychas, 2006) as well as in the domestic environment (Humphrey et al., 2001; Choi et al., 2012). The surfaces of equipment used for food handling, processing and storage are considered as major sources of microbial contamination (Bae et al.,

2012). Several studies have shown the ability of microorganisms to attach to surfaces commonly found in the food processing environment, such as stainless steel, polystyrene, hydroxyapatite, rubber, glass and wood (Soares et al., 1992; Barnes et al., 1999). Additionally, if certain microorganisms remain on a given surface for a relatively long time, they can continue to replicate and eventually form biofilms (Uhlich et al., 2006). The microbial attachment and the eventual biofilm formation, acting as reservoir of spoilage and pathogenic species, increase significantly the risk for food contamination (Valeriano et al., 2012). In fact, microorganisms can be easily detached from surfaces and/or biofilms and contaminate foods, causing reduced product shelf-life and disease transmission (Shi and Zhu, 2009). Several studies have shown that various foodborne pathogens including *Escherichia coli* and *Listeria monocytogenes* can survive for hours or even days on utensils and equipment surfaces (Humphrey et al., 2001; Wilks et al., 2005, 2006; Martinon et al., 2012). On the other hand, *L. monocytogenes* and *E. coli* are among the most frequently involved bacterial species in foodborne diseases (Scallan et al., 2011; Oliveira et al., 2012). Consequently, controlling the longevity of microorganisms in surfaces is fundamental in reaching food safety standards and improving food quality and shelf-life (Nitschke et al., 2009).

Several chemical detergents and disinfectants are commonly used and their application depends on their efficacy, safety and toxicity, corrosive effects, ease of removal and the subsequent sensory impact on the final products (Møretrø et al., 2009). Many of these chemicals are corrosive to equipment and toxic to humans if over exposure occurs (Lee and Pascall, 2012). In addition, conventional cleaning and disinfection regimes may also contribute to antimicrobial resistance dissemination (Lunden et al., 2003; Minei et al., 2008; Ryu and Beuchat et al., 2005; Surdeau et al., 2006; Cruz and Fletcher, 2012).

Therefore, new sanitizing strategies based on the use of bio-solutions containing enzymes, phages, inter-species competitions, antimicrobials of microbial origin and natural plant molecules are constantly emerging (Simões et al., 2010; Chorianopoulos et al., 2008). The growing negative consumer perception against synthetic chemical compounds favors the research of such natural alternatives (Davidson, 1997). Essential oils (EOs) are volatile, natural, complex compounds characterized by a strong odor and formed by aromatic plants as secondary metabolites. They have been studied for their antimicrobial activity against many microorganisms, including several pathogens (Dorman and Deans, 2000; Delaquis et al., 2002).

The activity of oils from *Lamiaceae* (Tassou et al., 2000; Gunduz et al., 2010) has been investigated in model and real food systems in order to understand the action of single constituents, their cell targets and to balance their intrinsic variability. Moreover, EOs and their bioactive components have been recently studied also for their antibacterial activity on surface adherent microorganisms in order to evaluate their potential as disinfectants in the food industry (Chorianopoulos et al., 2008; Oliveira et al., 2012) and as promising anti-biofilm agents (Amalaradjou and Venkitanarayanan, 2011). *Origanum vulgare* essen-tial oil has been largely studied for this purpose and its composition, in relation to its geographical origin, dry and extraction methods, has been investigated (Mockute et al., 2001; Teixeira et al., 2013; Figiel et al., 2010). In fact, it is well known that the oil composition, and particularly the presence of phenolic content, can increase its antimicrobial properties. Thus, information regarding the oil composition and the effectiveness of its bioactive components in killing pathogenic species on food contact surfaces is needed to aid in the development of optimal sanitation conditions for food industries.

The aims of this study were:(i) to evaluate the efficacy of killing *L. monocytogenes* and *E. coli* in solution, calculating the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of oregano essential oil, reported to have antimicrobial activity against a large variety of microorganisms (Marino et al., 2001; Viuda-Martos et al., 2007) and (ii) to evaluate the oregano EO efficacy in reducing pathogenic cell loads on food contact surfaces such as wood and stainless steel. Most of the food processing industry's surfaces such as machinery, pipelines and working surfaces are made of stainless steel. This material is traditionally selected in the kitchen for food preparation because of its mechanical strength, corrosion resistance and longevity (Carrasco et al., 2012). Wood, although less employed in food industry than in domestic food preparation, is often used as cutting boards (Soares et al., 2012). Different contamination levels and contact times were

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Abbreviation: MIC, Minimum inhibitory concentration; **MBC,** minimum bactericidal concentration; **EO,** essential oil; **SPME,** solid phase micro extraction.

assessed for each tested surface.

MATERIALS AND METHODS

Strains

L. monocytogenes Scott A and *E. coli* 555, used in this work, belong to the strain collection of the Department of Agricultural and Food Sciences, University of Bologna. The strains were maintained at -80°C and cultured in brain heart infusion (BHI) broth (Oxoid, Basingstoke, Humpshire, UK) for 24 h at 37°C. Before experiments, the strains were sub-cultured, on BHI broth for 24 h.

Essential oils

In this work, the oregano (*Origanum vulgare)* essential oil was obtained from Flora s.r.l. (Pisa, Italy).

Characterization of oregano essential oil using GC/MS-solid phase micro extraction (SPME)

Oregano EO in amount of 0.5 mL was placed into a 10 mL vial and sealed through a PTFE/silicon septum. Three different samples were prepared for each EO. The samples were conditioned for 30 min at 25°C. An SPME fiber covered by 50 mm divinylbenzene-carboxen-poly (dimethylsiloxane)- (DVB/CARBOXEN/PDMS StableFlex) (Supelco, Steiheim, Germany) was exposed to each sample at room temperature (25°C) for 20 min, and finally, the adsorbed molecules were desorbed in the GC for 10 min. For peak detection, an Agilent Hewlett-Packard 6890 GC gas-chromatograph equipped with a MS detector 5970 MSD (Hewlett-Packard, Geneva, Switzerland) and a Varian (50 m×320 μm×1.2 μm) fused silica capillary column were used. The temperature program, starting from 50°C, increased to 230°C at 3°C/min, this temperature was maintained for 1 min. Injector, interface, and ion source temperatures were 200, 200 and 230°C, respectively. Injections were performed with a split ratio of 30:1 and helium as carrier gas (1 mL/min). Compounds were identified by the use of the Agilent Hewlett-Packard NIST 98 mass spectral database.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination of oregano EO against *L. monocytogenes* and *E. coli*

For the determination of MIC values, 150 μL of BHI broth inoculated at three different levels (2, 4 or 6 log cfu/mL) of the tested pathogens (*L. monocytogenes* and *E. coli*), were added to 200 μL microtiter wells (Corning Incorporated, NY, USA). Oregano essential oil was properly diluted in ethanol 96% (VWR international, PROLABO, France) and 50 μL of the different dilutions were added in the microtiter wells, in order to obtain oregano EO concentrations ranging between 50 and 400 mg/L. Microtiter plates were incubated at 37°C and checked after 48 h. The MBC were determined by spotting 10 μL of each well after 48 h, onto BHI agar plates. Minimum inhibitory concentration (MIC) was defined as the lowest concentration of the compound preventing visible growth of the inoculated cells after 48 h (MIC48h). The MBC was defined as the lowest concentration of the compound that caused the death of the inoculated cells and therefore there was no growth after 48 h of incubation at 37°C of a 10 μL spot plated onto BHI agar.

Sanitization tests on surfaces

Stainless steel and wood surfaces were used for decontamination experiment with EOs. The sizes of the surfaces were 1 and 2.25 cm^2 for stainless steel and wood, respectively. Before use, the surfaces were sterilized by autoclave at 121°C for 15 min.

The target microorganisms chosen for this experiment were *E. coli* and *L. monocytogenes*. Both target microorganisms were inoculated at a concentration of 6.2 log cfu/cm^2 for wood and 7 log cfu/cm^2 for stainless steel. The inoculum was prepared from the pre-inoculum by making serial dilution in aphysiological solution, and the surfaces were inoculated with 10 (stainless steel) or 100 μL (wood). The inoculated surfaces were dried at room temperature for 0, 15, 30 and 60 min before treatments with Oregano EO. The treatments were performed by the immersion of the surfaces in 20 mL of Oregano EO solutions used at concentration of 125 mg/L for the treatment of the surfaces inoculated with *L. monocytogenes*, and 250 mg/L for the surfaces inoculated with *E. coli*. Oregano EO was delivered through 1% of ethanol. The duration of treatments was 10 min and the surfaces were removed from the solutions and placed into 10 mL of physiological solution, to determine viable bacteria by plate counting. *E. coli* was determined on Violet Red Bile Agar (VRBA, Oxoid, Basingstoke, Hants, England) with addition of MUG (Oxoid) supplement while Listeria Selective Agar based (Oxford formuladion) (Oxoid, Basingstoke, Hants, England) was used to detect *L. monocytogenes*.

Data processing and statistical analysis

The cell load data were analyzed by means of ANOVA one way by using Statistica for Windows.

RESULTS AND DISCUSSION

GC/MS-SPME characterization oregano essential oil

Preliminarily, oregano EO was characterized using GC/MS-SPME. This technique was chosen because it gives a measure of the volatile molecules of the oil and the preliminary condition for the antimicrobial effects of EO is the contact between the antimicrobial molecule and the target cells. The contact is favored if the molecules are in their vapor phase, that corresponds to their most hydrophobic state, because this improves their partition in the cell membranes. In addition, this technique provides a volatile profile fingerprinting fundamental to standardize the EO composition in terms of the most effective molecules and consequently to standardize antimicrobial activity of the essential oils. In fact, the EO composition, and consequently the volatile molecule profile, can notably vary with plant variety and origin, extraction modality, agronomic practices, etc (Nannapaneni et al., 2009). Table 1 shows the total area of the GC peaks and the percentage (on the basis of the relative peak area) of each compound present in the headspace of the oregano

Table 1. GC/MS-SPME characterization of oregano *(O. vulgare)* essential oil.

Molecule	Total peak area	Area (%)
α-Pinene	29616709	3.43
Camphene	3431254	0.40
β-Pinene	1664439	0.19
3-Carene	1693569	0.20
β-Myrcene	22216747	2.57
α-Phellandrene	2456150	0.28
α-Terpinene	33644575	3.89
Limonene	8547408	0.99
β-Thujene	5079423	0.59
γ-Terpinene	72693569	8.41
p-Cymene	309885246	35.86
Terpinolene	3166331	0.37
Ylangene	1739261	0.20
α-Cubebene	7410171	0.86
β-Bourbonene	3635866	0.42
Linalol	322546	0.04
Caryophyllene	49654406	5.75
(+)-Aromadendrene	1836300	0.21
Carvone	122563	0.01
α-Caryophyllene	1293689	0.15
γ -Muurolene	2910065	0.34
α-Terpineol	140525	0.02
Borneol	1637263	0.19
Copaene	311791	0.04
β-Farnesene	1325112	0.15
α-Muurolene	237200	0.03
δ-Cadinene	3114797	0.36
γ-Cadinene	1189108	0.14
Anetol	529966	0.06
Calamenene	525772	0.06
p-Cymen-8-ol	244657	0.03
p-Timol	1092970	0.13
Thymol	41459717	4.80
Carvacrol	249347302	28.85

EO, as well as the cumulative percentages of the classes of compounds (monoterpenes, sesquiterpenes, oxygenated monoterpenes, aliphatic alcohols, aliphatic aldehydes, esters and ketones). The volatile profiles of the used oregano essential oil was characterized by the presence of 34 identified molecules belonging to different chemical classes. The main components of this type of oregano were terpinolene, carvacrol and p-cymene accounting for about 70% of the total area of the identified molecules. These data are in agreement with the data of Ortega-Nieblas et al. (2011), Russo et al. (1997) and Bisht (2009) who found carvacrol as one of the major components. Also, according to Teixeira et al. (2013), who studied the composition of oregano essential oil from Portuguese origin, carvacrol, terpinene and thymol were the main components. This is positive because a wide literature attributed to carvacrol and to monoterpenes the great antibacterial activity of oregano EO (Burt, 2004; Gutierrez et al., 2008; Oussalah et al., 2006). In fact, such molecules can interact with some cellular structures causing the inhibition of cell growth or cell death. However, according to Caccioni et al. (1998), to evaluate the antimicrobial activity of an EO it is fundamental to use a holistic approach due to

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of oregano *(Origanum vulgare)* essential oil against *L. monocytogenes* and *E. coli* in relation to the inoculum level.

Microorganism	Cell concentration (log cfu/mL)					
	6 log cfu/mL		4 log cfu/mL		2 log cfu/mL	
	MIC 24 h (mg/L)	MBC (mg/L)	MIC 24 h (mg/L)	MBC (mg/L)	MIC 24 h (mg/L)	MBC (mg/L)
L. monocytogenes	175	225	175	225	125	150
E. coli	350	350	300	325	250	250

synergistic or antagonistic actions among the different EO components.

MIC and MBC determination

The MICs and the MBCs of the oregano EO against *L. monocytogenes* Scott A and *E. coli*555 were assessed after incubation at 37°C with three levels of the target microorganisms (Table 2). Differences in the MICs and MBCs were observed in relation to species and the inoculum level taken into consideration. In fact, increasing the inoculation level increased the MIC and MCB values for both microorganisms considered. This data are in agreement with literature (Belletti et al., 2010). Oregano EO showed the highest antimicrobial activity against *L. monocytogenes* with respect to *E. coli*. In fact, the *L. monocytogenes* MIC ranged between 125 and 200 mg/L while those for *E. coli* ranged between 250 and 350 mg/L. This behavior for Gram-negative bacteria can be due to the presence of the outer membrane, which acts as an efficient permeability barrier against macromolecules and hydrophobic substances, as well as to the high content in cyclopropane fatty acids of the inner membrane (Chang and Cronan, 1999).

Effects of oregano EO in decontaminating stainless steel and wood surfaces inoculated with *L. monocytogenes* and *E. coli*

To evaluate the decontamination efficacy of oregano EO, stainless steel and wood coupons previously sterilized were inoculated at level of 7 and 6.2 log cfu/cm^2 with *L. monocytogenes* and *E. coli*, respectively. Immediately after the inoculation and after 15, 30, 60 min at room temperature (about 25°C), the coupons were treated with 20 ml of oregano EO treatment solutions at concentration of 125 ppm for *L. monocytogenes*, or 250 ppm for *E. coli*., corresponding to the MIC values previously determined in antimicrobial assay. After 10 min of contact between the coupons and the EO solution, the surfaces were removed from treatment solutions and were placed into 10 ml of physiological solution, which was used for the determination of the surviving *L. monocytogenes* and *E. coli* cells. In Figures 1 and 2, the results obtained for *E. coli* and *L.* monocytogenes, respectively, are shown. A decrease of viability over time was observed independently of microorganisms and oregano EO supplementation. The viability decreases were more pronounced on wood material than in steel coupons, independently of the treatment time and EO supplementation. 60 min after inoculation, *E. coli* and *L. monocytogenes* were present on the control steel coupons (untreated) at cell loads of 6.6 and 5.9 log cfu/cm^2, respectively. Significantly lower counts (3.6 and 4.2 log cfu/cm^2, for *E. coli* and *L. monocytogenes,* respectively) were recorded in the control wood coupons 60 min after inoculation. Earlier research indicate that survival of microorganisms on surfaces is affected by many factors including temperature, microbial species (Rusin et al., 2002), nature of surfaces (Gill and Jones, 2002), time lapsed post-inoculation, moisture level and inoculum size (Monville and Schaffner, 2003).

The addition of oregano EO speed up the viability decrease of both microorganisms. The treatment with EO, at the concentration used, reduced, after 10 min of contact, *E. coli* cell loads of 1.9 and 1.2 log cfu/cm^2 in steel and wood, respectively while *L. monocytogenes,* immediately after the inoculation on steel and wood coupons reduced its counts of about 2 and 1 log cfu/cm^2, respectively.

When the treatment with the EO was performed after 30 and 60 min from the inoculation of the coupons, lower microbial counts were recorded with respect to treatment carried out immediately after the inoculation. This phenomenon was more pronounced in wood in comparison with steel.

This result can be due to the porosity of the wood where the microbial cells might penetrate under the surface of the wood. On the other hand, several authors make remarks on the problem of recovery of microorganisms from porous or damaged surfaces (De Vere and Purchase, 2003). Earlier research indicates the decreased number of microorganisms over time deliberately inoculated on wood surfaces (Carpentier, 1997). For example Abrishami et al. (1994) observed a reduction of 98% 2 h after inoculation of new wood by *E. coli*,

Figure 1. Recovery of *Escherichia coli* cell loads (log cfu/cm^2) inoculated onto stainless steel (washed or not with oregano essential oil) and wood coupons (washed with oregano essential oil or not). The treatment with oregano essential oil was performed for 10 min after that the recovery of the pathogenic strain was performed immediately after treatment (0), after 15, 30, 60 min. For each group considered, different letter represent significant differences (p<0.005).

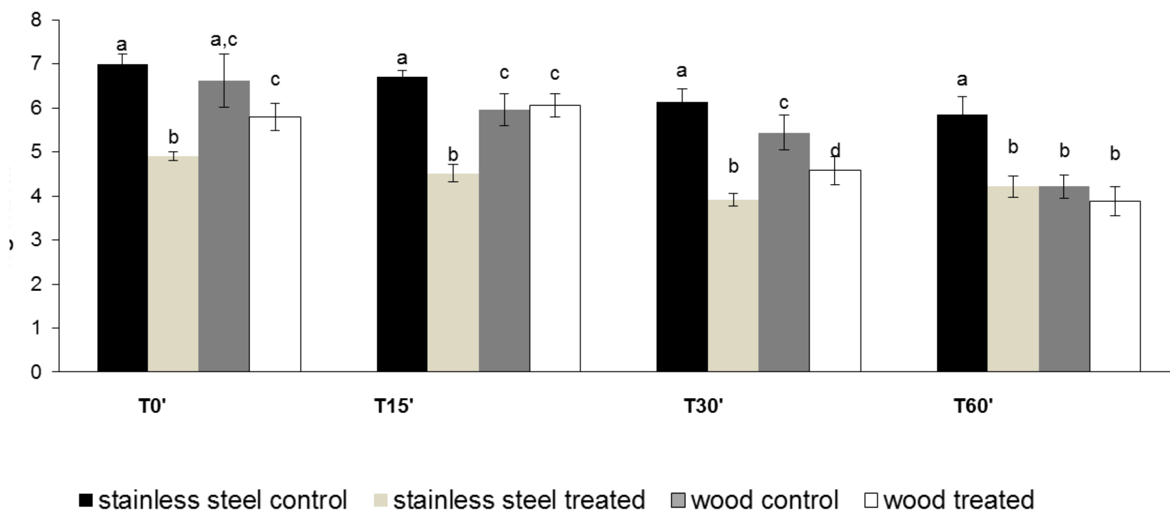

Figure 2. Recovery of *Listeria monocytogenes* cell loads (log cfu/cm^2) inoculated onto stainless steel (washed or not with oregano essential oil) and wood coupons (washed with oregano essential oil or not). The treatment with oregano essential oil was performed for 10 min after that the recovery of the pathogenic strain was performed immediately after treatment (0), after 15, 30, 60 min. For each group considered, different letter represent significant differences (p<0.005).

while Ak et al. (1994) observed a reduction of 99.9% of *L. monocytogenes* after 2 h. Also Milling et al. (2005) showed a consistent viability loss of the inoculated micro-organism on wood surfaces. These authors showed that the survival of the bacteria on wood was dependent on various factors such as the wood species, the type of the inoculated bacterium, the ambient temperature, and humidity and attributed it to the better hygienic perfor-mances of pine and oak with respect to plastic in combination with the hygroscopic properties of wood and the effect of wood extractives. Similar results were observed by Gehrig et al. (2002) and Schonwalder et al.

(2002) who concluded with the possibility that bacteria are transferred into the wood surface by absorption with no evidence of a subsequent release.

Conclusion

This research shows the good potential of the used oregano essential oil to inhibit pathogenic microorganisms both when tested as planctonic cells and when inoculated onto surfaces of industrial interest. In particular, the trials of surface decontamination have highlighted the ability of this type of oregano essential oil to inactivate *L. monocytogenes* and *E. coli* after just 10 min of contact, independently of the surface considered. The reductions obtained, representing more than 90% of the population, are very promising, also taking into account that the inoculation levels tested exceeded significantly those present on industrial surfaces. The American Public Health Association recommends that chemical sanitizers are able to reduce the pathogenic species and mesophilic bacteria of stainless steel surfaces up to 0.3 log cfu/cm^2. The trials we performed inoculating *L. monocytogenes* and *E. coli* at level of 10-100 cfu/cm^2 of surface and treating with oregano essential oil permitted reaching cell loads under the detection limit after 10 min of contact. According to APHA, the sanification level is acceptable when the coliform cell loads are under 5 cfu/cm^2 and acceptable when ranging between 5-100 cfu/cm^2. Moreover, according to Lelieveld e al. (2003), an ideal sanitizer should have characteristics such as wide action spectrum, environmental resistance, toxicity and corrosiveness absence. In our opinion, oregano essential oil could be considered as new tool to prevent or delay colonization of food contact surfaces. However, its use at industrial level still requires additional investigations on the ability of removing it and on its organoleptic impact.

Conflict of Interest

The authors did not declare any conflict of interest.

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Full Length Research Paper

Molecular serotyping of foot and mouth disease outbreaks in Ethiopia

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This study was conducted in five regional states of Ethiopia from January 2011 to March 2012 with the objective of identifying the serotypes of foot and mouth disease by molecular technique in Ethiopia. Epithelial tissue samples were collected from cattle and swine found in the foot and mouth disease outbreak areas of the country and submitted to the National Veterinary Institute, DebreZeit, Ethiopia and World Reference Laboratory for Foot and Mouth Disease, Pirbright, UK. Thus, virus isolation and serotype identification were performed. From a total of 59 samples, cytopathic effect was observed in 43 (72.88%) samples in BHK-21 cell culture. Serotyping of foot and mouth disease viruses were done by applying agarose gel-based RT-PCR at the National Veterinary Institute, and by cell culture ELISA at World Reference Laboratory for Foot and Mouth Disease. Serotype O was recorded throughout the country where outbreaks occurred. Regular investigation of foot and mouth disease outbreaks is important to have more detailed information on the serotypes and topotypes circulating in Ethiopia and for effective vaccine development.

Key words: Ethiopia, foot and mouth disease (FMD), serotype.

INTRODUCTION

Foot and mouth disease (FMD) is a severe, highly contagious viral disease of livestock with significant economic impact. The main effect of the disease is its economic losses resulting from the loss of milk production, retarded growth, loss of draught power, abortion in pregnant animals, and deaths in calves, kids and lambs. In areas of the world where food and draft animals are essential for subsistence agriculture, FMD can affect nutrition. In countries with highly developed animal industry and free trade, outbreaks are responsible for economic devastation (OIE, 2007).

Foot and mouth disease virus (FMDV) was identified by Loeffler and Frosch in 1898 as the first filterable viral agent to cause animal disease. The virus responsible for FMD is a member of the *Aphthovirus* genus in the *Picornaviridae* family (Alexandersen and Mowat, 2005). There are seven immunologically distinct serotypes: O, A, C, South African Territories 1 (SAT-1), SAT-2, SAT-3 and Asia 1 and over 60 strains within these serotypes. New strains occasionally develop spontaneously. Early indications of the disease include fever, excessive salivation and vesicles on the tongue especially in small ruminants

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in which clinical signs are often milder, depending on the strain of the virus. The disease spreads rapidly among non-immunized animals, because of very high morbidity rates, whilst mortality is low except in young animals (Cameron et al., 1999; Geering and Lubroth, 2002; Ryan et al., 2007).

The disease affects cattle, swine, sheep, goats and other cloven hoofed ruminants. Furthermore, elephant and giraffe are susceptible to FMD (Kitching, 2005; Mahy, 2005). Depending on the conditions, FMDV can become aerosol and spread to susceptible animals. FMD is a notifiable disease because the exports of infected livestock and animal products could easily cause outbreaks in countries currently free from FMD. Recently, FMD has been endemic in several parts of the world, particularly in Asia, South Africa, the Middle East, and South America (Mahy, 2005).

FMD is probably the most important livestock disease in Ethiopia in terms of economic impact. Recently, the disease had become the major constraint hampering export of livestock and livestock products to the Middle East and African countries; the Egyptian trade ban of 2005/2006, in which Ethiopia lost more than US$14 million, being a recent reminiscence (Leforban, 2005). Livestock are at risk of endemic strains as well as antigenic variants prevailing in neighbouring countries.

Serotype identification of FMDV in Ethiopia were done mostly by 3ABC ELISA, but the recent detailed knowledge of the molecular characteristics of FMDV major antigenic sites have been helpful to identify serotype, strains and transmission events, to characterize biodiversity and effective quarantine measures against reintroduction (Samuel and Knowles, 2001), and to develop specific diagnostic tests and protective vaccine. Genetic analysis of the viral protein 1 (VP1) region of FMDV has been extensively used to investigate the molecular epidemiology of the disease worldwide. The techniques have assisted in studies of the genetic relationship between different FMDV isolates, geographical distribution of lineages and genotypes, and the establishment of genetically and geographically linked topotypes and tracing the source of virus during outbreaks (Knowles and Samuel, 2003; Sangare et al., 2003)

Molecular techniques to identify FMDV have been studied in details in different countries of the world. In Ethiopia, however, records from the National Animal Health Diagnostic and Investigation Center (NAHDIC) and the National Veterinary Institute (NVI) indicated that serotypes O, A, C, SAT-1 and SAT-2 were responsible for FMD outbreaks during 1974-2008 (Sahle et al., 2004; Gelaye et al., 2005; Legess, 2008; Gelagay, 2009; Haileleul et al., 2010). Continuous research is needed to identify FMDV isolates using molecular methods. Therefore, the objective of this study was to identify the serotypes of FMD viruses causing outbreaks in Ethiopia by a molecular technique.

MATERIALS AND METHODS

General description of study areas

This study was conducted from January 2011 to March 2012 in five national regional states of Ethiopia: Amhara, Oromia, Southern Nation Nationalities and People's (SNNP), Tigray, and Addis Ababa.

