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*Full Length Research Paper*

# **Prevalence, cytotoxicity and antibiotic susceptibility of *Campylobacter* species recovered from retail chicken meat in Mansoura, Egypt**

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This study was performed to determine the prevalence of *Campylobacter* species in retail chicken meat and chicken by-product, determine their *in vitro* cytotoxicity, as well as, examine their susceptibility to different antimicrobials. A total of 300 raw chicken meat samples were collected from different retail chicken meat outlets located at Mansoura city, Egypt classified into 120 thighs, 120 breasts, and 60 livers. All samples were subjected to conventional culture techniques and confirmed as *Campylobacter jejuni* by real time polymerase chain reaction (PCR). Antimicrobial susceptibility of *Campylobacter* species was determined using disc diffusion method to determine their susceptibility to 12 different antimicrobial agents. In addition, *C. jejuni* isolates were examined for their cytotoxicity against Vero cells. The overall prevalence of *Campylobacter* spp. was 10.3% (31/300) classified into 20 (18.2%) *C. jejuni* and 11 (10.7%) *Campylobacter coli*. Among *C. jejuni* isolates (n=20), 15 strains belonged to biotype I and 5 isolates belonged to biotype II. The isolation rate from chicken thighs, breasts and livers was 12.5, 10 and 6.6%, respectively. A total of 15 (75%) *C. jejuni* strains revealed cytopathic effect (CPE) against Vero cells. *Campylobacter* spp. displayed a high antimicrobial resistance against penicillin G, gentamicin, trimethoprim-sulfamethoxazole, cephalothin, erythromycin, and chloramphenicol. On the other hand, *Campylobacter* spp. displayed high sensitivity to ciprofloxacin and nalidixic acid. Multidrug resistance was observed in 85 and 81.82% of *C. jejuni* and *C. coli* isolates, respectively. High frequency of cytotoxicity and multidrug resistance in *Campylobacter* spp. from chicken meat indicates an important epidemiological role of *Campylobacter* spp. in human infections which necessitate proper hygienic measures on poultry farms and control measures during carcass slaughtering and processing.

**Key words:** *Campylobacter*, retail chickens meat, real time polymerase chain reaction (PCR), cytotoxicity, antimicrobial susceptibility.

## **INTRODUCTION**

*Campylobacter jejuni* is a major zoonotic pathogen that causes food-borne gastroenteritis worldwide (Bronowski

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et al., 2014). Poultry meat and poultry by-products are the major sources of infection to humans (EFSA, 2016). *Campylobacter* is considered a part of saprophytic microflora in the digestive tract of poultry and frequently transmitted from contaminated chicken meat either by ingestion of undercooked or raw chicken meat contaminated with the *Campylobacter* or handling of poultry meat and meat products during food processing procedures (EFSA, 2016).

*Campylobacter* infections in human are self-limiting and the infection usually lasts for only one week, but the illness may relapses in some untreated cases. *Campylobacter* infections symptoms range from mild to severe symptoms. It usually started after 2 to 5 days after ingestion of the contaminated food including, fever, headache, nausea and diarrhea (Campbell et al., 2006). Infrequently, *Campylobacter* infections cause life threatening infection if it spread to blood stream and causes multiple diseases all over including, pseudoappendicitis, abdominal cavity, central nervous system, gallbladder, or urinary tract infection (Campbell et al., 2006). *C. jejuni* infection may result in serious post-infectious sequelae, the most important sequel are Reiter's syndrome or Hemolytic Uremic Syndrome (HUS), and rarely resulted in neurological disorder known as Guillain-Barré syndrome (GBS), which manifests as sever neurological signs and paralysis which may result in respiratory dysfunction, and eventually death (Murray et al., 2007; Nachamkin, 2008).

*Campylobacter* has numerous virulence factors which contribute to its survival and establishment of the disease, but four major virulence factors have been identified which include motility, adherence, invasion and toxin production and these toxins had biological activity on tissue culture cell lines (Wassenaar, 1997).

The excessive and misuse of antibiotics in the treatment of infections, prophylaxis, as well as a growth promoters in Egypt has resulted in the development and spread of drug resistances which represents a public health problem (Levy and Marshall, 2004). Hence, the aims of this study were to recognize the prevalence and antimicrobial susceptibility of *Campylobacter* species isolated from retail chicken meat and chicken products sold in Mansoura city outlets, and to investigate the cytolethal distending toxin (CDT) activity of *C. jejuni* against Vero cells.

## MATERIALS AND METHODS

### Collection of samples

A total of 300 raw chicken meat samples and chicken products classified into 120 thighs, 120 breasts and 60 livers were collected from retail outlets in Mansoura city, Dakahlia Governorates, Egypt. Samples were obtained from three street markets (n = 100), four supermarkets (n = 100), and two slaughterhouses (n = 100). Poultry samples were collected, 20 specimens by visit during the period between March and August, 2016. Each chicken sample was

individually packed into a clean polyethylene bag and transferred directly to the laboratory in an ice box under aseptic conditions.

### Isolation of *Campylobacter* spp.

Isolation of *Campylobacter* from the chicken meat samples was performed according to ISO 10272-1:2006 (ISO, 2006); briefly, 10 g of chicken meat was aseptically taken and placed into a clean sterile plastic bag. The plastic bag was filled with 90 ml of Bolton broth (CM0983, Oxoid) with selective supplement (SR0183, Oxoid), and the samples were mixed in a stomacher for 1 min and incubated at 37°C for 4 to 6 h under microaerobic conditions (Campygen, Oxoid) followed by 41.5 ± 0.5°C for 48 h. Approximately, 10 µl of the previous enrichment broth was streaked into the surface of mCCDA (PO5091A, Oxoid, Basingstoke, UK) with supplement (SR0155, Oxoid) plates media and incubated under microaerobic conditions at 41.5 ± 0.5°C for 48 h. Presumptive colonies of *Campylobacter* were purified onto Columbia blood agar (Oxoid) plates and incubated under microaerobic conditions at 41.5 ± 0.5°C for 24 h. Presumptive colonies that displayed typical growth on the mCCDA, Gram-negative with corkscrew-like darting motility, oxidase-positive were considered to be *Campylobacter*. Biotyping of *C. jejuni* were performed according to Benjamin and Skirrow (1980). To confirm the biochemical identification, the isolates were subjected to Real Time PCR targeted hippuricase enzyme encoded by *hipo* gene.

