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

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Full Length Research Paper

Sociodemographic, microbial and phylogentic studies of *Mycobacterium tuberculosis* cases diagnosed in El-Minia governorate, Egypt

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Egypt is considered one of the high-burden countries in WHO's Eastern Mediterranean region. This study determined the sociodemographic, microbial and phylogentic characteristics for *Mycobacterium tuberculosis* cases diagnosed in El-Minia governorate in upper Egypt. Fifty clinical samples were collected (male-to-female sex ratio 1.5 and 50% within the 21- to 40-years old) with complete history of patients; samples were cultured and identified by biochemical tests. DNA was extracted and tested for plc gene. Seven isolates were subjected to sequencing analysis of the amplified *plc* fragment and a phylogentic tree for these strains was formed. Out of 50 samples, 24 samples were culture positive, all grown isolates were identified as *M. tuberculosis* according to physical characters, rate of growth, biochemical tests. Direct polymerase chain reaction (PCR) method based on the amplification of 350 bp region of *plc gene* was used for the detection of *M. tuberculosis*, to differentiate it from *Mycobacterium bovis* in clinical samples. It was found that all isolates were positive for *plc gene*. Direct sequencing of 350 bp region of plc gene revealed many substitution mutations along the entire sequenced fragment. The main cause for such condition may rely on inadequate treatment regimen. The phylogenetic tree of the seven sequenced isolates were classified into three distinct nodes. We concluded that all cases were positive for *M. tuberculosis* and most of them were from rural area. There was different substitution mutations in the *plc* gene which may be due to the intermittent treatment regimens that made the tested strains under stress resulting in mutation.

Key words: *Mycobacterium tuberculosis*, plc gene, phylogentic tree, mutation.

INTRODUCTION

According to WHO (2015), more than 2 billion people, equal to one-third of the world's population are infected with TB. Out of this, 1 in 10 will go on to develop TB during their lifetime. Out of the overall, 13 million TB cases in 2013, 9 million were new cases but consistently 3 million people were not diagnosed and not treated or officially not registered by national TB programmes (NTPs). Many of these missed cases might either die or

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follow some unknown treatment but most of them were thought to be a source of infection for others (WHO, 2015).

In Egypt, tuberculosis is one of the important public health problems. It considers as one of the major public health problems after HCV and bilharziasis. The incidence and prevalence of tuberculosis in Egypt has been declining due to increased efforts of the Ministry of Health and Population (MOHP). Prevalence dropped from 88/100,000 population in 1990 to 24 in 2008, while incidence dropped from 34 in 1990 to 19 in 2008. Although tuberculosis is one of the important public health problems in Egypt, limited information on the *Mycobacterium tuberculosis* genotypes circulating in Egypt is available (Abd-El Aal et al., 2013).

M. tuberculosis and *Mycobacterium bovis* are considered the most important pathogens from the *M. tuberculosis* complex (MTC). Both are highly related TB that can cause infections in humans and other mammals (Vordermeier et al., 2002). *M. tuberculosis* cause mainly active TB among millions of people every year (Brosch et al., 2002), but *M. bovis* has a broader host range causing infections in domestic and wild animals (WHO, 2011). In addition, it was reported that *M. bovis* can infect humans and causing zoonotic TB (Radostits et al., 1994). *M. bovis* is transmited from infected animals to humans by the ingestion of contaminated raw milk or the inhalation of the aerosol released from infected animals (Boulahbal et al., 1978; Cosivi et al., 1998; Michalak et al., 1998; Alexander et al., 2002; Sternberg et al., 2002; Michel et al., 2003; Pavlik et al., 2003; Ocepek et al., 2005; Pavlik et al., 2005; Une and Mori, 2007; Berg et al., 2009). Many studies showed that *M. tuberculosis* can be isolated from domestic or wild animals. As farmers with active TB who share their house with animals can infect animals through their sputum, urine or feces (Michalak et al., 1998; Alexander et al., 2002; Sternberg et al., 2002; Michel et al., 2003). In addition, there are some factors that facilitate the transmission of the disease between animals and humans such as dietary habit of people, close physical contact between humans and animals, rise in the incidence of immunosuppressive diseases, and inadequate disease control measures in animals and humans (Radostits et al., 1994). Our study was done in upper Egypt area which is an agricultural area with climate condition that is suitable for practicing cattle farming and crop cultivation (Ameni et al., 2003, 2008, 2011).

The key to control the spread of tuberculosis includes proper case finding, rapid diagnosis, immediate initiation of effective therapy and contact tracing to arrest further transmission. Recent developments in DNA technology and molecular biology methods have led to rapid detection of mycobacterial infection (Abd-El Aal et al., 2014).

DNA sequencing of variable genomic regions offers a more rapid and accurate identification of mycobacteria compared with conventional phenotypic methods. In addition, it is also capable of providing phylogenic information about the relatedness of the isolated strains. The most routinely used and reliable method of this kind is the amplification and sequence analysis of hyper variable regions of the gene encoding 16S r RNA (Somoskovia et al., 2002).

Although the worldwide use of live attenuated vaccine and several antibiotics to prevent and treat tuberculosis, *M. tuberculosis* is considered one of the infectious diseases of high mortality rate. So, it is essential to gain a better understanding of the pathogenicity and the virulence factors of *M. tuberculosis* (Goudarzi et al., 2010).

M. tuberculosis carries different virulence factors, which allow proliferation of the pathogen in the host cell, cell-to cell spread, and evasion of immune response. Among the most known virulence factors, phospholipases C (PLCs) stand out in several intracellular bacteria, including *Clostridium perfringens, Corynebacterium pseudotuberculosis Pseudomonas aeruginosa, Staphylococcus aureus and Listeria monocytogen*. Due to their role in the virulence mechanisms of bacterial pathogenicity, the relevance of PLCs during mycobacterial infection has been the subject of investigation (Assis et al., 2014). Phospholipase C genes include A, B, C and D segments. Three of these genes, *plcA*, *plcB* and *plcC*, are located at 2351 position of the genomic map of *M. tuberculosis* H37Rv, and are organized in tandem (locus *plcABC*). The fourth gene *plcD*, is located in a different region (Goudarzi et al., 2012).

The present study demonstrates demographic, genetic and phylogenetic study of mycobacterium strains that isolated from in-and out-patients treated in the chest hospital at El- Minia governorate.

MATERIALS AND METHODS

Sampling

This study was conducted in El- Minia governorate. A total of 50 early morning sputum samples were collected between March 2011 until December 2011 from patients attending El-Minia chest hospital and suspected to have pulmonary TB through X ray radiography and Mantoux test. The complete case history of these patients were taken. No patient suffered from immunodeficiency illness or treated with immunosuppressant drugs.

Specimen processing and culture

Sputum samples (3 to 5 ml) were homogenized for 15 min in a shaker using an equal volume of 4% NaOH containing 0.5% NALC decontamination solution at room temperature. After centrifugation at 3,000 rpm for 15 min, the deposit was neutralized with 20 ml of sterile distilled water. The samples were again centrifuged at 3000 rpm for 15 min (Petroff et al., 1915; Tripathi et al., 2014). Then samples (N=50) were inoculated on LJ media and liquid medium Middlebrook 7H9. The observations like rate of growth at different

temperature (28, 30, 37 and 42°C), colony character and pigment production (if any) in light and dark (on LJ slants) for colonies were noted.

Identification of *M. tuberculosis*

Smears were prepared using the dissolved sediment from all specimens, stained by Ziehl-Neelson (ZN) method, and examined for presence of Acid-Fast Bacilli (AFB) with a light microscope. Both the samples sediment before culture on LJ, then the sub-cultured samples on LJ media was subjected to microscopical examination. Colonies of *M. tuberculosis* isolates were identified on the basis of niacin production, nitrate reduction, catalase production (semi quantitative and heat resistant), Tween-80 hydrolysis, Iron uptake, Growth in presence of thiophen-2 carboxylic acid hydrazide (TCH), growth on presence of sodium chloride (5%), Arysulfatse test , Urease test.

DNA extraction

One hundred mg (wet weight) of the cell pellet of each isolate was resuspended in 0.5 ml TE buffer, allowed for 2 cycles freezing and thawing. The cells were then homogenized in glass homogenizer, and then incubated 4 h at 37°C with 5 µl lysozymes (final concentration 100 µg/ml). Proteinase-K was added 25 µl/0.5 ml (final concentration 100 µg/ml) and incubated for further 3 h at 56°C with shaking. DNA was then extracted using Trizol reagent.

One milliliter Trizol was added and after 5 min of incubation at room temperature (RT), 0.4 ml of chloroform was added, vortex for 15 s, kept at RT for 3 min. Then centrifuged at 14,000 rpm for 10 min at 4°C. The upper aqueous phase containing RNA was completely discarded and the DNA in the interphase was precipitated with 0.6 ml absolute ethanol and kept at room temperature for 3 min before centrifugation at 4000 rpm for 5 min at 4°C.

The supernatant was completely discarded and the DNA pellet was washed twice with 1 ml of 0.1 sodium citrate in 10% ethanol. At each time, the DNA pellet was kept in the washing solution for 30 min at room temperature with periodical mixing and centrifuged at 4000 rpm for 5 min at 4°C. Following the 2 washes, the DNA was resuspended in 2 ml of 75% ethanol, kept at RT for 20 minutes with periodical mixing and then centrifuged. The DNA pellet was finally dried briefly for 5 min under vacuum and redissolved in 50 µl of 8 mM NaOH. The pH was then adjusted at 8 by adding 115 µl/ml of 0.1 M HEPES. To DNA was added 10 µl of RNAase and incubated at 37°C for 1 h. The mixture was stored at -20°C till used (Sambrook et al., 1989; Soliman et al., 2004). DNA was purified by using Wizard DNA clean up system (Promega, Catalog no. A7280).

PCR amplification of plc gene

Sequences for plcABC from *M. tuberculosis* H37Rv were obtained from GenBank *(http://www.ncbi.nlm.nih.gov/). plc* gene specific primers were 5'-GGATTCCTGGACTGGCGTTG as forward primer and 5'-CCCACCCAAGAAACCGCAC as reverse primer. The two primers include a 350-bp region. The reaction volume was 25 µl containing, 5 µl of the extracted DNA (100 ng/µl), 1 µl of Taq polymerase (5u/µl), 2.5 µl of 10x enzyme buffer, 2 µl of dNTPs, 10 µl of Q-Solution, 0.1 µl of each of the forward and reverse primers, then the volume was completed with bidistilled water to 25 µl.

The mixture was placed in the thermal cycler (T3000 professional BiometraInc, Germany), which was programmed for 40 cycles with initial denaturation at 95°C for three minutes. Each cycle was performed with denaturation at 95°C for one minute, annealing at 56°C for 45 s with an extension at 72°C for one minute. At the end of the last cycle, Final extension at 72°C for 10 min. The PCR product (6 µl aliquot) was separated by electrophoresis in 1% agarose gel at 100 V for 40 min in Tris-acetate buffer visualized by ethidium bromide staining illuminated by UV transilluminator (Sambrook et al., 1989 and Soliman et al., 2004).

Automated DNA sequencing

The PCR product (16 µl aliquot) of 7 isolates were separated by electrophoresis in 1% low melting agarose gel at 30 v for 60 min at 4°C in Tris-acetate buffer then visualized by ethidium bromide staining, illuminated by UV transilluminator to visualize amplified plc gene from each isolate (Sambrook et al., 1989) and then was sliced of and placed separately in 1.5 ml nuclease free tubes. PCR products were purified from gel slices by using Biospin kit catalog no.BSC02S1 .

Sequencing of PCR products were performed using a MJ Research PTC-225 Peltier Thermal Cycler and the Big Dye Terminator sequencing kit v 3.0 (Applied Biosystems) in accordance with the manufacturer's protocol. Subsequent analysis was performed on an ABI PRISM 3730XL Analyzer (Applied Biosystems). Single-pass sequencing was performed on each template using the primer (forward primer) used for PCR amplification. The resulted sequences were aligned using CLC bio software version 6.7.1.

Phylogenetic analysis

The relationships among the sequenced samples were studied by using CLC bio software version 6.7.1. with using maximum likelihood with the following parameter: Starting Tree: Neighbour Joining, Substitution rate model: Jukes Cantor, Include rate variation: Yes, Number of substitution rate categories: 4, Initial Gamma distribution parameter: 1.0, Estimate Gamma distribution parameter: Yes, Estimate substitution rate parameter(s): Yes, Estimate topology: Yes created.

RESULTS AND DISCUSSION

In this study, we evaluated the prevalence of Mycobacterium species in the upper Egypt area which is mainly an agricultural area.

The relation between different parameters (age, sex, Place of Residence, family history, co-morbid disease) and smear microscopy positivity and culture positivity were shown in Table 1. Our results showed that all sputum samples (50) were acid fast bacilli (AFB) positive. Out of the 50 smear positive samples, 24 samples (48%) were culture positive by using modified Petroff's method. The smear positivity was higher than culture positivity in our study which may be due to that Acid-fast bacterium seen in the smear may represent non-tuberculous mycobacteria. Also, smear positive culture-negative specimens can be resulted from Laboratory errors, prolonged specimen decontamination, shortened incubation times of culture, cross-contamination of smears, using water or stains contaminated with acid-fast organisms or obtaining samples from patients under antimycobacterial therapy (Dunlap et al., 2000).

Sharma et al. (2012) reported that culture positivity obtained by modified Petroff's method (46.7%) were less

Table 1. The relation between different parameters and smear microscopy positivity and culture positivity.

Parameter	Smear microscopy positive (N=50)	Culture positive (N=24)
Gender		
Male	20 (40%)	9 (37.5)
Female	30 (60%)	15 (62.5)
Age		
>20	13 (26%)	5 (21%)
21 – 46	25 (50%)	11 (46%)
41-60	9 (18%)	6 (25)
>60	3 (6%)	2 (8%)
Co-Morbid disease		
Yes	5 (10%)	4 (17%)
No	45 (90%)	20 (83%)
Family history		
Yes	15 (30%)	7 (29%)
No	35 (70%)	17 (71%)
Place of residence		
Rural	38 (76%)	20 (83%)
Urban	12 (24%)	4 (17%)

Table 2. The distribution of tuberculosis patients according to age and sex.

Sex	Age in years								Total (n = 50)	
	< 20		21 – 40		41 - 60		> 60			
	No	%*	No	%*	No	%*	No	%*	No	%*
Male	3	6	9	18	7	14	1	2	20	40
Female	10	20	16	32	2	4	2	4	30	60
Total	13	26	25	50	9	18	3	6	50	100

*Percentage was correlated to the total number of tuberculosis patients.

than NALC-NaOH treatment (63.7%). This mean that modified Petroff's treatment method may result in killing mycobacteria in specimens, so the cultures give negative results which may explain the low positivity of culture in our study. On the other hand, Wulandari et al. (2011) reported that although modified Petroff's method using 4% NaOH was more toxic to mycobacteria than NALC-NaOH method, but if the procedure was done properly and carefully, a good rate of decontamination (2-5%) will be obtained. On contrary, maximum number of cultures positivity were obtained by Modified Petroff's method in studies done by Chaudhary and Mishra (2013) and Tripathi et al. (2014).

Table 2 showed the prevalence of tuberculosis in patients according to their age and sex. Females represented 60% while males were 40% which may be due to some factors such as poverty, pregnancy, ignorance, inadequate anta-natal care in pregnancy. Also, Table 2 showed that TB infection was more common in patients aged 21 to 40 (50%) years, and least in patients aged > 60 years (6%) meaning that pulmonary tuberculosis affected productive age group more. Many studies, done in El-Minia, Port Said, Dakahlia showed also that the highest prevalence of TB was among individuals aged 15 to 30 years (El Zeheiry, 2012; George, 2013; Abu Shabana et al., 2015). Also, Ahmad et al. (2015) found that females were more susceptible to infection 56.21% than males 43.79 and 23.54% of pulmonary TB cases were reported in the age between 15 to 24 years. On the contrary, there are many studies reported that males were more affected than females (Acharya et al., 2007; Jethani et al., 2014; Ifeanyi et al., 2015).

By studying the relation between TB infection and the

presence of underlying diseases (Diabetes mellitus, hypertension asthma, chronic kidney disease (ckd)), it was found that about 10% of smear positive cases and 17% of culture positive cases had Co-Morbid diseases. Gupta et al. (2011) found that Diabetes mellitus (DM) was the most underlying risk factor followed by smoking, alcoholism in patients with pulmonary tuberculosis. In a study carried by Golsha et al. (2009), Diabetes mellitus was found to be the most prevalent condition (23.05%) followed by chronic renal failure, corticosteroid consumption and malignancy ranking second, third and forth in the list (5.8, 2.5 and 2% respectively) in patients with pulmonary tuberculosis.

Also, Shetty et al. (2006) and Reis-Santos et al. (2013) found strong relation between TB infection and the presence of diabetes in their studies. Smoking is another factor that increases the chance of infection with TB. It was found that 80% of male cases were smokers for cigarette and Shisha (Goza). Which indicates that smoking may be an important predisposing factor for the infection with *M. tuberculosis.* In agreement with our results, Gupta et al. (2011) reported that 16.9% of pulmonary TB patients were smokers. Also, the same results were showed by Shetty et al. (2006). The present study found that 30% of smear positive cases and 29% of culture positive cases had family history of TB and one or more of the family members were infected. In a study by Abdelwahab et al. (2009) showed that family history is the most important parameter that increase the rate of the infection by *M. tuberculosis.*

Results showed that 76% of smear positive patients and 83% of culture positive patient were from rural area. Most of these cases sharing house with another family members infected with TB and cattles. This high percent in rural area may be attributed to the low socioeconomic level, high family size, and poor socio-economic status. Poor education is associated poor knowledge of tuberculosis, inadequate and delayed availability of health care also associated with the prevalence of tuberculosis. Similar findings have been reported by George (2013) in the El-Minia governorate who explain increasing tuberculosis cases in rural areas due to poverty and bad social conditions and milk sanitation also agricultural workers may acquire occupational infection by bovine bacilli from the infected animals. Hindi (2009) found that rural cases (76.2%) were significantly higher in comparison with urban cases (23.8%) in retrospective study at the Benha chest hospital over the period from 2002 to 2006. Also, Abdelghany (2010) conducted a similar study in the Menoufia governorate (1992 to 2008), he concluded that rural cases (80.05%) were also significantly higher than urban cases (19.95%) during all years of the study. on other hand, Arya et al. (2013) who reported that sputum positivity for *M. tuberculosis* using Zeihl Neelsen staining for samples obtained from rural and urban were nearly the same (15.8 and15.2%).

Although, most of cases were from rural area, physical and biochemical identification tests to the 24 mycobacterial isolates showed that the prevalent strain was *M. tuberculosis* while *M. bovis* was not isolated (Table 3). Ameni et al. (2013) studied the prevalence of Mycobacterium among Ethiopian farms who shared animals in their housed. They found that All mycobacteria (141) isolated from farmers were *M. tuberculosis*, while only five of the 16 isolates from cattle were members of the *M. tuberculosis* complex (MTC) while the remaining 11 were members of non-tuberculosis mycobacteria (NTM). Further speciation of the five MTC isolates showed that three of the isolates were *M. bovis* (strain SB1176), while the remaining two were *M. tuberculosis* strains (SIT149 and SIT53). Also, they found that transmission of TB from farmers to cattle by the airborne route sensitizes the cows but rarely leads to TB. Similarly, low transmission of *M. bovis* between farmers and their cattle was found, suggesting requirement of ingestion of contaminated milk from cows with tuberculous mastitis.