The Amhara regional state is located in North-western and North central part of Ethiopia, with an estimated area of 170,752 km^2 (Central Statistical Authority, CSA, 2012). In Addis Ababa, which lies an altitude ranging from 2,000 - 2,800 m.a.s.l., there are about 5,200 dairy farms with some 58,500 cattle, and almost 50% are cross breed (CSA, 2012). In SNNP region, FMD outbreak occurred in Sidama Zone. Tigray regional state is located in Northern Ethiopia. The region has common boundaries with Afar and Amhara regional states at the eastern and southern parts, respectively, and international boundaries with Sudan and Eritrea at the western and northern parts, respectively. It covers 54,548.32 km^2. FMD outbreaks were also investigated in the Oromia regional state, which covers 366,000 km^2, accounting for 31.17% of the total area of Ethiopia.

Study population and sampling method

The study population consisted of cattle and swine that manifested clinical signs of FMD in the outbreaks. Five regions, eight administrative zones, and thirteen areas were included for the occurrence of FMD outbreaks. Sampling was purposive and based on temporal feasibility to investigate. Cattle and swine of all age groups, sex, breeds and different management practices were recorded. Accordingly, a total of 59 epithelial tissue samples were collected.

Study methodology

Clinical examination

Cattle and swine were carefully examined for the presence of characteristic clinical signs of FMD. In each outbreak, animals manifesting vesicular lesions (ruptured vesicles) in oral cavity and on the feet and teats, salivation, lameness and rise in temperature were considered as clinically affected by FMD. Other animals in the herd without these signs were similarly examined, but sampling of epithelial tissue in such instance was done only when lesions were suggestive of FMD.

Sample collection

During the study period, epithelial tissue samples were collected from FMD suspected animals in different areas of Ethiopia (veterinary clinics, institutes, and farms) and submitted to the NVI, Debre Zeit, Ethiopia. Bovine and swine epithelial tissue samples were collected from where outbreaks occurred. Samples were transported from the collection site to the NVI in 0.04 M phosphate buffered saline solution (pH 7.2-7.6) with glycerol and antibiotics at 4°C and stored at -20°C until processed (OIE, 2007). Samples which were tested at the NVI were also submitted to the World Reference Laboratory (WRL) for FMD, Pirbright, UK. A total of 59 epithelial tissue samples were collected from 13 outbreaks during the study periods.

Virus isolation and serotype identification

Virus isolation was established under laminar air flow hood class II

Table 1. Summary of cytopathic effect (CPE) observed on tissue cultures.

Animal species	No. of tested samples	No. of CPE positive samples	Percentage of CPE positive samples
Bovine	54	38	70.37%
Swine	5	5	100%
Total	59	43	72.88%

Table 2. FMDV serotype identified in different outbreaks of Ethiopia.

Site of outbreak	No. of sample	CPE positive	Serotype by		Final result
			Agarose gel-based RT-PCR	Cell culture ELISA	
Alage Dairy Farm (Oromia)*	7	1	O	-	O
Alaba (SNNP)*	3	1	O	-	O
AdamituluJidokombolcha (Oromia)*	1	1	O	-	O
Debre Zeit Swine Farm (Oromia)	5	5	O	O	O
Behylu Dairy Farm (DebreZeit) (Oromia)	2	1	O	O	O
Tigest Dairy Farm (Debre Zeit) (Oromia)	4	3	O	O	O
Malga (Sidam Zone) (SNNP)	7	7	O	O	O
EMDTI (Debre Zeit) (Oromia)	5	5	O	O	O
Adama (Oromia)	4	2	O	O	O
Akaki-Kaliti (Addis Ababa)	2	2	O	O	O
Mekele Universty Farm (Tigray)	8	8	O	O	O
Enderta (Tigray)	3	3	O	O	O
Debre Berehan (Amhara)*	8	4	O	-	O
Total	**59**	**43**			

*Not sent to the World Reference Laboratory for FMD, Pirbright, UK.

on baby hamster kidney-21 (BHK-21) cell layers inoculated with 1 ml of filtered tissue suspension and incubated at 37°C for 1 h for virus adsorption, then flashed with 2% Modified Essential Medium and finally incubated at 37°C and 5% CO_2 in a humidified incubator for 24-48 h. Cytopathic effect (CPE) was observed after 48 h (or even less) in positive cases. If no CPE was detected, the cells were frozen and thawed, used to inoculate fresh cultures and examined for CPE for another 48 h before the samples were declared to be negative (Buxton and Faser, 1977; OIE, 1990; Yoseph et al., 1991). Samples not exhibiting CPE by 72 h post-infection on the second step were considered virus negative. Serotyping of FMDV was made by applying Agarose gel-based RT-PCR at the NVI (Vangrysperre and De Clercq, 1996; Mehran et al., 2006) or/and by cell culture ELISA at the WRL for FMD (Buxton and Faser, 1977). According to Kitching and Donaldson (1987), specimens were submitted to the WRL for FMD using the recommended international standard format of three letter, indicating the country code, isolate number and year of isolation (for example, ETH/02/2012).

RESULTS

Virus isolation

Forty-three (72.88%) out of the total 59 bovine and swine epithelial tissue cultured samples showed FMDV CPE on BHK-21 monolayer cell cultures (Table 1). The CPE was characterized by a fast destruction of monolayer cells, and infected cells were found singly and round shaped. Complete destruction of the cell sheet was mostly seen within 48 h of inoculation. Of the 43 samples that showed CPE, 36 samples were sent to the WRL for FMD for further serotyping analysis.

FMDV serotype identification

Only FMDV serotype O was found both by agarose gel-based RT-PCR at the NVI and by cell culture ELISA at the WRL for FMD on samples collected from outbreaks that showed CPE (Table 2).

DISCUSSION

In this study, FMDV was isolated from most of the samples collected from outbreaks. Forty-three (72.88%) out of the total of 59 epithelial tissue-cultured samples showed FMDV CPE on BHK-21 monolayer cell cultures for FMD virus suspected tissue, while the other 16 tissue cultured samples had no CPE. This might be due to im-

proper transportation from the field to the NVI laboratory since some outbreaks occurred in areas where vehicle is inaccessible and some may be due to death of the virus during transportation.

As previously reported (Gelaye et al., 2005; Ayelet et al., 2009; Haileleul et al., 2010), our results confirmed that serotype O, the most prevalent serotype worldwide (Klein, 2009), was the dominant serotype from bovine and swine samples collected from different district of Ethiopia.

Conclusions and recommendations

FMD is endemic in Ethiopia due to factors such as the presence of high number of susceptible domestic animals, free movement of livestock and livestock products in different regions and states across the country and free cross borders between neighbouring countries. Moreover, lack of control of animal movements and ineffective vaccination measures may contribute to the occurrence of FMD and the difficulty in controlling the outbreaks. Only serotype O was identified during the study period throughout the Ethiopia where outbreaks occurred. Most of the samples collected showed CPE in BHK-21 cell culture. Restriction of animal movement across the regions, importation/movement of livestock and livestock products across the border areas, regular investigation of FMD outbreaks and further phylogenetic analysis should be done to have more detailed information on the serotypes and topotypes circulating in Ethiopia.

Conflict of Interest

The authors have not declared any conflict of interest.

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Full Length Research Paper

Evaluation of Drigalski agar supplemented with ceftazidime (2 mg/L) for selective isolation of extended-spectrum beta-lactamase (ESBL) producing *Enterobacteria*

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The purpose of this study was to evaluate the performance of the Drigalski agar supplemented with 2 mg/L of ceftazidime (DC) for selective isolation of expanded-spectrum beta-lactamase (ESBL) producing *Enterobacteria* in clinical samples. The Drigalski agar supplemented with 2 mg/L of ceftazidime was compared with the chromID™ ESBL medium (bioMérieux, Marcy l'Etoile, France). Six strains from the collection of reference center for antibiotics of the Pasteur Institute of Paris and 247 clinical samples were used to calculate the performance and the intrinsic characteristics of DC medium, respectively. The main ESBL producing *Enterobacteria* consist of *Escherichia coli* (n = 31; 44.3%), *Klebsiella pneumoniae* (n = 19; 27.1%) and *Enterobacter cloacae* (n = 12; 17.1%). ESBL producers were confirmed by synergy testing. The sensitivity (Se), specificity (Sp), positive predictive value (PPV) and negative predictive value (PPN) after 24 h of incubation at 37°C were respectively 98.6, 98.7, 98.6 and 98.7%. The study shows that DC agar is a sensitive and specific medium for selective isolation of ESBL producing *Enterobacteria*, but the character chromogenic agar chromID™ ESBL remains an additional advantage in the identification of strains.

Key words: Expanded-spectrum beta-lactamase (ESBL), Enterobacteriaceae, sensitive culture medium, specific culture medium, predictive culture medium.

INTRODUCTION

Beta-lactams are the main family of antibiotics that include the largest number of molecules and the most used in the world. This widespread use is due to their broad spectrum of activity, low toxicity, effectiveness and affordability for certain molecules (Livermore, 1995; Dosso, 2000). In recent years, many studies have shown

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Table 1. Reference strains.

Number	Strain	ESBL genes or Cephalosporinase genes
U2A 1446*	*Salmonella*sp	Tem1, SHV_{12}
U2A 2252*	*Klebsiellapneumoniae*	Ges 1
U2A 2239*	*Klebsiellapneumoniae*	Tem1
U2A 2240*	*Klebsiellapneumoniae*	DHA1
U2A 1528*	*Escherichia coli*	AAC6'
U2A 1878*	*Proteus mirabilis*	Cit/Fox
ATCC 25922	*Escherichia coli*	-

- : No resistance genes.

the increase around the world, of ESBL infections. The increase in the relative frequency of ESBL producing *Enterobacteria*, very epidemiogenic was observed in both treatment centers (Kim et al., 2002; Pitout et al., 2004) and community (Arpin et al., 2003; Woodford et al., 2004). In addition, the involvement of ESBL strains in both community and nosocomial infections are increasing (Colodner et al., 2004; Brigante et al., 2005; Paterson and Bonomo 2005; Guessennd et al., 2008). Multidrug resistance of ESBL is a step towards the therapeutic impasse due to the accumulation of acquired resistance to several classes of antibiotics. They are sensitive to only a small number of antibiotics (Dosso et al., 2000; Bronzwaer et al., 2002). These resistors are therefore a major public health problem with consequences for both the individual and the economy (Akoua et al., 2004; Guessennd et al., 2008). They are a significant cause of morbidity and mortality worldwide (Beaucaire, 1997). Currently, in Ivory Coast, there are very little data on the overall cost of treatment of infections caused by multi-resistant bacteria. The ESBL plasmid associated with fluoroquinolone resistance were studied in Ivory Coast in 2008 and this study showed a prevalence of 27.2% of *qnr* genes associated with producing extended-spectrum beta-lactamase (Guessennd et al., 2008). The recent emergence and spread of new types of ESBL pose an additional challenge for clinical microbiology laboratories for their detection (Livermore et al., 2005; Ben-Ami et al., 2006). Several phenotypic tests were recommended to search for and confirm ESBL, but these tests are only effective on bacteria isolated after culture (Pfaller and Segreti, 2006). The identification of an infection caused by ESBL promptly in a patient is a critical step in the management of patients to avoid treatment failure and spread of these bacteria, and for the detection of ESBL infections in several culture media existing in the market, such as chromID™ ESBL medium (bioMérieux, Marcy l' Etoile, France). Although, the often too long delivery associated to with high cost, the expiration date often too short and often random conservation conditions are obstacles and difficulties that make these media inaccessible to most southern laboratories for routine research on ESBL. The objective of this study was to validate Drigalski agar supplemented with ceftazidime (2 mg/L) named (DC) to detect within a reasonable time (24 h), the presence of ESBL in clinical samples.

MATERIALS AND METHODS

Collection of clinical samples

Clinical samples (78 urines samples, 24 blood cultures, 22 pus and 26 others) were provided by the Clinical Bacteriology Unit (CBU) of the Institute Pasteur in Ivory Coast. A total of 247 clinical samples from hospitalized and not hospitalized patients (community patients) were used from January 2013 to May 2013 for the study.

Medium to validate

The Drigalski agar (ref 64664 from Bio-Rad ® France) supplemented with 2 mg/L of ceftazidime was assessed as compared to chromID ® ESBL medium (43481 reference BioMérieux, Marcy l'Etoile, France) as a reference. Ceftazidime powder used in the experiment was provided by the manufacturer SIGMA reference C0690500.

Reference strains

Reference strains of ESBL-producing *Enterobacteria* (U2A) with different resistances genes and a strain of none ESBL -producing *Enterobacteria* (ATCC) used for the validation of culture media are from the Collection of the Centre of Reference for antibiotics at the Pasteur Institute in Paris (Table 1).

Reference strains culture

All reference strains (Table 1), lyophilized was revived in Brain Heart Infusion (BHI) for three hours. They were then transferred onto agar Eosin Methylene Blue (BIO-RAD, France) and then on ordinary agar. Susceptibility testing was performed by disk diffusion method on strains in which the 3rd generation cephalosporins, ceftriaxone (CRO), cefepime (FEP), cefotaxime (CTX), ceftazidime (CAZ) and aztreonam (ATM) were placed at a distance of 1.5 cm from center to center around the disk of amoxicillin + clavulanic acid (AMC) (Jarlier et al., 1988). This allowed the identification of the Expanded- Spectrum Beta-Lactamase (ESBL) production by the strain, resulting in a synergy image "champagne cork" (synergy testing).

Preparation of stock solution of ceftazidime

A quantity of 2 mg of ceftazidime was weighed then added to 5 ml sterile distilled water to give an initial concentration (Ci) of 0.4 mg/mL (ceftazidime stock solution). The antibiotic solution was stored in a freezer at -20°C.

Preparation of medium Drigalski + 2 mg/L of ceftazidime

After preparation of Drigalski agar according to the manufacturer's instructions, 20 mL were distributed in flasks. The flasks were autoclaved at 121°C for 15 min and then kept at 45°C in a hot-water bath.

Volume (Vi) of the stock solution of ceftazidime to be taken for each flask containing 20 mL of Drigalski agar was determined according to the following formula 1:

$$CiVi = CfVf \Longrightarrow Cf = \frac{CiVi}{Vf} \quad (1)$$

Ci = initial concentration of the antibiotic solution; Vi: volume of the initial solution to be taken for antibiotic; Cf: final concentration of Drigalski agar; Vf: volume of the final Drigalski agar.

For a Drigalski agar at a concentration of 2 mg/L of ceftazidime, 100 uL of the antibiotic stock solution at concentration of 0.4 mg / mL was incorporated in each flask containing 20 mL of Drigalski agar. After homogenization, the content of each flask was poured into a Petri dish of 90 mm.

Test sterility testing

This test was performed according to standard NF EN 1040. Sterility of media was assessed after incubation at 37°C and after storage in a refrigerator at 4°C. After preparation of each batch of Drigalski agar supplemented with Ceftazidime, two Petri dishes containing the first Petri dish casting, the last Petri dish casting were incubated at 37°C as sterility control batch. The absence of colonies on the agar showed the sterility of the culture media. The results were taken after 24, 48 and 72 h of incubation.

Sterility of Petri dishes stored in a refrigerator at 4°C and the reference medium Petri dishes (CHROMID^{TM} ESBL) was monitored for a month. Every weekend, indicators such as the growth of microorganisms on agar plates (Petri dishes soiled) and the appearance of agar were noted (color, consistency agar, surface dried or not). Rate (T) Petri dishes rejected according to the different indicators was calculated using formula 2. Some characters such as the capacity of Drigalski agar supplemented of Ceftazidime to select ESBL, lactose fermentation and the color of the agar were also recorded.

$$T = \frac{n}{N} \times 100 \quad (2)$$

n: Number of Petri dishes corresponding to the indicator considered after a week; N: total number of Petri dishes stored.

Fertility testing

This test was also done according to Standard NF EN 1040. It was made with extended-spectrum beta-lactamase (ESBL) producing *Enterobacteria* (reference strains) and one reference strain non-producing beta lactamase (ATCC 25922). The comparison was made with a reference medium used for the selective isolation and presumptive identification extended spectrum beta-lactamase *Enterobacteria* producing (chromID^{TM} ESBL ref 43481) Bio-Mérieux and synergy testing.

The enumeration of bacterial colonies on Drigalski supplemented with ceftazidime and the reference medium was made by plating after seeding 100 µL of 10^{-3} and 10^{-4} dilutions bacterial suspension calibrated to 0.5 McFarland using densimat ® of bioMérieux. The Petri dishes were incubated at 37°C for 24 h. The calculation of the number of colonies, expressing in Forming-Colony Unit (FCU/mL) per milliliter was adapted (Anonymous, 2006) and results were expressed as log10.

Calculation of performance Drigalski medium containing ceftazidime

The performance p (%) of Drigalski agar supplemented with ceftazidime on the strains tested, estimated relative to the reference medium chromID ESBL ®, is obtained by the following equation (Denton et al., 1998):

$$P\,(\%) = \frac{\text{Log}\,(FCU\ on\ CHROMID\ ESBL\ medium) - \text{Log}\,(FCU\ on\ Drigalski\ with\ antibiotic)}{\text{Log}\,(FCU\ on\ CHROMID\ ESBL\ medium)} \times 100$$

Any value of p < 0 means that the Drigalski agar supplemented with ceftazidime is more efficient than the reference medium (chromID ® ESBL). Otherwise, the comparison medium is most efficient on the test strains concerned. A standard deviation of 10% is allowed between the two compared media.

Determination of the intrinsic characteristics of Drigalski agar supplemented with ceftazidime

After doing mobility and morphology tests by microscopy of clinical samples, DC agar and chromID^{TM} were seeded simultaneously. The incubation was performed at 37°C for 24 h. The strains were identified using the API 20E of bioMérieux. The production of extended-spectrum beta-lactamase by strains was confirmed by synergy testing.

Knowing the prevalence (p0) of the extended-spectrum betalactamase producing *Enterobacteriae* in clinical samples, the intrinsic characteristics such as sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), Youden index J, the accuracy E (the proportion of correct results) and likelihood ratio (LR) expressing the likelihood of a diagnosis in case of positive test were determined (Adjidé et al., 2009).

Sensitivity (Se): proportion of Drigalski supplemented containing ceftazidime with culture positive (true positives, TP) when the reference strains have growth "chromID ESBL positive".

Se = TP / (TP + FN) and 95% of Se = 1.96 ± √ ln (Se * (1- Se) / n)

Specificity (Sp): proportion of Drigalski supplemented containing ceftazidime with negative culture (true negatives, TN) when the reference strains have no growth "chromID ESBL negative".

Sp = TN / (TN + FP) and 95% Sp = Sp ± 1.96 √ (Sp * (1- Sp) / n)

Positive predictive value (PPV): conditional probability that there are ESBL in the sample when the culture is positive:

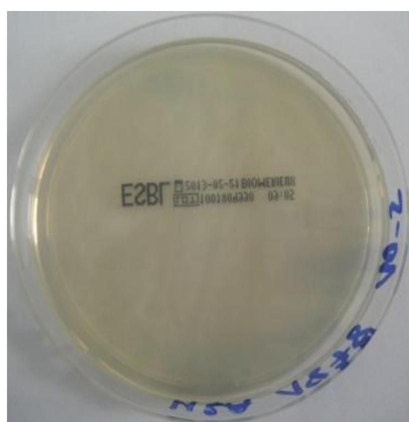
VPP = [TP / (TP + FP)] × 100 = P0 × Se / [p0 × Se + (1- p0) × (1 - Sp)]

Negative predictive value (NPV): conditional probability that there is no ESBL in the sample when the culture is negative.

Table 2. Growth of reference strains after storage for 1 month at +4°C.

Reference strains	Growth	Lactose fermentation	Colonies size
*Salmonella*sp U2A 1446	Good	Lactose $_{-}$	Unchanging
K. pneumoniae U2A 2252	Good	Lactose $_{+}$	Unchanging
K. pneumoniae U2A 2239	Good	Lactose $_{+}$	Unchanging
K pneumoniae U2A 2240	Good	Lactose $_{+}$	Unchanging
E. coli U2A 1528	Good	Lactose $_{+}$	Unchanging
P. mirabilis U2A 1878	Good	Lactose $_{-}$	Unchanging
E. coli ATCC 29922	Complete inhibition	ND	ND

ND: Not determined.



(a)

(b)

Figure 1. Appearance of chromID™ ESBL medium (a) and Drigalski agar (b) before seeding.

VPN = [TN / (TN + FN)] × 100 = 1 - P0 × Sp / [p0×Sp + p0 × (1- Se)]

Youden index J = Se + Sp - 100

The correct E = (TP + TN) / (TP + FP + TN + FN)

Likelihood ratio LR = Se / (1- Sp)

RESULTS

Sterility test

Incubated Petri dishes comprising of the first and last casting Petri dish in each batch of prepared medium (50 Petri dishes) showed no sign of contamination after 24 h, 48 and 72 of incubation at 37°C in an oven.

After four weeks of storage in a refrigerator at 4°C, the appearance of agar remained the same and no growth of microorganisms on the media was observed.

The results of the culture of reference strains on the Petri dishes stored in the refrigerator are shown in Table 2.

The Drigalski agar have a green color when the pH is neutral and a blue color when the pH is in the range of basic pH (Figure 1).

Growth and colony size reference strains were respectively right and unchanging on different culture media after four (4) weeks of storage in a refrigerator at 4°C (Figure 2).

Fertility tests

The main strains ESBL producing *Enterobacteria* (ESBLE) isolated from clinical samples were *Escherichia coli*, *Klebsiella pneumoniae* and *Enterobacter cloacae* (Table 3).

Performance reflects the ability of the medium to selectively and quantitatively promote ESBL culture as compared to chromID™ ESBL medium (Table 4).

Evaluation of Drigalski agar with clinical samples (ability to select ESBLE) was used to estimate the Se, Sp, VPP and VPN as well as the positive likelihood ratios

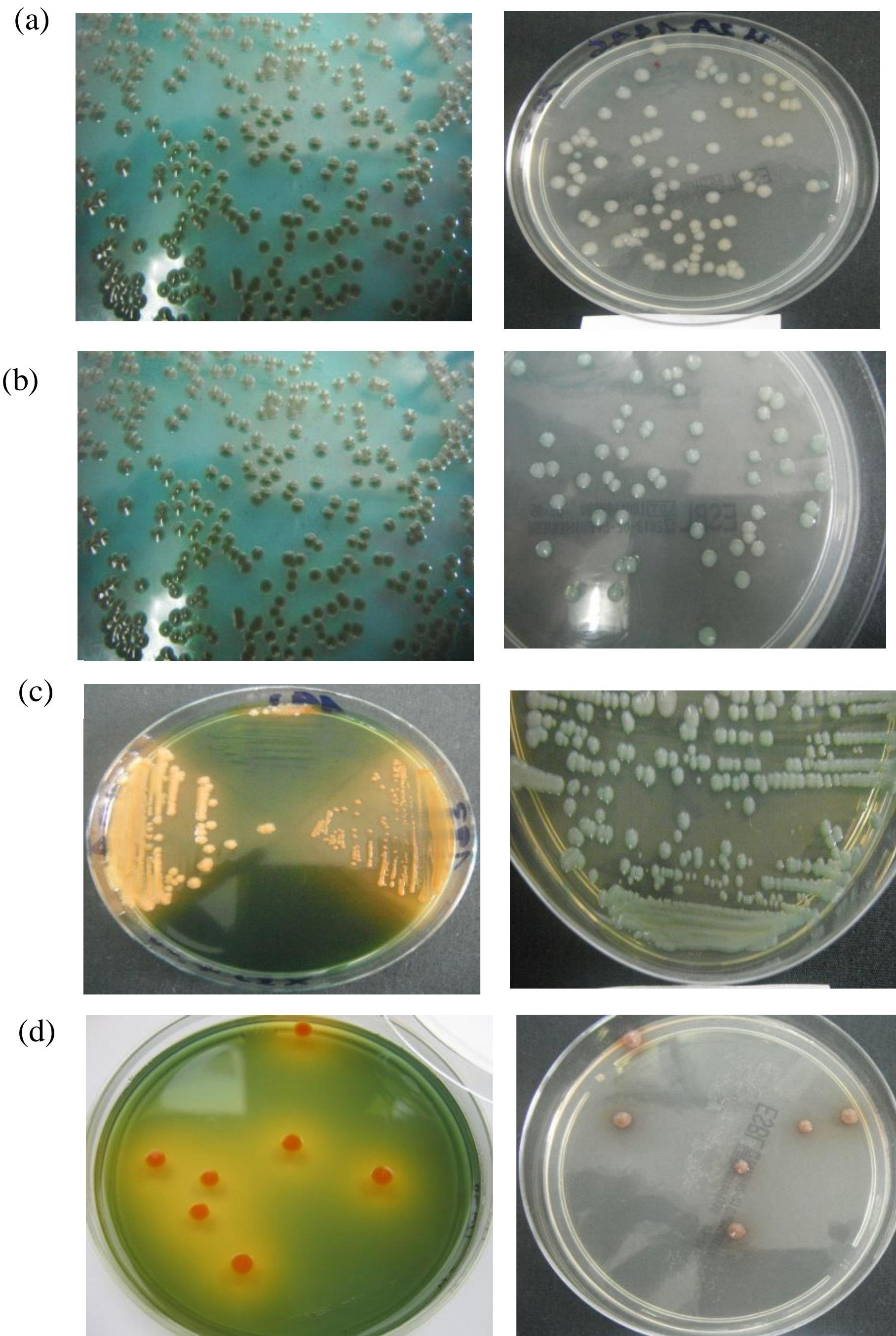


Figure 2. Appearance of reference strains on Drigalski agar supplemented with ceftazidime and chromID^{TM} ESBL before and after four weeks storage in the refrigerator. (a) *Salmonella* spp. U2A 1446; (b) *Proteus mirabilis* U2A 1878; (c) *Klebsiella pneumoniae* U2A 2239; (d) *Escherichiacoli* U2A 1528.

Table 3. Frequency of main strains ESBL producing *Enterobacteria* in clinical samples.