### Real time PCR for *C. jejuni*

A real-time probe based quantitative PCR (qPCR) reaction was used for the confirmation of *C. jejuni* isolates. DNA extraction of *C. jejuni* was performed using boiling method according to De Medici et al. (2003). The sequences of primers and probe used for amplification of *hippurhipO* gene specific for *C. jejuni* are listed in Table 1 (Benson et al., 2002). PCR reaction was performed in a total volume of 25 µl containing 0.4 mM of each dNTP, 1x reaction buffer, 2.5 mM MgCl<sub>2</sub> (Thermo Fisher Scientific, USA), and 1 U Platinum Taq DNA Polymerase (Life Technologies, USA). A positive control tube was also included. PCR condition concerning the MX3005P of Stratagene Cycle included initial denaturation at 94°C for 30 s followed by denaturation at 94°C for 30 s (40 cycles), annealing and extension ranged from 57 to 64°C for 60 s (Benson et al., 2002). The probes were conjugated with the fluorescent reporter dyes FAM and VIC (DNA Technology, Aarhus, Denmark) for *C. jejuni*, respectively, at the 5' ends and with the quencher dye MGBNFQ (Minor groove binder-non fluorescent quencher) at the 3' ends. The nucleotide sequences were retrieved from the Gene Bank sequence database under accession numbers Z36940 (HipO).

### Cytopathic effect of *C. jejuni* live cells and their cytotoxins on Vero cells

#### Extraction of cytotoxins

*Campylobacter* cells growth were harvested in 10 ml of Brucella broth (Difco) supplemented with *Campylobacter* growth supplements (Oxoid), and then incubated at 42°C for 48 h (Misawa et al., 1994). Bacterial density was determined from absorbance measurement at 55 nm (Schmicz, Germany) and correlated to colony forming units (cfu). Then, cultures were centrifuged at 5000 g/15 min and the supernatant was filtrated through a 0.22 Mm Nitrocellulose Membrane filter (Millipore). The resultant supernatant was tested for sterility using *Campylobacter* selective media (mCCD).

**Table 1.** Primers sequences and fluorogenic probe used for detection of *hipO* gene in *C. jejuni*.

Target species	Primer and probe	Type	Target gene	Sequence (5 – 3)
<i>C. Jejuni</i>	<i>hipO</i> -F	F-primer	hipO	5-CTGCTTCTTTACTTGTGTGGCTTT -3_
	<i>hipO</i> -R	R-primer		5-GCTCCTATGCTTACAACCTGCTGAAT-3_
	<i>hipO</i> -P	CJ-probe		5-FAM-CATTGCGAGATACTATGCTTTGMGBNFQ-3

**Table 2.** Prevalence of *Campylobacter* species isolated from examined chicken samples.

Type of sample total	No. of examined samples	<i>C. jejuni</i>			<i>C. coli</i>	Total
		No. (%)	Biotypes		No. (%)	No. (%)
			Biotype 1 (%)	Biotype 2 (%)		
Thighs	120	10 (8.3)	8 (80)	2 (20)	5 (4.1)	15 (12.50)
Breasts	120	8 (6.6)	5 (62.5)	3 (37.5)	4 (3.3)	12 (10.00)
Livers	60	2 (3.3)	2 (100)	0 (0.00)	2 (3.3)	4 (6.6)
Total	300	20 (18.2)	15 (75)	5 (25)	11 (10.7)	31 (10.30)

### Vero cells

Determination of cytotoxicity of *C. jejuni* isolates obtained was performed on Vero cells. They were used for live bacterial cells as well as for cytotoxins assay and they were from African green monkey kidney (Vero) cells which is being supplied from Animal Health Research Institute, Dokki, Giza, Egypt. The cell viability was determined by trypan blue dye uptake. Suspension of all cell lines were prepared in eagle minimal essential media (MEM, Sigma) supplemented with 7.5% sodium bicarbonate, 10% fetal calf serum, 3% glutamine, 100 I.U/ml penicillin and 100 Mg/ml streptomycin. Cells were seeded in sterile screw capped glass Leighton tube (KIMAX) and incubated at 37°C for 24 h to allow adhering under normal atmosphere condition. When the cells become confluent, the growth medium was removed. Confluent monolayer of these cell lines were incubated with bacterium free supernatant fluids of various dilutions at 37°C (two fold dilutions of supernatant tested 1/2 to 1/4). The control groups included sterile Brucella broth was inoculated into cell culture and incubated at 42°C for 48 h, under the same condition described earlier. The cover slip was fixed with 95% methanol for 5 min and stained with 10% Giemsa stain for 20 min, then, it was washed with water and used cover slips were air-dried (Al-Delaimi, 2009).

### Antimicrobial susceptibility of *Campylobacter* isolates

*Campylobacter* isolates were subjected to antimicrobial susceptibility testing including 20 *C. jejuni* and 11 *Campylobacter coli* strains tested using agar disk diffusion method (CLSI, 2014) on Muller-Hinton agar (Oxoid, CM0337) supplemented with 5% defibrinated horse blood. Plates were incubated at 42°C for 48 h under microaerobic condition. Antimicrobial agents used in this study included 12 different antimicrobials belonging to different classes including, penicillin (10 µg), ampicillin (10 µg), amoxicillin/clavulanic acid (30 µg), erythromycin (15 µg), oxytetracycline (30 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), cephalothin (30 µg), gentamicin (10 µg), streptomycin (10 µg), and sulphamethoxazole/trimethoprim (25 µg) and chloramphenicol (30 µg).