Saifi et al. (2013) reported that 80% *M. tuberculosis*, 6% *M. bovis* and 14% NTM strains were obtained from positive cultures isolates. Species identification by conventional cultural methods and biochemical tests are of limited value because of the comparatively low yield of the culture and long time to grow. Even the so-called rapid growers may require 1 to 3 weeks to grow. The differentiation between *M. tuberculosis* and *M. bovis* is

Table 3. Results of physical and biochemical identification of the isolated acid fast bacilli.

Test or property	Result
Number of isolates	24
Zeal Nielsen staining	Acid fast bacilli
Growth at 28°C	-ve
Growth at 35°C	+ve
Growth at 37°C	+ ve
Growth at 42°C	±Ve
Colony morphology	Rough
Dark pigmentation	non-chromogen
Photo pigmentation	non-chromogen
Niacin production	+ve
Nitrate reduction	+ve
Tween 80 hydrolysis 10 days	-ve
Catalase semiquantitative < 45	-ve
Catalase PH7.0 -68°C	-ve
Arylsulfatase (3days)	-ve
Arylsulfatase (2 weeks)	-ve
Urease	+ ve
Iron uptake	-ve
Growth on TCH (2µg/ml)	+ve
Growth on NaCl 5%	-ve

*Percentage was correlated to the total number of tuberculosis patients.

Table 4. The distribution of Mycobacterium tuberculosis infection according the type of patients and sex.

Type of infection / Sex	New case	%*	Reactivation	%*	Relapsed	%*
Male	18	36	1	2	1	2
Female	26	52	4	8	-	-
Total	44	88	5	10	1	2

*Percentage was correlated to the total number of tuberculosis patients.

not always conclusive by Niacin production and PZA sensitivity tests. To differentiate between *M. tuberculosis* complex and non-tuberculous mycobacteria, PCR was a very good tool. If it was used in conjunction with culture and biochemical tests; it would give best results as already mentioned by Gupta et al. (2010).

Results shown in Table 4 showed that 88% of patients were new cases, while 10% were reactivated cases. Most of the reactivated cases were for female which may be due to pregnancy, the presence of active cases in their houses. One of these females was sharing house with 4 family members active cases.

Also, some of the reactivated cases were suffering from depression due to losing someone they love in their families or due to the presence of psychological stress of any other reason. The relapsed case for a patient who was treated from TB infection with his sister in the same time. He did not complete his course of treatment because he was a prisoner. In addition, We found that all new cases were treated by first line regimen that include pyrazinamide, Ethambutol, Rifampicin, Isoniazide. The relapsed case was treated by first line regimen plus streptomycin.

The standard regimen for treatment of new cases of pulmonary tuberculosis consists of 6 months treatment, with four drugs in the initial phase including isoniazid, rifampicin, pyrazinamide, and either ethambutol or streptomycin, followed by two drugs in the continuation phase including isoniazid and rifampicin. For retreatment cases (reactivated, relapsed), the recommendation is a 9-month standard regimen starting with five drugs including isonazid, rifampicin, pyrazinamide, ethambutol and streptomycin for the initial 3 to 4 months, followed by isoniazid and rifampicin in the continuation phase (Tam, 2006). WHO recommended drug susceptibility testing (DST) at the start of therapy for all previously treated patients. As, previously treated patients are defined by their likelihood of MDR-TB. The retreatment regimen with first-line drugs is ineffective in MDR-TB; it is therefore critical to detect MDR-TB promptly so that an effective regimen can be started. So, samples for culture and drug susceptibility testing (DST) should be obtained from all previously treated TB patients at or before the start of treatment. DST should be performed for at least isoniazid and rifampicin (WHO, 2010).

In our study, we found that 60% of reactivated cases were treated by first line regimen and other 40% were treated with first line regimen plus streptomycin. It mean that not all reactivated cases were treated by one regimen and no drug susceptibility testing was done that may suggest the presence of multi drug resistant-TB (MDR) strains in the hospital. As all patients in our study were in the same hospital and treated in the same period.

Plc gene is considered as one of the virulence factor for *M. tuberculosis*, the 350 bp region of this gene was amplified and sequenced. All the 24 grown isolates gave nearly the same amplicon size (Figures 1, 2 and 3 represents a selected sample of the amplified plc gene from some isolates). Direct PCR method based on the amplification of 350 bp region of plc used for detection of *M. tuberculosis*, to be differentiated it from *M. bovis* in clinical samples. The result of our study is in accordance with the study made by Behr et al. (1999). Also, Vera-Cabrera et al. (2001) in their study found out that the main genetic difference known between *M. tuberculosis* and *M. bovis* was the presence of the mtp40 sequence. mtp40 was part of the mpcA gene, which encodes a phospholipase C.

Goudarzi et al. (2010) reported that Phospholipase C genes could be important in pathogenesis of Beijing strains of *M. tuberculosis*. The Beijing MTB strain has attracted special attention because of its global emergence and resistance to multiple drugs.

Seven isolates were subjected for sequencing analysis of the PCR amplified plc fragment. Sequence alignment revealed that many substitution mutations (SNPs) were found along the entire sequenced fragment denoting the heterogeneity of the isolated strains (Figure 4). Although these strains were isolated from the same governorate and within a short period of time therefore homogeneity of the isolated mycobacterial strains were assumed to be, and that in contrary of the data presented here. The main cause for such condition may rely on inadequate treatment regimen such as intermittent dosing during the initial intensive treatment phase (Li et al., 2005; Burman et al., 2006) or discontinued treatment (Nolan et al., 1995; Bradford et al., 1996). In a study by Ramaswamy and Musser (1998) found that inadequate treatment and non-compliance lead to the development of selected mutated resistant and MDR-TB. Drug stress induced

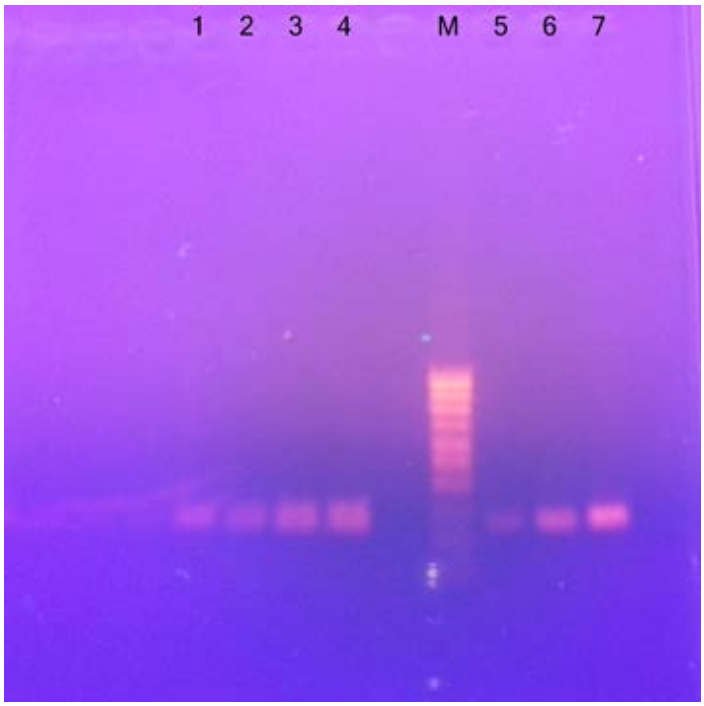

Figure 1. Amplified plc gene migrating about 350 bp from isolated *M. tuberculosis* strains, M= 100 bp DNA ladder, Lane 1 to 4 represents isolate number 1, 2, 7, 9 and Lane 5 to 7 represents isolate number 10 , 11 and 12.

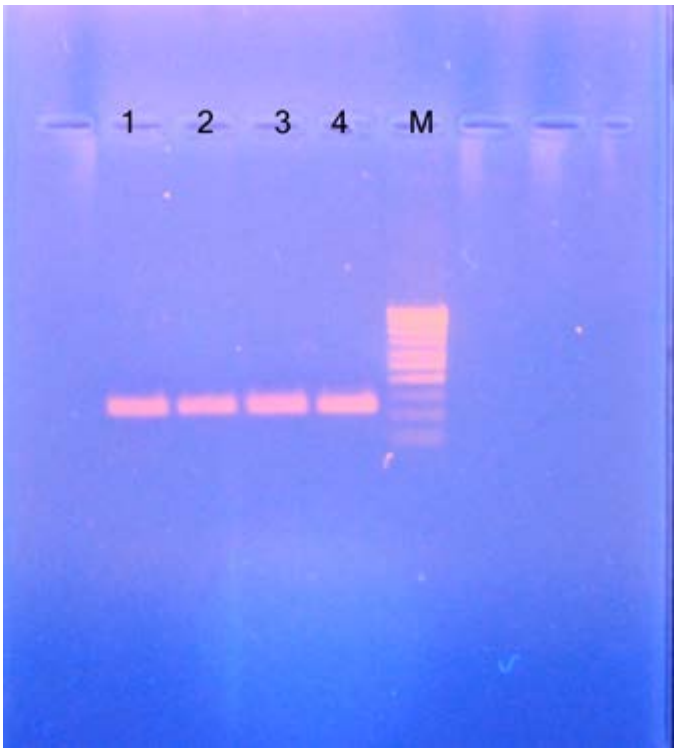

Figure 2. amplified plc gene migrating about 350 bp from isolated *M. tuberculosis* strains, M= 100 bp DNA ladder, Lane 1 to 4 represents isolate number 13, 21, 22 and 31.

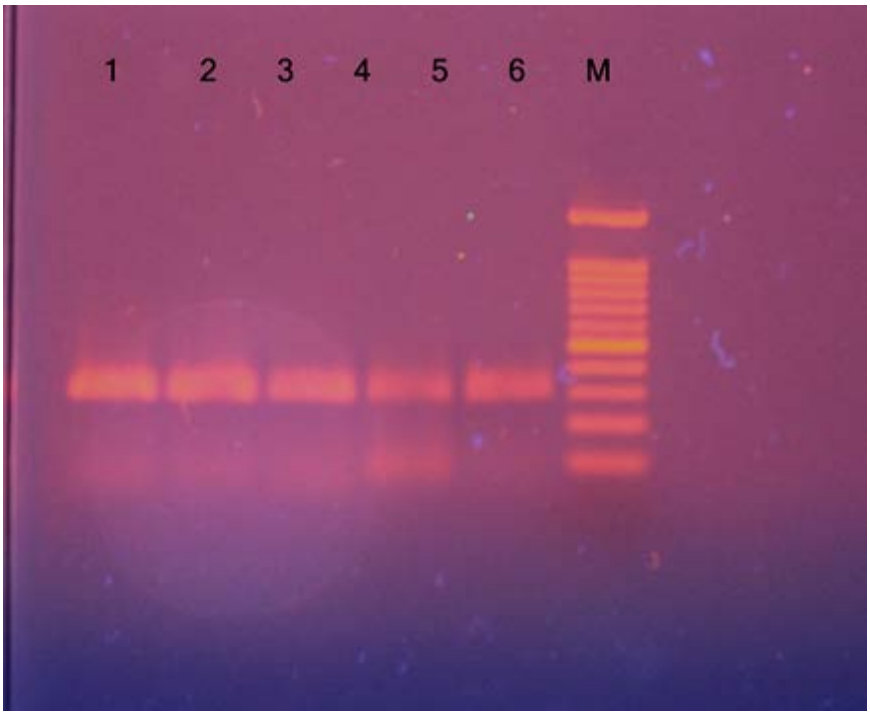

Figure 3. Amplified plc gene migrating about 350 bp from isolated *M. tuberculosis* strains, M= 100 bp DNA ladder, Lane 1 to 6 represents isolate number 32, 38, 39, 40, 41, and 42.

mutation is one of the main methods by which the mycobacteria can escape the devitalizing state caused by the drug or the immune system to survive in the tissues and because plc gene is one of the important virulence factor as described earlier so it would be reasonable to be target for mutations .

Unsurprisingly, the phylogenetic tree (Figure 5) sub classed the 7 sequenced isolates into 3 distinct nodes: node 1 (isolates 31 and 1), node 2 (with isolates 43 and 22), and node 3 (isolates 49, 40 and 2). Each node contain samples for patients that may share some socio-demographic characters and differ in some. As patients of node 2 differ in their marital state (43 is married while 22 is single) such condition may arise from different source of infection, different responses to the drug regimen, non-adherent to the drug regimen. Such cause would raise a question about the usefulness of the same drug regimen to all patients.

Table 5 showed that most of patients were from rural area, they all shared in the treatment regimen which was first line consisting of isoniazid, rifampicin, pyrazinamide and ethambutol.

LIMITATIONS OF THE STUDY

This study has certain limitations; the sample size was limited as it covered only a proportional number of the total reported cases during the study period. Also 7 selected isolates were sequenced only. Other studies on large sample size may be needed to give precise data about the prevalence of mycobacterial strains in El-Minia governate and on other governates in Egypt. In future, we can study the prevalence of MDR strains in the environment of the hospital.

Conclusion

(1) *M. tuberculosis* was the prevalent strain in El Minia governate.
(2) Tuberculosis was most prevalent in female especially at age group 21-40 due to some factors such as poverty, pregnancy, ignorance, inadequate anta-natal care in pregnancy, the lack of female education, carelessness.
(3) Most of cases were from rural area which may be due to their lifestyle, ignorance, large number of family members sharing the same house and they can share their houses with animals. In addition to the presence of psychological stress.
(4) Smoking, Family history of TB infection, Co morbid disease (especially Diabetes mellitus) are an important predisposing factors for the infection with *M. tuberculosis*.
(5) To choose the effective treatment regimen and detect the presence of Multi-drug resistance (MDR) strains, drug susceptibility tests should be done to the reactivated,

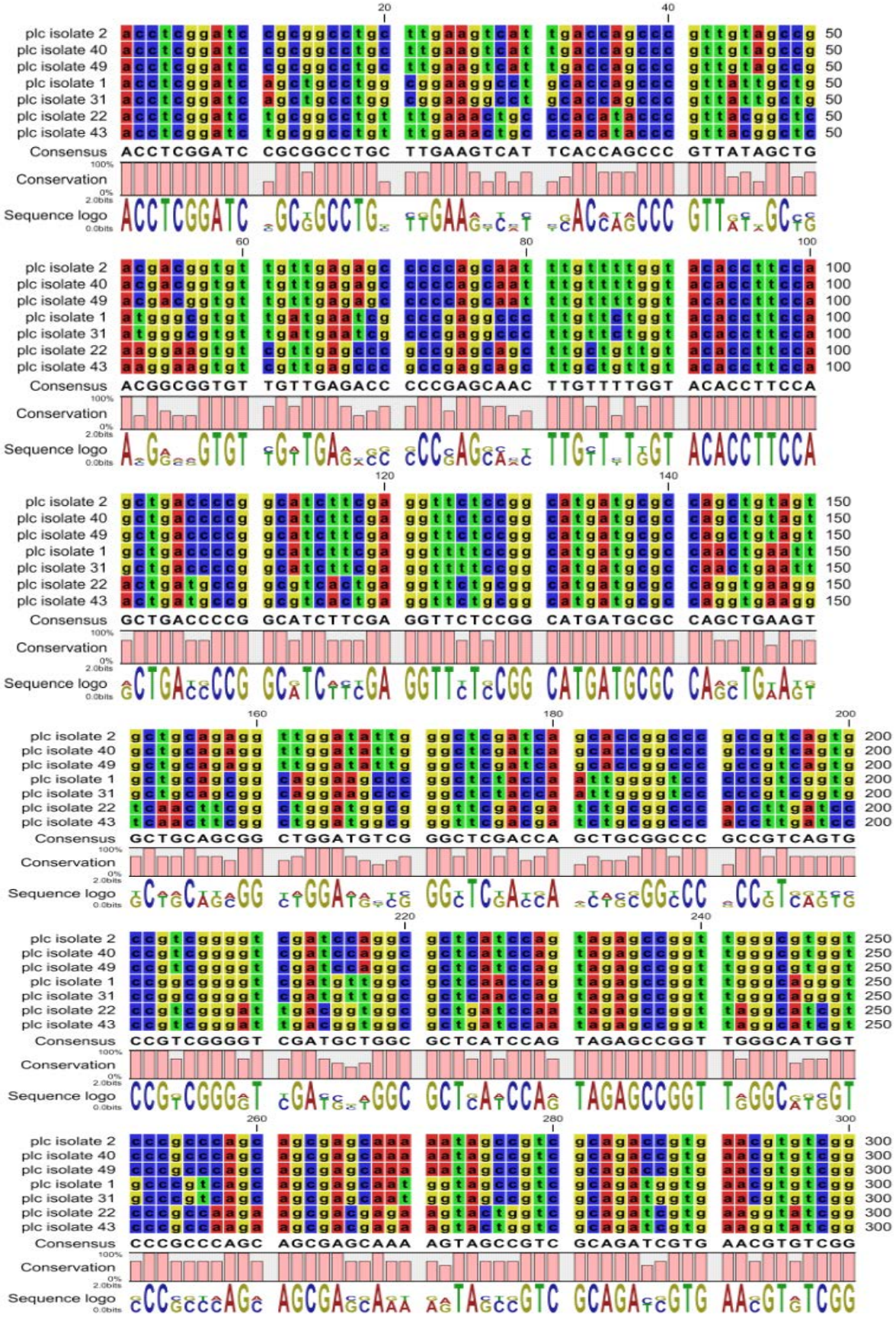

Figure 4. The sequence alignment of the plc gene amplified from 7 selected Mycobacterium *tuberculosis* isolates.

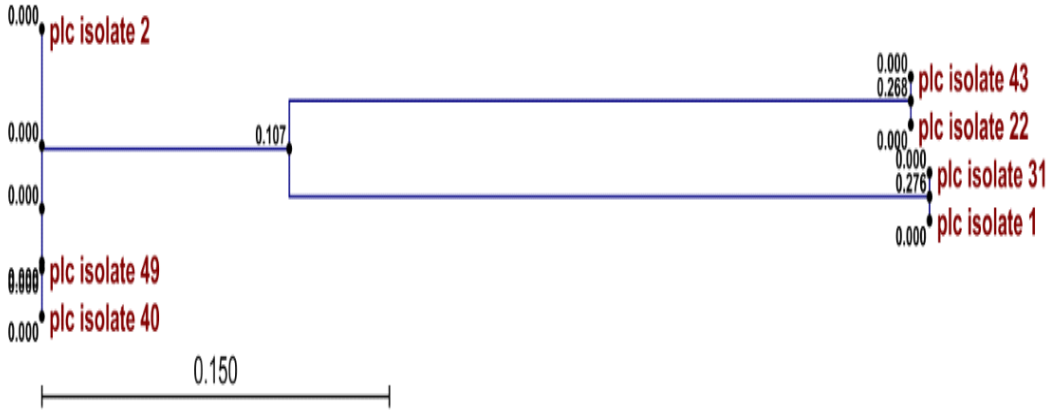

Figure 5. The phylogenetic tree of the sequenced plc gene amplified from the 7 selected *M. tuberculosis* isolates.