Enterobacteria	**Strain ESBLE/total number of ESBLE**	**Frequency (%)**
Escherichiacoli	31/70	44,3
Klebsiellapneumoniae	19/70	27,1
Enterobactercloacae	12/70	17,1

Table 4. Comparative Drigalski agar supplemented with ceftazidime and chromID™ ESBL medium performance.

Reference strains	Number of viable bacteria obtained on each media Log10 (CFU / mL)		Performance agar Drigalski and chromID™ ESBL
	Drigalski supplemented with Ceftazidime	chromID™ ESBL	
*Salmonella*sp U2A 1446	7.78	7.76	- 0.26
K. pneumoniae U2A 2252	7.71	7.77	0.77
K. pneumoniae U2A 2239	7.45	7. 5	0.67
K pneumoniae U2A 2240	7.35	7.24	-1.52
E. coli U2A 1528	7.76	7.76	0.00
P. mirabilis U2A 1878	7.74	7.70	- 0.51
E. coli ATCC 29922	(-)	(-)	(-)

(-): No growth.

Table 5. Intrinsic characteristics of the medium Drigalski agar supplemented with ceftazidime on clinical samples.

Characteristics of Drigalski + ceftazidime as compared to CHROMID ESBL	Value
Sensibility (Se%) (IC 95%)	98.6
Specificity (Sp%) (IC 95%)	98.7
Predictive positive value (PPV%) (IC 95%)	98.6
Predictive negative value (PNV%) (IC 95%)	98.7
Accuracy E (%)	98.7
Positive likelihood ratio (L)	∞
Negative likelihood ratio (λ)	0
The Youden index (J%)	0.97

(L) and negative (λ) and Youden index (Table 5).

DISCUSSION

Lactose Drigalski Agar (Figure 1a) without antibiotic selective medium is similar to the differential Agar and MacConkey agar based media deoxycholate. It is used as a differential selective medium for Gram-negative bacilli in general (*Enterobacteriaceae* and certain non-fermenters such as *Pseudomonas*) and inhibits Gram-positive bacteria. It is recommended to use it with clinical samples such as urine and other samples that may contain a mixed microbial flora (Dupeyron et al., 1986).

Enteric Gram-negative bacteria are differentiated lactose fermenters (yellow colonies) and non- lactose fermenting (blue colonies) through the combination of lactose and pH indicator (bromothymol blue) (Zajc-Satler et al., 1993). Supplemented with ceftazidime or cefotaxime, two extended-spectrum cephalosporins, lactose agar agar Drigalski can be used to isolate Enterobacteriaceae (*E. coli*, *K. pneumoniae*, *E. cloacae* and *Citrobacter freundii*) producing beta-lactamase extended spectrum in hospitalized patients (De Champs et al., 1993; Komatsu, 2000).

All ESBLE reference strains used had good growth, good fermentation of lactose for lactose-fermenting strains. Colony size remained unchanged after seeding the DC agar kept in the refrigerator at 4°C (temperature controlled) for four weeks (Figure 2). These results reflect the stability of the DC agar when stored at a temperature between +2 and 8°C.

During the study period, the main ESBLE strains isolated from clinical samples (urine, blood culture and pus) were *E. coli* (44.3%), *K. pneumoniae* (27.1%) and *E. cloacae* (17.1%). These results are similar to those of Cady et al. (2006). Hélène et al. (2008) have isolated these bacteria during their work on the isolation of ESBL from rectal swabs, urine and bronchial aspirations on chromID™ ESBL and ESBL Agar Medium (AES) in France.

These same bacteria were also described by Glupczynski et al. (2007) in their work on the comparison of chromID - Bx and Mac Conkey on different clinical samples.

Microbiological performance on reference strains are almost equivalent or even superior for some reference strains to the chromID ESBL agar used for the isolation of ESBL. The Drigalski agar supplemented with ceftazidime showed, from the viewpoint of the rate of contamination of clinical samples with ESBL, 46.7% during the study period. This rate is significantly higher than those observed in the hospital and community infections in many African countries (South Africa 15.9%, Cameroon 12%), Europe (4.7% in Northern Europe and 13.5% southern Europe) and Asia (4.8% in Korea , 12% in Hong Kong) (Pai et al., 1999; Ho et al., 2000; Bouchillon et al., 2004; Gangoué-Piéboji et al., 2005).

The intrinsic characteristics define, for a given medium, is its ability to find the desired item when present and not to believe that the item is present when it is absent. VPP (98.6%), VPN (98.7%), combined with the accuracy (98.7%), the positive likelihood ratio (∞) and the Youden index (0.97) are DC agar sensitive and specific medium, predictive and accurate for the detection of ESBL in clinical samples. DC agar has intrinsic characteristics of Se (98.6%), Sp (98.7%) and predictive values giving its capacities to isolate ESBL identical to those of the reference medium, chromID™ ESBL. The intrinsic characteristics of the DC agar are higher than those observed by Hélène et al. 2008 on chromID ESBL and ESBL agar medium with sensitivities respectively of 88 and 85% after 24 h of incubation. By opposition, some characteristics such as sensitivity are almost identical to those found by Glupczynski et al. (2007) chromID agar-BX (97.7%), but lower than that of MacConkey agar supplemented 2 mg/L of ceftazidime (84.1%). Sánchez-Carrillo et al. (2009) showed values of sensitivity, specificity, positive predictive values, negative predictive value respectively to be 100, 92.6, 85.8 and 100%.

Some *Pseudomonas* strains resistant to ceftazidime were also isolated from DC agar and chromID™ ESBL agar. No strains of Enterobacteriaceae producing a high-level cephalosporinase were isolated during this study. The production of expanded-spectrum beta-lactamase strains isolated on DC agar was confirmed by synergy testing according to the recommendations of the susceptibility committee of the French Society for Microbiology (CASFM, 2012).

Conclusion

This study showed that the Drigalski agar containing 2 mg/L of ceftazidime is a sensitive and specific medium for research of Enterobacteriaceae producing extended-spectrum beta-lactamase. Based on this good sensitivity and specificity, this medium can be introduced in routine laboratories for research on ESBL in organic products in southern countries. The use of DC agar could even be considered in the search for ESBL in various ecosystems such as hospital environment, hospital effluents, sewage and animals in low-income countries. In addition to the sensitivity and specificity, the chromogenic properties of the medium chromID ESBL confer an advantage as compared to our study agar, which guides the user in identifying different strains. In addition, the DC agar can be kept in the refrigerator for at least a month.

Conflict of Interest

The author(s) have not declared any conflict of interest.

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Full Length Research Paper

Occurrence of *Listeria monocytogenes*, total coliforms, *Escherichia coli*, and production and storage processes of raw milk from dairy farms in the state of São Paulo, Brazil

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In the present study, the occurrence of *Listeria monocytogenes*, total coliforms and *Escherichia coli* in refrigerated raw milk from 75 dairy farms distributed in three regions (São Carlos- A, Pirassununga- B, and Piracicaba- C) of São Paulo State, Brazil was assessed. The production and storage conditions as well as milking procedures of raw milk were also evaluated. The analysis of *L. monocytogenes* was performed according to the method established by the Food and Drug Administration (FDA). The Official Method was used for the analysis of coliforms using the SimPlate system. *L. monocytogenes* was not isolated from raw milk samples (n=286), although *Listeria innocua* has been isolated in the milking environment. Total coliforms counts above 10^3 MPN/mL were found in 86% (n = 85), 75% (n = 71) and 72% (n = 66) of samples from regions A, B and C, respectively. *E. coli* was found in 66% of samples in region A, 65% in region B and 46% in region C. Of the 75 farms surveyed, 77.3% showed inadequate conditions for milk production as well as insanitary milking equipment and utensils which certainly compromises the quality of milk and dairy products.

Key words: *Listeria, Escherichia coli,* milk quality, raw milk, microbiological quality.

INTRODUCTION

Sanitary factors at milking and of equipment, as well as the use of skilled workers are essential to produce milk hygienically and with good microbiological quality, which is required for the technology used in milk and milk products (Citadin et al., 2009). In Brazil, the regulations adopted by the Ministry of Agriculture, Livestock and Supply in recent years have compelled milk producers to improve the microbiological quality of raw milk, especially by requiring the cooling of raw milk on the dairy farms. The first regulation, Normative Instruction (IN) 51 (Brazil, 2002), was updated in 2013 by IN62 (Brazil, 2013), and the detailed criteria for the production, identification,

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quality and rating of milk in Brazilian dairy farms were established. According to the IN51 and IN62, the raw milk need to be refrigerated in the farm in bulk tank milk or in jar immersed in chilled water.

To comply with the new regulations, several investments on techniques for dairy production were done in Brazil, which have contributed in the improvement of the microbiological quality of milk and increasing milk production in dairy farms, especially in intermediate and large scale operations. However, nearly 30% of the total milk produced in Brazil comes from small scale farms (up to 400 L/day) (Battaglini et al., 2013; Paixão, 2013). The microbiological quality of milk from these dairy farms is rather variable, mainly because of lower investments as compared to large scales farms, lack of educational programs regarding hygiene procedures during milking and storage, and lack of milk refrigeration during transportation to dairy plants (Fagundes et al., 2011; Paixão, 2013).

Coliforms are good indicators of the sanitary conditions of production and storage of milk. As they are typically found in environments of the milking, inadequate hygiene practices can result in high coliform counts in raw milk (Bramley and McKinnon, 1990). The most used microbiological indicator of fecal contamination is *Escherichia coli* (Roitman et al., 1988), although several pathogenic microorganisms can also contaminate raw milk, especially *Listeria monocytogenes* (Van Kessel et al., 2004). *L. monocytogenes* is an important human pathogen, mainly because of the severity of the disease, listeriosis, which results in high mortality rates. *L. monocytogenes* is usually destroyed by pasteurization of milk, but recontamination can occur along the milk production chain (Waak et al., 2002). In dairy plants, raw milk can be an important source of *L. monocytogenes* contamination. Moreover, factors related to milking hygiene conditions in dairy farms were significantly associated with the contamination of raw milk with *L. monocytogenes* (Sanaa et al., 1993). In Brazil, previous studies have reported the incidence of *L. monocytogenes* in milk and dairy products (Destro et al., 1991; Casarotti et al., 1994; Moura et al., 1993; Silva et al., 2003; Nero, 2005; Arcuri et al., 2006; Barancelli et al., 2014). However, there is little information on the raw milk quality and production characteristics indairy farms in Brazil, especially after regulations IN 51 (Brazil, 2002) and IN 62 (Brazil, 2013) have been enforced. Therefore, the aim of this study was to evaluate the presence of *L. monocytogenes,* total coliforms and *E. coli* in samples of raw milk and their relation to different milking practices in small and intermediate scale dairy farms in São Paulo Brazil.

MATERIALS AND METHODS

The experiment was conducted in dairy farms in the regions of São Carlos (A), Pirassununga (B) and Piracicaba (C) of the northeastern region of São Paulo State, Brazil, between October 2008 and September 2009. Seventy-five dairy farms were analyzed, 25 from each region and the farms were visited four times at intervals of approximately two months. Questionnaires were used to characterize the farms in terms of milking conditions and the raw milk storage system. They were applied on site and were based on current regulations (Brazil, 1997, 2002) and Spexoto (2003).

A total of 286 samples (500 mL) of refrigerated raw milk were collected from region A (N = 99), region B (N = 95) and region C (N = 92), directly from the tanks or drums, after homogenization with the aid of sterile ladles and placed in sterilized jars. Nine farms (three per region) were selected for milk collection with a Moore's strand (Lacen, 2000), which remained in the tank of raw milk for about 12 h before the procedure, performed in sterile packaging in order to increase the chance of isolation of *L. monocytogenes.* Before collection and after homogenization, the milk temperature was measured with a digital thermometer. From three selected farms (one per region), samples from the milking environment were collected including drains (N = 6), floor of the milking area (N = 3), liners (N = 10), floor of the cooling room (N=5), udders and teats surface of lactating cows (N = 18), silage (N = 2) and surface milk in the storage tank (N = 1). For the collection of environmental samples, sponges (Inlab) moistened in saline (0.85%) and peptone (0.1%) were used, added with neutralizing sanitizers: 0.01% sodium thiosulfate (Silva et al., 2003), 0.5% polysorbate (Tween 80) and 0.07% soybean lecithin (Evancho et al., 2002). After collection, the sponges were placed in bags with 60 ml of Listeria Enrichment Broth Buffered (BLEB) (Difco). The samples were transported in coolers with ice to the Laboratory of Hygiene and Dairy College of Agriculture "Luiz de Queiroz" (ESALQ) where they were analyzed.

Samples of raw milk and the Moore's strands and environmental samples (collected with sponges) were analyzed according to the methodology recommended by the Food and Drug Administration (Hitchins, 2003). For the isolation of *L. monocytogenes* in raw milk and the milking environment, 50 ml of milk was inoculated into 450 ml of BLEB. Swabs and the Moore's strands were inoculated with 225 mL BLEB. The sponges were homogenized in a Stomacher strands before incubation. The samples were incubated at 30°C/48 h. After 4 h from the start of incubation, acriflavine (10 mg/L) and nalidixic acid (40 mg/L) and cycloheximide (50 mg/L)were added. After incubation, the striation in the Oxford agar (Oxoid) and Listeria agar was carried out according to Ottaviani and Augustine(ALOA - AES Chemunex), which were incubated at 35°C/24-48 h and 37°C/24-48 h, respectively. Three characteristic colonies from each medium were purified on Trypticase Soy Agar (TSA) (Oxoid) with 0.6% yeast extract. For the biochemical confirmation of the suspect colonies, the analyses of catalase, Gram stain and characteristic motility at 25°C were performed, and the ApiListeria Kit (BioMérieux) was used to characterize the species. The strain of *L. mocytogenes* ATCC 7644 was used as a positive control. For the enumeration of total coliforms and *E.coli*, successive dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) of the milk in saline solution (0.85%) and peptone (0.1%) were prepared. The analyzes were performed on SimPlate CEC (BioControl Systems, Inc.) according to the Official Method 2005.03 (AOAC, 2005). Aliquots of raw milk and its dilutions were used for the analyses with the hatching plates of 32°C/24 h. The wells with a purple color were considered positive for total coliforms and those that were fluorescent under ultraviolet light (366 nm), positive for *E. coli.* The most probable number (MPN) was determined in the appropriate table and the result expressed as MPN/mL of milk. The statistical analysis was done by comparing the counts of coliforms and *E. coli* with the selected questionnaire items using multiple comparisons of means (Tukey test), adjusted for the level of significance ($p< 0.05$).

RESULTS AND DISCUSSION

Table 1 shows the daily milk production, type of milking

Table 1. Characteristics of milk production in farms in São Carlos (A), Pirassununga (B) and Piracicaba (C) regions, Brazil.

Characteristic	A n (%)	B n (%)	C n (%)
Daily milk production			
Up to 100 L	11(44)	1 (4)	10 (40)
>100-500 L	11(44)	18 (72)	14 (56)
>500-1000 L	0 (0)	4 (16)	1 (4)
>1000-3000 L	2 (8)	2 (8)	0 (0)
>3000-5000 L	1 (4)	0 (0)	0 (0)
Type of milking			
Mechanical - canalized	1 (40)	5 (20)	0 (0)
Mechanical - bucket at foot	12 (48)	12 (48)	15 (60)
Manual	12 (48)	8 (32)	10 (40)
Refrigeration system			
Bulk tank milk	11 (44)	22 (88)	22 (88)
Jar of milk immersed in chilled water	14 (56)	3 (12)	3 (12)

n: Number of farms (total number of farms studied: 25 in each region).

and refrigeration system of the 75 farms studied. The three regions had a predominance of small producers, with 86.6% (N = 65) producing up to 500 L/day. This characteristic of small-scale production is also typical in other regions in Brazil (Nero et al., 2005; Monteiro et al., 2007; Brito et al., 2004). In 45 farms (60%), the milk was obtained by mechanical milking devices, while 30 farms (40%) had manual milking. Regarding the refrigeration system, 55 farms (73%) used bulk milk tanks, and 25 farms (33%) used milk jars immersed in chilled water for cooling the raw milk. In a study conducted in Paranapanema, also in São Paulo state, Furlaneto et al. (2008) found a higher percentage of dairy farms with manual milking (77%). This practice is also widely used in other states in Brazil, such as Rio Grande do Sul, where Moraes et al. (2005) found 50% of 41 farms using manual milking, and in the Northeastern states, where 88% of 41 farms use manual milking, and only 24.4% used community bulk tanks (Monteiro et al., 2007). The community bulk tanks are used by a group of small producers, from different farms, to cool the raw milk in a unique place. This has been a valuable strategy adopted in Brazil to reduce costs, aiming to improve the price of milk for producers with increasing scale (Pereira and Magalhães, 2012).

Table 2 presents the milking practices in dairy farms from the three regions, indicating that most producers did not comply with basic and critical points to avoid the risk of milk contamination, such as washing and drying procedures of teats, and use of pre-and post-dipping. The appropriate handling of milking procedures is one of the most important strategies to ensure good quality of raw milk (Fonseca and Santos, 2000). Our results are in agreement with data reported in previous studies showing unsatisfactory production conditions in dairy farms in various regions of Brazil (Monteiro et al., 2007; Silva et al., 2008; Zegarra et al., 2007; Arcuri et al., 2006). In the present study, high temperatures of raw milk stored in the farms were reported, mainly in region B, with temperatures between 2-14°C, and even in cooling tanks, the temperatures reached 14°C. These data are consistent with the temperatures up to 14°C reported by Tebaldi et al. (2008) in cooling tanks in Minas Gerais State, Brazil.

L. monocytogenes was not isolated from the raw milk samples analyzed (n = 286). Importantly, in the current study, a higher number of samples of raw milk was analyzed, hence confirming the low prevalence or absence of *L. monocytogenes* in raw milk collected in dairy farms in Brazil, as observed previously by Casarotti et al. (1994) (n = 20); Nero (2005) (n = 240); Arcuri et al (2006) (n = 42) and Barancelli et al. (2014) (n = 16). In contrast, Moura et al. (1993) isolated *L. monocytogenes* from 9.5% of raw milk samples (n = 220) from São Paulo State. Moreover, highest prevalence rates were reported in raw milk collected from processing plants in Brazilian Northern states. Catão and Cebalos (2001) obtained 37.8% (n = 45) of positive samples, and Silva et al. (2003) found the pathogen in 16.7% (n = 6) of samples. Differences in the occurrence of the pathogen can be explained by the geographical distribution of the genus *Listeria* (Van Kessel et al., 2004). Furthermore, the detection of *L. monocytogenes* in raw milk can be difficult because of low numbers of bacteria and bacterial

Table 2. Milking practices (presence/absence) in farms in São Carlos (A), Pirassununga (B) and Piracicaba (C) regions, Brazil.

Milking practice	A		B		C	
	Presence n (%)	Absence n (%)	Presence n (%)	Absence n (%)	Presence n (%)	Absence n (%)
Use of proper uniform	2 (8)	23(88)	4 (16)	21(84)	1(4)	24(96)
Any washing of the teats	25(100)	0 (0)	24(96)	1(4)	23(92)	2 (8)
Use of pre-dipping	7 (26)	18(72)	12(48)	13(52)	14(56)	11(44)
Any drying of the teats	24 (96)	1 (4)	20(80)	5(20)	22(88)	3(12)
Use of paper towel	4 (16)	21(84)	9(36)	16(64)	9(36)	16(64)
Use of post-dipping	5 (20)	20(80)	9 (36)	16(64)	16(64)	9(36)
Use of gloves for milking	3 (12)	22(88)	3(12)	22(88)	0(0)	25(100)
Paved floor in corral waiting area	15 (60)	10(40)	13(52)	12(48)	21(84)	4 (16)
Cleaning of corral waiting area	5 (20)	20(80)	4(16)	21(84)	1(4)	24(96)
Flies in the waiting area	21 (84)	4 (16)	22(88)	3(12)	24(96)	1(4)
Paved floor in milking parlor	17 (68)	8 (32)	17(68)	8(32)	23(92)	2(8)
Cleaning of waiting room	5 (20)	20(80)	4(16)	21(84)	1(4)	24(96)
Washing of equipment/ utensils in hot water	7 (26)	18(72)	6(24)	19(76)	3(12)	2(88)
Use of sanitizing rinse in equipment/utensils	7 (26)	18(72)	5(20)	20(20)	13(52)	12(48)

n: Number of farms (total number of farms studied: 25 in each region).

Table 3. Distribution of total coliforms and *Escherichia coli* in samples of raw milk in São Carlos (A), Pirassununga (B) and Piracicaba (C) regions, Brazil.

Range (MPN/mL)	A n (%)	B n (%)	C n (%)
Total coliforms			
$<10^2$	3 (3)	8 (9)	7 (7)
10^2-10^3	11 (11)	17 (18)	19 (19)
$>10^3$-10^4	13 (13)	15 (16)	19 (19)
$>10^4$-10^5	17 (17)	23 (24)	21 (21)
$>10^5$	55 (56)	33 (34)	26 (26)
Escherichia coli			
<1	35(34)	33 (3)	48 (52)
>1-10	17(17)	17 (18)	17 (18)
>10-10^2	22 (24)	27 (28)	17 (18)
$>10^2$-10^3	9 (9)	5 (5)	4 (4)
$>10^3$	16 (16)	13 (14)	6 (6)

n: Number of samples. Total number of samples analyzed in each region: 99 (A), 95 (B) and 92 (C).

microflora competition (Meyer-Broseta et al., 2003). The uneven distribution of bacteria in large volumes, as in raw milk tanks, can also hinder their isolation. Thus, the absence of *L. monocytogenes* in samples does not mean that the pathogen was not present in the batches of raw milk analyzed. Regarding the milking environment, *L. innocua* was isolated in two points: the floor drain and a farm milking room in region C, representing 4.4% of the environmental samples.

The total coliforms and *E. coli* counts in samples collected in the three regions studied are shown in Table 3. In region A, 72 milk samples (73%) presented $>10^4$ MPN/mL of coliforms, hence indicating poor hygienic conditions of raw milk, considering that coliform bacteria are not part of the native micro flora of milk (Roitman et al., 1988). High coliforms counts ($>10^4$MPN/mL) were also found in 56 (68%) and 47 (47%) raw milk samples from regions B and C, respectively. *E. coli* was found in 64 (65%), 62 (65%) and 44 (46%) samples from regions A, B and C, respectively, indicating risk to human health, fecal contamination and possible presence of intestinal pathogens. In the United States, Van Kessel et al. (2004) found a higher percentage of raw milk samples contaminated with fecal coliforms (93% of 859 samples), and approximately 40% of 419 samples with populations between 10-10^2colony forming units/mL (CFU/mL). In the present study, *E. coli* counts higher than 10^2 MPN/mL were found in 25 (25%), 18 (19%) and 10 (10%) samples from regions A, B and C, respectively. Also, a significant difference ($p< 0.05$) between coliform counts in raw milk from the same farm in different sampling times was observed, indicating that there is no standardization or consistency in milking practices. The total coliforms counts in raw milk were not different ($p>0.05$) in farms with mechanical or manual milking and teat washing, which is similar to the results described by Moraes et al. (2005) and Gottardi et al. (2008). However, the average counts of total coliforms was significantly lower ($p<0.05$) for the farms that performed procedures for pre and post-dipping, had paved floor in the milking parlor, and had milk tank equipped with a cooling system.The coliform

Table 4. Total coliforms and *Escherichia coli* in samples of raw milk, according to the daily milk production of dairy farms in São Carlos, Pirassununga and Piracicaba regions, Brazil.

Milk production (L/day)	N	Total coliforms[1] (MPN/mL)	*Escherichia coli*[1] (MPN/mL)
<100	64	$3.9 \times 10^4 \pm 0.64 \times 10^4$	$1.3 \times 10 \pm 0.35 \times 10$
500-1,000	6	$2.2 \times 10^5 \pm 1.95 \times 10^5$	$7.2 \times 10^2 \pm 2.3 \times 10^2$
1,000-3,000	4	$1.5 \times 10^4 \pm 0.83 \times 10^4$	$6.9 \times 10 \pm 0.1 \times 10$
3,000-5,000	1	$3.9 \times 10^3 \pm 0.1 \times 10^3$	$1.2 \times 10^2 \pm 0.1 \times 10^2$

[1]Results are expressed as mean ± standard deviation; N: Number of farms (total number of farms studied: 25).

Table 5. Total coliforms and *Escherichia coli* in samples of raw milk, according to the overall hygienic category of dairy farms from São Carlos, Pirassununga and Piracicaba regions, Brazil.

Hygienic category	N	Total coliforms[1] (MPN/mL)	*Escherichia coli* (MPN/mL)
1	2	$5.6 \times 10^3 \pm 0.2 \times 10^3$	$1.7 \times 10^2 \pm 0.63 \times 10$
2	15	$1.1 \times 10^5 \pm 1.1 \times 10^5$	$3.8 \times 10^2 \pm 0.75 \times 10$
3	58	$4.9 \times 10^4 \pm 1.1 \times 10^4$	$2.0 \times 10^2 \pm 1.10 \times 10$
Total	75	$1.4 \times 10^4 \pm 1.1 \times 10^4$	$2.5 \times 10^2 \pm 0.83 \times 10$

[1]Results are expressed as mean ± standard deviation. N: Number of farms (total number of farms studied: 25).

counts found in the present study were lower than those reported by Moraes et al. (2005), who found nearly 100% of raw milk samples from 42 farms in the state of Rio Grande do Sul with counts ranging from 2.3×10^3 to 3.0×10^5 CFU/mL. Tebaldi et al. (2008) analyzed milk from 16 farms in the state of Minas Gerais, and found total coliforms in all samples with counts around 10^5 MPN/mL.

Table 4 presents the distribution of total coliforms and *E. coli* in samples of raw milk according to the daily milk production of dairy farms studied. The counts of total coliforms were higher in raw milk from producers with less than 1,000 L/day, especially in the 500-1,000 L/day category. However, *E. coli* counts were higher in dairy farms from 500-1,000 and 3,000-5,000 L/day categories. The reasons for the differences are difficult to access at this time. The hygienic procedures may be easily implemented in larger than in smaller operations, as a result of greater investments, but the higher number of lactating cows in large dairy farms also requires more equipment to sanitize and extend milking procedures, which can facilitate the gaps and opportunities for contamination of milk. Regardless of the daily milk production, results of this trial indicate the need for effective educational programs on good agricultural practices addressed to dairy farms in Brazil, in order to prevent the contamination of raw milk.