## RESULTS AND DISCUSSION

Poultry and meat products represent the main vehicle for the distribution of *Campylobacter* infection (Pitk"anen, 2013). In this study, the prevalence rate of *Campylobacter* spp. in chicken meat was 10.3% (31/300) classified into *C. jejuni* 64.5% (20/31) and *C. coli* 35.48% (11/31) which was in agreement with Nisar et al. (2018). All *C. jejuni* isolates were confirmed by real time PCR (Figure 2). The distribution of *Campylobacter* spp. in thighs, breasts and livers samples was 12.5, 10 and 6.6%, respectively (Table 2). Among *C. jejuni* samples, 15 strains belonged to biotype I and five strains belonged to biotype II (Table 3). The occurrence of *Campylobacter* spp. in chicken meat could be explained by unhygienic slaughter techniques including searing of carcasses with feces and rinsing, which leads to contamination of carcasses. A higher prevalence of *Campylobacter* spp. (56.0%) in poultry meat was recorded by Bardo'n et al. (2011) in Czech Republic. Moreover, Strachan et al. (2012) recorded a high prevalence of *Campylobacter* spp. (81.0%) of chicken livers in broiler chickens at retail. In Northern Poland, Andrzejewska et al. (2015) assessed the prevalence of *Campylobacter* spp. in poultry meat and recovered a total of 309 (41.6%) *Campylobacter* isolates. In France, Guyard-Nicod'eme et al. (2015) examined 361 chicken products samples and recovered *Campylobacter* from 76.0% of the examined samples. In Estonia, M"aesaar et al. (2014) reported 89.0% prevalence rate of *C. jejuni*. In addition, a literature survey conducted by Suzuky and Yamamoto (2009) on the presence of *Campylobacter* in retail poultry meats and meat by-products, the results showed high detection frequencies ranging between 28.1% in South Africa and 100% in Argentina, Belarus and Russia. Diversity in the

**Table 3.** Results of inoculation of live *C. jejuni* and their cytotoxins on Vero cells.

<i>C. jejuni</i> and cytotoxins	Incubation period (h)	Morphological changes on Vero cell
Live <i>C. Jejuni</i>	24	Cell destruction Rounding and detachment of Vero cell
1/2 Cytotoxins dilution	48	Degenerative change Detachment of Vero cell
1/4 Cytotoxins dilution	48	Elongation of epithelial cell Shrinkage with cytoplasmic vacuolation
1/4 Cytotoxins dilution	72	Pleomorphic cells Pyknosis and multinucleated giant cell

prevalence rates of *Campylobacter* from retail chicken meat may result from the difference in the sanitation level during handling and processing of chicken, the sampling time of the year (hot or cold season), the sampling design, as well as diagnostic methods followed (Shin, 2000; Willis and Murray, 1997); which is definitely, the first contamination rate of poultry meat depending on post slaughter treatments, temperature control and hygiene management during the food processing or storage (Campbell et al, 2006). In the present study, *C. jejuni* was the most prevalent species identified from chicken samples which is in close agreement with those reported world-wide in different studies in which *C. jejuni* was the most prevalent than other *Campylobacter* spp. (Andrzejewska et al., 2015; Guyard-Nicodème et al. 2015; Suzuki and Yamamoto, 2009; Whyte et al., 2004)

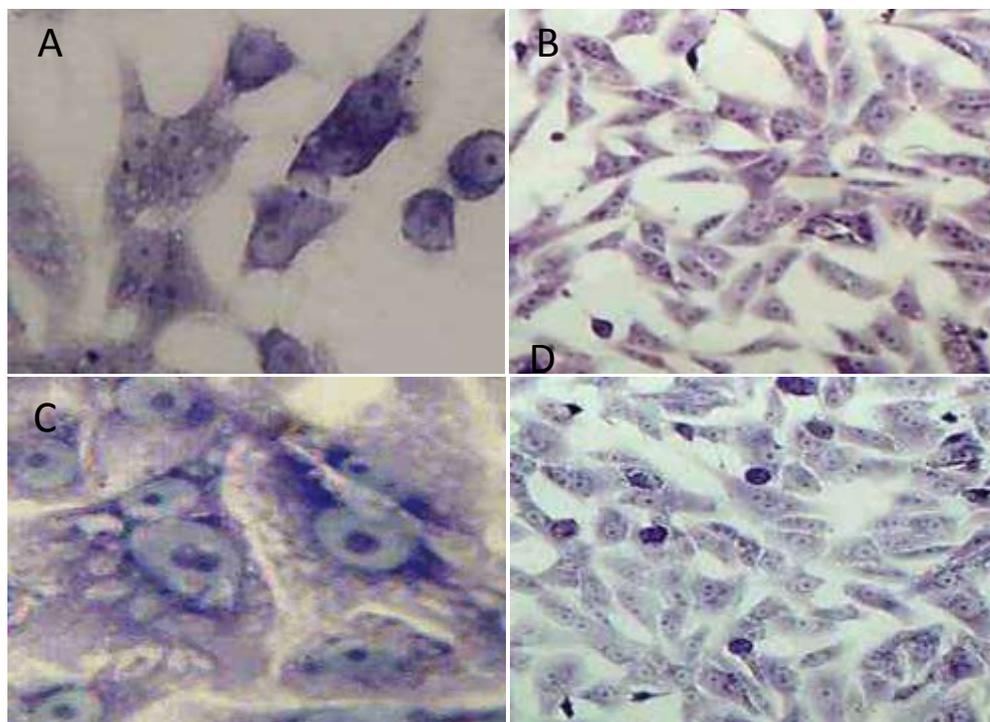
Among *C. jejuni* positive samples, *C. jejuni* biotype I was the predominant biotype detected in the current study shown in Table 2. Similarly, Adesiyun et al. (1992) and Shaheen et al. (1994) concluded that *C. jejuni* biotype I was the predominant *C. jejuni* biotype isolated from poultry meat and poultry meat products and being frequently associated with human enteric infection.