Table 5. Socio-demographic data for the tested cases in each node of the phylogenetic tree.

	Sample no.	Sex	Age	Place of residence	Marital state	Presence of house animals	Treatment	Co-morbid disease	Family history	Case
Node 1	1	F	58	Rural	Married	Yes	First line	Yes	No	New
	31	F	21	Rural	Married	yes	First line	No.	yes	Reactive case
Node 2	43	F	20	Rural	Married	Yes	First line	No	No	New
	22	F	24	Urban	Single	No	First line	No	No	New
Node 3	2	F	21	Rural	Married	No	First line	No	No	New
	49	M	59	Urban	Married	No	First line	No	No	Reactive case
	40	M	44	Rural	Married	No	First line	Yes	No	New

F: Female, M: Male.

relapsed cases and patient who had family history of TB infection before the beginning of treatment.

(6) There was different substitution mutations in the plc gene which may be due to the intermittent treatment regimens that made the tested strains under stress resulting in mutation.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Seropositivity and risk factors for *Brucella* in dairy cows in Asella and Bishoftu towns, Oromia Regional State, Ethiopia

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A cross-sectional study was conducted in Asella and Bishoftu towns of Oromia Regional State of Ethiopia to determine seropositivity and associated risk factors exposing dairy cattle to brucellosis from December, 2013 to March, 2014. A total of 570 dairy cattle from 35 herds were purposely selected for inclusion in the study based on abortion history. From 35 farms studied, 80, 55.56 and 100% of the farm owners in small, medium and large herd sizes responded as they were aware of brucellosis, respectively. It was also found out that all farm owners of the study area were dependent on culling of the known *Brucella* infected animals, while most of the farm owners dispose the after birth to open dump in small and medium herd size farms. All sera sample collected were tested and confirmed serologically using the card test (CT), rose Bengal plate test (RBPT), indirect enzyme linked immuno sorbent assay (i-ELISA) and complement fixation test (CFT). Out of 570 samples tested in the present study, an overall sero prevalence was estimated 1.4% (95% CI: 0.241, 3.461) by complement fixation test (CFT). Among the tested samples, 13 (2.28%), 15 (2.63%) and 16 (2.81%) were found positive by the aforementioned tests, respectively. The higher seroprevalence, 3.23% (95% CI: 3.0, 7.4) was observed in Asella compared to Bishoftu (0.52%) town. A Chi-square computed statistical analysis indicated that origin ($\chi 2=6.63$; $P<0.05$), breed type ($\chi 2= 8.49$; $P<0.05$), abortion history ($\chi 2=92.43$; $P<0.001$) and abortion period ($\chi 2=192.97$; $P<0.001$) were the major risk factors for *Brucella* infection in the study areas. Multivariable logistic regression statistical analysis revealed that origin and breed type were significantly associated with *Brucella* seropositivity ($P<0.05$). Consequently, origin was statistically identified to be the major risk factor for brucellosis to occur in relation to other factors (OR=7.56). In conclusion, the prevailing *Brucella* seropositivity in most of the dairy farms of the study areas signifies the economic importance of brucellosis in the dairy cattle industry and the potential public health implication for human population. Therefore, more proactive measures should be taken to protect the cattle populations from *Brucella* infection to reduce its economic impact to the dairy industry and the risk of zoonotic infection in exposed human population in the study areas.

Key words: Asella, Bishoftu, brucellosis, dairy cattle, seropositivity, risk factors.

INTRODUCTION

Brucellosis is endemic in many developing countries and is caused by *Brucella* species that affect man, domestic and some wild animals, and marine mammals (Seleem et al., 2010). It causes abortion and sterility in livestock leading to serious economic losses and has even more serious medical impact in humans, leading to more than 500,000 infections per year worldwide (Godfroid et al., 2005).

Bovine brucellosis is an infectious and contagious disease known for its impact on reproductive performance of cattle in Africa and is predominantly a disease of sexually mature animals (Rahman et al., 2012; Asmare et al., 2013). The disease is primarily caused by *Brucella abortus* and occasionally by *Brucella melitensis* where cattle are kept together with infected sheep or goats and characteristically associated with abortion at first gestation ("abortion storm" in naïve heifers) and is mainly caused by biovars (mainly biotype -1) of *B. abortus* (OIE, 2009a; Godfroid et al., 2010). Chronic infection of the mammary glands due to *Brucella suis* has also been reported (Lopes et al., 2010). Clinically bovine brucellosis is characterized by impaired fertility specifically with abortion, metritis, orchitis and epididymitis (Seleem et al., 2010).

The mode of transmission of the bacteria varies with the epidemiological area, the animal reservoir and the occupational exposed groups (Seleem et al., 2010). Sources of infection for the transmission of the bovine brucellosis are aborted fetuses, the fetal membranes after birth, and vaginal discharges and milk from infected animals (Radostits et al., 2000; Tolosa et al., 2010). The most common route of transmission is the gastrointestinal tract following ingestion of contaminated pasture, feed, fodder, or water, and after birth, fetuses, and newborn calves, all of which may contain a large number of the organisms and constitute a very important source of infection. The bacteria can be transmitted to humans through direct contact with infected tissue via breaks in skin, ingestion of contaminated tissues or milk products, and inhalation or mucosal exposure to aerosolized bacteria (Seifert, 1996; Radostits et al., 2007).

The prevalence of brucellosis is influenced by a number of risk factors related to production systems, biology of the individual host and environmental factors. These include age, herd size and composition, hygienic status of the farm, rate of contact between infected and susceptible animals, farm biosecurity and climate (Radostits et al., 2007; McDermott and Arimi, 2002).

A precise diagnosis of *Brucella* spp. infection is important for the control of the disease in animals and consequently in man. Clinical diagnosis is based usually on the history of reproductive failures in livestock, but it is a presumptive diagnosis (Poester et al., 2010) that must be confirmed by laboratory methods (Nicoletti, 2002; Poester et al., 2010). Although blood and tissue cultures remain the 'gold standard' for diagnosis, they show low sensitivity, are time consuming, and represent a risk for laboratory personnel (Bricker et al., 2002; Navarro et al., 2004).

Serology is a standard method for the epidemiological surveillance of brucellosis (Köppel et al., 2007; Leuenberger et al., 2007). However, cross-reactions between *Brucella* species and other Gram- negative bacteria, such as *Yersinia enterocolitica* O:9, *Francisella tularensis*, *Escherichia coli* O:157, *Salmonella urbana group* N, *Vibrio cholerae* and *Stenotrophomonas maltophilia*, are a major problem of the serological assays (Muñoz et al., 2005; Al Dahouk et al., 2006). The source of antigenic cross reactions is the O-chain of the smooth lipopolysaccharide (S-LPS) present on the surface of the bacterial cell, which shows great similarity in smooth *Brucella* spp. and the above mentioned bacteria (Hinić et al., 2009). False positive serological results are due only to *Y. enterocolitica* O: 9 affect up to 15% of the cattle herds in regions free from brucellosis, generating considerable additional costs for surveillance programs (Muñoz et al., 2005). False negative results have also been observed in serological diagnosis of brucellosis (Godfroid et al., 2002; Tessaro and Forbes, 2004). They occur mostly due to the fact that the antibody response is dependent upon the stage of infection during sample collection (Hinić et al., 2009).

Since the first report of brucellosis in the 1970s in Ethiopia, the disease has been noted as one of the important livestock diseases in the country (Asfaw, 1998; Eshetu et al., 2005; Kebede et al., 2008; Ibrahim et al., 2010). Prevalence of bovine brucellosis varies widely across Ethiopia, with reported seroprevalences ranging from 0.2% in south-western Ethiopia (Tolosa, 2004) to 38% in western Ethiopia (Rashid, 1993).

The dairy industry has been growing to meet an ever increasing demand for milk and milk products in the country. Crossbreeding indigenous cattle with high yielding exotic cattle is the main policy established by the Ethiopian government to bridge the gap between supply and demand for dairy products. Owners of dairy cattle and institutions promoting the dairy industry require current, reliable scientific data on such important diseases as brucellosis. Therefore, it is of paramount importance to know the magnitude of brucellosis, major

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potential risk factors exposing to the disease and use of different serological tests which in turn assist in controlling and eradicating the disease and devising baseline information to develop national wide brucellosis information.

The main aims of this study were: i) to determine seropositivity of brucellosis in dairy cattle and major potential associated risk factors, ii) to assess knowledge-attitude and practices (KAP) of the farm owner's regarding this disease, and iii) comparison of four different serological test agreement in diagnosing brucellosis.

MATERIALS AND METHODS

Description of the study area

The study was conducted in two purposely selected sites in central Ethiopia, Bishoftu, East Shewa Zone and Assela, East Arsi Zone. These study areas were selected based on the abundance of dairy farms that constituted the known milk sheds (Land O'Lakes, 2010).

Bishoftu is located at 47 km southeast of Addis Ababa. The area is located at 9°N latitude and 40°E longitudes at an altitude of 1850 meters above sea level in the central high land of Ethiopia. It has an annual rainfall of 866 mm of which 84% is in the long rainy season (June to September). The dry season extends from October to February. The mean annual maximum and minimum temperatures are 26 and 14°C respectively, with mean relative humidity of 61.3% (ADARDO, 2007). Farmers in the vicinity of Bishoftu town use a mixed crop and livestock farming system. Moreover, Bishoftu and its surrounding area have variable and yet representative agro-ecologies of the country. These agro-climatic zones are inhabited with different plant and animal species (Conway and McKenzie, 2007).

The second study area was Asella, which is located at 175 km southeast of Addis Ababa, and the altitude and annual rainfall of the area ranges from 502-4130 meters above sea level and 200-400 mm with mean annual temperature of 22.5°C, respectively. It is one of the highly populated areas in Ethiopia with estimated human population of 2,521,349 and livestock population of cattle-82,190; sheep-51,292; goat-8, 11,479; poultry- 5, 62,915; equine- 22,055 (Deselegn and Gangwar, 2011).

Definitions

In Ethiopia dairy cattle production systems are classified into rural smallholder (mixed crop-livestock) production, pastoral and agro-pastoral production, urban and peri-urban smallholder dairy production, and commercial dairy production systems (Asmare et al., 2013; Land O'Lakes, 2010). This study focuses on the latter two production systems. Urban and peri-urban dairy is one of the four dairy production systems in Ethiopia producing milk either as a full-time or a part-time business. These smallholder dairy farms predominantly keep a small number (≤10 animals) of cross-bred cows in a zero grazing system to produce milk for both home use and sale. Commercial dairy farms are also farms located in urban and peri-urban areas mainly in and around the major cities and produce milk exclusively for sale.

The farms were classified according to herd size and level of production into smallholder farms (<10 animals), medium farms (10 to 50 animals) and large farms with more than 50 animals (Megersa et al., 2011).

The disease is primarily caused by *B. abortus* in cattle. However, occasionally there were reports as the disease is caused by *B. melitensis* where cattle are kept together with infected sheep or goats (Godfroid et al., 2010) and chronic infection of mammary glands due to *B. suis* has also been reported when keeping cattle with pigs (Lopes et al., 2010). Though the above reports revealed that as the cattle were infected by different species of *Brucella* upon mixing with sheep, goats or pigs, in our study area, there were no sheep/goat/pig mixing practices with the dairy cows by the owner of the farms.

Risk factors assessment

In this study covariates (hypothesized explanatory variables) were assessed at both individual and farm level. Information was extracted from herd records where possible, and if this information was not available owners were interviewed using semi-structured questionnaires.

The presence of abortion history in the farm, separate parturition/maternity pen, separation of cows during parturition, awareness about brucellosis (knowledge), brucellosis test in the farm, frequent contact between animals with other herds were categorized as yes or no variables while breed of dairy cattle were categorized as Holestein-Friesian, cross or local. Breeding was characterized by service types (artificial insemination (AI), bull or both). The method of after birth disposal (placenta, aborted material and dead fetus) was also categorized into burying/burning, or thrown to open dump.

The culling criterion of animals from the farm was categorized as reproductive problems, non-reproductive problems or both variables. The method of cleaning of calving pen after parturition was categorized as flushing with water, disinfecting with detergents or both variables. The replacement stock of each farm was defined as buy in, raise own replacement or both. Culling, test and slaughter or both were considered as the measures taken against the known *Brucella* infected animals. Individual animals were categorized as young (≤ 36 months) and adult (>36 months), origin of each individual animal was defined as either Asella or Bishoftu while the location of the farm was classified as urban or peri-urban. Parity of the animals in the farm were categorized as primiparous, pluriparous or not applicable variables whereas the abortion stage was classified as first trimester, second trimester or third trimester.

Study design and sample size determination

A cross-sectional study design was conducted to determine the seropositivity of *Brucella* infection in dairy cattle in the two selected towns and to identify the potential risk factors associated with the seropositivity. Dairy cattle above six months of age were selected for this study. Relevant individual animal biodata and farm level information were collected using a semi-structured questionnaire.

The sampling was performed using a two level approach, selecting first individual farms with abortion history and then randomly selecting individual animals systematically inside each farm. About 57% of the sampled cattle were from smallholder farm (small herd size) while the remaining 43% were from medium and large herd size around urban and peri-urban including the commercial dairy farms. A list of dairy farms was prepared for each of the two study areas in collaboration with the respective district livestock health departments.

The sample size for cattle in Asella was calculated using a 14.14% seroprevalence of bovine brucellosis (Deselegn and Gangwar 2011), 95% confidence interval (CI) and 5% required precision (Thrusfield, 2007). In Bishoftu, 50% expected prevalence, 95% confidence interval and 5% required precision were used, resulting in a sample size of 384 cattle for this study. Hence, a total of 570 dairy cattle (186 from Asella and 384 from Bishoftu) were

considered for this study from 35 farms in the study areas.

Study methodology

Serological blood sample collection

Blood samples (10 ml) were collected from the jugular vein of each animal, using sterile needles and plain vacutainer tubes. The blood samples were allowed to stand overnight at room temperature and centrifuged at 1500 × g for 10 min to obtain the serum. Sera were decanted into cryovials, identified and transported to the National Animal Health Diagnostic and Investigation Center (NAHDIC), Sebeta, Ethiopia in ice packs and stored at -20°C until screened for antibodies against natural *Brucella* exposure using serological analysis.

Serological laboratory techniques

Rose Bengal plate test (RBPT)

All sera samples collected were initially screened by RBPT using RBPT antigen (Veterinary Laboratories Agency, New Haw, Addlestone, Surrey, KT15 3NB, United Kingdom) according to OIE (2004) and Alton et al. (1975) procedures. Briefly, sera and antigen were taken from refrigerator and left at room temperature for half an hour before the test to maintain to room temperature and processed following the recommended procedures.

Indirect enzyme linked immunosorbent assay (i-ELISA)

For further laboratory analysis, i-ELISA was performed using a commercial i-ELISA kit (BRUCELISA(160+400), (Veterinary Laboratories Agency, New Haw, Addlestone, Surrey KT15 3NB, United Kingdom) to detect circulating antibodies of *Brucella* in cattle serum sample, and the protocol provided by the developers was followed precisely. The test sera were analyzed at a final dilution of 1/200. The positive and negative controls were used at a dilution of 1/40 as has been indicated by the manufacturer. Following the addition of the conjugate and substrate-chromogen mixture at a recommended strength, the plate was incubated and examined for the intensity of reaction with an automated ELISA reader at 405 nm. Color development within a well indicates that the tested serum has antibodies to *Brucella.* A positive/ negative cut-off was calculated as 10% of the mean of the optical density (OD) of the eight positive control wells. Any test serum with an OD value equal to or above this value was considered positive.

Card test (CT)

The brucellosis card test is a macroscopic agglutination procedure utilizing disposable materials, a stained buffered whole cell antigen suspension of *B. abortus* strain119-3 and contained in compact kits of minimal size. The card test for brucellosis is a rapid, sensitive and reliable procedure for detecting serologic evidence of *Brucella* infection. This test was performed according to instructions of the manufacturer (United States Department of Agriculture (USDA), APHIS, Veterinary Services, USA). A positive serum showed characteristic agglutination, moderate to large clumps where as a negative one showed a pattern of dispersed particles without characteristic clumps and those showed no clumping.

Complement fixation test (CFT)

Sera that tested positive to the card test, i-ELISA and RBPT were further tested using CFT for confirmation using standard *B. abortus* antigen S99 (Veterinary Laboratories Agency, New Haw, Addlestone, Surrey KT15 3NB, United Kingdom). Preparation of the reagent was evaluated by titration and performed according to protocols recommended by World Organization for Animal Health (OIE, 2009b). Sera with a strong reaction, more than 75% fixation of complement (3+) at a dilution of 1:5 or at least with 50% fixation of complement (2+) at a dilution of 1:10 and above were classified as positive and lack of fixation/complete hemolysis was considered as negative.

Case definition

Animals were considered as seropositive on the complement tests result, i.e., an animal was considered positive if tested seropositive on CT/ RBT/ i-ELISA and CFT in serial interpretation. The test was regarded as valid if the negative control serum showed complete haemolysis and the positive control shows inhibition of haemolysis. Due to its high accuracy, complement fixation is used as confirmatory test for *B. abortus*, *B. melitensis*, and *Brucella ovis* infections and it is the reference test recommended by the OIE for international transit of animals (OIE, 2009a, b).

Data analysis

Data generated from the questionnaire survey and laboratory investigations were recorded and coded using a Microsoft Excel spreadsheet (Microsoft Corporation) and analyzed using STATA version 11.0 for Windows (Stata Corp. College Station, TX, USA). The seroprevalence was calculated as the number of seropositive samples divided by the total number of samples tested. To identify association of *Brucella* seropositivity with the risk factors (origin, age, management system, breed type, herd size, separate parturition, abortion history, abortion period and parity) were computed by Pearson's Chi-square test. After the association of exposure variables with *Brucella* seropositivity was analyzed at individual animal level by the Chi-square test, those variables significantly associated with *Brucella* seropositivity (origin, breed type, abortion history and abortion period) were further analyzed by multivariable logistic regression. A multivariable logistic regression model was used to identify the potential risk factors associated with *Brucella* infection in animal and variables with a p-value lower than or equal to 0.05 (in Chi-square analysis) were included in the multivariable logistic regression model. Further selection of variables was based on backward elimination procedure using a LR-test at 0.05 as cut point. Prior to building a final model, variables were tested for interaction effects using cross-product terms and for multiple-collinearity using the collinearity matrix index. The validity of the model to the observed data was assessed by computing the Hosmer-Lemeshow goodness-of-fit test. Finally, deviant covariate patterns and their influences on parameter estimates of the model were identified.

The agreement between CT, RBPT, i-ELISA with CFT, considering as gold standard test, were done using kappa test and interpreted according to the recommendations of (Dohoo et al., 2003) who states Kappa values as: <0.2: slight agreement, 02–0.4: fair agreement, 0.4–0.6: moderate agreement, 0.6–0.8: substantial agreement and >0.8: almost perfect agreement. The 95% confidence interval and a significance level of $\alpha = 0.05$ were used.

RESULTS

Knowledge, attitudes and practices (KAP) of the farm owner about brucellosis

From 35 farms studied, 80, 55.56 and 100% of the farm

Table 1. Knowledge, attitudes and practices (KAP) of farm owner's about *Brucella* infection in small, medium and large herd size in the study areas.