The data presented in Table 2 on the milking practices were used for classification of the 75 dairy farms in three categories in the overall hygienic conditions items, as follows: category 3 (n = 58, 77.3%), which comprised farms with poor conditions of production and hygiene of equipment and installations; category 2 (n =15, 20%), formed by farms with fair, intermediate conditions; and category 1, which had only 1 (2.7%) farm showing good hygienic conditions of milk production (Table 5). As expected, raw milk from dairy farms in the categories 2 and 3 showed higher mean counts of total coliforms than category 1. Of the 75 farms, only 2 (one from category 1 and one from category 2) fully met the IN 51/62 guidelines and had milk with better microbiological quality than the other 73 farms. However, *E. coli* counts were similar among the categories, indicating that fecal contamination of raw milk is not completely related to the environmental contamination during milking procedures in dairy farms.

The high coliforms and *E. coli* counts obtained indicate difficulties and/or lack of knowledge of the farmers to comply with the regulations of IN 51 (Brazil, 2002) and IN 62 (Brazil, 2013) as adopted in Brazil for raw milk. Although *L. monocytogenes* was not detected in raw milk samples, *Listeria innocua* was isolated in the milking environment, indicating that this site may be an important source of *Listeria* spp. Therefore, educational programs should be done to improve milk quality, especially in small and intermediate scale dairy farms.

Conflict of Interest

The authors have not declared any conflict of interest.

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Full Length Research Paper

Efficacy of *Pseudomonas fluorescens* on control of chilli fruit rot caused by *Colletotrichum capsici*

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The fungitoxic effects of 10 isolates of *Pseudomonas fluorescens* from various parts of Tamil nadu were evaluated under *in vitro* conditions on growth of *Colletotrichum capsici*, the causal agent of chilli fruit rot. The identity of *P. fluorescens* was confirmed by DNA sequence analysis of the isolates. *P. fluorescens* PI-1 was the most effective showing 75.6% inhibition of colony growth with minimum mean mycelial growth (4.7 cm) of the pathogen. The aim of this research work was to study the use of biocontrol agents as an alternative to fungicides in the control of fruit rot of chilli.

Key words: Polymerase chain reaction (PCR) analysis, disease management, plant growth promotion.

INTRODUCTION

Chilli (*Capsicum annuum* L.) is an important spice crop of India. It is affected by several fungal, bacterial and viral diseases, of which chilli anthracnose causes considerable damage, inflicting severe quantitative and qualitative losses. The estimated loss due to this disease ranged from 8 - 60% in different parts of India. The fungus C*olletotrichum capsici* infects both unripe (green) and ripe (red) chilli fruits and survives on seed as acervuli and micro sclerotia (Suthin Raj and Christopher, 2009; Suthin Raj et al., 2013). Infection of *C. capsici* is higher at the mature fruit stage than in the early fruit stage. The fungus pathogen is both seed borne and air borne and affects seed germination and vigour to a greater extent. Several fungicides have been reported to be effective in the management of fruit rot of chilli (Gopinath et al., 2006; Shovan et al., 2008). However, the indiscriminate use of fungicides leads to toxic residues on chilli products, development of fungicide resistance and also serves as a cause for environmental pollution (Suthin Raj et al., 2012). Therefore, under intensive chilli cultivation, there is an urgent need to develop alternative disease control measures. The present investigation screened various *P. fluorescens* isolates (confirmed by PCR analysis) against *C. capsici in vitro* and in a field trial on their efficacy to control *C. capsici*

MATERIALS AND METHODS

Collection of seed materials

Fresh seed samples of *C. capsici* were collected from the Department of Agronomy, Annamalai University, Chidambaram, Tamil Nadu.

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Isolation, maintenance and identification of *C. capsici*

Diseased chilli fruits showing typical symptom of anthracnose disease were collected fresh from 20 conventional chilli growing areas of Tamilnadu. The pathogens isolated from each of these localities formed one isolate of *C. capsici.* The pathogen was isolated onto potato dextrose agar (PDA) medium from diseased specimens showing typical symptoms. The infected portion of the fruit was cut into small pieces, surface sterilized in 0.1% mercuric chloride solution for 30 s and then washed in repeated changes of sterile distilled water and plated onto sterile PDA medium in 9 cm Petri dishes. The plates were incubated at room temperature (28 ± 2°C) for five days and then observed for fungal growth. Pure cultures were obtained using the single spore isolation technique (Rangaswami, 1958). Identification of the isolates was confirmed by comparing them with the pure culture obtained from ITCC, IARI, New Delhi and the purified isolates were maintained on PDA slants.

Evaluation of bacterial antagonism against *C. capsici*

Isolation of bacterial antagonist

Ten strains of *P. fluorescens* were isolated from the rhizospheric soil of healthy chilli cultivating fields. A sample of 10 g soil was suspended in 100 ml of sterile physiological water and shaken vigorously at 28°C for 30 min. Serial dilutions were plated on pneumococcal cell wall (PCW) isolation agar medium, and each dilution was incubated at 30°C until colonies were observed. Bacterial colonies that exhibited fluorescence at 365 nm (King et al., 1954) were selected and purified for further studies.

***In vitro* antagonistic activity**

Dual culture technique (Dennis and Webster, 1971)

P. fluorescens was multiplied on King's 'B' medium (20 g pblightease peptone, 1.5 g magnesium sulphate, 1.5 g dipotassium hydrogen phosphate, 15 g agar agar, 10 ml glycerol, 1000 ml distilled water and pH adjusted to 7.2). An 8 mm disc of the pathogen from an actively growing PDA culture was placed onto PDA plates 1.5 cm away from the edge of the plate, and incubated at room temperature (28 ± 2°C). After 48 h, actively growing cultures of the respective bacterial isolates were separately streaked onto the medium on the opposite side of the plate, 1.5 cm away from the edge of the plate. The inoculated plates were re-incubated at room temperature (28 ± 2°C). Three replications were maintained for each bacterial isolate. Control PDA plates were inoculated with the pathogen alone. The radial growth of the pathogen was measured after 48 h. The results were expressed as percent growth inhibition over control.

Mycelial dry weight

Potato dextrose broth was prepared in 250 ml Erlenmeyer flasks and autoclaved. Aliquots of 5, 10, 15 and 20 ml of culture filtrates of *P. fluorescens* taken from 48 h were added to 45, 40, 35 and 30 ml broth in flasks to give a final concentration of 10, 20, 30 and 40% of the culture filtrate in the broth. All the flasks were inoculated with 8 mm culture discs of *C. capsici* and incubated at 28 ± 1°C for 10 days. Flasks containing broth without any culture filtrate served as controls. Each treatment was replicated three times. After 15 days of incubation, the mycelial mat was harvested on a previously weighed filter paper and dried at 105°C for 12 h in a hot air oven, cooled in a desiccator and the mycelial weight was recorded and expressed as mg/50 ml broth.

The most effective isolates of *P. fluorescens* (P.I$_1$) were used for further study.

Identification *P. fluorescens* by PCR

DNA extraction

Template DNA was prepared by boiling 200 µl of bacterial suspension in milliQ (OD_{600}= 0.6) in safe lock Eppendorf tubes for 10 min. The tubes were immediately cooled on ice and centrifuged (20,000 g x 10 min, 5°C). The supernatants were subsequently kept on ice or at -20°C. A microlitre of template DNA suspension was used for each reaction.

PCR amplification

All the PCR amplification were performed in a volume of 50 µl containing ≈50 to 100 ng of bacterial genomic DNA solution, 5 µl of 10 Ml PCR buffer, 200 µM of each dNTP; 2 mM of $MgCl_2$; 0.5 µM of each primer and 0.5 U of taq polymerase (obtained from Genei, Bangalore). DNA 16S specific region for *P. fluorescens* amplification was performed using the primer set 16SPSEfluF and 16SPSER (16SPSEfluF 5'-TGCATTCAAA-ACTGACTG-3'; 16SPSER 5'-AATCACACCGTG-GTAACCG-3'). The forward primer is species specific, while the reverse is family specific. The primers were developed and compared with partial regions 16S of *P. fluorescens* belonging to group 1 (NCBI, National Center for Biotechnology Information) by software DNA sis 2.0 and the following thermal profile: 2 min at 94°C; 5 cycles consisting of 94°C for 45 s, 55°C for 1 min, 72°C for 2 min; 35 cycles consisting of 92°C for 45 s, 60°C for 45 s, 72°C for 2 min; final extension of 72°C for 2 min; and final cooling at 4°C. The amplification was performed in a DNA thermal cycle (Yercaud Biotech, Salem).

Following amplification, 7 µl of product were analysed by electrophoresis at 100 V (1% agrose gel 0.2 µg of ethidium bromide ml^{-1}) in TAE buffer. Photograph was taken with the Nikon COOLOIXSI0 VR Camera and Gel Documentation was done in DGelDAS Software analysis tool (Yercaud Biotech, Salem).

Evaluation of *P. fluorescens* for the management of *C. capsici* under field conditions

The field trials were conducted at Shathankudi Village, Perambalur-District between December 2009 and March 2010 in a field with a history of chilli fruit rot incidence. Trials were laid out in plots (33 x 13 feet) arranged in a randomized block design. Thirty day-old seedlings were planted into the field plots in rows with row/plants spacing of 60 x 30 cm and a total population of 210 plants/plot. Three replicate plots were maintained for each treatment. Treatment application details and experimental observation were the same as in the green house experiment with below mentioned treatment schedule. Regular cultivation practices were followed as per the recommendation.

Treatment details

T_1: Application of *P. fluorescens* (seed treatment); T_2: application of *P. fluorescens* (prophylactic spray at 25 and 75 DAT); T_3: $T_1 + T_2$; T_4: seed treatment with mancozeb + spraying 50 and 75 DAT; T_5: control.

Disease incidence

The fruit rot incidence was assessed 100, 125 and 150 days after transplanting. The intensity of fruit rot was calculated using the

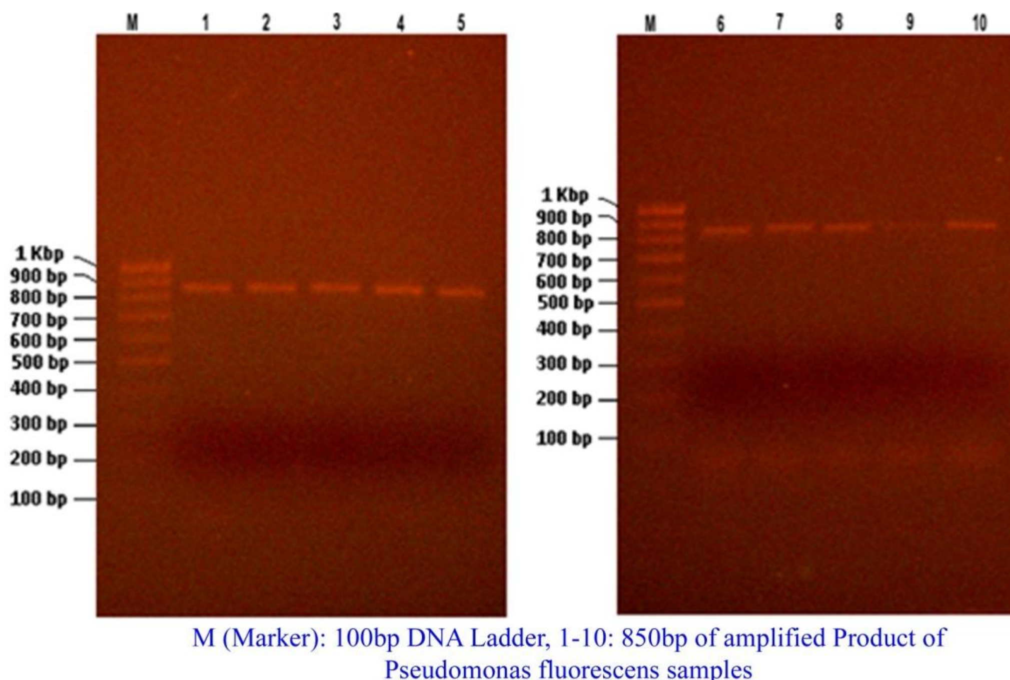

Figure 1. Agarose gel showing species-specific amplification for *P. fluorescens* using PCR.

percent disease index (PDI) grade chart proposed by Reddy et al. (2008); the percent disease index (PDI) was calculated using the Mc Kinney (1923) infection index:

$$\text{PDI} = \frac{\text{Sum of numerical ratings}}{\text{Total number of fruits observed}} \times \frac{100}{\text{Maximum category value}}$$

Plant growth parameters

Growth parameters *viz*., mean leaf area, mean plant height, mean no. of flowers/plant, mean no. of fruits/plant, mean fruit length and fruit yield were assessed for the plants at the time of harvest.

Experimental design and data analysis

The experiments were conducted using completely randomized design (CRD) with three replications. The significant difference, if any, among the means were compared by Duncan's multiple range test (DMRT). Whenever necessary, the data were transformed before statistical analysis following appropriate methods.

RESULTS

PCR assay to identify *P. fluorescens*

Ten isolates of *P. fluorescens* were collected from various parts of Tamilnadu and were confirmed by using PCR analysis. Primer sets 16SPSEfluF and 16SPSER (16SPSEfluF 5'-TG CATTCAAA-ACTGACTG-3'; 16SPSER 5'-AATCACACCGTG-GTAACCG-3') (obtained from Genei, Bangalore) were used to amplify the DNA 16S specific region for *P. fluorescens.* The size of the amplified single DNA fragment obtained was about 850 bp of 16SrRNA. All the tested isolates of *P. fluorescens* were amplified in a single DNA fragment of 850 bp of 16SrRNA (Figure 1). Hence all 10 isolates were confirmed as *P. fluorescens.*

In vitro evaluation of *P.fluorescens against C. capsici*

The results of the screening of 10 isolates of *P. fluorescens* against *C. capsici* on PDA plates are presented in Table 1. Among the isolates, *P. fluorescens* PI-1 appeared to be most effective against *C. capsici* showing 75.6% inhibition of colony growth and minimum mean mycelial dry weight in broth 146.5 mg/50 ml broth. It was followed by isolate PI-8 which showed 74.1% inhibition and minimum mean mycelial dry weight in broth of 153 mg/50 ml.

Isolates PI-9 and PI-10 showed minimum growth inhibition. All the isolates significantly had reduced mycelial growth of the pathogen over the control.

Mycelial dry weight

The mycelial dry weight of *C. capsici* decreased with increasing concentration of culture filtrates of *P. fluorescens* in all isolates and isolates P1 and P8 again showed the greatest inhibition (78 and 72%, respectively) on mycelia dry weight (Table 1).

Table 1. Evaluation of various isolates of *P. fluorescens* against *C. capsici* by dual culture technique.

Isolate	Linear growth (mm)		Growth inhibition (%)	Mycelial dry weight (mg/50 m/broth)				
	Antagonist	*C. capsici*		10%	20%	30%	40%	Mean
P. fluorescens (P. I_1) (TNAU-P.f_1)	68.00	22.00	75.56*^{a} (4.33)**	260 (5.56)	200 (5.30)	104 (4.65)	22 (3.13)	4.66^{a}
P. I_2 - Dharmapuri	60.60	29.40	67.32^{b} (4.22)	305 (5.72)	228 (5.43)	114 (4.74)	30 (3.43)	4.83^{bc}
P. I_3 - Marthandam	53.60	36.40	59.11^{d} (4.05)	326 (5.79)	235 (5.46)	119 (4.78)	34 (3.55)	4.89^{b}
P. I_4 - Aduthurai	51.20	38.80	56.89^{e} (4.50)	338 (5.82)	249 (5.52)	123 (4.82)	37 (3.63)	4.94^{b}
P. I_5 - Vallampadugai	54.50	35.50	60.55^{d} (4.11)	330 (5.80)	239 (5.48)	121 (4.80)	36 (3.61)	4.92^{b}
P. I_6 - Vadalore	62.00	28.00	68.88^{b} (4.24)	282 (5.64)	224 (5.41)	111 (4.71)	34 (3.55)	4.83^{bc}
P. I_7 - Kovilpatti	56.33	33.67	62.58^{c} (4.15)	313 (5.74)	231 (5.44)	116 (4.76)	32 (3.49)	4.86^{b}
P. I_8 - Annamalainagar	66.70	23.30	74.11^{a} (4.31)	266 (5.58)	208 (5.34)	107 (4.68)	27 (3.32)	4.72^{ab}
P. I_9 - Cuddalore	48.72	41.28	54.13^{f} (4.00)	354 (5.87)	256 (5.54)	136 (4.91)	40 (3.70)	5.01^{cd}
P. I_{10} - Sivapuri	49.60	40.40	55.11^{f} (4.02)	351 (5.86)	248 (5.51)	132 (4.89)	39 (3.68)	4.98^{c}
Control		90.00		540 (6.29)	540 (6.29)	540 (6.29)	540 (6.29)	6.29^{d}
Mean				5.79^{d}	5.52^{c}	4.91^{b}	3.76^{a}	

*Values in the column followed by common letters do not differ significantly by DMRT (P=0.05). **Figures in parenthesis are arcsin transformation.

Effect of *P. fluorescens* on incidence of fruit rot under field conditions

The results (Table 2) indicated that the application of *P. fluorescens* (seed treatment + prophylactic spray at 25 and 75 DAT) (T_3) significantly reduced the incidence of fruit rot at 100, 125 and 150 days after planting, respectively, as compared to the other treatments. This was followed by application of Mancozeb (seed treatment + prophylactic spraying 30 and 45 DAT) (T_4).

Effect of *P. fluorescens* and mancozeb treatments on growth and yield of *C. capsici* under field condition

Table 3 shows that all treatments have significantly enhanced the growth and fruit yield, as compared to the control. Among the combination, seed treatment + prophylactic spraying 25 and 75 DAT with *P. fluorescens* (T_3) significantly increased mean plant height, number of flowers/plant, mean number of fruits/plant, mean fruit length and fruit yield (375 g/plant) followed by spraying mancozeb (seed treatment + prophylactic spraying at 30 and 45 DAT) (T_4)

DISCUSSION

DNA sequence analysis has been used to characterize and analyze the taxonomic complexity of *P. fluorescens.* The present study revealed that the primer sets 16SPSEfluF and 16SPSER (16SPSEfluF 5'-TG CATTCAAA-ACTGACTG-3'; 16SPSER 5'-AATCACACCGTG-GTAACCG-3') (Genei, Bangalore) were used to amplify the DNA 16S specific region for *P. fluorescens*. The size of the amplified single DNA fragment obtained was 850 bp of 16S rRNA for *P. fluorescens*. All the tested isolates of *P. fluorescens* were amplified in single DNA fragments of 850 bp of 16SrRNA. A similar study was carried out by Scarpellini et al. (2004) who reported the amplification of single DNA fragment of 850 bp of 16S rRNA for *P. fluorescens*.

The results revealed that, all the ten isolates of *P. flourescens* (TNAU) and native isolates inhibited the growth of *C. capsici in vitro*. Similar results were observed by Vivekananthan et al. (2004, Bharathi et al. (2004), Srinivas et al. (2006), Muthukumar et al. (2010) and Anand et al. (2010). The above authors have reported that isolate *P. flourescens* as well as native isolates strongly inhibited the growth of *C. capsici* under laboratory condition.

Table 2. Effect of IDM formulation on fruit rot incidence under field condition.

Treatment	Fruit rot incidence on 100th day	Increase over control (%)	Fruit rot incidence on 125th day	Increase over control (%)	Fruit rot incidence on 150th day	Increase over control (%)
T_1 - Application of *P. fluorescens* (Seed treatment)	3.95[c]	49.68	7.44[cd]	49.04	9.70[c]	57.86
T_2 - Application of *P. fluorescens* (prophylactic spray at 25 and 75 DAT)	4.44[d]	43.43	8.05[d]	42.86	11.09[d]	38.82
T_3 - T_1 + T_2	2.50[a]	68.15	6.60[a]	54.79	8.33[a]	63.81
T_4-Seed treatment with mancozeb + spraying, 30 and 45 DAT	3.23 [b]	58.53	6.99 [b]	52.12	8.98 [b]	60.99
T_5 - Control	7.85 [e]		14.60[e]		23.02[e]	

*In a row under each treatment, value in the column followed by common letters do not differ significantly by DMRT (P = 0.05).

Table 3. Effect of IDM formulation on growth and yield attributes under field condition.

Treatment	Mean plant height (cm)	Mean no. of flowers/plant	Mean no. of fruits/plant	Mean fruit length (cm)	Fruit yield (g/plant)
T_1 - Application of *P. fluorescens* (seed treatment)	99.10[c]	162[c]	94[c]	7.10[c]	350[c]
T_2 - Application of *P. fluorescens* (Prophylactic spray at 25 and 75 DAT)	98.21[d]	160[d]	92[d]	6.76[d]	338[d]
T_3 - T_1 + T_2	109.06[a]	184[a]	110[a]	8.88[a]	375[a]
T_4 - Seed treatment with mancozeb + spraying, 30 and 45 DAT	101.29[b]	166[b]	96[b]	7.55[b]	352[b]
T_5 - Control	85.95[e]	150[e]	61[e]	4.20[e]	230[e]

*Values in the column followed by common letters do not differ significantly by DMRT (P=0.05).

In the present study, application of *P. fluorescens* (P.f1) (seed treatment + prophylactic spraying at 25 and 75 DAT) at 50 DAT significantly reduced the incidence of *C. capsici.* A significant reduction in *C. capsici* incidence from *P. fluorescens* (Pf-1) + chitin treatment was recorded in chilli plants by Bharathi et al. (2004). Similar conclusions on the management of plant diseases by different pseudomonad strains either as bacterial suspension or through different formulations have been reported by many workers (Vidhyasekaran et al., 1997; Viswanathan and Samiyappan, 1999). This may be due to fluorescent pseudomonas producing compounds like pseudobactin, HCN, salicylic acid, 2-hydroxy phenazine, oligomycin, pyoluteorin, pyrolnitrin, pyocyanin and 2,4-diacelyphloroglucinol which elicit induced systemic resistance in the host plant or interfere specifically with fungal pathogens (Ongena et al., 1999; Velazhahan et al., 1999; Dave and Dube, 2000; Gupta et al., 2001; Pandey et al., 2006; Hofte and Bakker, 2007; Reddy et al., 2008 and Muthukumar et al., 2010).

The results of the present experiment revealed the superiority of all the treatments in increasing the mean leaf area, mean plant height, mean number of flowers/plant, mean number of fruits/plant, mean fruit and fruit yield g/plant length over control. Application of P.f1 (seed treatment + prophylactic spraying at 25 and 75 DAT) to treated chilli plants was found to be more effective than the other treatments in improving growth

characters and yield parameters. Combination of bioagents *viz. P. fluorescens* (Pf-1) and *B. subtilis* along with soil application of organic amendments significantly increased growth characters and yield parameters (Reddy et al., 2008). Same phenomenon was observed by Almaghrabi et al. (2013). Combined application of *P. fluorescens* (Pf32, Pf93) + *B. subtilis* (B49) significantly increased the plant height, number of branches and number of bolls under field conditions in cotton (Salaheddin et al., 2010).

The present study indicated that application of *P. fluorescens* (seed treatment + prophylactic spray) will be effective in controlling the chilli fruit rot disease and increasing the plant growth and yield of chilli.

Conflict of Interest

The authors have not declared any conflict of interest.

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Full Length Research Paper

Effect of drying conditions on physicochemical parameters of powdered *Prosopis africana* condiment fermented with or without consortia

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Seeds of African mesquite (*Prosopis africana*) were purchased from Otukpo market in Benue state of Nigeria. *P. africana* seeds were precleaned and processed for fermentation. Processed unfermented seeds of *P. africana* (300 g) were transferred in three earth pots lined with aluminum foil. Mixed *Bacillus* species (5%) (*Bacillus subtilis* and *Bacillus pumilus*) of both standard and test strains were prepared as consortia and were calibrated using McFarland standard 7. Pot A was inoculated with standard strains of *B. subtilis* and *B. pumilus* (consortium A), while pot B was inoculated with test strains of *B. subtilis* and *B. pumilus* (consortium B), pot C was allowed to ferment without consortium. Fermentation in all the earth pots was allowed to progress at room temperature (25°C). It was observed that fermentation in earth pots with consortia fermented was faster (84 h) as compared to natural fermentation (96 h). Freshly fermented seeds were subjected to different drying conditions (solar drying, oven drying, vacuum drying, direct sunlight drying protected with a net and direct sunlight drying without net). Fermented dried seeds of *P. africana* were converted into powdered form using a sterile blender. Physicochemical analyses were carried out on powdered form under different conditions of drying. It was observed that *P. africana* powdered condiment subjected to hot air oven drying differed significantly from other drying methods and gave lowest values of moisture and peroxide contents as opposed to higher values in other drying conditions. Therefore, condiment of *P. africana* dried using oven will have extended shelf life stability during storage than *P. africana* condiment dried using other methods of drying.

Key words: *Prosopis africana*, fermentation, consortia, *Bacillus* earth pots, aluminum foil, solar dryer, vacuum dryer, hot air oven.

Introduction

In Nigeria, legume seeds such as African mesquite (*Prosopis africana)* are very good source of dietary proteins. Fermented condiment of *P. africana* is used as seasoning, in Africa and other parts of the world

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(Oniofiok et al., 1996). Fermentation of *P. africana* is carried out by the genus *Bacillus,* and the predominating species is *Bacillus subtilis* (Oguntoyinbo, 2010). Ouoba et al. (2003) and Dakwa et al. (2005) opined that, the genus *Bacillus* in the fermentation of locust bean seeds and other legumes like soybean, African mesquite and castor oil seeds has been established. Organism consortia if developed and used as starter cultures, will reduce fermentation time and inconsistencies that arises during fermentation of *P. africana*, thereby guaranteeing product quality that will be appreciated as a result of improved fermentation processes (Holzapfel, 2002). Fermentation improves digestibility, nutritive value and flavour of the raw seeds (Ogunshe et al., 2006).