*In vitro* demonstration of cytotoxins produced by *C. jejuni* isolates suggests a correlation between pathogenic virulence factors and clinical symptoms. Vero cells are employed to study the effect of microbial toxins and provide a useful, sensitive and reproducible experimental method for the study of pathogenic mechanism. Other investigators referred to association of cytotoxins production with a clinical history of bloody diarrhea, but enterotoxin production with watery diarrhea (Lee et al., 2000; Prasad et al., 2006). In this study, cytotoxin-producing capacity was detected in most of the strains tested (75%, 15/20). Moreover, Vero cells cytotoxicity was represented by rounding and detachment of cells. This activity was observed after 24, 48 and 72 h after incubation with titers which varied from 1/2 to 1/4 for cytotoxic isolates (Table 3 and Figure 1). High prevalence of cytotoxicity in *Campylobacter* spp. indicates a significant epidemiological role of *Campylobacter* in

human infections which was in agreement with many previous investigators (Klipstien et al., 1985; Johnson and Lior, 1986; Florin and Antillon, 1992).

In Egypt, due to the excessive use of antibiotics for treatment and prophylaxis as well as growth promotion in chickens, poultry meat is considered a serious vehicle of antimicrobial-resistant *Campylobacter* transmission to human. Antibiotic susceptibility rates of *Campylobacter* isolates are shown in Table 4. There was a remarkably high resistance rate displayed by *C. jejuni* and *C. coli* to penicillin (95 and 90.1%), chloramphenicol (90 and 90.1%) and gentamicin (80 and 81.81%), respectively. *Campylobacters* also revealed a high antibiotic resistance against trimethoprim- sulfamethoxazole (85 and 81.81%), cephalothin (75 and 72.72%), erythromycin (75 and 72.72%), ampicillin (70 and 54.5%), amoxicillin-clavulanic acid (65 and 63.63%), oxytetracycline (65 and 63.63%), and streptomycin (65 and 63.63%), while they revealed a lower resistance against nalidixic acid (30 and 36.36%) and ciprofloxacin (10 and 18.81%) for *C. jejuni* and *C. coli*, respectively. Multidrug resistance (Resistance to three or more classes of antimicrobials) was observed in 85% (17/20) and 81.82% (9/11) of *C. jejuni* and *C. coli* isolates respectively. While, none of the *Campylobacter* isolates were resistant to all of the antimicrobials tested. In this study *C. jejuni* showed remarkable higher incidence in antimicrobial resistance than *C. coli*. These findings were in agreement with that previously reviewed in many studies (Saleha, 2002; Sáenz et al., 2000; Aarestrup and Engberg, 2001; Taremi et al., 2006). In contrary, *C. coli* showed a higher prevalence of antimicrobial resistance than *C. jejuni* by Signorini et al. (2018)

In this study, *Campylobacter* isolates were more sensitive to ciprofloxacin which is in agreement with McDermott et al. (2002) and Moore et al. (2005) who stated that ciprofloxacin was the drug of choice for empirical therapy of bacterial food borne diarrhea, including that caused by *Campylobacter*. In addition, Kassa et al. (2007) found that *C. jejuni*, *C. coli* and *C. lari* isolated from food animals were sensitive to



**Figure 1.** (A) Localized cell destruction, rounding and detachment of Vero cells after 24 h incubation with live bacteria (X 1000). (B) CPE characterized by degenerative changes and detachment of Vero cells, incubated with 1/2 Cytotoxins dilution at 24 to 48 h (X400). (C) Elongation, shrinkage with cytoplasmic vacuolation after incubation with 1/4 Cytotoxins dilutions at 24 to 48 h (X1000). (D) Polymorphic cells, rounding with pyknosis and multinucleated giant cell with 1/4 Cytotoxins dilutions at 72 h (X 400).