Variables	Proportion of respondents (n)		
	Herd size		
	Small(n=20) n (%)	Medium(n=9) n (%)	Large(n=6) n (%)
Awareness about brucellosis			
No	4(20)	0)	0(0)
Yes	16(80)	5(55.56)	6(100)
Brucella infected animal			
Test and slaughter	0(0)	0(0)	0(0)
Culling	20(100)	9(100)	6(100)
Both	0(0)	0(0)	0(0)
After birth disposal			
Burrying/ Burning	1(5)	1(11.1)	5(83.33)
Open dump	19(95)	8(88.89)	1(16.67)

n= number.

owners in small, medium and large herd sizes responded as they were aware of brucellosis, respectively. It was also found out that all farm owners of the study area were dependent on culling of the known *Brucella* infected animals while most of farm owners dispose after birth to open dump in small and medium herd size farms (Table 1).

Farm characteristics

Of the 35 farms assessed by a questionnaire survey, it was found that all of the large herd size farms had bulls on their farms, whereas only 5 (25%) of small farms have bulls. The study revealed that all farms in the study area had no frequent contact with other herds. The majority (95%) of small farms and all of the large farms were using AI for breeding purposes. The practices of provision of separate parturition pens were 83.3% in large farms whereas they were only 5% for small farms (Table 2).

Seroprevalence of brucellosis

In the present study, an overall seroprevalence was estimated 1.4% (95% CI: 0.241, 3.461) by CFT. Among 570 tested samples, 13 (2.28%), 15 (2.63%) and 16 (2.81%) were found positive by RBPT, iELISA, and card test, respectively. The higher seroprevalence, 3.23% (95% CI: 3.0, 7.4) was observed in and around Asella town compared to Bishoftu (0.52%) (Table 3).

Comparison of serological test agreement

The kappa statistics showed that there was substantial agreement between the card test and RBT with CFT as gold-standard test, while almost perfect agreement was observed between i-ELISA and CFT (Table 4).

Chi-square analysis of association of the putative risk factors with *Brucella* seropositivity

A Chi-square analysis revealed that origin, breed, abortion history and abortion period were significantly associated (P<0.05) with seropositivity of bovine brucellosis than among other factors considered during the study (Table 5).

Multivariable logistic regression analysis of risk factors associated with *Brucella* sero positivity

The logistic regression analysis of the putative risk factors indicated that cattle those originated from Asella were more likely to be infected (OR= 6.4, 95% CI: 1.27 - 31.85) with *Brucella* than cattle from Bishoftu (Table 6).

DISCUSSION

In the present study, based on the questionnaire survey, most of the respondents were well aware about brucellosis and practice culling of the known *Brucella* infected animals in their farms. Among the prevention of brucellosis transmission, culling is the most known measures against animal brucellosis (Radostits et al., 2000). In addition, most of the respondents in this study with the small herd size (95%) did not bury afterbirth (aborted fetus, still birth and retained foetal membrane), rather they left them on open dump. Moreover, all the

Table 2. Summary of the proportion of variables in the three herd (farm) size.

Variables category	Herd size		
	Small (n=20) Frequency (%)	Medium (n=9) Frequency (%)	Large (n=6) Frequency (%)
Bull			
No	15 (75)	2 (22.2)	0
Yes	5 (25)	7 (77.8)	6 (100)
Frequent contact with other herd			
No	20 (100)	9 (100)	6 (100)
Yes	0 (0)	0 (0)	0 (0)
Service type			
AI	19 (95)	7 (77.8)	6 (100)
Bull	0 (0)	0 (0)	0 (0)
Both	1 (5)	2 (22.2)	0 (0)
Parturition pen			
No	19 (95)	5 (55.6)	1 (16.7)
Yes	1 (5)	4 (44.4)	5 (83.3)
Cleaning of calving pen			
Flushing with water	15 (75)	6 (66.7)	1 (16.7)
Both*	4 (20)	2 (22.2)	5(83.3)
Replacement stock			
Buy in	0 (0)	0 (0)	0 (0)
Raise own stock	19 (95)	7 (77.8)	5 (83.3)
Both	1 (5)	2 (22.2)	1 (16.7)

Both* = Flushing with water and disinfection with detergent.

Table 3. Results of CT, RBT, iELISA and CFT of brucellosis by origin.

Origin	N	Card test	RBT	i-ELISA	CFT
		Number positive (%)	Number positive (%)	Number positive (%)	Number positive (%)
Bishoftu	384	4(1.04)	3(0.78)	3(0.78)	2(0.52)
Asella	186	12(6.45)	10(5.38)	12(6.45)	6(3.23)
Total	570	16(2.81)	13(2.28)	15(2.63)	8(1.40)

N=number of animal tested.

respondents did not use protective gloves while handling calving or aborting animals. These factors combined with the poor cleaning practice by the owners could pose a great risk of the spread of the disease to unaffected animals (Tolosa, 2004).

The present study revealed that the overall seroprevalence of bovine brucellosis was 1.40%. This finding is consistent with the earlier reports of Degefu et al. (2011) (1.38%) in agro pastoral areas of Jijjiga zone of Somali Regional State. Comparable to this finding, Asmare et al. (2007) reported (1.92%) in Sidama zone, southern Ethiopia, Tolosa et al. (2012) in Jimma area(1.97%) and Tesfaye et al. (2011) (1.5%) in Addis Ababa.

On the other hand, there were reports with a relatively higher seroprevalence of bovine brucellosis in other parts of the country (Hunduma and Regassa, 2009) (11.2%); (Megersa et al., 2012) (8.0%). However, most of the reports were from the extensively managed herds, where cattle from several owners mingle at grazing or watering

Table 4. Kappa test for agreement between CT, RBPT, i-ELISA and CFT.

Variables	CFT		Kappa Value	Kappa value interpretation	P-value
	-	+			
Card Test			0.660		0.001
Negative	554	0		Substantial agreement	
Positive	8	8			
Rose Bengal Test			0.758		0.001
Negative	557	0		Substantial agreement	
Positive	5	8			
i-ELISA			0.839		0.001
Negative	559	0		Almost perfect agreement	
Positive	3	8			

*Common interpretation of kappa: <0.2 = slight agreement, 0.2 to 0.4 = fair agreement, 0.4 to 0.6 = moderate agreement, 0.6 to 0.8 = substantial agreement, >0.8 = almost perfect agreement.

Table 5. Association of risk factors with *Brucella* seropositivity.

Variables	Level	Number tested	Number positive	χ^2 (*P*-value)
Origin	Bishoftu	384	2(0.52%)	6.63(0.010*)
	Asella	186	6(3.23%)	
Age	Young	131	0(0)	2.42(0.120)
	Adult	439	8(1.82%)	
Management	Extensive	8	0(0)	1.51(0.469)
	Semi-intensive	178	1(0.56%)	
	Intensive	384	7(1.82%)	
Herd size(farms)	Medium	163	0(0)	3.73(0.155)
	Small	85	1(1.18%)	
	Large	322	7(2.17%)	
Breed type	HF	94	0(0)	8.49(0.014*)
	Cross	468	7(1.50%)	
	Local	8	1(12.5%)	
Abortion history	No	524	0(0)	92.43(0.001**)
	Yes	46	8(17.39%)	
Separate parturition	No	160	0(0)	3.17 (0.075)
	Yes	410	8(1.95%)	
Abortion period	First trimester	19	0(0)	192.97(0.001**)
	Second trimester	5	0(0)	
	Not applicable	523	0(0)	
	Third trimester	23	8(34.78%)	
Location	Urban	100	0(0)	1.73(0.189)
	Peri-urban	470	8(1.70%)	
Parity	Not applicable	160	0(0)	4.66(0.097)
	Primiparous	119	1(0.84%)	
	Pluriparous	291	7(2.41%)	

*, statistically significant;**, statistically highly significant.

Table 6. Multivariable logistic regression analysis of putative risk factors with *Brucella* sero positivity.

Variables	Level	No. of animal tested	Prevalence (%)	Crude OR (95%CI)	Adjusted OR (95% CI)
Origin					
	Bishoftu	384	0.52	1	1
	Asella	186	3.23	6.37(1.27,31.85)	7.56(1.48,38.61)
Breed type					
	HF	94	0	1	1
	Cross	468	1.50	-	-
	Local	8	12.50	0.11(1.02,86.99)	0.19(0.53,52.45)

OR, Odds ratio; CI, Confidence interval; 1, Reference.

points. Hence, the low seroprevalence observed in this study could possibly be explained by the developed awareness and instituted informal culling practice, as well as proper disposal of afterbirths as it has been also suggested by Tesfaye et al. (2011) and/or the prevailing management differences between the intensive, semi-intensive and extensive production systems (McDermott and Arimi, 2002; Matope et al., 2011).

The present study revealed that origin of dairy cattle was significantly associated with brucellosis in dairy cattle (P<0.05) and the results showed higher individual animal seroprevalence in Asella (3.23%) when compared to Bishoftu (0.52%). The reasons for the variations in brucellosis seroprevalence among the study areas might be related to the difference in management practice performed in the two study sites. At the onset of the dairy schemes in Asella, farm owners purchased *Bos taurus* cattle from commercial farms, but the screening of these for brucellosis was not done due to limited availability of veterinary services, while the practice of screening for brucellosis was developed before purchasing cattle in most of Bishoftu dairy farms. According to the report of different studies, purchasing of cattle from commercial farms without screening for brucellosis increases the chances of contact with infected herds (Muma et al., 2007).

In addition, different studies revealed that the seroprevalence of brucellosis is lower in low land agro-climate which is unsuitable for survival of *Brucella* organisms than highland (Radostits et al., 1994). Therefore, the practice of purchasing cattle from commercial farms without screening for brucellosis together with other agro-ecological factors could partly explain the observed higher seroprevalence of dairy cattle brucellosis in Asella as compared to Bishoftu.

In the present study, the higher seroprevalence of dairy cattle brucellosis was observed in large herd size in the study sites. This study finding was in line with that of Asfaw (1998) in which he found significant association between *Brucella* seropositivity and large herd size. However, in contrary to this, Kebede et al. (2008) reported that the risk of *Brucella* seropositivity was independent of herd size in small holder farms from Wuchale Jida district of East Wollega zone of Ethiopia. Higher seropositivity in large herd size can be explained by the fact that an increase in herd size is usually accompanied by an increase in stocking density, one of the determinants for exposure to *Brucella* infection especially following abortion or calving (Crawford et al., 1990).

Even though age was not significantly associated with *Brucella* seropositivity (P> 0.05), a sero prevalence of 1.82% was found among the adult age group whereas no *Brucella* seorpositivity was observed in the young age group of dairy cattle in the study sites. Several previous reports have indicated that higher seroprevalence of brucellosis in adult age group of cattle (Magona et al., 2009) similar to the findings of this study. This could be explained by sexual maturity and pregnancy due to the influence of sex hormones and placenta erythritol on the pathogenesis of brucellosis (Radostits et al., 2007).

The finding of this study revealed that higher seroprevalence of brucellosis was observed in intensive production systems. Similar to this finding, previous reports have indicated that higher seroprevalence of *Brucella* was found among dairy cattle in intensive production systems in highland areas of Ethiopia (Jergefa et al., 2009; Asmare et al., 2010). The higher seroprevalence of brucellosis in intensive production systems particularly in Asella could be explained by the fact that there is a greater chance of contact between infected and healthy animals in these systems, or between healthy animals and infectious materials, since most of farm owners' do not follow hygienic practices which was in agreement with the report of Jergefa et al. (2009).

The present study revealed that a history of previous abortions was significantly associated (P<0.001) with *Brucella* seropositivity. A seroprevalence of 17.39% was recorded for the occurrence of previous abortion in these study areas based on questionnaire survey. This finding was consistent with Tolosa (2004) who reported 17.6% in selected sites of Jimma Zones. This could be explained by the fact that abortions or stillbirths and retained

placentas are typical outcomes of brucellosis (Radostits et al., 1994; Swell and Brocklesby, 1990). In addition, in highly susceptible non-vaccinated pregnant cattle, abortion after the 5th month of pregnancy is a cardinal feature of the disease (Radostits et al., 2000). In contrary to this finding, a relatively lower seroprevalence (6.1%) was reported by Tesfaye (1996) in Mekele dairy cattle and 6.7% by Yayeh (2003) in North Gondar, Ethiopia.

There is still a controversy among different researchers on the issue of breed susceptibility to brucellosis. This study revealed that significant difference between breed type and *Brucella* sero positivity in dairy cattle. This might be due to the origin of the animal from the previously infected or exposed herds (Deselegn and Gangwar, 2011). In spite of the small sample size of local breed in the present study as potential limitation, a higher seroprevalence of 12.50% was found in local bred cattle in the study sites. In contrast to our finding, Yohannes et al. (2012) reported a seroprevalence of 1.7% in local bred cattle in Asella, Oromia Regional State, Ethiopia. In contrary to the present study, Jergefa et al. (2009) found that breed of cattle has significant effect on the serological prevalence of brucellosis and he reported higher seroprevalence of brucellosis in cross-bred than in indigenous (local) ones.

There was statistically significant association ($P<0.05$) between abortion period and sero positivity of brucellosis in the present study. This could be explained by the presence of higher seropositivity in cows in the last trimester which may be due to the preferential localization of *Brucella* in the uterus, in which allantoic fluid factors such as erythritol could stimulate the growth of *Brucella* and elevate in the placenta and fetal fluid from about the 5th month of gestation (Radostits et al., 2007; Coetzer and Tustin, 2004).

On the basis of parity, the difference observed in seroprevalence was statistically insignificant. Similar observations were made by Berehe et al. (2007). Though there is insignificant association between parity and brucellosis seropositivity, the higher seroprevalence was observed in pluriparous (2.41%) than primiparous cattle (0.84%) in the study areas. The higher seroprevalence of brucellosis in the pluriarous cattle of this study was in line with Asmare et al. (2013) who reported 2.5% in pluriparous dairy cattle and breeding farms with special emphasis on cross and exotic bred. With regard to serological test comparison, almost perfect agreement with significant association was observed between i-ELISA and CFT (K=0.839) whereas substantial agreement was found between CT (K=0.66) and RBPT (K=0.76) with CFT. This finding is inconsistent with Asfaw (1998) who reported a moderate agreement (K=0.44) between RBT and CFT. On the other hand, almost perfect agreement (K=0.98) was reported between RBPT and CFT by Abay (1999).

In conclusion, serological findings indicated that bovine brucellosis is an established disease in Asella and Bishoftu dairy farms. Higher seropositivity of *Brucella* was observed in Asella dairy farms compared to Bishoftu. Origin, local breeds, cattle with history of abortion, and the third trimester abortion period were the risk factors significantly associated with *Brucella* seropositivity in the study areas. Moreover, origin was statistically identified as the major potential risk factor for brucellosis to occur in relation to other factors. Therefore, more proactive measures should be taken to protect the cattle populations from *Brucella* infection and to reduce its economic impact to the dairy industry and the risk of zoonotic infection in exposed human population in the study areas.

Conflict of Interests

The authors have not declared any conflict of interests.

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Abbreviations: AI, Artificial Insemination; **CFT,** Complement Fixation Test; **CI,** Confidence Interval; **CT,** Card Test; **HF,** Holestein Fresian; **iELISA,** Indirect Enzyme Linked Immuno Sorbent Assay Test; **KAP,** Knowledge, Attitude and Practices; **NAHDIC,** National Animal Health Diagnostic and Investigation Center; **OD,** Optical Density; **OR,** Odds Ratio; **RBPT,** Rose Bengal Plate Agglutination Test; **S-LPS,** Smooth Lipopolysaccharide; **USDA,** United States Department of Agriculture.

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Correlation between biliary bacteriology and types of gallstones in uncomplicated symptomatic cholelithiasis

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The aim of this study was to identify the microflora in the gallbladder of patients undergoing cholecystectomy for different types of gallstones and the antibiotic susceptibility pattern of the isolates. Between January 2005 and December 2008, 136 samples of bile [cholesterol stones (CS)] and 175 pigment stones (PS) were collected from 213 women and 98 men, who were between 18-95 years (median age: 52.5±12.0) by laparoscopic or open cholecystectomy at the University Surgical Unit Duzce in Turkey. The bile samples were aerobically cultured to assess microflora and their antibiotic susceptibility. 210 (54%) of the 311 patients with gallstones had bacterial isolates; 78 isolates (37.2%) were from cholesterol stone-containing bile and 132 isolates (62.8%) from pigment stone-containing bile (*P*<0.01). The overall bacterial isolates from bile samples revealed *Escherichia coli (E. coli)* predominantly, followed *by Pseudomonas aeruginosa (P. aeruginosa)* and *Enterococcus* spp. In one sample, we found *Candida lusitaniae* (*C. lusitaniae*). None of the Gram negative strains were resistant to antibiotics but Gram positive strains were highly resistant to penicillins (100%). Looking at the microflora of the gallbladder and the susceptibility pattern of our isolates, we would suggest that antibiotic prophylaxis recommended for laparoscopic or open cholecystectomy for gallstones be reviewed and the role of bacteribilia in the surgical management of cholelithiasis requires further study.

Key words: Microflora, bile culture, gallstones, cholecystectomy.

INTRODUCTION

Gallstone disease is a common and costly disorder worldwide; it is often asymptomatic, biliary colic, cholecystitis and entails life-threatening complications such as obstructive jaundice (Stringer et al., 2013). The pathophysiological role of bacteria in the formation of gallstones was proposed long ago. Bacteria are often found in high concentrations in pigment and less in cholesterol gallstones. Although it is intriguing to hypothesize that cholesterol stones (CS) formation is non-bacterial in nature and essentially different from the pathogenesis of “infectious” pigment stones (PS), it is more likely that significant overlap exists between the two

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processes. This is in keeping with the fact that most gallstones are mixed in nature (Swidsinski and Lee, 2001).

Cholecystectomy is among the top 10 surgical procedures performed each year in Western societies; 2 with more than 500 000 patients are performed annually in the United States, consuming resources estimated at $6.5 billion (Stringer et al., 2013). In the present study, we demonstrated that gallbladders excised from patients with symptomatic cholelithiasis, even without any evidence of inflammation, might host bacteria in over 20% of cases (Gosling et al.,1996). Prompt administration of the appropriate antibiotics is crucial in the management of biliary tract infection and antimicrobial treatment is commonly administered pre- or perioperatively and often inhibits bacterial growth. Moreover, it is suggested that the recovery of bacteria in bile cultures is affected by toxicity of bile salts. Thus, traditional culturing methods of bile might miss a large number of underlying bacterial infections that could lead to acute or chronic cholecystitis (Grill et al., 2000; Cheng et al., 2002; De Boever and Verstraete, 1999; Kurtin et al., 2000; Manolis et al., 2008). Culture of bile from the gallbladder of patients with uncomplicated cholelithiasis during cholecystectomy has shown principally *E. coli*. Others include *Pseudomonas* spp*., Enteroccocus faecalis, Streptococcus* spp*.* and *Klebsiella* spp. (Darko and Archampong, 1994; Gold-Deutch et al.,1996).

Bile from the gallbladder of patients with uncomplicated choleilthiasis who have different types of gallstones is rarely studied. Also, investigations of type and prophylactic antibiotic sensitivity of microflora in bile from the gallbladder are rare in patients with different types of gallstones. This study aimed to determine the nature of microorganisms and the antimicrobial susceptibility of bacteria in bile from patients with uncomplicated symptomatic cholelithiasis who have different types of gallstones.