Removal of moisture content from freshly fermented legume seeds of *P. africana* must be carried out to extend shelf life stability of products (FAO, 2013). Direct sun drying method is usually practiced in villages because of lack of availability of modern drying facilities. The use of solar dryer, hot air oven and vacuum drying have been developed to dry and produce products devoid of contaminants as opposed to direct sun drying methods. Therefore, the use of modern drying techniques for freshly fermented seeds of *P. africana* is recommended for obtaining dried and hygienic products.

MATERIALS AND METHODS

Seeds of African mesquite (2 kg) were purchased from Otukpo market in Benue state of Nigeria. These seeds were packaged into cleaned polythene bags and transported to the laboratory, Department of Microbiology, Ahmadu Bello University, Zaria.

Revalidation and characterization of *Bacillus* isolates (consortia)

Preliminary characterization of isolates

Test strains of *Bacillus* species: *B. subtilis* (TS001) *and Bacillus pumilus* (TS002) obtained from the Department of Microbiology, Ahmadu Bello University, Zaria were compared by re-culturing in nutrient agar broth. The strains were incubated at 37°C for 24 h. Compared cells were sub-cultured on aerobic plates of nutrient and plate count agars and were incubated at 37°C for 24 h. This was carried out along side with standard strains of *B. subtilis* (SX1BS) and *B. pumilus* (SX1BP) obtained from Federal Institute of Industrial Research, Oshodi (FIIRO) Lagos, which was used as control. Representative colonies of microorganisms which developed on the aerobic plates of both nutrient and plate count agar were subjected to initial staining and microscopic examinations. The isolates were subjected to the following biochemical tests (catalase, coagulase, motility, indole, hydrogen sulphide production, growths at different sodium concentrations and sugar utilization) using standard methods as described by Gordon et al. (1973).

Preparation of *Bacillus* inoculum

The inoculum used for each fermentation contained 2.7×10^7 cells/ml; the cell population was calibrated using McFarland standards (No 7) which was prepared by adding 0.7 ml of 1% anhydrous barium chloride ($BaCl_2$) to 9.3 ml of 1% sulphuric acid (H_2SO_4) (Todder, 2009). The inoculum used formed 5.0% of fermenting materials and consisted of (15 ml of 24 h old cultures of organism into 300 g of unfermented seeds) consortium A (standard strain mixture of *B. subtilis* and *B. pumilus* combined) and consortium B (test strain mixture of *B. subtilis* and *B. pumilus* combined).

Preparation of *P. africana* seeds for fermentation

P. africana seeds obtained from the market were pre-cleaned by sorting out stones and debris. This was followed by washing and boiling in water for 24 h, renewing the water intermittently until the seeds became soft. The soft seeds were dehulled by removing seed coats with finger tips (Ogbadu, 1988). The cotyledons were reboiled for four hours and were allowed to cool to 35°C in an earthen pot lined with sterile aluminum foil.

Controlled fermentation of *P. africana* seeds with and without consortia

The fermentation process was set up using both consortium A and B, separately. The organisms were inoculated into 300 g of the unfermented seeds of *P. africana* and were wrapped with sterile aluminum foil and placed in an earthen pot with cover. Thermometers were inserted in each of the earth pot to monitor fermentation temperature. Initial temperature of earth pots with unfermented seeds was 35°C. Another fermentation process of *P. africana* was set up to ferment without consortia. Both fermentation processes of *P. africana* were allowed to progress at room temperature (24°C) in the laboratory of Department of Microbiology, Ahmadu Bello University, Zaria.

Microbiological monitoring of fermentation

Microbiological analysis was carried out at intervals of 12 h to monitor growth of starter cultures from the start to the end of the fermentation process. During the 120 h of fermentation, samples of ten grams of *P. africana* with consortia were taken aseptically at intervals of 12 h and were transferred into 90 ml sterile peptone water. The suspension was shaken vigorously for one minute to dislodge microorganisms, thus forming the stock concentration. Serial dilution was prepared to obtain dilutions up to ten folds. Aliquots of 0.1 ml of 10^{-5} and 10^{-6} dilutions were plated in duplicates on nutrient agar plates (Oxiod), plate count agar (Oxiod); for isolation and determination of count of bacteria. Potato dextrose agar containing chloramphenicol (0.5 mg/ml) to suppress growth of bacteria was used for isolation of fungi. The plating was done using a hockey glass stick spreader. The nutrient and plate count agar plates were incubated at 37°C for 24 h. Potato dextrose agar plates were incubated at room temperature (24°C) for one week.

Drying of freshly fermented seeds of *P. africana* using different methods

Fifty grams of freshly fermented seeds of *P. africana* containing consortia, and a control without consortia, were weighed into Petri dishes cleaned with ethanol. Petri dishes containing fermented samples of *P. africana* were subjected to drying conditions using the following methods; oven drying, vacuum drying, direct sun drying, drying using a solar dryer and sun drying protected with a net.

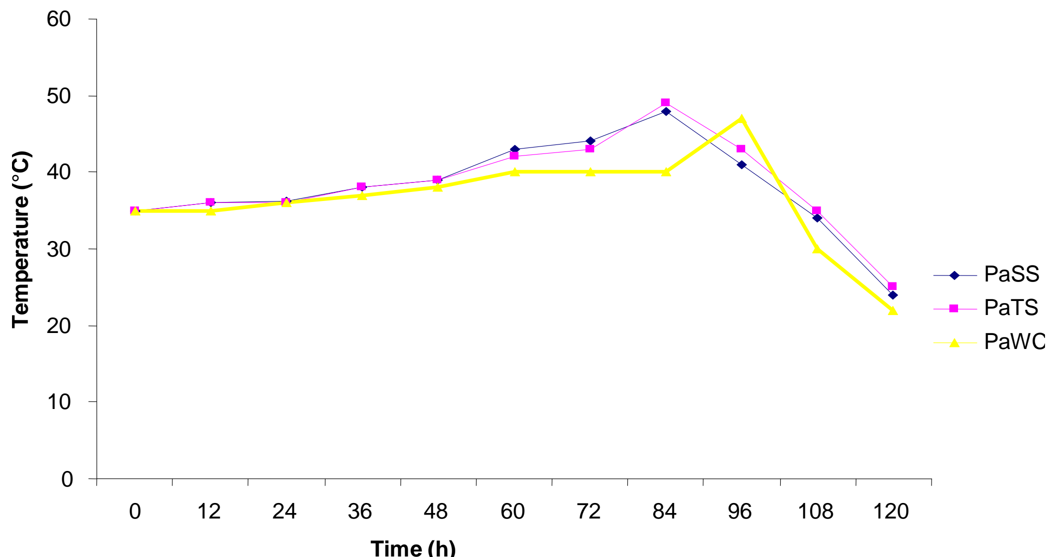

Figure 1. Temperature changes during fermentation of *P. africana* seeds with and without consortia. PaSS- *P. africana* seeds fermented with mixed culture of standard strain of *B. subtilis* and *B. pumilus* (consortium A); PaTS- *P. africana* seeds fermented with mixed culture of test strain of *B. subtilis* and *B. pumilus* (consortium B); PaWC- *P. africana* seeds fermented without consortia.

Powdering, blending and packaging of dried fermented seeds of *P. africana*

Dried fermented seeds of *P. africana*, were blended into powder using a sterile blender. Ten gram of each type of powder was packaged into small plastic containers with seals sterilized with 70% ethanol. The packaged condiments were stored at refrigeration temperature (9±2°C).

Determination of physicochemical parameters of *P. africana* powdered condiment

pH

A Pye Unicam pH meter, model 291 equipped with a glass electrode was first calibrated using standard buffers of pH 4.0 and 9.2. Readings were also taken at intervals of 12 h. This was done by mixing one gram of *P. africana* powder in 10 ml of sterile distilled water. The pH of the suspension was then determined. Moisture content, peroxide value and titratable acidity were analyzed adopting the methods of AOAC (2007).

RESULTS AND DISCUSSION

Microbial consortia can be found everywhere in nature and are implicated in processes of great importance to human, one of which is assistance in food processing (Brenner et al., 2008). Microbial consortia can perform complicated task and endure more changeable environment than monoculture (Brenner et al., 2008). A monoculture of *Bacillus,* namely *B. subtilis* can initiate and end fermentation of legume seeds as reported by Odunfa (1981). Experience has shown that *Bacillus* consortia enhance fermentation activities more than monoculture. Holzapfel (2002) reported that *Bacillus* species were found to be associated with fermentation of plant seeds, such as *P. africana* and other legume seeds. The development and introduction of combined *Bacillus* species as consortia is to speed up fermentation activities.

Figure 1 shows that as fermentation time progressed, there was a rise in temperature from 35°C to the peak (49°C) with seeds inoculated with consortia and 47°C with seeds that were allowed to ferment without consortia. The increase in temperature is due to increase in metabolic activities during which heat was evolved (Odunfa, 1981). Temperature dropped drastically to 22°C for seeds with consortia and 20°C for seeds without consortia. The drop in the temperature is as a result of reduced metabolic activities (Odunfa, 1981).

It was also shown in this study that fermenting mashes inoculated with consortia A and B fermented faster (84 h). Mashes that fermented without consortia, completed fermentation process within 96 h (Figure 1). This is because, *Bacillus* consortia developed as starter cultures optimized production processes and they speed up fermentation by their abilities to break down protein to amino acids faster than seeds fermented without consortia. Another reason for fermentation been faster with consortia may be due to the fact that, each species brought unique set of enzymes or metabolic pathways. They may not be able to break down protein in the seeds alone, but together, all the necessary enzymes are present for breaking down proteins to amino acids

Table 1. Effect of drying conditions on physicochemical parameters of *P. africana* fermented with or without consortia.

Drying condition	Powdered condiment of *P. africana*	Means of fermentation	pH	Moisture content (%)	Peroxide value (meqkg)	Titratable acidity (mg/actic acid g)
	P. africana	Consortium A	5.23±0.00	0.19±0.02	4.29±0.01	1.10±0.00
Solar	*P. africana*	Consortium B	5.23±0.00	0.20±0.00	4.27±0.04	1.12±0.00
	P. africana	Without consortia	5.24±0.00	0.20±0.02	4.30±0.02	1.11±0.01
	P. africana	Consortium A	6.24±0.00	0.10±0.02	3.10±0.01	1.02±0.05
Oven	*P. africana*	Consortium B	6.23±0.00	0.10±0.01	3.11±0.04	1.05±0.00
	P. africana	Without consortia	5.29±0.00	0.14±0.00	3.15±0.00	1.10±0.01
	P. africana	Consortium A	5.20±0.00	0.20±0.02	4.30±0.00	1.10±0.00
Vacuum	*P. africana*	Consortium B	5.20±0.00	0.21±0.02	4.31±0.01	1.11±0.00
	P. africana	Without consortia	5.21±0.00	0.25±0.02	4.29±0.00	1.10±0.01
	P. africana	Consortium A	5.23±0.05	0.27±0.05	4.33±0.02	1.11±0.03
Sun (N)	*P. africana*	Consortium B	5.20±0.05	0.20±0.05	4.30±0.02	1.12±0.13
	P. africana	Without consortia	5.25±0.00	0.24±0.02	4.27±1.01	1.12±0.00
	P. africana	Consortium A	5.23±0.05	0.20±0.05	4.32±0.02	1.12±0.03
Sun (D)	*P. africana*	Consortium B	5.20±0.05	0.24±0.01	4.30±0.02	1.12±0.03
	P. africana	Without consortia	5.25±0.01	0.24±0.01	4.26±1.04	1.10±0.01

Values are means of triplicate determinations. Consortium A- seeds fermented with standard strains of *B. subtilis* and *B. pumilus*; consortium B- seeds fermented with test strains of *B. subtilis* and *B. pumilus;* Sun (D)- direct sun drying; sun (N)- sun drying covered with net.

Table 2. Mean separation using Duncan multiple range table for different drying methods used in drying freshly fermented seeds of *P. africana*

Duncan grouping	Mean	N	Factor
A	5.5767	27	Oven drying
B	5.2726	27	Vaccum drying
BC	5.2681	27	Solar drying
BC	5.2578	27	Sun drying (N)
C	5.2541	27	Sun drying (D)

(N), Sun drying covered with net; (D), Direct sun drying.

faster, resulting to shorter fermentation time.

Different drying conditions (solar drying, sun drying with net protection, direct sun drying without net, vacuum drying and oven drying) were used to dry freshly fermented condiment of *P. africana*. Interactions of drying conditions on condiment fermented with consortium A (standard strains of *B. subtilis and B. pumilus*) and consortium B (test strains of *B. subtilis and B. pumilus*) showed that physicochemical analyses on condiments with and without consortia showed no significant difference with one another. Highest pH value of 6.24±0.00 was recorded in oven dried condiment. Lowest pH value of 5.20±0.00 was recorded in vacuum drying. Yeast and moulds grow best at lower pH. A higher pH of 6.24±0.00 will affect growth of yeast and moulds. Lowest moisture value of 0.10±0.10 was recorded in oven dried condiment; growth of microorganisms is always affected when moisture content is low. Peroxide and titratable acid values were low in oven dried condiment (Table 1). Peroxide and titratable acid levels when high (20-40 meq kg) in condiment posed spoilage threat to condiment (Kolapo et al., 2007). The fact that oven drying condition differed among other drying conditions (Table 2), makes physicochemical parameters obtained from oven dried condiment also optimal. Using Duncan multiple range table, oven drying differed greatly from other drying methods with the highest mean of 5.5767 and Duncan grouping of A. The lowest mean value of 5.2541 was recorded in direct sun drying with Duncan group of C (Table 2).

Conclusion

From the analyses of this research work, it has been concluded that *P. africana* seeds inoculated with 5% *Bacillus* consortia fermented faster (84 h) as opposed to fermentation without consortia (96 h). Oven drying, out of the different drying conditions (solar dryer, vacuum dryer, sun drying without net and sun drying with net) used in drying freshly fermented *P. africana* seeds gave powdered condiment with lower values of moisture, titratable

acid and peroxide values as compared to higher values obtained from other drying conditions. Powdered condiment of *P. africana* obtained from oven drying methods produced condiment with physicochemical properties that will contribute in preserving the condiment for a very long time.

Conflict of Interest

The authors have not declared any conflict of interest.

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Full Length Research Paper

Antibacterial activity and phytochemical screening of extracts of *Lippia alba* (Mill). NE Brown

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The use of natural products as an alternative for treatment and prevention of diseases has been increasing daily. *Lippia alba* is a shrub widely distributed throughout Brazil and its aqueous extracts, as infusion and decoction made from its leaves, are currently being used in popular medicine. Thus, the objective of the study was to do a phytochemical screening of these solutions as well as evaluate *in vitro* antimicrobial activity of these solutions in the reduction of: *Streptococcus mutans* (ATCC 25175), *Streptococcus mitis* (ATCC 49456), *Staphylococcus aureus* (ATCC 12600) and *Lactobacillus casei* (ATCC 27216). The negative and positive controls were respectively, BHI and Chlorhexidine. Statistical analysis was performed using the Mann-Whitney and Kruskal-Wallis test with Bonferroni's penalization, showing significant difference between the products tested ($p<0.001$). We found some phenolic compounds in the solutions. In all the microorganisms, the decoction and the infusion were more effective as compared to other products, having significant difference ($p< 0.05$). Therefore, the aqueous extracts of *L. alba* as well as being natural and easy to handle, were more effective against oral bacterias than chlorhexidine, which is considered a gold standard bactericidal agent.

Key words: Phytotherapy, dentistry, antimicrobial activity.

INTRODUCTION

The knowledge on plants has always followed the evolution of human civilizations throughout the ages. The use of medicinal plants dates from ancient times, when they were used by ancient Egyptian and Chinese civilizations in approximately 3000 BC (Jeon et al., 2011). Currently, it is seen as a favored alternative due to the lower risk of toxicity and ease of preparation.

Phytotherapy has been recommended by the World Health Organization (WHO) since 1970. In Brazil, the use of teas in popular medicine is regulated by the Resolution Board 267/05, considered relevant by the National Medicinal Plants and Herbal Medicine Board and secured by the National Policy on Integrative and Complementary Practices in public health (Brazil, 2010).

In this context, *Lippia alba* (Mill.) NE Brown is one of the many plants that can be used as herbal medicine. It

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belongs to the Verbenaceae family and it is commonly found in the Americas, from southern United States to Argentina, being of Asian origin. The flowers have colors ranging from blue to purple (Henao et al., 2011), this being an important tool in the differentiation from *Melissa oficinallis.*

The decoction and infusion of the *L. Alba* leaves are currently used in popular medicine to combat various diseases. The extracts are used in the treatment of gastric diseases, diarrhea, fever, cough, asthma and as a tranquilizing drug (Sena-Filho et al., 2006). Furthermore, many authors have confirmed the antibacterial activity of *L. alba* against various bacteria such as *Staphylococcus aureus*, *Klebsiela pneumonia* (Sena-Filho et al, 2006), *Escherichia coli* , *Helicobacter pylori* (Henao et al., 2011) and *Vibrio parahaemolyticus* (Ara et al., 2009). Aguiar et al. (2008) demonstrated antimicrobial activity in the *L. alba* leaves against *Micrococcus luteus*, *Bacillus subtilis, Mycobacterium smegmatis* and *Monilia sitophila*

Due to its popularity and the scarcity of data on the antibacterial activity of *L. alba* against oral bacteria, this study aimed to analyze the inhibitory activity against bacterial strains colonizing the oral environment, *in vitro* studies have been realized with oral monocultures to evaluate the effects of various substances (Moon et al., 2011). Thus, we sought to contribute to the body of research on herbal medicines against oral microbiota, as there are no reports on the literature of activity from the decoction and infusion of *L. alba* against oral microorganisms.

MATERIALS AND METHODS

Microorganisms

Four strains of bacteria commonly found in the mouth were used: *Streptococcus mutans* (ATCC 25175), *Streptococcus mitis* (ATCC 49456), *Staphylococcus aureus* (ATCC 12600) and *Lactobacillus casei* (ATCC 27216), they were obtained by request from the Oswaldo Cruz Foundation (Rio de Janeiro/RJ) and then reactivated in the Microbiology Department of Odontology/CCS/UFRN. These bacteria were selected because they are directly responsible for the colonization of tooth surfaces and consequently the formation of biofilm, thus being responsible for caries. The *S. aureus* was used because it is a microorganism that co-exists in the mouth, having high pathogenic potential.

Plant material

The plant specimen was collected from the city of Ceará-Mirim, Rio Grande do Norte, Brazil, in November 2010. The pharmacogenes used were part of the plant, specifically the leaves. The plant was botanically identified by the Federal University of Rio Grande do Norte herbarium with the reference number 16583.

Preparation of extracts

The extracts (decoction and infusion) were prepared following the Brazilian regulations recognized by ANVISA – National Agency of Sanitary Supervision. These regulations aim to standardize the extracts and will be used by SUS– the national public health system. Accordingly, both extracts were prepared following ANVISA's decree 519/98 (Brazil, 1998), at a ratio of 1:10 (mass/volume) and filtered vacuum. Thus, for the decoction, 50 g of pulverized dried leaves were mixed with 500 ml of water and boiled for 5 min. For the infusion, 500 ml of water was boiled at 100°C for 5 min and then poured over 50 g of pulverized dried leaves, after the mixture was left to rest for 5 min. Since they are extractions (infusion and decoction) that have been prepared following the popular mode of preparation and intended for home use, the shelf life is limited to 24 h when left at ambient temperature, this is because both have water as vehicle extractor which favors the proliferation of fungal extracts and becomes more unstable with microbial growth leading to changes in color, odor and flavor. However, if stored in the refrigerator or freezer at -20°C (203.15 K) the period of consumption can be up to 90 days, provided that the preparation has been successful as advocated by specific legislation (Brazil, 2010).

Phytochemical analysis

The phytochemical screening was based on the Wagner and Bladst (2001) protocol. The thin layer chromatography (TLC) followed the protocol proposed by Julião et al. (2003) and Lionço et al. (2001), using Ferric Chloride at 5% and A natural was revealed.

Evaluation of antimicrobial activity

In dentistry, there are already many studies evaluating the minimum inhibitory concentration (MIC) of herbal products and this proposal was to present a new methodology adapted from the study of Christensen et al. (1985) and Stepanovic et al. (2000) which also evaluated the antimicrobial activity of medicinal plants but mimicked the mouth environment. There was no initial intention to evaluate the bactericidal kinetics or time of death of such microorganisms. The *in vitro* antimicrobial evaluation in similar research is usually carried out by dilutions of the extract and the same tests carried out on disks or wells (holes) made in Petri dishes of isolated bacteria. However, despite promising results in bacterial suspension monoculture our intention was to use *in vitro* sterile polystyrene microplates with inert flat bottom, containing 96 wells (Nunclon; InterMed) to simulate an environment closer to mouth reality. For this purpose, modifications were carried out to streamline the protocol. The culture medium used was BHI (DIFCO®, Michigan, USA) broth with 5% sucrose and the culture was diluted 1:100. The negative control that was used was 10 µL of bacteria + 990 µL of BHI. To evaluate the antimicrobial activity of *L. alba,* we used 10 µL culture + 90 µL of the extract + 900 µL of BHI. The positive control was 0.12% chlorhexidine (Periogard®, Colgate-Palmolive Company, New York, USA). This solution was applied to 200 µL in microtiter plate wells. The plate was incubated for 20 h at 37°C. The optical density (OD) growth was measured at 570 ηm. The experiment was done eight times for greater certainty in the results.

Statistical analysis and percentage

Statistical analysis was carried out with STATA (10.0) software using the Kruskal-Wallis and Mann-Whitney test with Bonferroni penalty. The quantitative results obtained after statistical analysis were converted into quality for better viewing of the antimicrobial activity from the products tested. Following the logic of the percentage inhibition (PI), a PI>90% was considered as very strong (+++); 90%>PI>1% was considered strong (+), PI < 1% inhibition very low (---).

Table 1. Phytochemical screening of the infusion and decoction of *L. alba.*

Test	Infusion	Decoction
Saponins	+	++
Phenols	+++	+++
Tannins	+	+++
Gum	+++	+++
Flavonoids	++	++
Heterosides	S	+++
Alkaloids	+ (Dragendorff e Bouchard) +++ (Bertrand)	+++ (Bertrand)
Resins	++	+++

Strong presence (+++); presence moderate (++), trace (+), reaction suspected (S).

Table 2. Antimicrobial activity of infusion and decoction of *L. alba.*

Product / Bacteria	BHI	Infusion	Decoction	Chlorhexidine
Streptococcus mutans (ATCC 25175)	---	+++	+++	+
Streptococcus mitis (ATCC 903)	---	+++	+++	+
Staphylococcus aureus (ATCC 25923)	---	+++	+++	+
Lactobacillus casei (ATCC 9595)	---	+++	+++	+

Intensity of inhibition of bacterial growth: very weak (---); strong (+); very strong (+++).

Table 3. Median (quartile 25/quartile 75) and statistical significance of the tested products.

	Infusion	Decoction	CHX	p
S. mutans	-0.308(-0.392/-0.238)[a]	-0.398(-0.574/-0.262)[a]	0.044(0.001/0.192)[b]	<0.001
S. mitis	-0.152(-0.172/-0.142)[c]	-0.478(-0.578/-0.399)[d]	0.022(0.009/0.036)[e]	<0.001
S. aureus	-0.190(-0.280/-0.088)[f]	-0.177(-0.470/-0.084)[f]	0.369(0.334/0.456)[g]	<0.001
L. casei	-0.274(-0.313/-0.218)[h]	-0.522(-0.620/-0.411)[i]	0.308(0.270/0.340)[j]	<0.001

Same letters indicate no statistically significant difference, different letters indicate statistically significant difference.

Thus, the PI of the products tested were found using the following formula:

PI = [1-(PTMed/NCMed)] x 100

Where, PTMed = Product tested median; NCMed = negative control median

RESULTS

The infusion and decoction, in general, showed very similar chromatographic profiles in all solvent systems used (Table 1). A chromatographic thin layer indicated the presence of ellagic acid in the infusion, which was not observed in the decoction.

As shown in Table 2, the infusion and decoction showed highly effective antibacterial activity against the bacteria whose percentages of inhibition were higher than the substance considered the gold standard in dentistry, chlorhexidine, which showed an inhibition of between 87-62%, indicating bacterial growth. In Table 3, the statistical significance of the results can be seen.

DISCUSSION

Given the strong presence of phenols, including tannins

and flavonoids in the chromatographic analysis, we decided to research these specific compounds, because many of these compounds have antimicrobial activity which is well known and has been studied since Duarte et al. (2006) found an inhibitory effect of phenols in the formation of *S. mutans* biofilms. Given the potential antimicrobial activity of these compounds, especially tannins, this finding is important as a possible justification for the pharmacological activity of these extracts.

Although both are aqueous and present similar chromategraphic profiles, the process of preparation is different, which can influence the antimicrobial activity. The presence of ellagic acid in the infusion shows antimicrobial activity not present in the decoction (absence of ellagic acid), tannin-infusion can be seen as one of the justifications for this statement. Some studies have shown that the extraction temperature influences the chemical distribution of secondary metabolites (Velloso et al., 2009; Mossi et al., 2004). However, the difference between boiling the plant and adding hot water to it should be considered as a factor that influences the withdrawal of the plant's secondary metabolites.

The mechanism of antimicrobial action of tannins can be explained by three assumptions. The first assumes that tannins inhibit bacterial and fungal enzymes and/or complexes with substrates of these enzymes; the second includes the action of tannins on the cell membranes of microorganisms by changing their metabolism; and the third is based on the complexation of tannins with metal ions, decreasing the availability of ions essential for microbial metabolism (Loguercio et al., 2005; Scalbert, 1991). Simões (2003) confirms that the antimicrobial activity present in some plants is directly related to the presence of the metabolite (tannin), which also possesses a bactericidal characteristic.

The flavonoids are composed of hydrosoluble phenolics and are important defense agents against phytopathogenic microorganisms like viruses, bacteria and fungus, acting as a natural defense in plants in the form of a chemical response against an invasion of pathogens. They, thus, possess numerous biological activities, of which we can highlight the antimicrobial and anti carcinogenic activity, which have a very low toxicity in human cells (Capasso et al., 2003; Zuanazzi, 2000).