**Table 4.** Susceptibility of *C. jejuni* and *C. coli* to different antimicrobial agents.

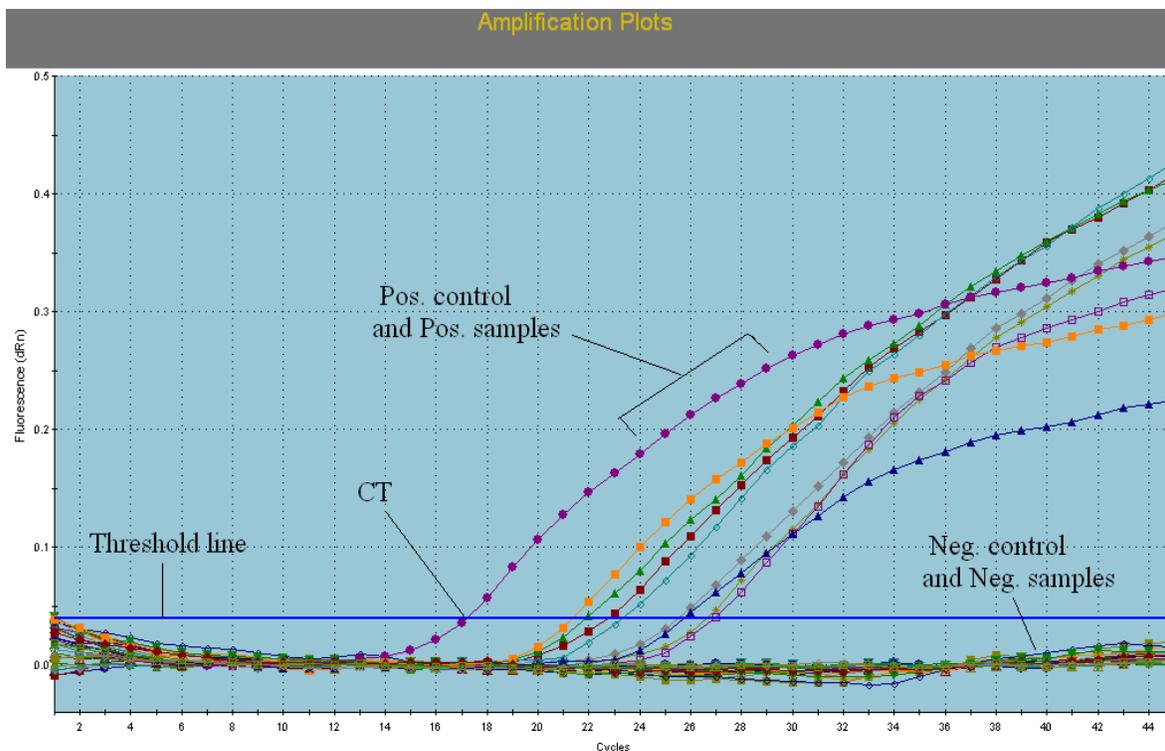
Antimicrobial agent	Antimicrobial class	<i>C. jejuni</i> (20)		<i>C. coli</i> (11)	
		Sensitive (%)	Resist (%)	Sensitive (%)	Resist (%)
Penicillin	Beta-Lactam	1 (5)	19 (95)	1 (9.1)	10 (90.1)
Ampicillin	Beta-Lactam	6 (30)	14 (70)	5 (45.4)	6 (54.5)
Amoxicillin-Clavulanic acid	Beta-Lactam	7 (35)	13 (65)	4 (36.3)	7 (63.63)
Erythromycin	Macrolides	5 (25)	15 (75)	3 (27.3)	8 (72.72)
Oxytetracycline	Tetracycline	7 (35)	13 (65)	4 (36.3)	7 (63.63)
Nalidixic acid*	quinolones	14 (70)	6 (30)	7 (63.6)	4 (36.36)
Ciprofloxacin	Fluorquinolones	18 (90)	2 (10)	9 (81.8)	2 (18.18)
Cephalothin	Beta-Lactam	8 (25)	12 (75)	3 (27.3)	8 (72.72)
Gentamicin	Amino glycosides	4 (20)	16 (80)	2 (18.2)	9 (81.81)
Streptomycin	Amino glycosides	7 (35)	13 (65)	4 (36.3)	7 (63.63)
Trimethoprim sulfamethoxazole	Folate Pathway Inhibitors	3 (15)	17 (85)	2 (18.2)	9 (81.81)
Chloramphenicol	Phenicols	2 (10)	18 (90)	1 (9.1)	10 (90.1)

\*Nalidixic acid and cephalocin were tested as recommended for the identification of *Campylobacter* isolates.

chloramphenicol and ciprofloxacin. Finally, a complete comparison in the susceptibility of *Campylobacter* to different antimicrobials is impossible as the strains numbers examined differ from one study to another.

## Conclusion

The results obtained from this study suggest an important role of chicken meat as a source of cytotoxic and



**Figure 2.** Amplification curve of suspected *C. jejuni* using probe based qPCR.

multidrug resistant *Campylobacter* spp.; therefore, there is a possible risk to human when dealing with the raw poultry carcass or consumption of undercooked chicken products. So effective vaccine against *Campylobacter* infection should be recognized to protect against infection by this group of organisms with appropriate hygienic measures during carcass slaughtering and processing. In addition, one of our future studies will be focused on developing strategies to decrease *Campylobacter* colonization in broilers chicken.

## CONFLICT OF INTERESTS

The authors have not any conflict of interests.

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*Full Length Research Paper*

## **Assessment of microbiota in root canals with pulp necrosis by means of Gram test**

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**The aim of this study was to evaluate the type of microbiota present in root canals with pulp necrosis, with and without periapical lesion. Nineteen patients were selected for the study and 30 root canals were analysed in uniradicular and/or multi-radicular permanent teeth, asymptomatic, with pulp necrosis, with or without periapical lesion, and no communication between root canal and oral cavity. Absorbent paper cones were introduced in the canal for 20 s. Then, the contaminated paper cones were inoculated in a Brain Heart Infusion Agar-BHI culture medium and incubated in an oven for 48 h at 37°C. The data were analysed by non-parametric statistical method of the Cochran-test. The results revealed that there was no statistically significant difference between the amount of Gram-positive and negative bacteria in endodontic infections, indicating that the microbiota of these endodontic infections is mixed.**

**Key words:** Microbiota, lipopolysaccharide, endodontics, root canals, pulp necrosis.

### **INTRODUCTION**

Treatment of infections in the root canal space of the teeth is a challenge in endodontics. One of the aims of endodontic treatment is the complete elimination of existing microorganisms, hence, the importance of chemical means of disinfection has aroused to accomplish complete sterilization of canal and to improve the treatment prognosis (Reddy et al., 2017). For this reason, the knowledge on endodontic microbiota, its organization and its distribution within the root canal system, is of vital importance in understanding the

infectious process and the establishment of therapeutic strategies that aims to eradicate the infection. Since some symptoms are associated with the presence of specific bacteria, if they are not completely eliminated from the root canals, they may perpetuate a pathological condition that causes treatment failure (Pajari et al., 1993; Lopes and Siqueira-Júnior, 2004).

However, microorganisms that cause endodontic infections are generally of low virulence. Their pathogenesis and survival are influenced by the release

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of lipopolysaccharide (LPS), bacterial toxins and synthesis of enzymes (Nair, 2004). The presence of bacteria within root canals contributes to the beginning of pulp necrosis and the possible involvement of the periapical region of the teeth (Dubei, 2016). These lesions are associated with a mixed microbiota, which consist of aerobic, anaerobic, Gram positive and negative microorganisms (Kakehashi et al., 1965; Cheung et al., 2001).