MATERIALS AND METHODS

Patients

Between January 2007 and December 2010, samples of bile (136 cholesterol stones and 175 pigment stones) were obtained from 311 patients (213 women and 98 men; aged 18-95, with a median age of 52.5±12.0 years) by laparoscopic (85.9%) or open cholecystectomy (3.8%) at the University Surgical Unit Duzce, Turkey. Cholelithiasis was diagnosed by abdominal ultrasonography or computer-aided topographic scan. The patients were followed up for a week and a month after being discharged from the surgical clinic.

For the selection of patients, excluded from the study were those who had previous episodes of cholecystitis or cholangitis.

Selection criteria for gallstones

Gallstones were classified according to visual appearance or color. The stones were separated into two groups: (1) black stones and brown stones were regarded as PS, (2) yellow stones were regarded as CS.

Collection of bile

As the gallbladder was removed at surgery, it was punctured under sterile conditions and 2.0 ml of bile was aspirated. The sample was transferred to a sterile container and transferred at room temperature in an hour to the laboratory for culture.

Method of culture and identification of bacterial colonies

Bile samples were cultured aerobically in blood agar (Oxoid, Basingstoke, UK), EMB agar (Difco,Detroit,Mich.,USA) and Sabouraud dextrose agar (Difco,Detroit,Mich.,USA). Direct inoculates of culture media and plates were incubated at 37°C under aerobic conditions. Direct gram staining was done to test for the presence of organisms and pus cells. The culture plates were examined at 24 and 48 h for bacterial growth. An aliquot of bile was inoculated into brain heart infusion (BHI) as an enrichment procedure. The broths were sub-cultured onto solid media following 24 h of incubation at 37°C. The plates were examined for bacterial growth at 24 and 48 h. Bacterial growth was identified by gram staining, culture characteristics, and biochemical test (API 32 E and API 32 GN, and kits, Bio- Merieux SA, Marcy I Etoile France).

Antibiotic sensitivity test

Antibiotic sensitivity testing of isolates was performed using Clinical Laboratory Standard Institution (CLSI) methods (CLSI 2012). For Gram-negative isolates, the following antimicrobials were used: ampicillin (10 µg),chloramphenicol (30 µg), ciprofloxacin (5 µg), amikacin (30 µg), gentamicin (10 µg), ceftazidime (30 µg), ceftrioxone (30 µg), cefotaxime (30 µg), cefepime (30 µg), ceftazidime (30 µg) and imipenem (10 µg); for Gram-positive isolates: oxacillin, penicillin (10 µg), vancomycin (30 µg), erythromycin (15 µg), ciprofloxacin (5 µg), co-trimoxazole (1.25/23.75 µg), gentamicin (10 µg), amikacin (30 µg), Clindamycin (2 µg), chloramphenicol (30 µg) and Rifampicin (30 µg).

Statistical analysis

Data were expressed as frequencies and analyzed using SPSS (SPSS 18.0, Chicago, Illinois, USA). The statistical significance of the incidence of positive and negative cultures from the patients and controls were compared using the paired *t* test. Significance was assigned a P value of <0.05.

RESULTS

In the 311 bile samples, 210 (54%) were shown to have bacterial isolates. 78 (37.2%) isolates were from CS-containing bile and 132 (62.8%) were from PS-containing bile (P<0.05). Over 50 years and sex were the signifcant pre-operative factors associated with positive bile culture containing stones (P<0.05). The overall bacterial isolates from bile samples showed 54.7% (115/210) *E. coli*, 23.3% (49/210) *P. aeruginosa*, 12.8% (27/210*) Enterococcus* spp*.,* 3.9% (8/210) *Klebsiella* spp*.*, 2.8% (6/210) *Staphylococcus epidermidis* (coagulase-negative) and the other microrganisms (*S. aureus,Streptococcus* spp*.,Candida* spp*.*) (Table 1). In one sample, we found *C. lusitaniae*. No bile samples from the controls showed bacterial growth after direct inoculation or after BHI

Table 1. Incidence of Bacteria in CS and PS- containing bile.

Microorganisms	Cholesterol stone containing bile (n/%)	Pigment stone-containing bile (n/%)	All (n/%)
E.coli	51/44.4	64/55.6	115/54.7
P.auregenosa	22/44.9	27/55.1	49/23.3
Enterococcus spp.	10/37.1	17/62.9	27/12.8
Klebsiella.spp	3/37.5	5/62.5	8/3.9
S.epidermidis	3/50.0	3/50.0	6/2.8
Other m.o*	2/44.4	4/66.6	6/2.4

**S. aureus*,Streptococcus spp., Candida spp.

Table 2. Antibiotic sensitivity pattern of Gram negative organisms (%).

Antibiotics	Cholesterol stone-		containing bile	Pigment stone-containing bile		
	S	MS	R	S	MS	R
Amikacin	100			100		
Ampicillin	98	2		98	2	
Ceftrioxone	98	2		100		
Cefotaxime	100			100		
Ceftriaxone	100			100		
Cefepime	100			100		
Ceftazidime	99	1		98	2	
Choramphenicol	98	2		99	1	
Ciprofloxacin	100			100		
Gentamicin	100			100		
Imipenem	100			100		

S=Sensitive; MS= Moderately sensitive; R=Resistant.

enrichment. Polymicrobial infection was encountered in 4.7% (10/210) and did not show any difference in the bile containing CS and PS *(P*>0.00).

Gram negative and Gram positive bacterial isolates from the bile containing CS and PS did not show any difference in sensitivity to antibiotics (Tables 2 and 3) (*P*=0.01). None of Gram negative bacterial isolates were resistant to antibiotics but Gram positive bacterial isolates were highly resistant to Penicillins (100%) (Tables 2 and 3).

DISCUSSION

There are conflicting reports in the literature regarding the significance of bacterial infection in both normal subjects and patients with cholelithiasis. It has been suggested that the discordant reports were because of the differences in grouping of patients, in sampling and cultural techniques (Csendes et al., 1996a).

Bile cultures during biliary surgery for patients with risk factors include age above 70 years, previous biliary tract operation, jaundice, chills and fever within one week of operation, and operation performed within one month of an acute attack of cholecystitis (Csendes et al.,1996a; Csendes et al., 1975; Nichols, 1984). Antimicrobial prophylaxis is recommended when one or more of these risk factors are identified preoperatively, although their relative importance in laparoscopic cholecystectomy is undetermined (Nord, 1990; den Hoed et al.,1998). In our study, over 50 years and sex were the significant pre-operative factors associated with positive bile culture containing stones *(P*<0.01). Isolation rates of bacteria in the bile of patients with gallstones ranging from 3 to 72% have been reported in the literature (Csendes et al., 1996a; Csendes et al., 1975; Won et al., 2008; Mahafzah et al., 2009).

The role of bacteria in the pathogenesis of CS and PS-containing bile has been rarely studied. Direct evidence of bacterial presence is not sufficient because of failure to

Table 3. Antibiotic sensitivity pattern of Gram positive organisms (%).

Antibiotics	Cholesterol stone-		containing bile	Pigment stone-containing bile		
	S	MS	R	S	MS	R
Amikacin	100			100		
Chloramphenicol	100			100	2	
Ciprofloxacin	99	1		100		
Clindamycin	100			100		
Methicilline/Cloxacillin	100			100		
Erythromycin	100			100		
Gentamicin	100			100	2	
Penicillin	100			100	1	
Rifampicin			100		100	
Co-trimoxazole	100			100		
Vancomycin	100			100		

S=Sensitive; MS= Moderately sensitive; R=Resistant.

culture bacteria from CS (Darko and Archampong, 1994). Bacteria are often found in high concentrations in pigment and less in cholesterol gallstones. Although it is intriguing to hypothesize that CS formation is non-bacterial in nature and essentially different from the pathogenesis of“infectious” PS, it is more likely that significant overlap exists between the two processes. This is keeping with the fact that most gallstones are mixed in nature (Swidsinski, 2001). According to our study, In 311 bile samples, 210 (54%) were shown to have bacterial isolates, 78 (37.2%) isolates were from CS-containing bile and 132 (62.8%) were from PS-containing bile (*P*=0.01).

The majority of the organisms isolated were enteric coliforms and *P. aeruginosa* followed by Gram positive bacteria such as Enterococcus, Staphylcoccus, Streptococcus (Bistgani and Imani, 2013; Li and Ji, 2009; Sattar et al., 2007; Flores et al., 2003). Similar to our study, the most common organisms were *E. coli, P. aeruginosa, Enterococcus* spp*., Klebsiella* spp*.* and *Staphylococcus epidermidis (coagulase-negative).* Enteric organisms have often been suspected to cause cholelithiasis, and intestinal flora has frequently been recovered followed by intervention on the biliary tree (Abd-Alkareem, 2011).

An interesting finding in this study is the identification of *C. lusitaniae*. Candidial cholecystitis is seen especially in patients with malignancies. According to Yildirim et al. (2008), in the review of the literature, this is the first report on acut calculous cholecystitis by *C. lusitaniae*. *C. lusitaniae* is intrinsic or secondary resistance to amphoteracin B. This patient was treated with flucanozale.

No scientific data support antibiotic treatment of biliary microflora. There are conflicting reports on the usefulness of bile cultures at the time of surgery and the association of positive bile cultures with post-operative wound infection and septic complications (Darko and Archampong, 1994; Gold-Deutch et al.,1996; Abeysuriya et al., 2008). There is a higher incidence of postoperative morbidity and infectious complications in patients with pathogenic bacteria in gallbladder bile than in patients with no bacterial growth or opportunistic bacteria (Csendes et al., 1996).

Antibiotic sensitivity patterns of bacterial isolates in the bile of patients in this study were similar to those reported elsewhere. Gram negative and Gram positive isolates from the bile containing CS and PS did not show any difference in sensitivity to antibiotics (*P*=0.01).

Bistgani and Imani (2013) in their studies concluded that the most susceptible antibiotic for microorganisms on the whole was Amikacin but our studies show that none of Gram negative bacteria were resistant to antibiotics and Gram positive bacteria were highly resistant to penicillins (100%).

Vancomycin and teicoplanin showed 100% sensitivity. Antibiotic sensitivity patterns of isolated organisms were similar, irrespective of the type of stone in the bile. Imipenem and vancomycins may be used as second choice to treat the infection which other drugs cannot treat.

In conclusion, bacterial isolates were significantly more common in PS-containing bile than in CS-containing bile in this study.

Our study shows an association between gallstone formation and the presence of bacteria in bile. From the microfora of the gallbladder and the susceptibility pattern of our isolates, we would suggest that antibiotic prophylaxis recommended for laparoscopic or open cholecystectomy for gallstones needs to be reviewed and the role of bacteribilia in the surgical management of cholelithiasis requires further study.

Conflict of interests

The authors have not declare any conflict of interest.

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Anti-candida and anti-enzyme activity and cytotoxicity of 2-phenyl-4H-chromen-4-one

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This study determined the fungicidal concentration (MFC), anti-enzymatic (production of proteinases and phospholipase), and cytotoxic activities of 2-phenyl-4*H*-chromen-4-one against different strains of *Candida sp.* The results were: MIC> 62.5 µg\ml and MFC>250 µg\ml for *C. albicans*; MIC/MFC = 31.25 µg\ml for *C. parapsilosis*; MIC/MFC= 62.5 µg/ml for *C. famata*; MIC/CFM =125 µg\ml for *C. glabrata* and MIC = 15.62 µg\ml for *C. lipolytica*. Values for phospholipase and proteinase before and after exposure of yeast to the compound had no statistical differences. For the cytotoxicity test, there were no statistical differences between the tested and control groups. Data from this study provide evidence that 2-phenyl-4H-chromen-4-one could be an alternative source for the treatment of fungal infections caused by *Candida*.

Key words: *Candida albicans*, antifungal activity, antifungal agents, anti-enzyme activity, flavone.

INTRODUCTION

Species of *Candida* spp. are often cited as opportunistic microorganisms and / or commensal found in the normal flora of the digestive and genitourinary tracts of humans and animals (Kam and Xu, 2002). Lately, these species have become clinically important, because they are responsible for superficial and deep infections (Saghrouni et al., 2013) and due to the increasing number of immunocompromised patients and the advances in intensive medical care (Pfaller and Diekema, 2007). Some species have become important pathogens of nosocomial infections, including *C. albicans*, which is associated with high mortality rates of above 30% despite the availability of already established (azoles) or new antifungal treatment options (Pfaller and Diekema, 2007; Horn et al., 2009). Some authors emphasize the need to implement appropriate control measures, which requires a high knowledge of the biology, epidemiology, and antifungal treatment of *Candida* species, including *C.*

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albicans, which are recognized as being particularly complex (Saghrouni et al., 2013).

However, the interaction between antifungal drugs and fungi, molds, and hosts as well as between the antifungal drug and the host are very complex. The establishment of the antifungal activity of a new drug offers only an *in vitro* of the information needed to predict the outcome of treating infection with a new drug. The improvement of *in vitro* assays and susceptibility testing against the constant emergence of new drugs makes for indispensable *in vitro* tools and meaningful contributions to the selection of the most appropriate antifungal agent (Johnson, 2008).

Medicinal plants have been used to fight diseases since the early days of mankind, and today these plants serve as a guide in supporting drug discovery. One of the main groups of phenolic compounds found in plants is the flavonoids. It has been known for many years that this class of compounds of plant origin has potential bioactivity. Flavonoids are subdivided into flavones according to their chemical structure. Flavones are heterocycles of six members which contains one oxygen and ketone group. The general methods to obtain flavones are the Allan–Robinson reaction, Auwers synthesis and Baker–Venkataraman rearrangement (Eicher and Hauptmann, 2003). The flavones are a class of naturally occurring compounds that are widely distributed in vascular plants, which are well known for having a wide range of pharmacological activities such as antiallergic, antibacterial, antidiabetic, antiinflammatory, antiviral, antiproliferative, antimutagenic, antithrombotic, anticarcinogenic, hepatoprotective, oestrogenic, insecticidal, and antioxidant activities (Fesen et al.,1994; Duthie and Crozier, 2000; Midleton et al., 2000; Fukai et al., 2002; Sohn et al., 2004; Cushnie and Lamb, 2005; Isobe et al., 2006). However, the antifungal properties of the flavone, 2-phenyl-4H-chromen-4-one, remain unknown.

The aims of this study were to evaluate the antifungal activity and the anti – enzymatic activity of 2-phenyl-4H-chromen-4-one against different strains of *Candida* and to evaluate their cytotoxicity. The hypothesis to be tested is that 2-phenyl-4H-chromen-4-one will show antifungal activity against *Candida* and low cytotoxicity.

MATERIALS AND METHODS

Mass spectrometry

The mass spectra were obtained on a Shimadzu GCMS-QP2010 SE with a split–splitless injector and equipped with a Rtx-5MS capillary column (30 m x 0.25 mmID); helium was used as the carrier gas (56 Kpa).

Infrared analysis

The IR spectra were taken on a Agilent Technologies Cary 640 FTIR spectrometer in KBr pellets.

Nuclear magnetic resonance

NMR spectra were recorded on a Bruker DPX 300 spectrometer (300.13 MHz for ^{1}H and 75.48 MHz for ^{13}C) at 300 K.

Strains

Candida albicans (33), *C. parapsilosis* (2), *C. famata* (2), *C. glabrata* (2) and *C. lipolytica* (2) strains were used for determining the *in vitro* anti-*Candida* activity of 2-phenyl-4H-chromen-4-one. These strains of *C. albicans* were collected from patients with Chronic Atrophic Candidiasis (CAC) who use dentures and who were enrolled in the Center for Diagnosis of Diseases of the Mouth (CDDB), School of Dentistry, Federal University of Pelotas (FOP-UFPel). The diagnosis of CAC was clinical, and microbiological as previously described (Lund et al., 2009).

In vitro anti-candida activity

Inoculum

The strains of *C. albicans* and *non-albicans* Candida were sub-cultured onto Sabouraud Dextrose Agar with 0.2 mg/mL of chloramphenicol at 36°C for 24 h. After incubation, these strains were individually inoculated into tubes containing 5 mL of a sterile 0.85% saline solution and the yeast suspension was adjusted to a 0.5 McFarland standard (which is approximately 10^6 CFU/mL). After that, the inoculum was re-suspended to obtain a final concentration of 0.5×10^3–2.5×10^3 in RPMI 1640 medium (Sigma, St. Louis, MO, USA) buffered to pH 7.0 with 165 mmol L^{-1} of morpholinepropanesulfonic acid (MOPS; Vetec).

Determination of MIC

The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of 2-phenyl-4H-chromen-4-one was determined by using broth microdilution techniques as described by the Clinical and Laboratory Standards Institute for yeasts (M27-A2). The stock solution of 2-phenyl-4H-chromen-4-one was prepared in a 70% ethanol solution at the concentration of 500 $\mu g.mL^{-1}$. A powder consisting of 2-phenyl-4H-chromen-4-one was previously weighed and dissolved in ethanol at the concentration of 500 µg mL^{-1}.The solutions were diluted in RPMI medium, and the final drug concentrations ranged from 0.49 to 250 $\mu g.mL^{-1}$. Two replicates were made for each concentration of the tested compounds.

After 48 h of incubation at 35°C, the MIC was determined visually by comparison with the drug-free growth control well. The MIC was defined as the lowest concentration of the antifungal agent that prevented visible fungal growth.

Determination of MFC

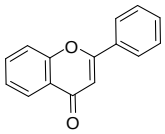
Each inoculum from the previous test that did not show growth was subcultured on agar plates. After 24 h of incubation, the reading was determined by the visible growth of strains. The CFM was considered to be the lowest concentration that prevented visible growth.

In vitro anti-enzyme activity (Carvalho et al., 2015)

Phospholipase

Reduced egg yolk agar (Reya) was used and the plates were

Table 1. *In vitro* antifungal activity of 2-phenyl-4*H*-chromen-4-one dissolved in ethanol 70% against *Candida albicans* and *non-albicans Candida*.

Structural formula		*C. albicans*	*C. parapsilosis*	*C. Famata*	*C. glabrata*	*C. lipolytica*
	MIC(ug/ml)	>62.5	31.25	62.5	125	15.6
	MFC(ug/ml)	>250	31.25	62.5	125	15.6
2-phenyl-4*H*-chromen-4-one						

inoculated (4 per isolate) with a 5 µL suspension of each isolate (108 cells/mL) and then cooled until they were dry. After the plates were incubated at 37°C aerobically for 48 h, the diameters of zones of precipitation around the colonies were measured. The enzyme activity was determined by the radius hyaline zone/diameter of the colony (Pz). Pz values equal to 1 (Pz=1) indicated the absence of enzyme activity, while values greater than 1 (Pz>1) indicated positive levels of phospholipase. Each experiment was repeated twice.