The strong antimicrobial activity against *S. aureus* is consistent with Soares (2001) study. However, Caceres et al. (1991) has already studied the power of antibacterial ethanolic extracts from *L. alba* against this bacterium. It is important to note that there is no literature to date referring to the antimicrobial activity of aqueous extracts of *L. alba* against *S. mutans, S. mitis, L. casei* and *S. aureus,* and, therefore, this is a pioneering study in this regard. On the other hand, it is common to find streptococcal species implicated in endocarditis and it is capable of inducing cell death (Okahashi et al., 2011), showing the importance of mouth health to systemic health.

Conclusion

The antimicrobial activity observed in the infusion and decoction of the leaves of *L. alba* encourages new research on single substances in order to establish constituent chemicals that are responsible for this activity. However, more research is necessary, especially *in vivo* and *in situ*, to establish conclusive evidence for the efficiency and clinical applications of these compounds in the prevention of dental caries.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Production and partial characterization of glucose oxidase and catalase from xerophytic strain of *Aspergillus niger*

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Six *Aspergillus niger* strains isolated from semi-desert of Coahuila State (Mexico) were screened for an extracellular catalase (CAT) and glucose oxidase (GOX) production using solid-state and submerged fermentations. The best enzymes producer, *A. niger* ASPN 1.1, was selected for enzymes production under submerged fermentation condition. The higher activity was observed at 24 and 48 h of submerged fermentation, respectively. The enzymes were partially purified to a yield of 98 and 48%, and fold 9.8 and 5, corresponding to specific activity of 589.60 and 0.60 U/mg, respectively for CAT and GOX, using ultrafiltration with 100 kDa filter. The enzymes showed high affinity for H_2O_2 and D-glucose with a K_m value of 80 and 4.24 mM, respectively. Both enzymes exhibited a greater catalytic activity at pH 6. Optimum temperature for glucose oxidation was 50°C, while for peroxide decomposition, it was 45°C. The enzymes showed a high thermostability at 50°C with a half-life time of 99 and 86 min, respectively, for GOX and CAT. These characteristics suggest the use of xerophytic *A. niger* strain as a potential producer for both enzymes, which have analytical and industrial application.

Key words: Catalase, glucose oxidase, xerophytic, *Aspergillus niger*.

INTRODUCTION

Xerophytic fungi grow in absence of free water due to use of water vapor from the air for growth. Fungi from semi-desert are characterized by this aptitude in addition to their ability to resist temperature changes of arid environment. The arid and semiarid regions of the world are recognized as one of the least explored niches occupied by fungi. It is well-known that xerophytic fungal strains are characterized by their adaptability to extreme conditions such as long drought periods, high mid-day temperatures, low night temperature, high osmotic pressures and changes in humidity level. Moreover, their ability to produce variety of enzymes with extraordinary

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Table 1. Morphological characteristics of selected fungi and their response to GOX and CAT qualitative tests.

Strain	Colonia	Vesicles	Hyphae	Phialides	Conidia	GOX test	CAT test
ASPN 1.1	Black and granular texture	Round	Septate hyaline	Primary densely covering vesicle	Dark brown, globular, smooth, radially	+	+
ASPN 3	Black and cottony texture	Round	Septate hyaline	Primary covering the entire vesicle and racemes	Round and radiated, hyaline	+	+
ASPN 4	Black and cottony texture	Round	No septate hyaline	Covering the entire vesicle	Globular, dark brown	+	+
ASPN 5	Brown and cottony texture	Round	No septate hyaline	Primary and secondary covering the entire vesicle	Globular, radial-shaped, dark brown	+	+
ASPN 7´	Dark brown, cottony texture, little sporulation	Round	Septate hyaline	Primary covering the entire vesicle	Globular, dark brown, with conidia exiting the conidiophore	+	+
ASPN 12	Black and granular texture	Round	No septate hyaline	Primary	Globular, dark brown	+	+

characteristics has been reported (Cruz-Hernández et al., 2005; Cruz-Hernández et al., 2006; Flores-Gallegos et al., 2012). In the present study, the strains of *Aspergillus niger* isolated from the Mexican semi-desert were screened for their ability to excrete extracellular enzymes catalase (CAT, H_2O_2: H_2O_2 oxidoreductase, EC 1.11.1.6) and glucose oxidase (GOX, β-D-glucose: oxygen-1 oxidoreductase, EC 1.1.3.4).

Both enzymes have various applications in industry, as well as in analytical chemistry (Tzonka et al., 2006; Wang et al., 2008; Singh and Verma, 2013). For example, they are applied as additives for food preservation, because they promote the elimination of residual oxygen after packaging. The fungus *A. niger* is a widely used source for obtaining GOX and CAT, since it has many biotechnological advantages, it is not pathogenic and can grow with a wide variety of nutrients (Schuster et al., 2002; Cruz-Hernández et al., 2006). This is one of the reasons to study this type of fungi. Furthermore, to our knowledge, the production of these enzymes by xerophytic fungi was not reported previously.

To characterize studied fungi, we applied traditional methods involving morphological and microscopical characterization, as well as rDNA identification for the strain with highest activities (Arnaud et al., 2012). The operational properties of enzymes were characterized using partially purified extracellular extract. Ultrafiltration with a semi-permeable membrane with pore sizes enough to permit movement of molecules smaller than 100 kDa and to prevent the passage of proteins with higher molecular weight was applied to this. This method used centrifugation to induce the movement of water and small molecules through the membrane under centrifugal force. This method is fast, allows separating one part of proteins, estimate molecular weight of enzymes, and results in concentration of the protein sample (Tauro et al., 2012).

The goals of the present study are: 1) to screen the collection of xerophytic *A. niger* strains for an extra-cellular catalase (CAT) and glucose oxidase (GOX) production; 2) partial purification of enzymes from one strain exhibiting greater enzymatic activities, and 3) to characterize some operational properties of both enzymes.

MATERIALS AND METHODS

Strains morphological characterization

Fungal strains used for the present study were obtained from Fungi Collection of Food Research Department, School of Chemistry, Autonomous University of Coahuila (Saltillo, Mexico). These cultures were originally isolated from soil or stems of semi-desert plants from semi-arid zone of Coahuila State (Mexico) and stored on potato dextrose agar (PDA) slants. The fungal strains were further transferred/sub-cultured by growing on freshly prepared PDA plates, as well as under slide culture conditions (Sarma et al., 2002). Morphological and microscopic characteristics described in Table 1 were noted to verify *A. niger* properties.

Screening for glucose oxidase and catalase production in solid-state fermentation

To select the fungi with glucose oxidase production, the fungi were grown on PDA medium and applied to solid diagnostic test, which allowed estimating the glucose oxidase activity by the presence of the brown zones of *o*-dianisidine (100 g/L) oxidation developed around the colonies in the presence of horseradish peroxidase type VI (15 U) (Fiedurek and Gromada, 2000).

Catalase activity was screened by applying a drop of 30% (v/v) H_2O_2 with a syringe to the edge of each colony. Catalase producing strains were selected due to appearance of oxygen bubbles (Kim et al., 1994).

Screening for catalase and glucose oxidase production in submerged fermentation

Promising *A. niger* strains were grown in submerged mode at 30°C and 320 rpm in the liquid medium (Fiedurek and Gromada, 2000), which contained (g/L): glucose, 80; peptone (type I, Sigma), 3; $CaCO_3$, 35; $(NH_4)_2HPO_4$, 0.388; K_2HPO_4, 0.188; and $MgSO_4 \cdot 7H_2O$, 0.156. This medium was inoculated to a density of 10^5 spores/mL. The enzymatic activities and protein concentration were monitored for 60 h after liquid aliquot filtration. Fungal mycelium grown for 60 h was collected by filtration, washed, dried at 90°C to a constant weight. The culture filtrate was used for the respective assays of enzymes partial purification and characterization.

Catalase was assayed by spectrophotometric measurement of the decomposition of H_2O_2, (Zeng et al., 2010). Sample (0.5 mL of culture medium) was added to 0.95 mL of H_2O_2 (0.05 M in phosphate buffer 0.02 M, pH 7). After stirring, the decrease in absorbance at 240 nm was measured for 3 min (Cary-50 UV/VIS Spectrometer). The initial rate of decomposition was determined after fitting the kinetic curves. The absorption coefficient at 240 nm for H_2O_2 was taken to be 40 M^{-1} cm^{-1}. One unit of catalase activity was defined as the amount of enzyme required to decompose 1.0 μmol of H_2O_2/min at 25°C.

Glucose oxidase activity was determined in aliquots of culture liquids by using a coupled *o*-dianisidine-peroxidase reaction (Singh and Verma, 2013). One unit (U) of enzyme activity was defined as the amount that produces 1 μmol H_2O_2/min at 30°C. The reaction mixture contained 0.6 mL of 0.01 M glucose, 0.33 mL of *o*-dianisidine (0.16 g/L), 0.12 mL of horseradish peroxidase type VI at 20 μg /mL (Sigma-Aldrich). All reactants were previously dissolved in 0.02 M phosphate buffer at pH 6. The reaction was initiated by addition of 0.12 mL of the extract containing the enzyme. The absorbance increase was detected continuously in Cintra-20 spectrophotometer at 436 nm for 105 s. The activity was calculated from the linear portion of the curve using a molar extinction coefficient of 8300 $M^{-1}min^{-1}$. Protein concentrations were estimated by Bradford method using bovine serum albumin as the standard.

Confirmation of fungal strain by rDNA identification

Fungal DNA, which was selected due to greater enzymatic activities, was isolated using the protocol previously reported by Barth and Gaillardin (1996). In particular, chopping of fungal mat (0.4 g on dry mass basis) was done using pestle and mortar with liquid nitrogen. Chopped material was transferred to 50 mL centrifuge tube containing 5 mL of extraction buffer TES (100 mM Tris-HCl of pH 8.0, 20 mM EDTA, and 0.8 % SDS) along with 2.5 mL of 5 M sodium acetate of pH 5.2 and 5 M NaCl. Centrifuge tube was placed at -20°C for 20 min. After centrifugation, done at 14800 rpm for 20 min at 4°C, supernatant was transferred to a new tube and an equal volume of isopropanol was added to the tube. Pellet DNA was obtained after 5 min centrifugation at the same conditions as earlier. Pellet of DNA was washed thrice using ethanol. After washing, the pellet was dissolved in 0.05 mL of 0.1X TE buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8). Electrophoresis was carried out at 100 V in 1% agarose gel with 1xTAE buffer (40 mM Tris, 20 mM acetic acid and 1 mM EDTA, pH 8) and ethidium bromide. The marker Hyperladder I (0.002 mL of the marker and 0.003 mL of TE buffer, pH 8.0) was used (Hovda et al., 2007; Rojas et al., 2008; Moreno-Dávila et al., 2010). The concentration of extracted DNA was checked on NanoDrop spectrophotometer using 0.001 mL of sample.

PCR conditions and primers for amplification of 18S rDNA were used as reported by Melchers et al. (1994). The primers were nu-SSU-0817-5´ (foward) (5'- TTAGCATGGAATAATRRAATAGGA-3') and nu-SSU-1193-3´ (reverse) (5´- TCTGGACCTGGTGAGTTTCC -3´). The PCR mixture consisted of 200 mM Tris-HCl pH 8.4, 2.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.4 μM of each primer, 0.2 μg of DNA and 1.0 U of Taq polymerase. The PCR system (Biometra®) was used for amplification. Initial denaturation, annealing and extension steps were performed at 95, 57 and 72°C, respectively. Analysis of the PCR products was performed by electrophoresis on 1.8% agarose gels using standard conditions and Hyperladder V marker.

The 18S rDNA product was extracted from agarose gel with the help of gel extraction kit (Fermentas). The PCR product was sent to Macrogen (USA) for sequencing. The alignment of the sequences was performed using the software BioEdit and taxonomic classification, as well as determination of the nearest neighbors by the NCBI database: *microbes*. Alignments (BLAST) were performed with each of the sequences obtained from the amplified 422 bp using the database "fungi-genomes" NCBI.

Partial enzymes purification

The enzymatic extracts obtained by submerged fermentation were filtered through Whatman No. 41 filter under vacuum. Then they were concentrated on Amicon Ultra-15 units (Millipore) containing the membrane for separation of 100 kDa proteins. The procedure was performed as follows: 15 mL of crude extract were placed in Amicon tube, which was centrifuged at 10,000 rpm, 4°C for 15 min. The extract obtained on the filter was separated to be applied in the subsequent assays. The protein concentration was detected by the Bradford method. The GOX and CAT activities were evaluated in all recovered samples as described above.

Partial enzymes characterization

Effect of pH on glucose oxidase and catalase activities was determined by assaying the enzymes as mentioned before with the difference that the activity was determined at different pH ranging from 1-8 using various buffer solutions as described by Tzonka et al. (2006). Temperatures ranging from 35-65°C, activation energy was determined from the Arrhenius plot. Thermal stability was evaluated applying activity assays, the enzymes pre-incubated at 50°C before activity measurements. To determine the kinetic parameters (V_{max} and K_m), the enzymatic reactions were carried out using different substrate (peroxide or glucose, respectively for catalase and glucose oxidase) at pH 7 and 6, respectively. The data was analyzed according to Lineweaver-Burk plot.

RESULTS

Different fungal strains were tested to select the fungi with characteristics of *A. niger*, as well as GOX and CAT production. The 6 strains of fungi that met the morphological characteristics of *A. niger* (Table 1) were selected due to the positive response on GOX and CAT qualitative tests.

Considering the evidence of GOX and CAT activity, submerged fermentation was carried out using selected strains. Kinetic data corresponding to the activities of both extracellular enzymes detected during this assay are shown in Figures 1 and 2. Both activities were detected from 12 h of fermentation, and were characterized by the presence of maximum values at the time different for each strain (Figures 1, 2 and Table 2).

The relation between catalase and glucose oxidase is significant for fungi due to hydrogen peroxide production

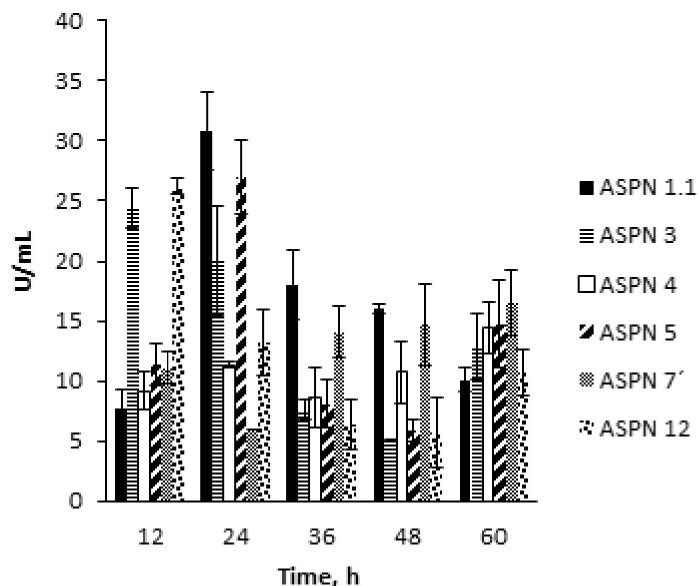

Figure 1. Glucose oxidase activity expressed by *Aspergillus niger* strains at different times of submerged fermentation.

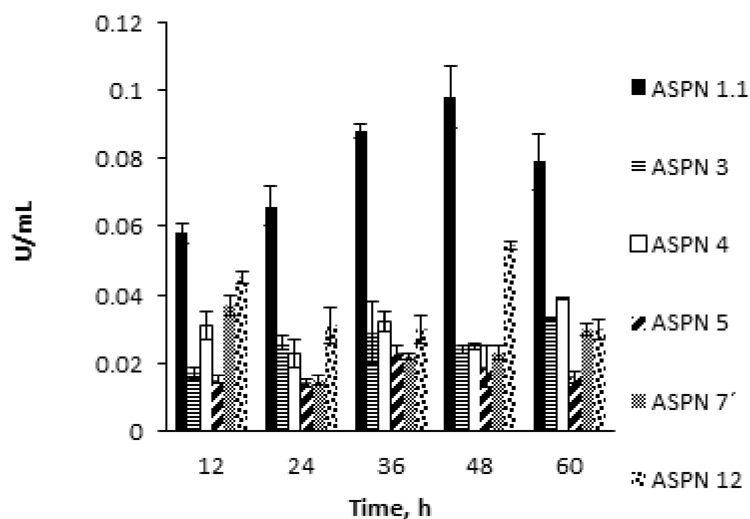

Figure 2. Catalase activity expressed by *Aspergillus niger* strains at different times of submerged fermentation.

Table 2. Comparison of enzyme extracts at the point of higher enzyme activity.

Strain	Time (h)	GOX volumetric activity (U/mL)	GOX specific activity (U/mg)	Time (h)	CAT volumetric activity (U/mL)	CAT specific activity (U/mg)
ASPN 1.1	48	0.10	0.8	24	30.83	560.5
ASPN 3	60	0.03	0.3	24	24.42	228.3
ASPN 4	60	0.04	0.6	60	14.46	241.0
ASPN 5	36	0.02	0.4	24	27.07	466.7
ASPN 7´	12	0.04	0.2	60	16.56	215.1
ASPN 12	48	0.05	0.4	60	26.24	430.2

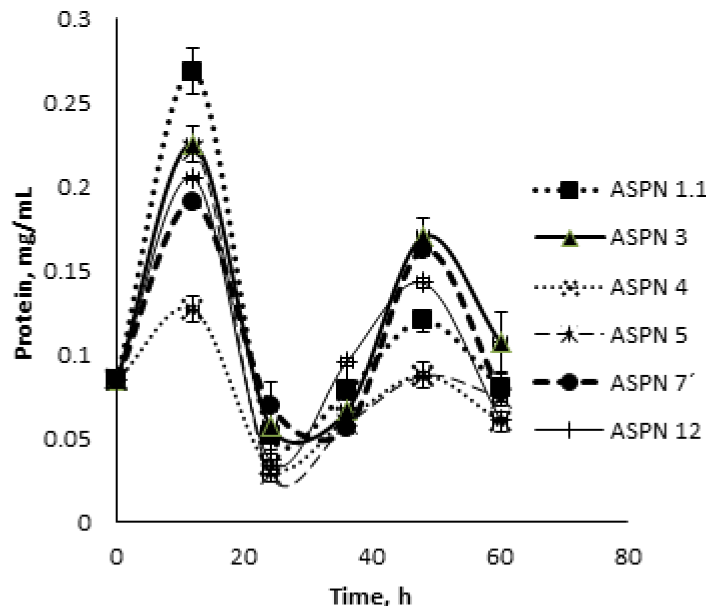

Figure 3. Protein concentration detected in culture medium during submerged fermentation of *A. niger* strains.

by GOX activity, the level of which is controlled by higher CAT activity (Singh and Verma, 2013). The CAT activity is considerably higher than GOX activity, even higher than that reported in some studies (Fiedurek and Gromada, 2000). However, in the case of three of six fungi (*A. niger* ASPN 1.1, ASPN 3 and ASPN 5), the maximum values of enzymatic activities were detected at different times: higher catalase activity was detected earlier than glucose oxidase (Figures 1, 2 and Table 2). Commonly, the maximum activities of both enzymes are detected at the same time as in the case of *A. niger* ASPN 4, or CAT appears later than GOX (Fiedurek and Gromada, 2000) as in the case of ASPN 7´and 12. This may be attributed to specific behavior of some xerophytic fungus *A. niger*, which can differ from common fungi in profile of enzyme excretion, as well as enzyme production.

The greatest GOX, as well as CAT activity was quantified in the case of *A. niger* ASPN1.1. In this case, the GOX maximum value of 0.1 U/mL was observed at 48 h, while CAT higher activity of 31 U/mL at 24 h of fermentation was observed.

For all studied strains, the change of proteins concentration was characterized by kinetic curves with two maximum values at 12 and 48 h. At 12 h, the higher concentration was detected for *A. niger* ASPN 1.1 strain, while at 48, for ASPN 5 and ASPN 7´strains (Figure 3). Using values of detected protein concentration, specific activity was calculated (Table 2). The comparison of characteristics of enzymes contained extracts obtained at the time corresponding to their maximum activity is shown in Table 2. The greater specific activities of GOX and CAT also were observed for strain ASPN 1.1.

Although the fermentation was performed using the same concentration of inoculum, quantity values of biomass recovered after 60 h fermentation were different. The strain ASPN 1.1 showed a similar level to the biomass quantized for strain ASPN 12, providing both higher detected values (Figure 4). Taking into account the higher values of CAT and GOX activities, the strain *A. niger* ASPN 1.1 was selected for subsequent assay, previously performing molecular confirmation of fungal species.

The identification of presumptive *A. niger* for strain ASPN 1.1 was performed by PCR using the primers (forward) (5'-TTAGCATGGAATAATRRAATAGGA-3') and (reverse) (5´- TCTGGACCTGGTGAGTTTCC-3´). The PCR products were visualized by electrophoresis and a 422 pb fragment was obtained (Figure 5A). The obtained sequence was compared to those reported at "Basic Local Alignment Search Tool" (BLAST, http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi/) (National Center for the Biotechnology Information, NCBI, USA) (Figure 5B). This analysis shows that the resulting fungus match the nucleotide sequence of *A. niger* (CBS 513.88 superconting An03, 18S ribosomal RNA) with an 99-100% identity.

Partial purification of crude extract was performed by means of ultrafiltration with 100 kDa filter to separate 99% of protein present in the sample. The enzymes were partially purified to a yield of 98 and 48%, and fold 9.8 and 5 corresponding to specific activity 589.60 and 0.60 U/mg, respectively for CAT and GOX (Table 3).

Glucose oxidase from *A. niger* was active within the pH

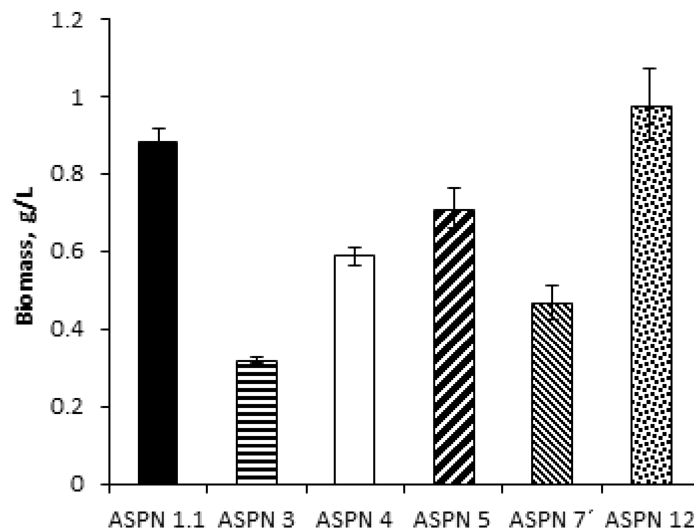

Figure 4. Biomass dry weight of *A. niger* strains after 60 h of submerged fermentation.

range of 1-7 and catalase of 1-8, while maximum activity was observed at pH 6.0 (Figure 6). The optimum temperature for glucose oxidase and catalase catalyzed reactions was found to be 50 and 45°C (Figure 7), and activation energies (E_a), calculated from initial part of obtained curves, were 32.33 and 73.5 kJ/mol, respectively.

Partially purified enzymes samples having a protein content of 0.48 mg/mL were used for the kinetic characterization (Figure 8). The K_m and V_{max} values obtained from Lineweaver-Burk plot for glucose oxidase were 4.24 mM and 3.2 M/min, and for catalase, 80 mM and 0.15 mM/min (Table 4).

Thermostability is the ability of enzyme to resist thermal treatment in substrate absence. GOX and CAT from xerophytic *A. niger* showed appreciable stability at 50°C (Figure 9). The linearization of kinetic curves in semi-logarithmic coordinates was applied to quantify inactivation constant and subsequently a half-life time, which were estimated as 0.007 and 0.008 1/min, as well as 99 and 87 min, respectively for GOX and CAT. Table 4 shows the data which compare between the enzyme from xerophytic *A. niger* and enzymes from some other sources.

DISCUSSION

Morphological characterization of studied fungi allowed the selection of *A. niger* strains. The conidia heads of all selected strains are characteristic to distinguish *A. niger* strains (Table 1). These heads are formed by conidiophores, vesicle, and a series of primary sterigma, followed by a second series of secondary sterigma of which the conidia sprout. The major morphological distinction of *A. niger* from other species of *Aspergillus* is the presence of carbon black or dark brown spores on biseriate phalides, which are arranged in a globule head radiating from a vesicle conidiophore.

A. niger is a filamentous fungus belonging to phylum Ascomycota. This fungus is commonly found in mesophilic environments such as soil, plants and enclosed air environments, but it is also capable of surviving in various environments: high or low temperatures, as well as humidity, etc. In the present work, xerophytic *A. niger* strains were studied.

A. niger fermentation is commonly accepted as safe by the United States Food and Drug Administration under the Federal Food, Drug and Cosmetic Act (Schuster et al., 2002) so the enzymes produced by *A. niger* can be used in food and medical industries without any objection. GOX and CAT are important enzymes due to a vast number of applications in various fields. The most important application of glucose oxidase is in diagnostics, as a part of colorimetric diagnostic kits for the determination of glucose in blood, serum or plasma. CAT is used in textile industry and environmental monitoring (Kim et al., 1994; Chun et al., 2008; Wang et al., 2008).

Submerged fermentation of selected fungi (Figures 1 and 2) led to obtain high activity levels for both extracellular enzymes. In the case of the *A. niger* strains ASPN 1.1, ASPN 3 and ASPN 5, the maximum activity of CAT was detected before the maximum activity of GOX, while for strains ASPN7´and ASPN12, was upside down, and in the case of *A. niger* ASPN 4, at the same time (Table 2). Production of more than one enzyme in one step suggestively increases the effectiveness and advances the process economy.