The aim of this study was to analyse the type of microbiota present inside the necrotic root canals with or without periapical lesions. The microbiota was identified through the coloured Gram method and its morphological classification.

## MATERIALS AND METHODS

### Sample selection

Nineteen patients were selected, regardless of age and sex. The research was performed at the College of Odontology, of the Federal University of Alagoas (UFAL), after the project approval by the Ethics Committee of Research of the same unit (No. 007418/2009-35). For this study, 30 root canals were analysed, taking into account, the sampling error, approximately 0.05%, with permanent teeth single-rooted or multi-rooted, asymptomatic, with pulp necrosis, with or without periapical lesion, and not presenting communication between root canal and the oral cavity. Only one exclusion criteria that the patients did not use any antibiotics for a minimum period of three months (Weiger et al., 1995).

### Clinical procedures

After signing the consent term, a clinical file of the patient was filled up and an evaluation of the patient's general health was performed. For diagnosis, radiographs were examined and pulp sensitivity to cold was tested.

Prior to intervention, the patient made a mouthwash with 10 ml of 0.12% chlorhexidine (Periogard®, Colgate - Palmolive Industry and Trade LTDA, São Paulo, Brazil). After that, the carious tissue was removed. After isolating the tooth, the working station (tooth, clamp, rubber dam and arch) was cleansed with NaOCl 2.5% (Brilux, Pernambuco, Brazil), followed by a surgery to access the pulp chamber.

### Collection of samples

Two to four sterile absorbent paper cones (Dentsply Industry and Commerce Ltda., Petrópolis, Brazil) were introduced into the canal for 20 s. In cases where the canal was dry, it was moistened with sterile distilled water (Laborclin Laboratory Products Ltda., Paraná, Brazil) to ensure a viable sample. Then, the contaminated paper cones were inoculated in a Brain Heart Infusion (BHI) Agar (Interlab Scientific Products Distributor S / A, São Paulo, Brazil) culture medium and incubated in an oven for 48 h at 37°C.

### Laboratory analysis

The analyses were done in the Laboratory of Biochemistry and Physiology in the Department of Antibiotics, at Federal University of Pernambuco (UFPE).

The Gram methodology was used according to the methodology of Winn et al. (2005). A thin scrub was prepared and the material

was left to air dry. After this step, the material was passed 3 to 4 times by a flame of a Bunsen burner to be fixed on a lamina, so that it will not be detached during the colouring process. Then, the material was covered with ethanol for 4 min. Next, it was put on a holder and its surface was covered with crystal violet solution for 1 min, washed with distilled water and covered with Gram iodide solution for 1 min. Subsequently, it was washed again with distilled water. Then, acetone was dropped on the material until the purple colour disappeared, within 10 s, washed with distilled water. The scrub surface was covered with safranin for 1 min and washed with distilled water. After this step, the material was placed in an upright position on a holder and left to dry. The stained lamina was examined through an optic microscope with objective lens, 100x, using a drop of immersion oil. Gram-positive bacteria were coloured in dark blue and Gram-negative, in pink-red. Optical microscope (Nikon E200) observation of the lamina for morphological classification of microorganisms was performed with an objective lens, 100x.

### Statistical analysis

The data were analyzed by non-parametric statistical method of Cochran-test, using the software Assisat, Beta version 7.0; the data do not follow a normal distribution ( $p < 0.05$ ).

## RESULTS

Thirty root canals with pulp necrosis were analysed, from which 24 presented periapical lesions. All the root canals with pulp necrosis and periapical lesions examined showed Gram-negative bacilli (100%), whereas in the canals of 23 teeth, it was possible to find cocci and Gram-positive bacilli (95.8%). In 13 teeth canals with pulp necrosis and periapical lesions, yeast infections were observed (54.1%) as described in Table 1. In the 6 cases evaluated with pulp necrosis without periapical lesions, there was a balance in the presence of Gram-positive and negative microorganisms (100%). However, yeast cells were found in three of these six root canals (50%,  $p < 0.05$ ) evaluated (Table 1).

For 24 teeth studied with periapical lesions, five cases of granuloma were diagnosed through radiographs and all of them presented Gram-negative and positive microorganisms (100%). Furthermore, one canal also had yeast cells (20%). In the five cases of chronic abscess with fistula, A balance of Gram-positive and negative bacteria was also observed in all the root canals (100%) and in 3 out of 5 canals, leavens cells were found (60%). Studying the six cases of chronic apical periodontitis, all of them presented Gram-positive microorganisms and yeast cells (100%), and in 5 out of the 6 canals, Gram-negative bacteria (83.33%) were found. Moreover, seven canals were diagnosed with thick apical periodontal, all these canals presented Gram-positive and negative microorganisms (100%), and in 3 canals, yeast cells (42.85%) were found. In this research, one canal with phoenix abscess was diagnosed. In this canal, Gram-negative and positive microorganisms (100%) were found. However, yeast cells were not present (Table 2).

**Table 1.** Comparison of the presence of microorganisms in root canals with or without periapical lesions ( $p < 0.05$ ).