Proteinase

Clinical isolates were inoculated in tubes containing 5 mL of YPD medium and incubated at 37°C for 18 h. After the incubation, aliquots of 1.5 mL of culture were transferred to Eppendorf® tubes and centrifuged at 3000 rpm for 5 min at 4°C. The cell pellets were re-suspended in saline (NaCl 0.9 wt%) and centrifuged using double speed that was used for removal of debris through cultivation. The concentrations of the suspensions of the strains were standardized, using the index range of 0.5 MacFarland (Approximately 1x106 CFU/mL), and 1 mL volumes were inoculated at equidistant points in the middle Proteinase Agar. The plates with different inocula were incubated at 37°C for four days. The tests were performed in duplicate. The values of the Precipitation Zone (PZ) were obtained by dividing the diameter of the colony by the diameter of the colony plus the precipitation zone. (PZ = 1, Negative; PZ ≥ 0.64 <1, Middle-Positive, Positive-PZ ≤ 0.63 High).

Cytotoxicity assay (Carvalho et al., 2015)

The cell culture medium was Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2% L-glutamine, penicillin (100 U/ml), and streptomycin (100 mg/ml). Mouse fibroblasts of the 3T3/NIH immortalized cell line were maintained as a stock culture in DMEM and incubated at 37ºC in a humidified atmosphere of 5% CO_2 in air until subconfluency was reached. Cells were incubated with the same concentrations of 2-phenyl-4H-chromen-4-one were used in the antifungal assay. These compounds were dissolved in DMSO and added to the DMEM supplemented with 10% FBS to the desired concentrations.

The 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) assay was used to assess cell metabolic function according to mitochondrial dehydrogenase activity. Mouse fibroblasts (3T3/NIH; 2×10^4/well) were maintained in DMEM in 96 well plates for 24 h. DMEM was removed and replaced with 200 µL of extract from different groups with 10% FBS. Cytotoxicity produced by the different extracts was assessed at 24 h after cell exposure time. After removing the extracts, the cells were washed with phosphate-buffered saline (PBS), and then 200 µL of medium in 20 µL of MTT solution (5 mg of MTT/ml DMEM) were added to each well. After 5 h of incubation at 37ºC in the dark, the blue formazan precipitate was extracted from the mitochondria using 200 µL/well of dimethyl sulfoxide (DMSO) on a shaker for 5 min at 150 rpm. The absorption at 540 nm was determined spectrophotometrically.

The cell viability was analyzed using SigmaStat 3.5 (Systat Software Inc., San Jose, CA, USA) statistical analysis software. The statistical analysis was performed by one-way ANOVA with the level of significance set at $p<0.05$.

RESULTS AND DISCUSSION

The results obtained for the minimum inhibitory concentration and minimum fungicide are shown in Table 1. The results show respectively, MIC and MFC 62.5 µg\ml for *C. albicans*; MIC 0.48µg\ml and fungistatic behavior for *C. parapsilosis*; MIC and MFC 62.5 µg\ml for *C. glabrata* and *C. famata*; MIC 15.62 µg \ ml and fungistatic behavior for *C. lipolytica*. The syntheses of flavones usually start from o-hydroxyacetophenones. The 2-phenyl-4H-chromen-4-one was obtained conform literature (Munawar and Groundwater, 1999). The Flavone was crystallized in colourless needles of mp 97°C; yield of isolated product was70%. The structure of flavone was confirmed by infrared analysis, mass spectrometry and nuclear magnetic resonance of ^{1}H and ^{13}C; MW: 222.06. CG-MS m/z (%): 120 (M^+, 75), 194 (M^+, 60), 222 (M^+, 100). NMR ^{1}H: 7.73-8.21 (Ph, 5H); 7.13 (H_1, 1H); 7.89-8.17 (Ar, 4H). NMR ^{13}C: 131.7; 129.0; 126.2; 131.1; 123.2; 155.7; 118.4; 134.2; 125.5; 124.7; 177.0; 106.9; 162.5. IR: v (cm^{-1}) 3100-3200; 1600-1700, 1300-1400, 700-800.

The research hypothesis was confirmed since data from this study provide evidence that 2-phenyl-4H-chromen-4-one could be an alternative source for the treatment of fungal infections caused by *C. albicans* and *non-albican Candida*. The fungal species chosen for this study were *C. albicans*, *non-albicans Candida (C. parapsilosis, C. famata, C. glabrata, C. lipolytica).* The Candida species were specially chosen because they are present in 75% of the commensal microflora of normal healthy individuals (Mulu et al., 2013) and infections caused by the same have increased dramatically in the last three decades, setting up a current clinical challenge (Samaranayake et al., 2002).

Several factors determine the severity and type of

infection that may be caused by this type of yeast, but the mechanism by which the transformation occurs from a harmless commensal to an aggressive pathogen is not completely understood (Santos and Braga, 2013). However, it is known that *C. albicans* virulence has attributes that promote adaptation, interaction with host structures, and the circumvention of host immune responses (Santos and Braga, 2013). One attribute of virulence is the secretion of hydrolytic enzymes such as phospholipase and proteinase. Thus, compounds that inhibit or decrease the release of these enzymes could prevent the establishment and proliferation of yeast, preventing the development of the disease. Therefore, it was necessary to test the activity of phospholipase anti-enzyme and proteinase with the administration of flavone.

Moreover, the diagnosis and the inappropriate and indiscriminate use of antifungals cause a predisposition to recurrent infections as a consequence of the acquisition of antifungal resistance by common pathogenic microorganisms (Akpan and Morgan, 2002). Two types of resistance are detectable in fungi: innate resistance, as in *C. krusei* against fluconazole, or acquired, as in after an antifungal treatment, as in the case of *C. albicans* being responsible for oral infections in HIV-positive patients treated with azole compounds (Yang and Lo, 2001). Because these episodes of resistance to conventional antifungals it is essential to search for new treatment alternatives in order to ensure the death or inhibition of the growth of pathogenic microorganisms or the inhibition of the enzymes that are responsible for their adhesion and subsequent colonization.

Flavonoids are polyphenolic compounds that are biosynthesized through the phenylpropanoid pathway and the acetate (Mann, 1987). The main sources of Flavonoids are fruits like grapes, cherries, apples, currants, citrus fruits and vegetables like peppers, tomato, spinach, onions, broccoli (Barnes et al., 2001). Medicinal and spice plants that contain these flavonoids have been used for thousands of years in Oriental medicine, but have still not spread to the West (Midleton et al., 2000). Flavones are a class of flavonoids that are divided in two groups. Flavones constructed by two-phenylchromone are characterized by a natural product of low molecular weight that is a participant in the photosynthetic reaction. Some important biological characteristics are already linked to the flavones, including antioxidant, anti-inflammatory, antiviral, antitumor, and antiatherosclerotic properties (Midleton et al., 2000). However, since the antifungal and anti-enzymatic activities of this compound have not yet been reported in the scientific literature, it is necessary to fill this gap. Based on the methodology applied in this study, the results show significant antifungal activity. Moreover, in this study, most of the compounds have similar results for MIC and MFC, as drugs behaving as fungicide. Immune depression in patients with only the inhibition of fungal growth may not be sufficient to prevent the spread of *Candida* (Elewiki and Ohio, 1993).

The choice of a broth dilution test to determine the sensitivity of yeasts to antifungal therapy, as recommended by The National Committee for Clinical Laboratory Standards (M27-A2 protocol), was chosen for the advantages of easy reproducibility (Arthington-Skaggs et al., 2002), low cost, and sensitivity. In addition, this method requires only a small amount of each sample, which is used in various tests (Ostrosky et al., 2008). The methodology of the Standard M27-A2 recommends the reading of the results for *Candida* after 48 h (NCCLS). In our study, we chose to read at two times: 24 and 48 h. This was due to a lack of knowledge about the action and behavior of this novel compound. The two required readings and their comparison resulted in the observance of the occurrence of trailing and the partial inhibition of growth over a wide range of concentrations of the antifungal.

In addition, the antifungal activity of the compound 2-phenyl-4H-chromen-4-one was tested for its possible ability to inhibit the secretion of the hydrolytic enzymes, proteinase and phospholipase. Protein and enzyme secretions are considered an essential process in fungi survival, and the characteristics of these secreted proteins defined many of the functional capabilities of these microorganisms (Carvalho et al., 2015). These enzymes appear as important pathogenic factors of *C. albicans*. Values for phospholipase and proteinase (Pz) before and after exposure of yeast to the compound had no statistical differences. Figures 1 and 2 illustrate the dose-response curves of the action of flavone tested on the inhibition of proteinase and phospholipase.

The results of the test for cytotoxicity (Figure 3) indicate that these products showed a low toxicity against fibroblasts 3T3/NIH concentrations of the flavones tested. For a better interpretation of the results, these data were analyzed statistically and the wells containing the product were compared to the control. One way analysis of variance (ANOVA) was not statistically significant. In order to compare the action of the compounds on cells and control cells respectively we used the Duncan's statistical test. The results show that there was a significant difference between the wells in which compound was added and those in which it was not added.

Regarding cytotoxicity, according to the present findings, the compounds tested showed no significantly different results where there was the same inclusion. They showed low cytotoxicity, reinforcing the possible use of these antifungal agents. Among antifungal agents, therapeutic agents that are used in most topically in most cases of oral candidiasis, are polienios (Nystatin and amphotericin B) and azoles (itraconazole, miconazole and clotrimazole) (Akpan and Morgan, 2002; Yang and Lo, 2001). Even the most widespread treatments have certain limitations due to side effects such as toxicity and the emergence of resistant strains (Yagiela et al., 2004),

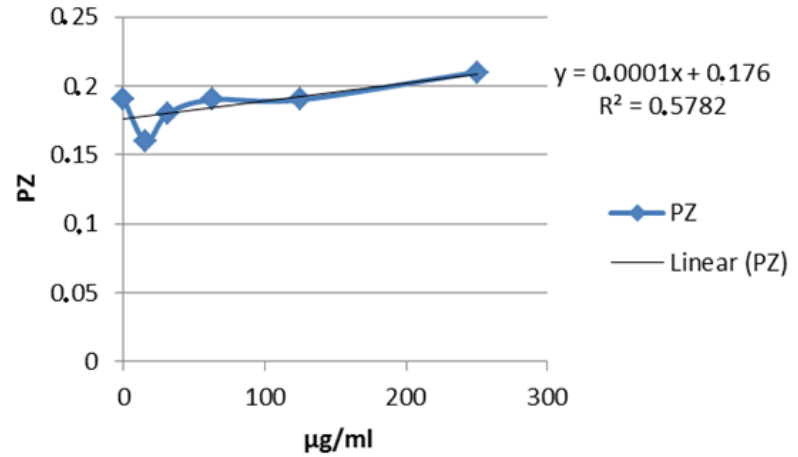


Figure 1. Dose response curve in the production of extracellular proteinase.

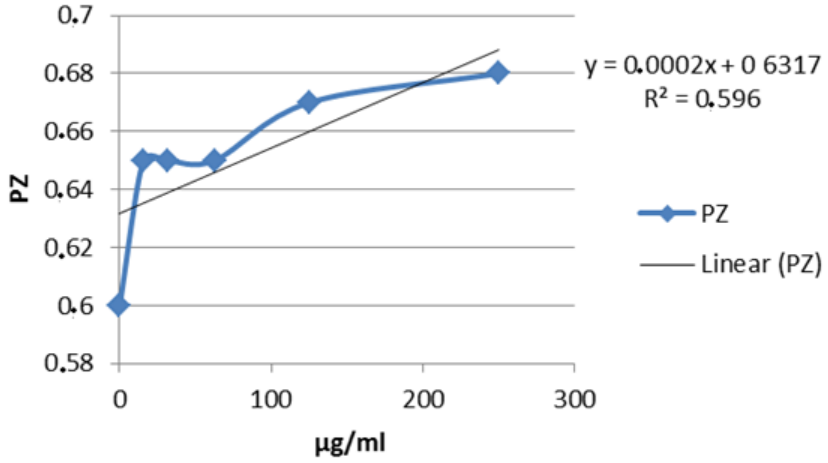


Figure 2. Dose response curve in the production of extracellular phospholipase.

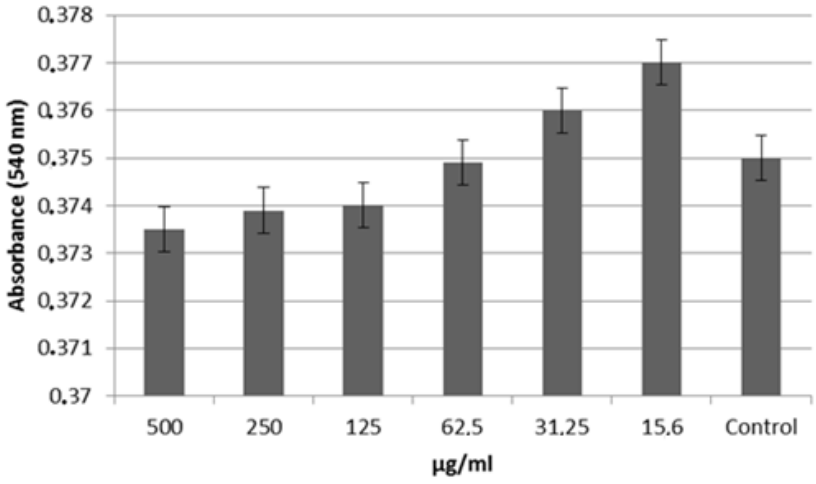


Figure 3. Test for cytotoxicity against fibroblasts 3T3/NIH concentrations of the flavones tested.

which confirms the need for new studies investigating other agents with potential for treating this mycosis and low cytotoxicity (Nobre et al., 2002).

Conclusion

Based on the methods employed, it was found that 2-phenyl-4H-chromen-4-one is a promising antifungal agent that has low cytotoxicity. However, there is a need for a specific study on the safety and efficacy of this *in vivo* use, and clinical trials are still needed to evaluate the practical relevance of in vitro results.

Authors' contributions

Simone Oliveira, Rafael Lund, Rodrigo V. Carvalho and Evandro Piva were responsible for the biological assays and writing and revision of the final scientific paper. Claudio de Pereira was responsible for the synthesis, purification and identification of flavone

Conflict of interest

The authors have not declared any conflict of interest

Abbreviations

CAC, Chronic atrophic candidiasis; **DMEM,** Dulbecco's modified eagle's medium; **DMSO,** dimethyl sulfoxide; **FBS,** fetal bovine serum; **IR,** infrared analysis; **MIC,** minimum inhibitory concentration; **MFC,** minimum fungicidal concentration; **MOPS,** morpholinepropanesulfonic acid; **NMR,** nuclear magnetic resonance; **RPMI,** roswell park memorial institute; **PZ,** precipitation zone; **YPD,** yeast peptone dextrose.

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Microorganisms associated with African star apple (*Chrysophylum albidum*) and their hydrolases

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Microorganisms associated with African star apple (*Chrysophylum albidum* Linn), their quality characteristics and hydrolases were investigated. The bacteria species were *Bacillus cereus, B. polymyxa, Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa* and *Staphylococcus aureus,* while the fungi species were *Aspergillus flavus, A. fumigatus, A. niger, A. repens, Fusarium* sp*., Mucor mucedo, Trichoderma viride* and *Rhizopus stolonifer.* Pathogenicity tests revealed that all the isolates were pathogenic on the fruits. Proximate analysis showed that microbial infections significantly reduced the carbohydrate, crude fibre, protein, moisture content and fat. However, mineral analysis accounted for an increased K, Ca, Mg, Na, Zn and P in an infected fruits compared with the apparently healthy fruits. The pH values ranged from 5.05 to 5.23. Massive infection leading to the deterioration of the fruits that could ultimately affect its quality posing health risk could be reduced by the early consumption of the fruits. All the microbial isolates produced the hydrolases which could be responsible to their enhanced abilities to deteriorate the fruit.

Key words: African star apple, fruits, hydrolases, pathogenic, infections, microorganisms.

INTRODUCTION

The African star apple (*Chrysophylum albidum* Linn.) is an angiosperm belonging to the order Ebernales, family Sapotaceae (Ehiagbonare et al., 2008). The plant has been reported to grow up to a height of 36.5 m and are known to occur in diverse ecological zones in Nigeria, Uganda, Niger Republic, Cameron and Cote d'Ivoire (Bada, 1997). It is commonly called African star apple fruit described as large beny containing 4 to 5 flattened seeds or fewer as a result of seed abortion (Keay, 1989). A detailed description of the apple has been documented in the reports of Adebisi (1997) and Amusa et al. (2003). The fleshy edible pulp is consumed as relished by the people (CENRAD, 1999) and for the purpose of stopping irritation, loss of appetite and salivation.

Asenjo (1946) reported that star apple fruit edible pulp is very rich in ascorbic acid even with about 100 times richer than of oranges and 10 times than that of cashew or guava. Studies have shown the fruit to be an excellent source of vitamins, iron, flavours to diet and raw materials to some manufacturing industries (Okafor and Fernandes,

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1987; Bada, 1997; Umelo, 1997; Adisa 2000). In addition to these qualities, is an acceptable com-position of moisture, ash, crude fibre, oil, protein, starch, sugars and ascorbic acid as cited in Adindu et al. (2003).

Recently, mineral analysis implicated the presence of K, P, S, Ca, Mg, Al and Zn (Chukwuemeka, 2006). The seed is also known to be an important source of oil, for diverse purposes (Amusa et al., 2003). The seeds are used for local games (Bada, 1997). The seed shell of the fruit has been adjudged to be an efficient adsorbent alternative material for the removal of heavy metals and organic matters from water and waste waters (Amuda et al., 2007; Oboh et al., 2009). However, the fruit is reported to contain 90% anacardic acid, used industrially to protect wood and wood materials, and a source of resin, while the leaves, roots and stem bark of the tree have medicinal purposes (Adewusi, 1997; Bada, 1997).

Fruiting season of the plant is usually in the months of December to April during which it is found both in rural and urban cities (Amusa et al., 2003). The fruits are not usually harvested, but left to naturally fall to the forest floor from where they are picked which tend to predisposes the fruits to microbial infections (Amusa et al., 2003). Worth mentioning is the fact that the fruits in its season has engaged some petty traders from which they enjoy some level of economic gains. Therefore, the fruit is fast becoming a fruit of economic value. This study focused on the isolation of microorganisms associated with African star apple fruits with the aim of establishing their quality, safety and extracellular hydrolases.

MATERIALS AND METHODS

Source of samples

Apparently fresh African star apple (*C. albidum* Linn.) fruits were obtained from Oja-Oba (Market), Akure, Nigeria. The samples were brought to the Microbiology Laboratory, The Federal University of Technology, Akure, Nigeria for further analyses and studies.

Sample treatments and isolation of associated microorganisms

The samples were surface sterilized with 75% ethanol, rinsed in several changes of sterile distilled water and placed inside sterile Petri dishes until symptoms of infections were noticed. For bacterial isolation, infected portions of the fruits were sliced into pieces, transferred into sterile distilled water from which serial dilutions were carried out. An aliquot of 0.1 ml of dilution 10^5 was plated on nutrient agar (NA) and incubated at 37°C for 24 h. Discrete colonies were observed, counted, recorded as colony forming unit per gram (cfu/g) and further sub-cultured to obtain pure cultures. The pure isolates were characterized and identified using the methods described by Holt et al. (1994).

A slight modification of the method of Amusa et al. (2003) was used for the isolation of associated fungi. In this case, a 0.1 ml aliquot of dilution 10^3 was dispensed on sterilized potato dextrose agar (PDA) in Petri dishes and incubated for seven days at ambient temperature. The fungal isolates were counted as spore forming unit per gram (sfu/g) and pure cultures of the fungi were examined with a stereo binocular microscope and identified by cultural, morphological and detailed descriptions in the references standard of Booth (1971), Barnett and Hunter (1972) and Webster (1980).