There are many reports on GOX and CAT production by fungal and bacterial strains (Federici et al., 1996;

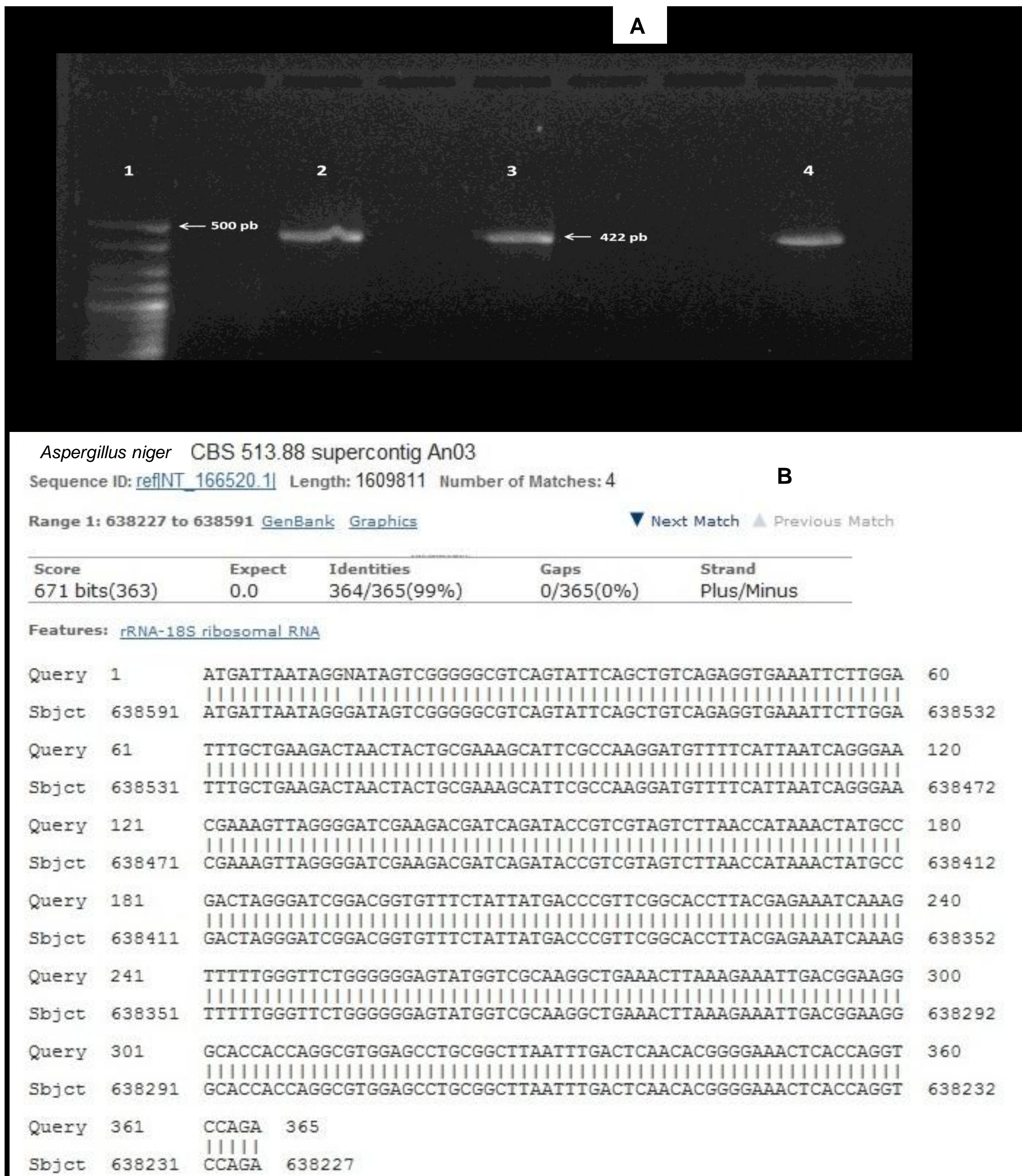

Figure 5. A) Agarose gel electrophoresis of PCR products amplified from DNA of *Aspergillus niger* ASPN 1.1: 1, molecular weight marker Hyperlader V; 2, 3, 4– PCR products from *A. niger* strain (repetitions). B) Sequence alignment of *A. niger* ASPN 1.1 (Query) with partial 18S rDNA sequence of *Aspergillus niger* (CBS 513.88 superconting An03) (Sbjct).

Fiedurek and Gromada, 2000; Wang et al., 2008), but few papers reported the simultaneous production of both enzymes, for example by submerged fermentation of *Penicillium variabile* P16 (Petruccioli et al., 1995), and with *A. niger* (Fiedurek and Gromada, 2000). The nature of the mechanisms responsible for the induction of enzymes synthesis has only been investigated in a few cases. For example, Fiedurek and Gromada (2000) reported that molecular oxygen increased expression of GOX and CAT probably at the transcriptional level, and

Table 3. Characteristics of partial GOX and CAT purification by ultrafiltration on 100 kDa filter (AMICON) assisted by centrifugation.

Fraction	GOX					CAT			
	Total activity (U)	**Total protein (mg)**	**Specific activity (U/mg)**	**Yield (%)**	**Fold**	**Total activity (U)**	**Specific activity (U/mg)**	**Yield (%)**	**Fold**
Crude extract	0.6	4.8	0.12	100	1	288.8	60.16	100	1
Fraction recuperated on 100 kDa filter	0.29	0.48	0.60	48	5	283.0	589.60	98	9.8

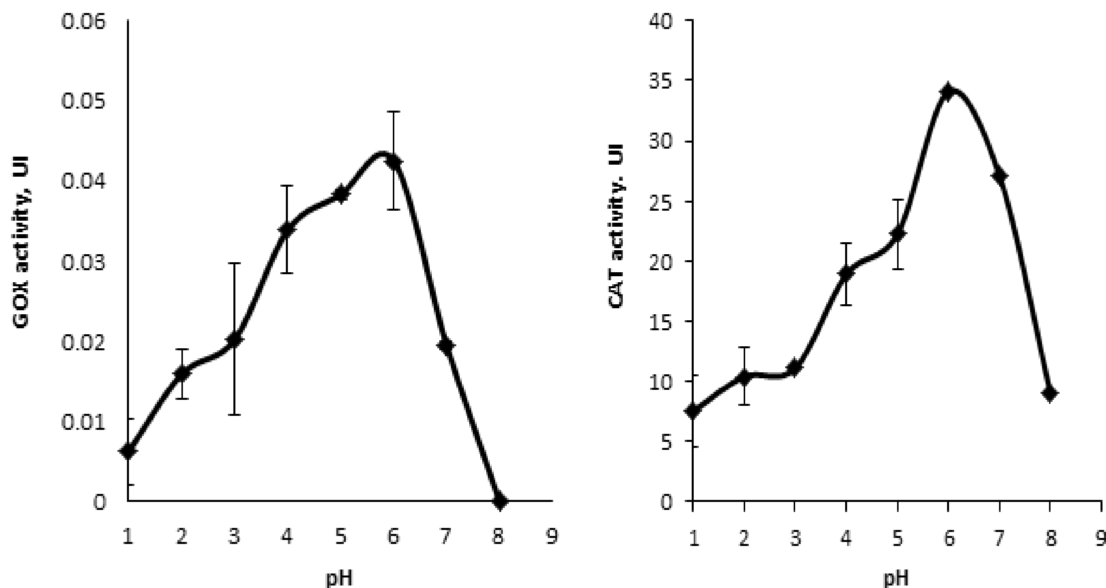

Figure 6. Effect of pH on activity of: left, - glucose oxidase; right, - catalase from xerophytic *Aspergillus niger.*

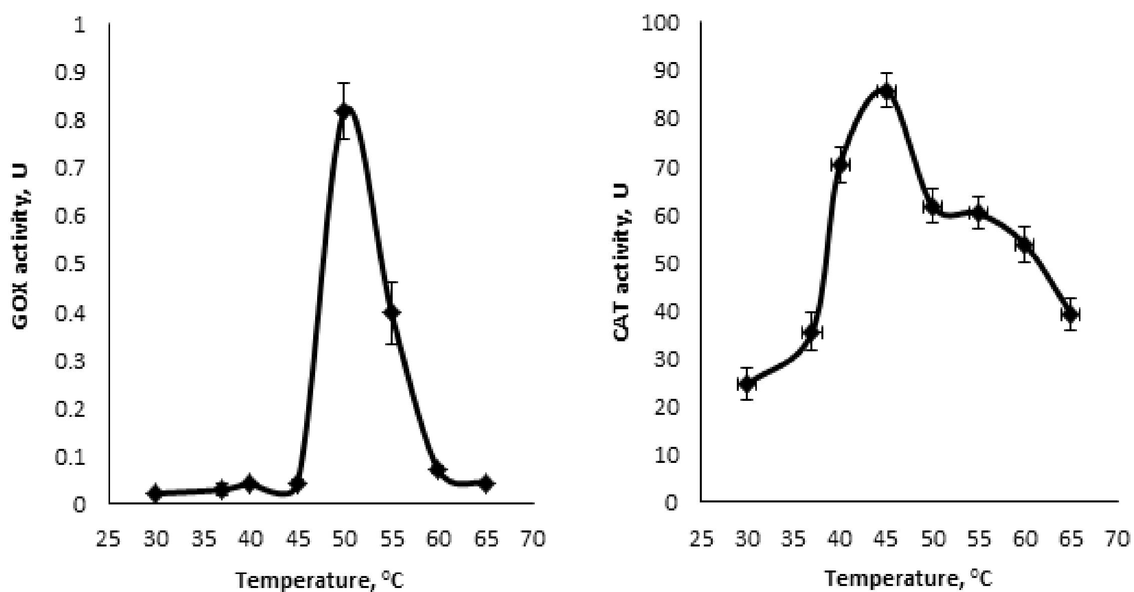

Figure 7. Effect of temperature on activity of: left, - glucose oxidase; right, - catalase from xerophytic *Aspergillus niger.*

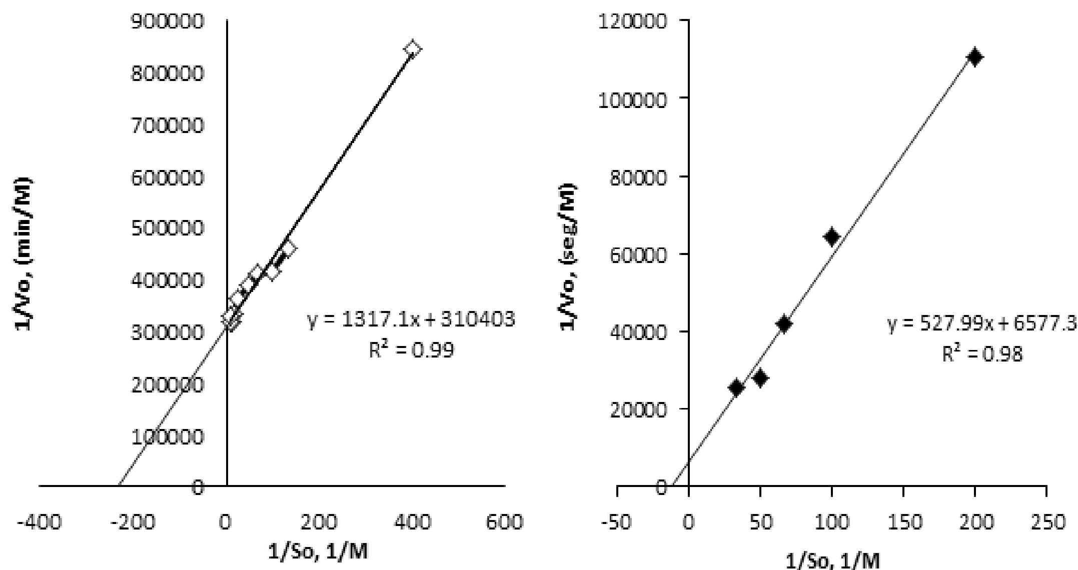

Figure 8. Linearization of Michaelis-Menten curve in Lineweaver- Burk coordinates: left, - for glucose oxidase; right, - for catalase.

Table 4. Comparison of various characteristics of partially purified glucose oxidase and catalase from xerophytic *A. niger* with other sources (the name of microorganism is noted when it is different from *Aspergillus* sp.)

Characteristics	GOX	GOX from other sources	CAT	CAT from other sources
Specific activity	0.6 U/mg	Obtained with ammonium sulfate at 0.75 U/mg (Singh and Verma, 2013)	589.6 U/mg	443.7 U/mg (Chandrashekar, 2011)
K_m	4.24 mM	10.3 mM (Garjonyte and Malinauskas, 2000) 25 mM (Bhatti et al., 2006) 2.56 mM (Zia et al., 2007) 5.7 mM (Zia et al., 2007) 10.5 mM H.N. (Bhatti and Saleem, 2009) 7.1 mM (Singh and Verma, 2013)	80.00 mM	599 mM (Switala and Leuwen, 2002) 127 mM (from *Proteus mirabilis*) (Lorentzen et al., 2006) 103.6 mM (from *Vibrio salmonicida*) (Lorentzen et al., 2006) 41.5 mM (from *Bacillus* sp. N2) (Wang et al., 2008) 29.7 mM (from *Serratia marcescens*) (Zeng et al., 2010) 10.5 mM (from *L. arborícola*) (Kapoor et al., 2013)
Optimum pH	6	5.5 (Bhatti et al., 2006; Zia et al., 2007) 5.4 (Bhatti and Saleem, 2009) 5.5-6 (Singh and Verma, 2013)	6	6 (Chandrashekar, 2011) 6.5 (Bayramoglu et al., 2011) 7-8 (from *L. arborícola*) (Kapoor et al., 2013)

that the metabolism of *A. niger* is changed from glycolysis to the pentose phosphate pathway after the addition of calcium carbonate. The tendency of higher levels of catalase activity in comparison with glucose oxidase, observed in the present study, was also reported previously (Fiedurek and Gromada, 2000).

The levels of activities detected in submerged fermentation were similar to those reported by Fiedurek and Gromada (2000) in the case of GOX, and considerably higher in the case of CAT. Moreover, the biomass generation (Figure 4) was significantly lower than in the case of *A. niger* mutants reported in literature

Table 4. Contd

Optimum temperature	50°C	40°C (Bhatti et al., 2006; Zia et al., 2007) 45°C (Bhatti and Saleem, 2009) 25-30°C (Singh and Verma, 2013)	45°C	20-50°C (from *Proteus mirabilis*) (Lorentzen et al., 2006) 30°C (from *Deinococcus radiodurans*) (Kobayashi et al., 2006) 0-10°C (from *Vibrio salmonicida*) (Lorentzen et al., 2006) 35°C (Senay et al., 2007) 25°C (from *Bacillus* sp.) (Wang et al., 2008) 20°C (from *Serratia marcescens*) (Zeng et al., 2010) 45°C (Bayramoglu et al., 2011) 45°C (Chandrashekar, 2011) 40-60°C (from *L. arborícola*) (Kapoor et al., 2013)
E_a	32.33 kJ/mol	15.46 kJ/mol (Zia et al., 2007) 44 kJ/mol (Ramos et al., 2011)	73.5 kJ/mol	11.28 kJ/mol (from *Scytalidium thermophilum*) (Kocabas et al., 2008)
k_{in} at 50°C **$\tau_{1/2}$**	0.007 min^{-1} 99 min	30 min (Bhatti et al., 2006) 0.012 min^{-1} and 58 min at 52°C (Zia et al., 2007) 0.015 min^{-1} and 46.2 min (Bhatti and Saleem, 2009) 60 min at 50°C (Singh and Verma, 2013)	0.008 min^{-1} 87 min	After 45 min at 60°C retained 20% (from *Klebsiella pneumonia*) (Goldberg and Hochman, 1989) After 5 min at 60°C had 0% of activity (from *Streptomyces coelicolor*) (Kim et al., 1994) After 15 min at 60°C had 0% of activity (from *Vibrio rumoiensis* S-1T) (Yumoto et al., 2000) After 5 min at 60°C retained 50% of activity (from *Vibrio salmonicida* LFI1238) (Lorentzen et al. 2006) After 50 min at 60°C retained 50% (from *Proteus mirabilis*) (Lorentzen et al., 2006) After 30 min at 60°C retained 30% (from *Deinococcus radiodurans*) (Kobayashi et al., 2006) After 15 min at 60°C retained 12% (from *Bacillus* sp.) (Wang et al., 2008)

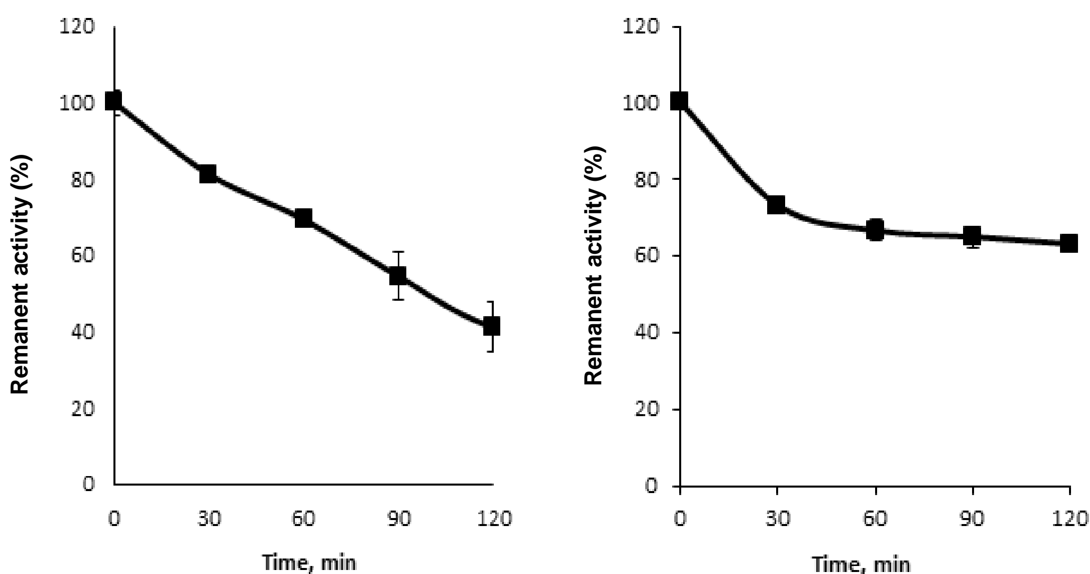

Figure 9. Kinetics of thermal denaturation of: left, - glucose oxidase; right, - catalase from xerophytic *Aspergillus niger* during enzyme pre-incubation at 50°C.

(Fiedurek and Gromada, 2000). However, these authors studied intracellular enzymes (Fiedurek and Gromada, 1997), while in the present study, the xerophytic *A. niger* strains, which produced extracellular GOX and CAT (Table 2), were investigated. The literature data (Chaouche et al., 2005) confirmed that in different fungal cultures, the excretion of catalase into the submerged culture began around 20 h after inoculation and increased as the time progressed, moreover the excretion was preceded by the intracellular catalase activity. GOX production from *A. niger*, for which excretion kinetics have been reported (Pluschkell et al., 1996), showed the presence of a signal peptide confirming that the glucose oxidase (GOD) is actively secreted in the culture medium. Chaouche et al. (2005) suggested that the intra- and extracellular catalase production was related neither to the fungal biomass nor to the size of pellet. However, it was demonstrated that this production may be directly related to the external layer of the pellet and precisely to the morphology of the hyphae in this region, and that secretion of proteins is primarily associated with the apical and subapical regions, called active region (Wongwicharn et al., 1999; Ramos et al., 2011).

As was mentioned above, the strain ASPN 1.1 was selected due to its greater activities levels in comparison with other studied strains. As compared to results obtained in the present study (Figures 1 to 4); in various previous reports, the *A. niger* strains produced extracellular GOX and CAT (Ojeda et al., 2011; Zoghbia et al., 2008) with lower activity, higher protein concentration and higher weight of generated biomass.

The *r*DNA identification (Figure 5) confirmed that the selected strain is *A. niger*. Genomic DNA sequence of *A. niger* strain CBS 513.88, its annotation and an initial gene expression study as well as genetic maps are described by Pel et al. (2007) and Arnaud et al. (2012).

The enzymes partial purification (Table 3) demonstrated that the molecular weight of GOX and CAT is higher than 100 kDa, because greatest part of both enzymes was concentrated in the filter with pores allowing separation of proteins with lower molecular weight. Various literature data report that molecular weight of GOX produced by fungi is around 160 kDa, while for CAT, is 210-280 kDa (Kirman and Gaetani, 1984; Singh and Verma, 2013).

The optimum pH of GOX (Figure 6) is slightly higher than that reported for other glucose oxidases from *A. niger*, while for CAT, is similar or slightly lower than the one reported for enzymes from different microbiological sources (Table 4). The effect of pH on enzyme activity is related to the ionization of essential active site amino acid residues, which participate in substrate binding and catalysis. Our results is consistent with those reported by Weibel and Bright (1971), who defined that GOX is working in the pH range of 4-7, and by Chandrashekar (2011), who demonstrated that pH optimum of catalase form *Aspergillus* sp. is equal to 6.

The plot (Figure 7) describes the effect of temperature on CAT and GOX activities; it is obvious that both enzymes had a single conformation up to transition temperature. The optimum temperatures are superior to the enzymes from various other sources (Table 4). This becomes more evident when comparing with bacterial catalase, as well as with various fungal GOX (Table 4). However, the E_a values are greater than that reported for enzymes from some sources that may be related to difference of enzymes structures.

Regarding properties of biotechnological relevance (Figure 8), the GOX of *A. niger* ASPN 1.1 exhibited a high affinity for D-glucose as it has low *K*m value for the substrate as compared to a high *K*m value of enzymes isolated from the *A. niger* reported earlier (Table 4). However, the K_m value quantified for CAT is higher in various cases than reported for enzymes from alternative sources (Table 4). Other important characteristic of studied enzymes is their high thermostability that is appreciated in lower values of inactivation constant and higher half-life time (Figure 9 and Table 4). The advantage that CAT from *A. niger* ASPN 1.1 has in comparison with enzymes from some bacterial sources (Table 4) is evident. High substrate affinity and specificity, in addition to its long-term stability at relatively high temperature, proved enzymes of *A. niger* ASPN 1.1 as a suitable biocatalyst for wide applications.

Thus, in the present study, different xerophytic *A. niger* strains were assayed for the presence of GOX and CAT activities. The strain with higher activity was selected to confirm its identification and to be used for enzymes partial purification and characterization. Partial purification was performed by means of ultrafiltration with 100 kDa filter. The enzymes showed high affinity for substrates, exhibited optimum catalytic activity at pH 6 and optimum temperature at 50 and 45°C, for GOX and CAT, respectively. Both enzymes showed a high thermal stability at 50°C having a half-life of nearly 90 min. These properties suggest the use of catalase and glucose oxidase from xerophytic *A. niger* strain for wide industrial, clinical and biochemical applications. However, further studies are required to optimize the fermentation process and obtain higher enzymatic activities.

Conflict of Interests

The authors have not declared any conflict of interest.

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Full Length Research Paper

Biological characterization and determination of comparative efficacy of an inactivated Newcastle disease virus vaccine prepared from velogenic strain

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This study was designed to characterize a field isolate of Newcastle disease virus (NDV) to check its suitability as an inactivated vaccine and to determine the comparative efficacy of this inactivated NDV vaccine with conventional live vaccines, Baby Chick Ranikhet Disease Vaccine (BCRDV, F strain) and Ranikhet Disease Vaccine (RDV, Mukteswar strain). The field isolate of NDV was identified as velogenic strain based on the pathogenicity indices {mean death time (MDT), intracerebral pathogenicity index (ICPI) and intravenous pathogenicity index (IVPI)}. Formalin precipitated inactivated NDV vaccine was prepared from the field isolate and adjuvanted with aluminium hydroxide. The comparative efficacy of three distinct vaccination schedules (Group A, inactivated vaccine three times only; group B, BCRDV followed by inactivated and group C, BCRDV followed by RDV) was then evaluated on the basis of haemagglutination inhibition (HI) antibody titre of sera samples collected at day 4, 27, 55 and 72 of post vaccinated chicks. The prepared inactivated vaccine with the field isolate induced satisfactory level of antibody following vaccination in chicken. Combined vaccination in chickens with live BCRDV followed by inactivated NDV vaccine induced better immune response than the live or inactivated NDV vaccine alone. The antibody titre though differed significantly (*P* <0.05, group B versus group C at 55- and 72-days and group B versus group A at 72 days) in the chickens among the vaccinated groups, the protection rate was 100% in all groups following virulent challenge infection.

Key words: Newcastle disease virus, vaccine, velogenic, haemagglutination inhibition (HI), antibody.

INTRODUCTION

Newcastle disease (ND) also known as Ranikhet disease (RD), is an acute infectious and contagious viral disease of birds that has a worldwide distribution including Bangladesh and has a serious economic impact on poultry

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production (Biswas et al., 2009). The factors that affect the disease may be host, species, age, immune status, infection with other organisms and environmental stress (Cheville et al., 1972; Lancaster, 1981; Campbell, 1986). The disease is characterized by sudden appearance and rapid spread within the flock with high morbidity and mortality. It may cause 100% mortality in young chickens and 80-90% in adult chickens (Brandly, 1950; Chowdhury et al., 1982). Newcastle disease is endemic in Bangladesh with prevalence of viscerotropic velogenic strains (Chowdhury et al., 1982; Islam et al., 2003).

The etiologic agent of ND is a member of the avian paramyxoviruses (APMV), which belongs to the *Avulavirus* genus and *Paramyxoviridae* family of the order *Mononegavirales* (Abolnik et al., 2004; Pedersen et al., 2004). Paramyxoviruses isolated from avian species have been grouped into nine serotypes (APMV-1 to APMV-9), and Newcastle disease virus (NDV) is referred to as APMV-1 (Alexander, 2003). Strains of NDV are categorized into five pathotypes according to the clinical signs observed in infected chickens: (i) viscerotropic velogenic, (ii) neurotropic velogenic, (iii) mesogenic, (iv) lentogenic and (v) asymptomatic (Alexander, 2004).