Microorganism	Root canals with periapical lesion (24)	Root canals without periapical lesion (6)
Gram+	23	06
Gram-	24	06
Yeast cells	13	03

**Table 2.** Association of periapical lesions with the presence of microorganisms in the root canals.

Types of periapical lesion (24)	Gram+ bacteria (%)	Gram- bacteria (%)	Leaven cells (%)
Periapical granuloma (5)	100	100	20
Chronic abscess with fistula (5)	100	100	60
Chronic apical periodontics (6)	83.33	100	100
Canals with thick apical periodontal (7)	100	100	42.85
Phoenix abscess (1)	100	100	0

## DISCUSSION

Due to its complexity, the endodontic microbiota has been extensively studied through macroscopic and microscopic observations, and also through the classification of the microorganisms by means of Gram colour and evaluation of the presence of yeasts, aerobic microorganisms, strict anaerobes and facultative bacteria, which supports the claim that endodontic infections are polymicrobial in nature (Jacinto et al., 2003; Pazelli et al., 2003; Pinheiro et al., 2003; Gomes et al., 2004; Vianna et al., 2006). In this research, the presence of a mixed microbiota colonizing the root canals with pulp necrosis was observed presenting aerobic, anaerobic, Gram positive, Gram negative microorganisms, Cocci, Bacillus and yeasts. In previous researches (Jacinto et al., 2003; Gomes et al., 2004; Pourhajibagher et al., 2017), there was a prevalence of Gram-negative microorganisms in the primary endodontic infections, both symptomatic and asymptomatic, which is in line with the data presented here. In the current study, there was no significant difference ( $p > 0.05$ ) in the presence of Gram-positive and negative microorganisms in the root canals with pulp necrosis, as reported by Nobrega et al. (2016) but in the study of Vianna et al. (2006) and Sakko et al. (2016), the prevalence of Gram-positive microorganisms was found. Other researches showed that the microbiota of untreated teeth is different from that in which failure in endodontic treatment occurred. In the case of retreatment of root canals, there is a prevalence of Gram-positive microorganisms. These findings indicate that the microbiota differs from primary to secondary endodontic infections (Gomes et al., 2004; Silva et al., 2006; Sousa, 2000).

In the analysis of teeth with periapical abscess, Siqueira and Rôças (2013) and Sousa (2000) found a

predominance of Gram-positive anaerobic bacteria in most of the root canals observed, although less frequent facultative anaerobic bacteria had also been found, demonstrating the presence of a mixed microbiota in the infected root canals associated with periapical abscesses. In the current research, a predominance of strict anaerobic bacteria was also verified, but there are no major Gram-positive or negative bacteria. However, Saini et al. (2004) observed the predominance of aerobic microorganisms in the abscesses. In another study, Penãrocha et al. (2007) evaluated microbiologically and histopathologically, 30 cases of periapical lesions. A total of 137 bacterial strains, including *Fusobacterium nucleatum* (Bacillus, Gram-negative, Anaerobic), Gram-negative anaerobic bacilli, *Peptostreptococcus* sp. (Cocci, Gram positive, Anaerobic), *Streptococcus mitis* Anaerobic facultative) and Gram-positive non-spore anaerobic bacilli were, in descending order, the most commonly isolated bacteria from the lesions. Histopathological analysis revealed the prevalence of periapical granuloma. However, in this study, the presence of Gram positive and negative bacteria in granulomatous lesions was observed.

On the other hand, study on the composition of the bacterial flora of infected root canals with apical periodontitis was carried out (Jacinto et al., 2003; Gomes et al., 2004). The presence of a mixed microbiota with the prevalence of Gram-negative anaerobic bacteria was observed, which corroborates the results of the current study study.

Gram-negative bacteria were found in all root canals of the chronic apical periodontitis affected teeth. Gram-positive bacteria was also found in 5 of the 6 evaluated teeth, corroborating with the findings of Foschi et al. (2005) and Vengerfeldt et al. (2014), which demonstrated the presence of Gram-positive coccus *E. faecalis*,

suggesting that these species play critical roles in endodontics pathology.

Pinheiro et al. (2003) and Pourhajibagher et al. (2017) examined teeth with failure in endodontic treatment and in the presence of periapical lesions, and observed *Candida albicans*. In the present study, the existence of *C. albicans* was observed in both canals, with and without periapical lesions. Therefore, fungi may play a significant role in the pathogenesis of periapical diseases, as they have the ability to adapt to a variety of environmental conditions, adhere to different surfaces, produce hydrolytic enzymes, form biofilms and modulate hosts immunity (Siqueira et al., 2004). In this way, Gomes et al., (2008) analyzed teeth with pulp necrosis (primary infection) associated with the periapical lesion and reported the presence of *C. albicans*. Lana et al. (2001) studied channels without periapical lesion and indicated the presence of fungi. These data confirmed the presence of fungi both in channels with necrotic pulp associated with periapical lesion or not.

## Conclusion

The result of this research showed a predominance of Gram negative microorganisms, in root canals with periapical lesion in comparison with Gram positive microorganisms and yeast.

In root canals without periapical lesions, there was a balance between the presence of Gram positive and negative microorganism; however, presenting a lower amount of yeast. In all the canals analysed with periapical lesion only in those affected by chronic apical periodontitis, there was a balance regarding the presence of Gram positive, negative and yeast bacteria.

The results obtained demonstrated that the microbiota of the root canals studied is mixed with a predominance of Gram negative bacteria.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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*Full Length Research Paper*

# **Thermotolerant bacteria of biotechnological potential from hot springs in Eritrea**

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**Thermophiles are excellent sources of enzymes that can withstand and carry out reactions efficiently under high temperatures. This study isolated and characterised thermotolerant bacteria that produce enzymes of potential industrial value from five hot springs in Eritrea. A total of 65 bacterial isolates were obtained from the five hot springs. Out of the 65 isolates; 19 isolates produced a positive reaction for amylases, 36 for carboxymethyl cellulases, eight for proteases, 10 for xylanases and 11 for pectinases. More than half (36 out of 65) were able to produce carboxymethyl cellulases. Six isolates which showed carboxymethyl cellulase activity were from the genus *Bacillus*, while those belonging to *Brevibacillus* were seven. BLAST analysis of the partial sequences showed that 19 out of the 24 isolates sequenced showed high similarity (> 99%) to those of reference strains of the genera *Bacillus* and *Brevibacillus* available in the Genbank and EZ-taxon databases. The five isolates (E5, G2, G8, M1 and M13) that showed moderate similarities (97.2-99%) to strains from the Genbank and EZ-taxon databases were further characterized. Physiological characterization of the five selected isolates based on tolerance to NaCl, temperature and production of hydrolytic enzymes indicated that these isolates are potentially novel. Isolates G8 and M13 showed significantly higher amylase activity ( $p < 0.05$ ) than the other three isolates. Caseinase activity recorded by the five isolates was the highest ( $p < 0.05$ ) compared to other enzyme activities. The enzymes produced by thermotolerant bacteria from the five hot springs may be potentially useful for catalysis under harsh operational conditions encountered in industrial processes.**