Pathogenicity test

Freshly harvested ripe African star apple fruits were surface sterilized with 75% ethanol and rinsed in several changes of sterile distilled water. The surface sterilized fruits were inoculated with 10^2 of 24 h old culture of bacterial isolates with sterile needle and syringe and incubated at 37°C. The spores of 48 h old fungal isolates were injected into the fruits and incubated at 28°C. The control fruits were injected with injection water. The extent of infection was determined according to the method of Amusa et al. (2003). The bacteria obtained from the infections were re-isolated, characterized and identified according to the methods earlier described, while wet mounts of fungal structures from the infected portions were stained with lactophenol in cotton blue and viewed under microscope for the presence of pathogen used in the inoculation.

Determination of nutrient composition

The fruits were initially kept in dried clean containers, cut opened, deseeded and weighed. The fleshy pulp was cut into pieces with sterile knife and dried in a dry cabinet at 60^oC for 5 days. The dried pulp was ground into powder and analysed for moisture, ash, protein, crude fibre, crude fat and carbohydrate content according to AOAC (2005) techniques. The mineral contents were determined using the standard method of AOAC (1990).

Determination of the pH

The pH of the African star apple fruit was determined using Jenway 3015 pH meter. The pieces of the fruit were homogenized for 30 min at 5 min interval inside a clean glass beaker with sterile distilled water. The electrode of the standardized pH meter was inserted into the homogenates and reading was taken and recorded.

Determination of the viscosity of African star apple fruits

The viscometer was cleansed with appropriate solution, rinsed in distilled water and drained dry. Ten milliliters of distilled water was added to the viscometer at 20°C. Using suction to draw water above the upper mark let the liquid level to fall and the timing started with a stop watch as meniscus passes the upper mark until it got to the lower mark. The viscometer was then rinsed and the samples applied and the time required for its passage between meniscuses was determined which was used to calculate the viscosity with the formula:

Viscosity (CP) = flow time of sample solution at 20°C x specific gravity of the sample solution x 1.002 / Flow rate of water at 20°C.

Determination of hydrolases from the microbial isolates

The amylase of the microbial isolates was determined according to the methods described by Alves et al. (2002) and Carrim et al.

Table 1. Microbial counts in days of African star apple (*Chrysophylum albidum*) fruits.

Day	Bacteria (cfu/g)	Fungi (sfulg)
2	3.40×10^5	5.60×10^2
4	3.20×10^5	4.20×10^2
6	1.60×10^3	6.00×10^2
8	2.50×10^4	3.80×10^2
10	4.00×10^5	2.80×10^2
12	2.00×10^4	4.50×10^2
14	ND	2.00×10^2
16	ND	4.00×10^2

Key: cfu = colony forming unit; / = per; g = gram; sfu = spore forming unit; ND=not detectable.

Table 2. Incidence and pathogenicity of microorganisms associated with African star apple Fruits

Microbial isolates	Incident rate (%)	Infection (Diam. mm)
Bacteria		
Bacillus cereus	28	15
B. polymyxa	35	20
Escherichia coli	30	20
Proteus mirabilis	20	15
Pseudomonas aeruginosa	35	25
Staphylococcus aureus	18	08
Fungi		
Aspergillus flavus	45	25
A. fumigatus	40	28
A. niger	20	12
A. repens	30	25
Fusarium sp.	38	22
Mucor mucedo	60	15
Trichoderma viride	20	10
Rhizopus stolonifer	65	19

% = percentage; Diam. = diameter; mm = milliliter.

(2006), while lipase was by the methods of Carrim et al. (2006) and Damaso et al. (2008). The method of Onyeocha and Ogbonna (1983) was used for protease and pectinase by Carrim et al. (2006), while cellulase was determined using the method of Nwodo et al. (2010).

RESULTS AND DISCUSSION

The highest bacterial count (4.0×10^5 cfu/g) was recorded on day 10 while day 6 recorded the least counts (1.60×10^3 cfu/g) (Table 1). These counts may not be unconnected with the nature of the associated bacteria. The highest fungal count (6.0×10^2 sfu/g) was recorded on day 6 while day 14 recorded the least counts (2.0×10^2 cfu/g) (Table 1). These counts could be a direct consequence of the fungi to easily utilize the nutrients in the fruits for growth. Fourteen microorganisms were isolated from the deteriorating African star apple fruits investigated. These included *Bacillus cereus, B. polymyxa, Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa* and *Staphylococcus aureus* for bacteria. The fungi isolated were *Aspergillus flavus, A. fumigatus, A. niger, A. repens, Fusarium* sp*., Mucor mucedo, Trichoderma viride* and *Rhizopus stolonifer* (Table 2). The array of these microorganisms could be due to the nutrient rich nature of the fruits, thus supporting the growth and proliferation of the organisms. Asenjo (1946) documented the richness in ascorbic acid

of the African star apple, while Bada (1997) and Umelo (1997) separately attested to the excellent sources of vitamins, iron, flavours to diet, raw materials for some industries and essential minerals (Amusa et al., 2003). Thus, the fruit could serve as nutrient source for the isolated microorganisms. The incidence of *Pseudomonas aeruginosa* could be adduced to its nutritional versatility (Oberhardt *et al*., 2008). Also, *Pseudomonas aeruginosa, E. coli, Proteus mirabilis* and *Staphylococcus aureus* were human pathogens cum flora (Awe et al., 2009), while *B. cereus* and *B. polymyxa* were associated with air and soil (Gravens et al., 1975; Awe et al., 2009). Therefore, the presence of the former group of bacteria could be traced to human contamination, while the later could have resulted from air and soil contamination.

The eight fungi isolated in this study were similar to isolates from the deteriorating African star apple fruits in Ibadan, Nigeria by Amusa et al. (2003) with the exception of *Aspergillus tamari, Penicillum sp.* and *Botryodiplodia theobromae.*

B. polymyxa and *P. aeruginosa* were the most frequently encountered bacteria and *Staphylococcus aureus* was least prevalent (Table 2). The poor hygienic standard and improper handling of the fruits could be accountable for the observed bacteria (Awe et al., 2009). Some of these organisms namely *E. coli* and *P. mirabilis* have been implicated in gastroenteritis (Nester et al., 2001). The incidence of *B. polymyxa,* and *B. cereus* are indicative of environmental contamination of the fruits as the fruits are constantly exposed to air, aerosols and dust particles during the course of selling the fruits which in most cases take days to weeks. Therefore, faulty foods and fruits handling techniques especially in between sales during which the fruits are stored at improper temperature (Gallo et al., 1992) and possibly in contact with contaminated surfaces could be adjudged for the incidences of *B. polymyxa* and *P. aeruginosa. Staphylococcus aureus* in fruits clearly attested to human contamination during handling. This singular organism is known to be associated with enterotoxin characterized by short incubation period, violet nausea, vomiting and diarrhea (Awe et al., 2009) when injested in foods, water, fruits and vegetables.

The predominant fungus was *R. stolonifer* followed by *M. mucedo*, *A. flavus, A. fumigatus,* while *A. niger* and *T. viride* least occurred. *A. niger* and *R. stolonifer* isolated in this study have been associated with field infection (Amusa et al., 2003). In addition, *Aspergillus* and *Rhizopus* species in this study and *Penicillum* species had been reported to play significant roles in melon pod rotting for seeds recovery (Uffonry and Achi, 1998; Kehinde and Ikenga, 2010). The micromycetes nature of the isolated fungi from the African star apple fruits tend to be a prelude to their ease of adaptation to changing environmental conditions and to infect and deteriorate different plants substrates hinged to specific biological properties (Luganskas, 2005). Aluded to this is the fact that many micromycetes are not only known as plant pathogens, but are sources of vital mycotoxins of interest in animal and human health (Placinta et al., 1999). These fungi isolated from the fruits were in no small measure indigenous to soil environment (Aboloma et al., 2009; Awe et al., 2009; Kehinde and Ikanga, 2010) from where they probably own their origin.

Table 3. Viscosity (%), nutrient composition (%), pH, titratable acidity (TTA) and mineral content (%) of African star apple fruits.

Parameter	Apparently healthy fruits	Spoilt fruits
Viscosity	12.32	4.25
Moisture content	66.45	40.14
Crude fibre	44.33	26.26
Crude protein	9.83	12.28
Ether extract (fat)	14.96	9.24
Ash	6.67	10.28
Carbohydrate	26.22	12.36
pH	5.25	6.62
TTA	1.65	1.24
Fe	1.28	0.45
Ca	46.25	52.12
Mg	38.34	46.35
Na	2.56	3.26
K	6.15	8.25
P	2.26	1.12
Zn	3.34	4.22

The nutrient analysis of the apparently healthy African star apple fruits lucidly had viscosity of 12.32%, moisture content of 66.45%, crude fibre of 44.33%, crude fat of 14.96% and carbohydrate of 26.22% that were higher than the spoilt fruits with 4.25, 40.14, 26.26, 9.24 and 12.36% for viscosity, moisture content, crude fibre, crude fat and carbohydrate, respectively (Table 3). However, crude protein of 12.28% and ash content of 10.28% were more in spoilt fruits than in apparently healthy fruits with 9.83 and 6.66% crude protein and ash content respectively (Table 3). In any case, Adelaja (1997) discovered lower carbohyradate content of 29.90%, crude protein of 8.80% and crude fat content of 17.10%. Similarly, Amusa et al. (2003) reported carbohydrate content of 29.60%, crude protein of 8.75%, crude fat content of 16.20% and moisture content of 42.10%. These observed changes could have been a function of time of harvest of the fruits as well as the environment where the plants are grown. Also, the nature of the nutrient content of the apparently healthy fruits compared with the spoilt fruits may not be unconnected with the activities of the microorganisms in the fruits cum time. Ajayi (2011) asserted the immense activities of microorganisms in the fermentation of African yam bean seeds that resulted in its nutritional composition.

Table 4. Degradative enzymes production in halo diameter (mm) of microbial isolates from African star apple fruits.

Microbial Isolate	Hydrolase activity (mm)				
	Protease	Amylase	Cellulase	Lipase	Pectinase
Bacillus cereus	3.10	10.26	0.00	1.18	0.00
B. polymyxa	0.00	12.00	4.40	0.00	6.50
Escherichia coli	4.20	6.28	0.00	0.00	0.00
Proteus mirabilis	5.25	4.46	0.00	1.26	3.45
Pseudomonas aeruginosa	8.62	14.00	10.00	3.20	6.15
Staphylococcus aureus	4.26	2.15	0.00	0.00	0.00
Aspergillus flavus	1.85	8.20	6.10	2.18	5.56
Aspergillus fumigatus	2.15	5.62	3.66	3.22	6.26
Aspergillus niger	3.21	8.05	8.05	4.26	5.66
Aspergillus repens	2.26	8.66	8.66	3.15	5.14
Fusarium sp.	0.06	3.25	4.40	0.00	2.40
Mucor mucedo	0.41	6.15	0.46	5.25	1.42
Trichoderma viride	3.32	4.25	2.40	3.50	4.64
Rhizopus stolonifer	0.00	5.50	1.15	6.45	1.00

The average pH values obtained show that the apparently healthy fruits are moderately acidic with pH of 5.25, while the spoilt fruits tend toward neutral with pH of 6.62. This in no small measure that indicated that the fruits could permit and tolerate the growth of bacteria and fungi (Frazier and Westerhof, 1967).

In addition, mineral content analysis revealed iron and phosphorous to be 1.28 and 2.26% respectively in the apparently healthy fruits more than the spoilt fruits which had iron of 0.45% and phosphorous of 1.12% (Table 3). This study also showed higher calcium of 52.12%, magnesium of 46.35%, sodium of 3.26%, potassium of 8.25% and zinc of 4.22% in spoilt fruits than healthy fruits which recorded calcium of 46.25%, magnesium of 38.34%, sodium of 2.56%, potassium of 6.16% and zinc of 3.34% (Table 3). The availability of these minerals in the fruits is an indication of the rich nature of the fruits with the essential elements. The more of these minerals in the healthy fruits as opposed to the spoilt and vice visa could have resulted from the concomitant uptake and released of these minerals in the course of the metabolic activities of the associated microorganisms. In their deductions from the previous studies, Amusa et al. (2003) posited that infection and deterioration of the fruits by pathogens might have lead to an increase in mineral content and decrease in metabolic synthetases of African star apple fruits.

The degradative enzymes production values of the microorganisms are illustrated in Table 4. Notably, all the bacterial isolates elaborated amylase, while *B. cereus, E. coli, P. mirabilis* and *Staphylococcus aureus* did not produce detectable cellulase. Also, all the bacterial isolates with the exception of *B. polymyxa, P. mirabilis* and *P. aeruginosa* were positive for lipase. In this study, all the fungal isolates expressed detectable hydrolases except *R. stolonifer* and *Fusarium* sp. without protease and lipase respectively. Therefore, the elaboration of these hydrolytic enzymes by the microbial isolates could be responsible for their proliferation in the fruits. This finding agreed with the fact that microorganisms are rich sources of enzymes (Akpan, 2004). Amylasaes are one of the most important enzymes used in biotechnological processes (Ajayi, 2011), particularly in starch hydrolysis. Cellulases have wide applications in textile, paper pulp as well as the feed industries (Nwodo et al., 2010). The protease and pectinase would have acted on protein content and cementing materials of the fruits, thus reducing the viscosity of the fruit juice. Microbial pectinases were reported to account for 25% of the global food enzymes sales (Jayani et al., 2005). The microorganisms from the fruits could be vital sources of these economically viable enzymes.

Conclusion

The microorganisms isolated are no doubt involved in the infection, degradation and cum deterioration of the African star apple fruits. Hence, the deteriorative activities of the microorganisms tend to exact its influence on the nutritive value of the fruits. The versatility of the array of enzymes elaborated by the microbial isolates could find their usefulness in techno-industrial applications if properly harnessed.

Conflict of interests

The authors have not declare any conflict of interest.

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Antibiogram of bacterial isolates and fungi associated with some creams and lotions sold in Zaria, Nigeria

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Skin moisturizing creams and lotions are external preparations that contain special additives that could serve as nutrients for microorganisms; it is possible that such products may get contaminated and serves as vehicles for pathogen transfer. Eight cosmetic products, consisting of 4 body creams and 4 lotions were randomly purchased from the local markets within Zaria and analyzed for their microbiological quality. Five out of the eight products examined were found to be contaminated with bacteria, which had counts ranging from 6.0×10^4 to 5.3×10^5 cfu/g. Three of the products showed evidence of fungal contamination, with counts ranging between 1.3×10^4 and 2.8×10^8 propagules/g. However, three of the products were free from both bacterial and fungal contaminants. Organisms isolated from the creams include *Staphylococcus aureus, Bacillus* sp., *Micrococcus* sp., *Escherichia coli, Pseudomonas aeruginosa, Aspergillus* spp., *Mucor*and *Penicillium* spp. The organisms showed a high level of antibiotic resistance. However, erythromycin had the highest activity against the Gram-positive isolates while gentamycin had the highest activity against the Gram-negative isolates. There was significant correlation between microbial contamination and package orifice diameter, with highest bacterial and fungal count observed in products with wider orifices. Based on the U.S. Food and Drug Administration (FDA) and U.S. Pharmacopeial Convention (USP) standard of 10^3 cfu g^{-1} for cosmetic products, it was evident that five products had counts above this level; thereby representing serious health hazard. As a result, manufacturers should adhere to good manufacturing practices.

Key words: Moisturizing cream, body lotions, microbiological quality, Zaria, Nigeria.

INTRODUCTION

The Food and Drug Administration (FDA) regulates cosmetics, which they define as products that are used for cleansing, beautifying, promoting attractiveness or altering the appearance without affecting the body's structure or function (Perry, 2011; Siegert, 2012; Noor et al., 2015). Included in this definition are products such as body creams, lotions, perfumes, lipsticks, fingernail polishes, eye and facial make-up preparations, shampoos, permanent waves, hair colors, toothpastes, deodorants and any material intended for use as a component of a cosmetic product (Özalp, 1998; USP, 2003; FDA, 2007).

Skin moisturizing creams and lotions are external preparations with different rheological properties that

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contain special additives like plant extracts, fatty acids and vitamins (Crowshow, 1997). As these additives could serve as nutrients for microorganisms, it is possible that such products may get contaminated and be vehicles for pathogen transfer (Gopalkrishna et al., 2010; Noor et al., 2015). They are therefore liable to microbial contaminations either in the course of their preparation, transportation and/or use by the consumers which may lead to their spoilage (Osungunna et al., 2010).

The warm humid conditions that are characteristic of a tropical environment are conducive for the growth of micro-organisms, which are responsible for a number of infectious diseases and the spoilage of food, cosmetic and pharmaceutical product (Ballereau et al., 1997; Okeke and Lamikanra, 2001).

Moulds, yeasts and pathogenic bacteria have been isolated from cosmetics (FDA, 2007; Okeke and Lamikanra, 2001; Gopalkrishna et al., 2010; Osungunna et al., 2010; Qasem et al., 2012; Aminu and Umar, 2012). According to FDA (2007), most cases of contamination are due to manufacturers using poorly designed, ineffective preservative systems and not testing the stability of the preservatives during the product's customary shelf-life and under normal use conditions.

The practice of controlling the microbial levels of industrially processed food and other products is an age-old one. For these products, microbial standards have since been established. Unfortunately, such standards appear to be non-existent with regards to cosmetics in general. However, as a result of this, some infectious diseases arise due to usage of contaminated moisturizing creams, body lotion and other cosmetic products. Some of these diseases include scabies, acne, eczemaand dyschromia (Parker, 1972; Brannan and Dille, 1990; Becks and Lorenzoni, 1995; Pollack, 2000; Mahé et al., 2003; Behravan et al., 2005). Contamination with *Clostridium tetani* and infection of neonates with *Pseudomonas aeruginosa* from contaminated cleansing solutions have been reported (Perry, 2001). It has also been shown that a contaminated hand cream could be a source of septicemia in intense therapy unit (Baird, 1977).

The incidence of skin diseases is likely to be frequent in the developing countries due to the unhygienic environment, dense population favoring contagious diseases, lack of awareness on cleanliness, improper sanitation practices, and finally the massive use of contaminated processing water (USP, 2003; Denyer et al., 2004; Cundell, 2005a, b; Prüss-Üstün and Corvalán, 2006).

Reports on the microbial quality evaluations of cosmetics and toiletries have mainly been from temperate countries (Morse and Schonbeck, 1968; Becks and Lorenzoni, 1995; Itin et al., 1998; Sánchez-Carrillo et al., 2009). As there is great difference in conditions between the temperate and the tropics, these results cannot be extrapolated to Nigeria as a tropical country. Some studies have however examined cosmetic creams and lotion marketed in Nigeria with the identification of potential pathogens such as *Escherichia coli, Salmonella* spp., *Pseudomonas* spp., *Bacillus* spp., *Micrococcus* spp., *Staphylococcus aureus, Klebsiella* spp., *Enterobacter gergoviae, Aspergillus flavus* and *Penicillin* spp. (Okore, 1992; Onwunali, 2000; Okeke and Lamikanra, 2001; Anelich, 2007; Osungunna et al., 2010). Only one of such study was conducted in Northern Nigeria (Aminu and Umar, 2012). The present study therefore aimed at evaluating the microbiological quality of some moisturizing creams and body lotions sold in cosmetic stores in Zaria, north-western Nigeria, where there is dearth of data.