Vaccination for ND is routinely practiced in countries where virulent strains of the NDV are endemic (Xiao et al., 2012). It has been practiced to control and prevent the ND from Bangladesh mainly by live NDV vaccine produced by Livestock Research Institute (LRI), Mohakhali, Dhaka, Government of the Peoples Republic of Bangladesh (two types of vaccines, BCRDV by F strain for baby chick and RDV by M strain for adult chicken). However, none of these strains were found to be used in controlling ND completely. Sometimes, reports on severe outbreak of ND are made even after vaccination of chicken with these live vaccines prepared from mesogenic and lentogenic (not local) strains and velogenic strain of NDV has been isolated from most of the outbreaks (Saha et al., 1998). The possible causes of outbreaks of ND in immunized flock were interfered by presence of maternal antibody and antigenic variation among the vaccine strains and field strains (Zhuo et al., 1998). Use of live vaccines in immuno-suppressed chicks is risky and conversely may produce disease. In Bangladesh, more than 70% rural households are involved in poultry keeping, where village vaccinators have been trying in going round villagers in their area vaccinating the family poultry and achieving high degree coverage. On the other hand, there are varieties of mode and means of transport and variation in maintenance of cooling system (4-8°C) at all stages of District, Thana, Union, Village and farm level in Bangladesh. This is one of the important reasons of vaccination failure causing economic loss to the farmers. To overcome these problems inactivated NDV vaccine is of growing interest and might be an efficient alternative which is already been practiced in many countries of the world (OIE, 2009). It has thus become important to isolate, identify and characterize the NDV, selection of a suitable vaccine strain and preparation of prophylactic agent from the selected isolate that would not adversely affect the maternal immunity as live vaccine and can be used in day-old chicks (Box et al., 1976). The present study was undertaken to characterize a local field isolate of Newcastle disease virus and to determine the comparative efficacy of inactivated Newcastle disease virus vaccine prepared from velogenic strain with conventional live Newcastle disease virus vaccines.

MATERIALS AND METHODS

Study area and duration

The present research work was carried out during the period of January to May 2012 in Virology Laboratory and in the Experimental Poultry Sheds of the Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh.

NDV isolate

Newcastle disease virus isolate (NDV/MBD/8/2012) was obtained from the repository of the Virology Laboratory, Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh as per record of where the virus was isolated from a natural outbreak and was identified as NDV using polyclonal sera raised in chicken against reference strains of NDV.

Reference NDV

Mukteswar, a Mesogenic strain of NDV was used as antigen in HI tests. Velogenic strain of NDV (NDV/DBD/1/2008) was used for challenge infection. The viruses were obtained from the repository of the virology laboratory, Department of Microbiology and Hygiene, Bangladesh Agricultural University (BAU), Mymensingh-2202.

NDV live vaccine

Lyophilized baby chick Ranikhet disease vaccine (BCRDV, F-strain) and Ranikhet disease vaccine (RDV, Mukteswar) produced by Livestock Research Institute (LRI) Mohakhali, Dhaka, Department of Livestock Services (DLS), Government of the People’s Republic of Bangladesh, were collected from the LRI, Dhaka.

Experimental chicks

A total of forty eight (48) day old Layer BB300 chicks were obtained from the Phenix Hatchery Ltd. Gazipur. The chicks were reared with feed and water *ad libitum* for 12 weeks maintaining strict biosecurity and in a well-ventilated experimental poultry shed of the Department of Microbiology and Hygiene, BAU, Mymensingh according to university animal care and use guidelines.

Pathogenicity indices of field isolate of NDV

The mean death time with the minimum lethal dose (MDT/MLD) was determined with 9-day-old chicken embryos following the procedure described by Hanson and Brandly (1955). The intracerebral pathogenicity index (ICPI) with day-old chicks was

determined following the method described in OIE (2009). The intravenous pathogenicity index (IVPI) with 6-week-old chickens was determined following the method described by Alexander (1998).

Titration and inactivation of the field isolate of NDV

The infectivity titre of NDV was determined by inoculating serial 10-fold dilutions (10^{-1} to 10^{-10}) of virus in the form of allantoic fluids, into embryonated chicken egg (ECE). The end point titre was expressed as 50% embryo lethal dose (ELD_{50}) per ml as calculated by the method described by Reed and Muench (1938). Infective allantoic fluid (AF) containing field isolate of NDV having ELD_{50} $10^{8.7}$/1 ml of AF was inactivated by treating with formalin at final concentration of 1:1000 following the method described by Koppad et al. (2011). Inactivation of NDV was checked by two serial passages in ECE resulting in live embryos with no HA activity in their allantoic fluids.

Preparation of inactivated NDV vaccine

The inactivated NDV vaccine was prepared following the method described by Tizard (1996). Briefly, after proper inactivation of the virus, aluminium hydroxide (AH) was added as adjuvant at the rate of 0.6 ml/1 ml of properly inactivated NDV vaccine (According to LRI) and was mixed properly by vortex machine.

Sterility and safety test of the inactivated NDV vaccine

The sterility and safety test of the inactivated NDV vaccine was done according to the OIE (2009) by using blood agar media and six 5-day-old chicks, respectively.

Experimental design

A total of forty eight (48) day-old Layer BB300 chicks were collected from the Phenix Hatchery Ltd. and used for this experiment. The chicks were divided into four (4) groups each containing 12 chicks viz group A, B, C and D. Chicks of different groups were vaccinated following three distinct vaccination schedules. Chicks of group A were immunized thrice with inactivated NDV vaccine through intramuscular (i/m) route at 5-, 28- and 56-days of age at the dose rate of 0.25, 0.5 and 1 ml/ bird, respectively. Chicks of group B were immunized with BCRDV through intraocular (i/o) route at 5 days of age at dose rate of 1 drop/bird and with inactivated NDV vaccine through i/m route at 28- and 56-days of age at the dose rate of 0.5 ml and 1 ml/bird respectively. Chicks of group C were immunized with BCRDV through i/o route at 5- and 28-days of age at dose rate of 1 drop/bird and with RDV through i/m route at 56 days of age at the dose rate of 1 ml/bird. Chicks of group D were kept as unvaccinated control. The comparative efficacy of the three vaccination schedule was then evaluated by measuring HI antibody titre of sera samples collected at day 4, 27, 55 and 72 of chicks.

Haemagglutination inhibition (HI) test

The HI test was performed following the method described by Anon (1971) to determine the HI antibody titre of the sera samples collected from all groups of vaccinated chickens at different intervals following administration of ND vaccines. The HI titre of sera samples of control group of chicks was also determined to measure the maternal antibody and its persistence. The test was conducted by using constant 4 HA unit antigen and decreasing serum method (beta-procedure). Briefly, 50 µl PBS was taken in all the wells of 96 well plate. Then 50 µl of heat inactivated (56°C for 30 min) serum was taken in the first well of the respective row and a two-fold serial dilution of the serum was prepared. Then, 50 µl of antigen suspension containing 4 HA units was added into all well except the last well of the row and mixed thoroughly. The last well of respective row was kept as control for respective sample. The serum antigen mixture was then incubated for 30 min at room temperature. Then 50 µl of 0.5% cRBC suspension was added into all well. Then the mixture was again kept at room temperature for 45 to 60 min. A compact mass of sediment cells covering the bottom of the plate was considered as positive for HI. Serum end point was determined as the highest dilution of serum, which inhibited the agglutination of the RBC in the test. The HI titre of each serum corresponded to reciprocal of highest original dilution of serum inhibiting agglutination of cRBC completely.

Protection test

Sixteen days after final immunization, the chickens were challenged through intranasal (i/n) route with 0.1 ml of allantoic fluid containing $2ELD_{50}$ of NDV which correspond to about 100% mortality in chickens of 10 weeks of age (Sarkar et al., 2012).

Analysis of data

All data (HI antibody titre) were expressed as mean ± SE and difference serum antibody titres among the groups of chickens were compared using one-way ANOVA. Statistical analysis was performed using SPSS software version 17 and significance level was set at $P \leq 0.05$. Survival rate among the different groups after challenge were analyzed by Mantel-Cox log rank test.

RESULTS

Pathogenicity indices

The results of the mean death time (MDT), intracerebral pathogenicy index (ICPI) and intravenous pathogenicity index (IVPI) of the field isolate of NDV were found to be 57.6, 1.81 and 2.69 h, respectively and values indicate the isolate is velogenic.

Sterility and safety test of the inactivated NDV vaccine

The inactivated NDV vaccine was found to be sterile as no bacterial growth was observed in inoculated blood agar media incubated at 37°C for 48 h. Following inoculation in naive chicken, the inactivated NDV vaccine was found safe as the chick showed no significant effects after administering double dose of prepared inactivated NDV vaccine through intramuscular (i/m) route and subcutaneous (s/c) route.

HI antibody titre of vaccinated and unvaccinated control chickens

The HI antibody titres (mean ±SE) of vaccinated chickens

Table 1. Comparative HI antibody titer of chickens of vaccinated groups and unvaccinated control, Group D.

Chickens groups	Age of chickens			
	Day 04	Day 27	Day 55	Day 72
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
Group A (Vaccinated with inactivated NDV vaccine)	77.33±9.20	85.33±9.09	181.33±19.03	469.33±28.77
Group B (Vaccinated with BCRDV and inactivated NDV vaccine)	69.33±8.66	90.67±9.51	213.33±18.19	597.33±57.53
Group C (Vaccinated with BCRDV and RDV)	72.00±8.00	96.00±9.65	160.00±16.71	490.67±21.33
Group D (Unvaccinated control)	85.33±9.09	50.67±4.76	26.67±2.27	4.33±0.33

Chickens of group A were immunized thrice with inactivated NDV vaccine through i/m route at 5-, 28- and 56-days of age. Chickens of group B were immunized with BCRDV through i/o route at 5 days of age and with inactivated NDV vaccine through i/m route at 28- and 56-days of age. Chickens of group C were immunized with BCRDV through i/o route at 5- and 28-days of age and with RDV through i/m route at 56 days of age. Chickens of group D were kept as unvaccinated control. Serum was collected from chickens of all vaccinated groups (Group A, B and C) and unvaccinated control group (Group D) at 4-, 27-, 55- and 72-days of age. Serum antibody titers of chickens against different NDV vaccines and control birds were determined by HI test. The table shows the mean ± SE values of serum HI antibody titre (n=12 chickens/group), where SE = Standard error, HI = Haemagglutination inhibition.

and unvaccinated control group are presented in Table 1. It was observed that serum antibody titre of chickens of all vaccinated group (Group A, B and C) was gradually increased up to 72 days of birds following primary, secondary and tertiary vaccination (Table 1). On the other hand, serum antibody titre (Maternal antibody titres) of chickens of unvaccinated control group D decreased gradually (Table 1).

Protection test

The results of the protection test is presented in Figure 4, All the chickens exhibited no signs of illness and survived after challenge infection throughout the period of observation of 10 days. On the other hand, all the control chickens challenged at the same day started to show typical clinical signs of ND such as gasping, greenish-dark watery diarrhoea, drowsiness, dropping of wings etc from 2nd day onward and started to die from day 3 onwards. Dead birds showed typical postmortem lesions on ND such as hemorrhage in the trachea, proventriculus and ulceration of intestinal mucosa. Based on the protection test, it appeared that chickens of vaccinated groups (Groups A, B and C) conferred 100% protection following virulent challenge (Figure 4).

DISCUSSION

The results of the MDT, ICPI and IVPI of the field isolate of NDV were found to be 57.6 h, 1.81 and 2.69, respectively. These findings suggest that the isolate belong to velogenic group and correlated with the criteria mentioned by several investigators (Adi et al., 2010; Munir et al., 2012; Courtney et al., 2013).

The mean ± SE of HI antibody titre of chickens of group C at 27- (22 days after primary vaccination) and 55-days (27 days after secondary vaccination) of age (Table 1 and Figure 1) showed that serum antibody level of chickens (Group C) following primary vaccination did not show any impetuous production of HI antibody. This lower level of antibody production was due to presence of high level of maternally derived antibody (MDA) as the chicks used in this study had comparatively high MDA (Sarkar et al., 2012; Cornax et al., 2012; Kapczynski et al., 2012). On the other hand, HI antibody titre of chickens after secondary vaccination increased significantly (P <0.01, day 27 versus day 55) and similar finding were reported by other investigators (Sarkar et al., 2012; Kafi et al., 2003; Shuaib et al., 2003). The mean ±SE of HI titre at 72 days of age (16 days after tertiary vaccination) of chickens of group C (vaccinated with RDV) indicated that serum antibody level of chickens (Group C) was increased significantly (P <0.001, day 55 versus day 72) following tertiary vaccination at 56 days of age (Sarkar et al., 2012; Banu et al., 2009; Chowdhury et al., 1981).

The mean ±SE of HI antibody titres of chickens of group A (Table 1 and Figure 1) vaccinated thrice with inactivated NDV vaccine through i/m route at 5-, 28- and 56-days of age were increased from day 4 to day 72 of age and the titre was increased significantly at 55- and 72-days (P <0.001, day 27 versus day 55 and day 55 versus day 72) of age. These HI antibody titres of chickens of group A is mostly similar to HI antibody titre of the chickens of group C (Fan et al., 2012; Iqbal et al., 2003 and Rajeswar and Masillamoni, 2002).

The mean ±SE of HI antibody titres of chickens of group B (Table 1 and Figure 2) vaccinated once with

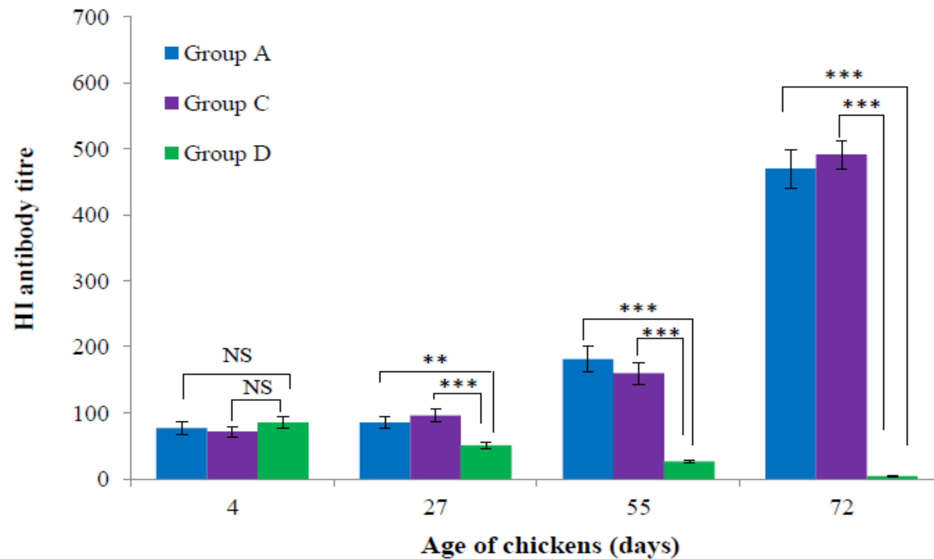

Figure 1. Comparative HI antibody titre of vaccinated groups (A and C) and unvaccinated control (D). Chickens of group A were immunized thrice with inactivated NDV vaccine through i/m route at 5-, 28- and 56-days of age. Chickens of group C were immunized with BCRDV through i/o route at 5- and 28-days of age and with RDV through i/m route at 56 days of age. Chickens of group D were kept as unvaccinated control. Serum was collected from chickens of vaccinated groups (Group A and C) and unvaccinated control group (Group D) at 4-, 27-, 55- and 72-days of age. Serum antibody titers of chickens against different NDV vaccines and control birds were determined by HI test. The graph shows the mean ± SE values of serum HI antibody titre (n=12 chickens/group). NS = Non significant, ***P* <0.01 and ****P* <0.001 by one-way ANOVA.

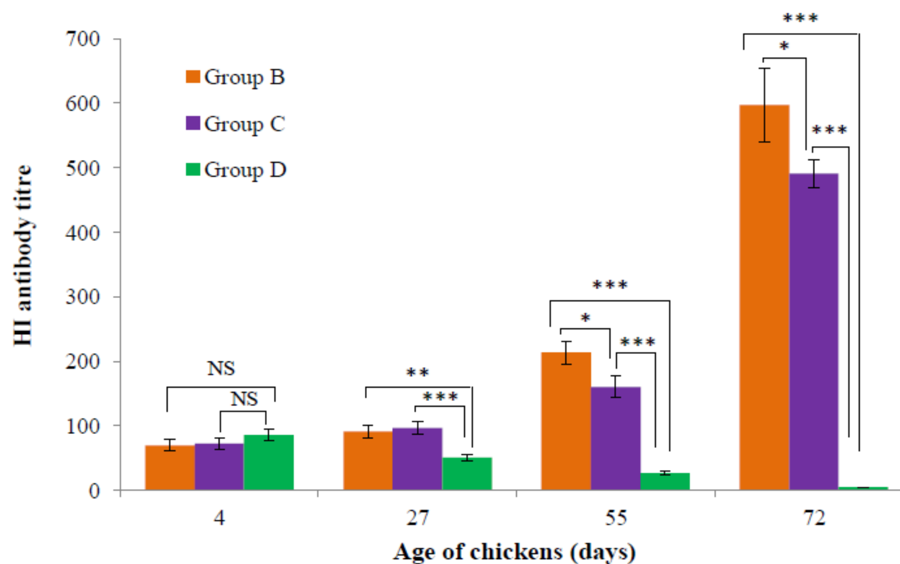

Figure 2. Comparative HI antibody titre of vaccinated groups (B and C) and unvaccinated control (D). Chickens of group B were immunized with BCRDV through i/o route at 5 days of age and with inactivated NDV vaccine through i/m route at 28- and 56-days of age. Chickens of group C were immunized with BCRDV through i/o route at 5- and 28-days of age and with RDV through i/m route at 56 days of age. Chickens of group D were kept as unvaccinated control. Serum was collected from chickens of vaccinated groups (Group B and C) and unvaccinated control group (Group D) at 4-, 27-, 55- and 72-days of age. Serum antibody titers of chickens against different NDV vaccines and control birds were determined by HI test. The graph shows the mean ± SE values of serum HI antibody titre (n=12 chickens/group). NS =Non significant, **P* <0.05, ***P* <0.01 and ****P* <0.001 by one-way ANOVA.

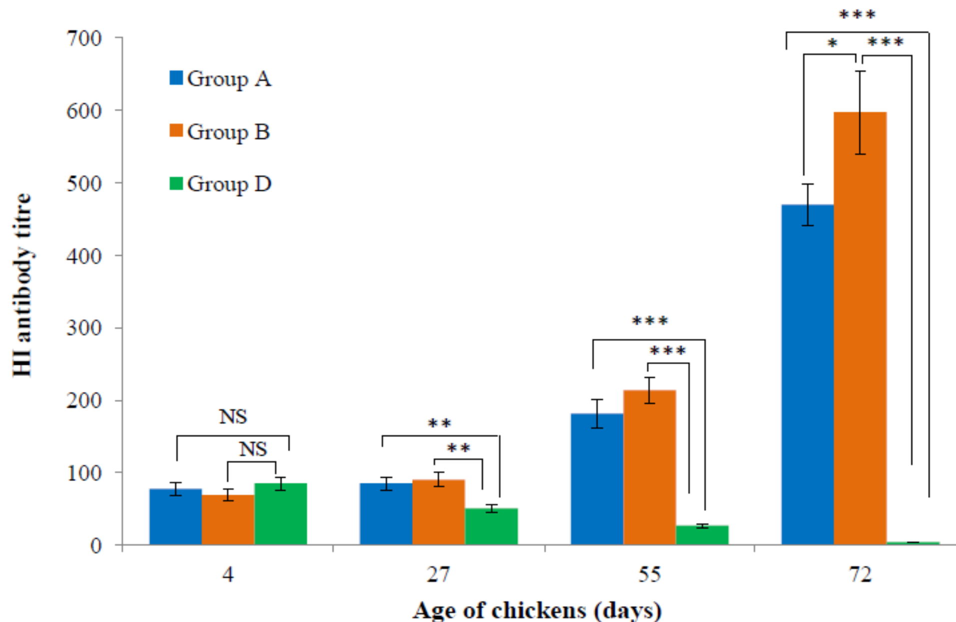

Figure 3. Comparative HI antibody titre of vaccinated groups (A and B) and of unvaccinated control (Group D). Chickens of group A were immunized thrice with inactivated NDV vaccine through i/m route at 5-, 28- and 56-days of age. Chickens of group B were immunized with BCRDV through i/o route at 5 days of age and with inactivated NDV vaccine through i/m route at 28- and 56-days of age. Chickens of group D were kept as unvaccinated control. Serum was collected from chickens of vaccinated groups (Group A and B) and unvaccinated control group (Group D) at 4-, 27-, 55- and 72-days of age. Serum antibody titers of chickens against different NDV vaccines and control birds were determined by HI test. The graph shows the mean ± SE values of serum HI antibody titre (n=12 chickens/group). NS =Non significant, **P* <0.05, ***P* <0.01 and ****P* <0.001 by one-way ANOVA.

BCRDV at 5 days of age through i/o route and twice with inactivated NDV vaccine through i/m route at 28 and 56 days were increased from day 4 to day 72 of age and the titre was increased significantly at 55 (*P* <0.01, day 27 versus 55 day) and 72 days (*P* <0.001, day 55 versus day 72) of age. But the HI titres of the chickens of group B were significantly higher (*P* <0.05) than the chickens of group A (vaccinated thrice with inactivated NDV vaccine) at 72 days of age and group C (vaccinated twice with BCRDV and once with RDV) at both 55- and 72-days of age (Figures 1 and 3). This result strongly supported the previous results of several scientists (Barbour et al., 2011; Wanasawaeng et al., 2009; Kafi et al., 2003). This result indicated that chickens vaccinated primarily with live NDV vaccine followed by inactivated NDV vaccines produce strong immune response.

To investigate the persistence of maternal antibody for any possible interference or hindrance to the vaccinates that might have occurred, sera samples obtained from the chickens of unvaccinated control group D at 4-, 27-, 55- and 72-days of age were analyzed. It was observed that the HI antibody titres of chickens of unvaccinated control group gradually decreased from day 4 to day 76 of age (Sarkar et al., 2012; Kai et al., 2012). Two live vaccines (BCRDV and RDV) are being used to control Newcastle disease in Bangladesh. Chicks having high maternal antibody titer interfered with the immune response when vaccinated with BCRDV (Sarker et al., 2012) so live vaccine (BCRDV) followed by live vaccine (RDV) do not induce strong antibody response. So farmers are using imported killed NDV vaccines which are expensive and urging to develop a killed vaccine which might be economic and save the growing poultry industry from Newcastle disease in Bangladesh. Inactivated vaccines were not imported, except farms rearing parent stock commercially. The benefit of using inactivated vaccine followed by live vaccine result in high and long lasting immune response. With the increase of commercial poultry farms in Bangladesh the demand for inactivated vaccine is on the rise as well.

Chickens of all vaccinated (Group A, B and C) and unvaccinated group were challenged at 72 days of age (16 days after tertiary vaccination) with virulent field isolate of NDV. The chickens (Groups C) vaccinated with conventional live vaccines (BCRDV and RDV) provided 100% protection (Sarkar et al., 2012; Cornax et al., 2012). The chickens vaccinated thrice with inactivated NDV vaccine (Groups A) and vaccinated with BCRDV followed by inactivated NDV vaccine (Group B) also conferred 100% protection, similar to chickens of group C

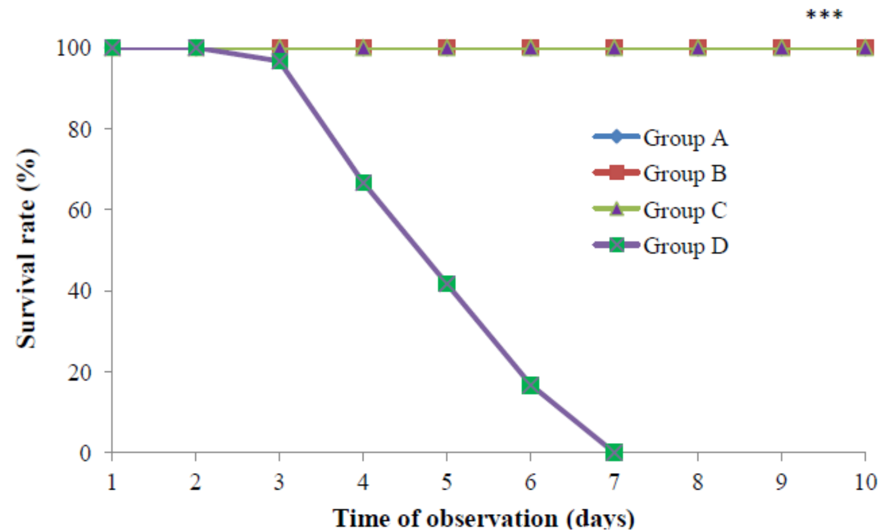

Figure 4. Survival rate of chickens of vaccinated groups (A, B and C) and unvaccinated group (D). Chickens of group A were immunized thrice with inactivated NDV vaccine through i/m route at 5-, 28- and 56-days of age. Chickens of group B were immunized with BCRDV through i/o route at 5 days of age and with inactivated NDV vaccine through i/m route at 28- and 56-days of age. Chickens of group C were immunized with BCRDV through i/o route at 5- and 28-days of age and with RDV through i/m route at 56 days of age. Chickens of group D were kept as unvaccinated control. Sixteen days (72 days of age) after final immunization (Tertiary vaccination) of chickens of all vaccinated group (Group A, B and C) and chickens of unvaccinated control group (Group D) were challenged with virulent field isolate of NDV at the rate of 0.1ml of $2ELD_{50}$ per bird intranasally and survival rate of chickens was observed for subsequent 10 days. (n=12 chickens/group). ****P* <0.001 by Mantel-Cox log rank test.

(Kai et al., 2012; Diaon et al., 1998). On the other hand, chickens of unvaccinated control group (Group D) had no protection capacity following virulent challenge infection (Figure 4).

From the above discussion it may be summarized that the field isolate of the NDV was velogenic and prepared inactivated vaccine from this isolate induced satisfactory level of antibody after vaccination in chicken. Combined vaccination in chickens with live (BCDRV) vaccine followed by inactivated NDV vaccine induced better immune response than the live or inactivated NDV vaccine alone. Although the antibody titre differs significantly (*P* <0.05, group B versus group C at 55- and 72- days and group B versus group A at 72 days) in the chickens among the vaccinated groups, the protection rate was 100% in all groups following virulent challenge infection.

Conclusions

Inactivated NDV vaccine prepared from velogenic strain of NDV provided similar protection as live NDV vaccine. So, inactivated NDV vaccine may be used for the prevention and control of ND in Bangladesh and vaccination program against ND should be rescheduled with live BCRDV vaccine followed by inactivated NDV vaccine.

Conflict of Interests

The authors have not declared any conflict of interests.

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