MATERIALS AND METHODS

Sample collection

A total of eight commercially available products, consisting of four body creams and four lotions most commonly used were employed in this study. The products were purchased from different market stalls within Zaria, Kaduna State Nigeria. The samples ranged from moisturizing creams to toning creams commonly used by people in this part of the country, and all were within their shelf life.

Media preparation

Plate count agar was used for bacterial count, nutrient broth was used for enrichment and nutrient agar was used for preservation of isolates in slants. Simmon citrate agar,mannitolsalt agar, MacConkey agar, eosin methylene blue agar and *Salmonella-Shigella* agarwere used for bacteria isolation and Sabouraud dextrose broth and Sabouraud dextrose agar were used for fungi isolation. All media used were prepared according to the manufacturer's instruction.

Analysis of sample

Enumeration of microorganisms

Ten grams of each sample was transferred into 90 ml distilled water and stirred thoroughly. Serial dilutions of the stock solution were made and appropriately labeled. 1 ml of the diluent 10^3 was inoculated on plate count agar, and incubated at 37°C for 24 h. Bacterial counts were determined by spread plate method. Also, 0.1ml of the 10^2 diluent was transferred each unto Sabouraud dextrose agar, and the plate was incubated at ambient temperature for 3 days after which observation was made and counting carried out upon the appearance of growth. The fungi count was carried out using the pour plate method. Sterility test was carried out by incubating freshly prepared media at room temp for 24h (Cheesbrough, 2005).

Cultural and morphological characterization of the bacterial and fungi isolates

For bacterial isolation, 1 g of each body cream was weighed and inoculated unto 9 ml of nutrientbroth, and incubated over night at 37°C for enrichment. After which, one loopful each was again taken from the enrichment culture and streaked on Mannitol salt agarand

Table 1. Microbial loads of the products analyzed.

Product analyzed	Orifice diameter	Bacterial count (cfu/g)	Fungal count (propagules/gram)	Manufacturing date	Expire date
P1	4mm	-	-	11/2011	11/2014
P2	Wide opening (60 mm)	3.5×10^5	2.7×10^4	04/2012	04/2015
P3	Wide cream opening (65 mm)	4.0×10^4	-	10/2011	10/2013
P4	3 mm	-	-	02/2012	02/2014
P5	6 mm	6.0×10^4	-	11/2011	11/2013
P6	5 mm	1.6×10^5	1.3×10^5	12/2011	12/2013
P7	Wide cream opening (70 mm)	5.3×10^5	2.8×10^4	12/2011	12/2014
P8	Wide cream opening (50 mm)	-	-	02/2012	02/2014

P = Product, - = no growth.

Mac Conkey agar and then incubated at 37°C for 24 h. The plates were examined for evidence of growth, colonies were sub-cultured unto nutrient agar slants, Gram stained and examined under 100x objective according to the methods outlined by Cheesbrough (2005) as described by Aminu and Umar (2012). Thereafter, isolates were identified by their colonial morphology and Gram reaction.

For fungi isolation, 1 g each of the body creams were weighed, transferred into 9ml of Sabouraud dextrose broth andincubated over night at 37°C for enrichment, this was to selectively favor fungi growth. After incubation, one loopful each was inoculated unto Sabouraud dextrose agar and the plates incubated at ambient temperature for 3 days. The plates were observed for evidence of growth and colonies were identified according to the methods outlined by Cheesbrough (2005) as described by Onwunali (2000).

Biochemical identification of the bacterial isolates

The bacterial isolates were further identified using conventional biochemical methods. Isolates that were Gram-positive cocci were subjected to coagulase, catalase and mannitol fermentation test, Gram-positive rods were subjected to indole, citrate, motility, VP,etc tests. While Gram-negative organisms were subjected to indole, citrate, MRVP and oxidase tests. Other biochemical test such as swelling of bacilli body, citrate, methyl red and growth on Cetrimide agar were also employed in bacteria identification.

Antimicrobial susceptibility test

Antimicrobial susceptibility pattern of each isolate was done using conventional disc diffusion method according to NCCLS standard. This was carried out using multiple antibiotic discs. A turbid suspension of the isolates was made in distilled water using McFarland Standard, prepared as a comparator. A sterile swab was dipped into the bacteria suspension, pressed on the side of the bottles to allow excess drip-off, and then used to evenly streak the entire surface of the Mueller-Hinton agar. Sterile forceps were then used to place the multiple antibiotic discs in a circular pattern on the media. The process was carried out for all the presumptively identified isolates, and the plates incubated at 37°C for 24h. After incubation, the zone of inhibition for each antibiotic was measured from the center of the disc to the point where clearing stopped.

Analysis of data

Data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan multiple range test using SPSS version16. All data were expressed as mean ± SDand p value < 0.05 was considered significant at 95% confidence interval.

RESULTS

Out of the eight products examined for bacterial contaminant, 5 (62.5%) yielded bacterial growth, with counts ranging between 6.0 x 10^4 and 5.3 x 10^5 cfu/g (Table 1). Both Gram-positive and negative bacterial were isolated from the products. Gram-positive organisms such as *Staphylococcus aureus* (3/5: 60%), *Bacillus* spp. (3/5: 60%) *and Micrococcus* spp. (1/5: 20%)were isolated from the products, while Gram-negative organisms such as *Escherichia coli* (2/5: 40%) and *Pseudomonas* spp.(1/5: 20%)were isolated (Table 2). One of the products from which Gram-negative organisms were isolated had the highest count.

Out of the eight products examined for fungal contamination, 3 (37.5%) showed evidence of fungal contamination, with counts ranging from 1.3 x 10^4 to 2.8 x 10^4 propagules/g (Table 1). Fungal contaminants isolated from the products include *Aspergillus* spp., *Penicillium* spp. and *Mucor* (Table 3). Table 4 shows the biochemical identification of the bacterial isolates.

Erythromycin had the highest activity against the Gram-positive isolates (Table 5), while Gentamycin had the highest activity against the Gram-negative isolates (Table 6). There was significant correlation between microbial contamination and package orifice diameter. Product six and seven with highest bacterial and fungal count had wider orifices.

DISCUSSION

Five out of the eight samples analyzed had evidence of microbial contamination. Generally, it is desirable that cosmetics and toiletries including body creams should contain <10^3cfu/g or cfu/ml of bacteria at the time they reach the consumer and they should also be free of

Table 2. Morphological characteristic of bacterial isolates.

Products analyzed	Colonial morphology	Gram reaction	Cellular morphology
P2 (A)	Creamy large macerated colonies.	+	Spherical, Scattered organisms (Cocci)
P2 (B)	Large, flat pale colonies	+	Rod like cells in single, pairs and clusters
P3	Large, flat pale colonies	+	Rod like cells in single, pairs and clusters
P5	Creamy large macerated colonies.	+	Spherical, clustered organisms (Cocci)
P6 (A)	Creamy large macerated colonies.	+	Spherically clustered organisms (Cocci)
P6 (B)	Pinkish smooth colonies	-	Rods in clusters and pairs
P6 (C)	Pale colonies	-	Rods in clusters and scattered
P7 (A)	Creamy large macerated colonies.	+	Spherical, clustered organisms (Cocci)
P7 (B)	Large, flat pale colonies	+	Rod like cells in single, pairs and clusters
P7 (C)	Pinkish smooth colonies	-	Rod like cells in Chain

+ = Positive - = negative, P = product.

Table 3. Morphological characteristic of fungal isolates.

Products	Colonial morphology	Microscopic morphology	Presumptive identification
P2 (A)	Black fluffy colony; Reverse: Creamy	Conidial head splits into columns	*Aspergillus* spp.
P2 (B)	White cottony colony Reverse: Cream/yellow, becomes grey on getting older	Non-septate hyphae, branched sporangiophore, with round sporangiospores	*Mucor* spp.
P6	Black fluffy colonies Reverse: Creamy	Conidial head splits into columns	*Aspergillus* spp.
P7 (A)	Blue green fluffy colony Reverse: Dark green	Branched conidia, resembling bushes	*Penicillium* spp.
P7 (B)	White cottony colony; Reverse: Cream/yellow, becomes grey on getting older.	Non-septate hyphae, branched sporangiophore, with round sporangiospores	*Mucor* spp.

P = Product.

Table 4. Biochemical identification of the bacterial isolates.

Products	Cat	Cog	FM	Mot	Cit	VP	SBB	SH	Ind	MR	OX	GCA	Presumptive Identification
P2 (A)	+	+	+	-	-	ND	ND	ND	ND	ND	ND	ND	*S. aureus*
P2 (B)	+	ND	ND	+	+	+	-	+	-	ND	ND	ND	*Bacillus* spp.
P3	+	ND	ND	+	+	+	-	+	-	ND	ND	ND	*Bacillus* spp.
P5	+	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	*Micrococcus* spp.
P6 (A)	+	+	+	-	-	ND	ND	ND	ND	ND	ND	ND	*S. aureus*
P6 (B)	ND	ND	ND	ND	-	-	ND	ND	+	+	-	-	*E. coli*
P (C)	ND	ND	ND	ND	+	+	ND	ND	-	-	+	+	*Pseudomonas* spp.
P7 (A)	+	+	+	-	-	ND	ND	ND	ND	ND	ND	ND	*S. aureus*
P7 (B)	+	ND	ND	+	+	+	-	+	-	ND	ND	ND	*Bacillus spp.*
P7 (C)	ND	ND	ND	ND	-	-	ND	ND	+	+	-	-	*E. coli*

P = Product, ND = not determined, Cat = catalase, Cog = coagulase, FM = fermentation of Mannitol, Mot = motility, Cit = citrate, VP = Voges-Proskauer, SBB = swelling of bacillary body, SH = starch hydrolysis, Ind = indole, MR = methyl red, OX = oxidase test, GCA = growth on Cetrimide agar.

Table 5. Antibiotic susceptibility patterns of Gram positive bacterial isolates.

Sample	AU	FX	AP	AM	CO	CX	GN	CD	CP	E
P2 (A)	R	R	S	S	S	S	S	S	S	S
P2 (B)	S	R	R	S	R	R	S	S*	R	S
Product 3	R	R	R	S	R	S	R	R	S	R
Product 5	S	S	R	R	S	S	S	R	S	S
Product 6 (A)	S	R	R	S*	R	R	R	S	S	S
Product 7 (A)	R	S*	R	S	R	S	S	R	S	R
Product 7 (B)	S*	R	R	R	R	R	S	R	R	S
Percentage sensitive (%)	57	28	14	72	28	57	72	43	72	72
Percentage Resistant (%)	43	72	86	28	72	43	28	57	28	28

R = Resistant, S = sensitive, * = intermediate resistance, AU = Augmentin, FX = Ceftriazone, AP = Cloxacillin, AM = Ampicillin, CO = Cotrimoxazole, CX = Cephalexin, GN = Gentamycin, CD = Clindamycin, CP = Ciprofloxacin, E = Erythromycin.

Table 6. Antimicrobial susceptibility patterns of Gram negative isolates.

Sample	N	CIP	TE	NF	AX	OF	C	CF	AM	GN
product 6 (B)	S	S	S	S	S*	S	R	S	S*	S
product 6 (C)	R	R	R	S*	R	R	S	S	R	S
Product 7 (C)	S	S*	S	R	S	R	R	S	S*	S
Susceptible (%)	67	67	67	67	67	33	33	100	67	100
Resistant (%)	33	33	33	33	33	67	67	0	33	0

R = Resistance, S = sensitive, * = intermediate sensitivity, AM=Ampicillin, CIP = Ciprofloxacin, TE = Tetracycline, NF = Norfloxacin, AX = Amoxicillin, OF = Ofloxacin, C = Chloramphenicol, CF = Cefuroxime, AM = Ampicillin, GN = Gentamycin N = Nitrofurantoin. Zone of Inhibition: 0 -13 mm = resistance; 14 -17 mm = intermediate sensitivity; 18 mm and above = sensitivity.

potentially pathogenic organisms (FDA, 1995; USP, 2003). The microbial count varied with the products. The bacteria counts ranged from 6.0×10^4 to 5.3×10^5 cfu/g. The bacteria count obtained in this study is slightly higher than that previously reported in Nigeria (Okeke and Laminaka, 2001) and similar to a study in same area where count of 5.4×10^3 to 1.7×10^5cfu/g was obtained (Aminu and Umar, 2012). The count contrast results of other studies with higher contamination rates (10^4 to 10^9cfu/ml) were reported (Okore, 1992; Onwunali, 2000). Similar rates of 10^5cfu/g (Gopalkrishna et al., 2010) and $>10^5$cfu/g have also been reported from other countries and in outbreaks where counts of facultative pathogens were in levels capable of infecting the immuno-compromised (Baird, 1977; Becks and Lorenzoni, 1995; Itin et al., 1998).

The variation of the counts may be due to non-uniformity in the processing of raw materials and procedures as well as handling and distributions by the various companies. Poor personal hygiene and level of awareness to maintain good manufacturing practice may also contribute to the total viable counts obtained. Although, the bacterial counts of the moisturizing creams and body lotions examined in this study were not higher than counts $>10^5$cfu/g previously reported in some studies, the counts were higher than the recommended level for cosmetic products by FDA and BP/USP. For example, in the case where cosmetic items are applied within the non-eye area, the total aerobic microbial count (TAMC) should notbe more than 10^3cfu g^{-1}; and for the items used within the eye area, the limit should not exceed 10^2cfu g^{-1} (Pollack, 2000; Behravan et al., 2005; Onurdağ et al., 2010).Therefore, there is the possibility that these products could be vehicles for pathogen transmission when shared.This is in view of the facts that, the preservative content of these products were apparently incapable of dealing with the organisms isolated and the counts obtained. Furthermore, bacterial contamination may cause spoilage of the product as earlier observed by Aminu and Umar (2012).

Both Gram-positive and negative bacteria were isolated in the present study. This result contrast the finding of Anelich and Korsten (1996) where only Gram-negative bacteria were isolated and that of Aminu and Umar (2012) where only Gram-positive bacteria were isolated. The present report is however similar to the findings of Okeke and Lamikanra (2001), Gopalkrishna et al. (2010), Osungunna et al. (2010) and Qasem et al. (2012) where both Gram-negative and Gram-positive bacteria were isolated.

Gram positive organisms predominated in the products examined in this study as previously reported (Onmunali,

2000), with *S. aureus* and *Bacillus* spp. isolated from three products each and micrococcus from one product. *S. aureus* was also one of the predominant bacteria isolated recently from body creams and lotions by Osungunnaet al. (2010). *S. aureus* being a normal flora of the skin easily contaminate products during handling and processing; and the heat resistance nature of *S.*spp. and *Micrococcus* spp. also contribute to their survival in processed products (Efiuvwevwere, 1988). The presence of *Bacillus* spp. which are spore formers is due to their resistance to moist heat even at temperatures as high as 120°C (Moedenhauer et al., 1995).

The products from which *S. aureus* were isolated can be considered unsuitable because the bacterium is an opportunistic pathogen and it was isolated from the product before use. *S. aureus* is associated with skin infections such as folliculitis (pimples) and furuncles (boils) (Arora and Arora, 2008). Although not many investigations have examined cosmetics as potential sources of infections, there are documented cases of eye infection such as conjunctivitis and blepharitis from cosmetics and toiletries particularly in immunocom-promised (Becks and Lorenzoni, 1995; Itin et al., 1998).

The Gram negative organisms identified were *E. coli* isolated from two products and *Pseudomonas* spp. isolated from one product. This result is similar to an earlier report by Okeke and Lamikanra (2001). *Pseudomonas* spp. was the predominant species isolated by Gopalkrishna et al. (2010). The presence of *E. coli* indicates the presence of faecal contaminants, while *Pseudomonas* spp. is an opportunistic pathogen that contaminate wound hence can serve as a source of wound infection, which could result in serious complications when such creams are applied.

The source of contaminants detected in these products are likely due to the raw materials used, conditions prevalent in the environment in which the products are manufactured and packaged as well as the storage conditions they are subjected to (Balsam, 1974). Water employed in the manufacture has been described as the most likely source of contaminants in cosmetics (Crowshow, 1997).

In this study, there was significant correlation between bacterial count and package orifice diameter and pack size as earlier observed (Brannan and Dille, 1990; Gopalkrishna et al., 2010; Aminu and Paul, 2012). People usually use their fingers to take the creams present in wide mouthed containers. Lack of growth seen in Product 1 may be probably due to the nature of its packaging and its narrow orifice, which prevented the contamination of the cream by microorganisms. None of the products packaged in narrow orifice containers and tubes were contaminated in the study conducted by Gopalkrishna et al. (2010). It has been earlier observed that the use of non-invasive packages (tubes, pumps or narrow orifice containers) and adequate preservation increase the chances that contamination level will remain low during storage and use of products (Brannan and Dille, 1990).

It has been shown that every time a bottle of cosmetic is opened, microorganisms in the air have the opportunity to rush in, but narrow orifices reduces the chances of exposure to air and contamination. In addition, adequately preserved products can kill a lot of the microorganism to keep the product safe. Indeed the presence of micro-organisms in body creams coming directly from the industries does not only indicate a possible threat to health but also possibility of economic losses due to spoilage during storage (Onmunali, 2000). The use of water and raw materials of suitable quality and good manufacturing practices should generally lead to the production of preparations with low microbial contamination. Adequate preservation and the use of non-invasive packages (such as tubes, pumps or narrow orifice containers) increase the chances that contamination levels will remain low during storage and use of the product (Brannan and Dille, 1990).

Based on the antibiotic susceptibility testing recorded from the isolates, it was seen that majority of *Staphylococcal* isolates showed resistant to a wide range of antibiotics, this is in agreement with the results of Osungunna et al. (2010) and Gopalkrishna et al. (2010), in which resistance as high as 90% was recorded for *S. aureus*. The characteristic resistance of *S. aureus* can be attributed to a previous encounter of the organisms with various antibiotics in which resistance was conferred (Osungunna et al., 2010). Likewise in the case of the Gram negative isolates, relatively high level of sensitivity was recorded, especially to antibiotics such as Gentamycin and Ciprofloxacin.

Conclusion

The microorganisms isolated from the products in the study were *Escherichia, Bacillus, Micrococcus, Staphylococcus, Pseudomonas, Aspergillus, Mucor* and *Penicillium,* indicating that these products might be unsafe for use. It is undisputable evident that contaminated creams and lotions are dominant in the Nigerian market. Based on the FDA and USP standard of 10^{3}cfu $g^{-1}$for cosmetic products, it was evident that out of the eight products sampled, only three were fit for use as the other five products had counts above this level, as well as fungal contaminants.

RECOMMENDATION

It is therefore recommended that special agencies should be put in place with stringent regulatory standards to check the manufacturing and processing of these widely used applications. Scrupulous attention should be given to all ingredients as well as microbial testing before their usage, so as to make them less sources of microbial

infection. Adherence to good manufacturing practice and aseptic handling of raw materials should be ensured to ensure optimum reduction of the microbial load of the finished products. Individuals responsible for maintenance and sales of the finished products should be aware of the storage conditions of each product.

Conflict of Interests

The authors have not declared any conflict of interests.

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