**Key words:** Thermophilic, bacteria, thermozyms, hot springs, Eritrea.

## **INTRODUCTION**

The discovery of extremophiles has been a remarkable impetus for biotechnology industries. The products

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secured from extremophiles such as proteins, enzymes (extremozymes) and compatible solutes have great biotechnological potential. The inherent stability of thermostable enzymes, which have been isolated mainly from thermophilic or thermotolerant organisms, has made them suitable candidates for a number of commercial applications (Singh et al., 2011). The chemical production of polymer intermediates, pharmaceuticals, speciality chemicals and agrochemicals is hindered by expensive processes due to low selectivity and undesired byproducts (Angelaccio, 2013). The lack of enzyme stability of mesophilic enzymes renders them inefficient for the harsh reaction conditions required in industrial processes. For this reason, the use of biocatalysts in organic reactions represents only a small fraction of the potential industrial market (Meyer et al., 2012). Thermotolerant bacteria have attracted industrial and biotechnological attention as their enzymes are well suited for harsh industrial processes (Abdel-Rahman et al., 2016; Archana et al., 2015; Verma et al., 2015). Proteases obtained from thermophilic or thermotolerant (Adhikari et al., 2015; Bozoglu et al., 2015; Lele and Deshmukh, 2016; Panda et al., 2013; Remigio et al., 2012; Verma et al., 2014) bacteria, for example, have found applications such as hide dehairing in the leather industry and stain removal in laundry detergents (Chandrashekhar and Narayan, 2015; Dudhgara et al., 2015; Jaouadi et al., 2013). Cellulases have showed great potential in the production of bioethanol and other speciality chemicals from renewable agricultural residues (Hardiman et al., 2010). Brewing and sugar production require  $\alpha$ -amylases that are stable at high temperatures for gelatinization and liquefaction of starch to run processes at a relatively low cost (Rasooli et al., 2008). Xylanases active at high temperature and pH have attracted special interest in the pulp and paper industry as they reduce the need for toxic chlorinated compounds (Srinivasan and Rele, 1999). Pectinases are of importance in cotton scouring in textile industries (Dhiman et al., 2008). However, only a few of actual applications of these biocatalysts have reached the market (Coker, 2016).

Hot springs are potential habitats for thermophilic microorganisms. During the last few decades, hot spring environments from around the world have been targeted for the isolation of novel thermotolerant or thermophilic microorganisms that produce stable thermozymes (Lele and Deshmukh, 2016; Shahinyan et al., 2015; Verma et al., 2014). Even though Eritrea is endowed with plenty of thermal springs, aside from their prospects in geothermal energy, the thermal hot springs in Eritrea have not yet been studied with respect to biotechnological prospects. In Eritrea, thermal springs are located scattered in the eastern low lands. This study aimed to isolate and characterize thermotolerant bacteria from hot springs in Eritrea that produce enzymes such as amylases, cellulases, pectinases, xylanases and proteases.

## MATERIALS AND METHODS

### Sampling

Five hot springs; Maiwooi, Akwar, Garbanabra, Gelti and Elegedi were selected for sampling from three different locations, Gahtelai area, Irafayle and Alid area (Figure 1). Maiwooi (15° 32' 53"N 39° 06' 38" E) and Akwar (15° 33' 34"N 39° 05' 37" E) are located near Gahtelai at elevations of 330.1 and 344.5 m, with temperatures of 51.9 and 49.0°C and pH range of 7.54 and 6.97 respectively (Figure 1). These are low energy hot springs which discharge near-neutral bicarbonate waters (Yohannes, 2010). Garbanabra (15° 03' 38"N 39° 46' 27" E) and Gelti (15° 03' 39"N 39° 46' 46" E) are located near Irafayle on the shore of Gulf of Zula at elevations of 0.0 and 0.0 m, temperatures of 51.0 and 52.7°C and pH range of 7.05 and 7.01 respectively. Elegedi (14° 52' 55"N 39° 55' 37" E) is located in Alid volcanic center at elevation of 512.7 m, with a temperature of 100°C and pH range of 7.19. Elegedi which is located about 30 km south of the Gulf of Zula and is associated with a high temperature geothermal system underlying the Alid volcanic centre in the northern Danakil depression of Eritrea (Yohannes, 2010). The bubbling water discharged from this hot spring is typical of the fumarolic steam condensate with high temperatures. Triplicate samples of water, wet sediment and microbial mat were collected from each hot spring.

Samples were collected in 20-ml autoclaved test tube containers and immediately placed in a thermoflask to keep the temperature of the water samples constant (Khalil, 2011). The samples were transported to the College of Health Science Laboratory in Asmara, Eritrea for further processing.

### Isolation and enumeration of thermotolerant bacteria

Five millilitre from each water sample was used to inoculate 100 ml of culture media in 250-ml Erlenmeyer flask. The enrichment culture media contained; 5 g of NaCl (BLULUX), 9 g of peptone (BLULUX) and 2 g of yeast (BLULUX) per litre. The inoculated flasks were incubated at 55°C with shaking at 240 rpm for 94 h.

One hundred microlitre of culture from each flask was spread on agar media containing; 5 g of NaCl (BLULUX), 9 g of peptone (BLULUX), 2 g of yeast (BLULUX) and 20 g of bacteriological agar (BLULUX) per litre and was incubated at 55°C in a bench top incubator for 24 to 48 h (Khalil, 2011). To obtain pure cultures, distinctive colonies were picked, transferred to fresh agar medium and incubated at 55°C for 24 and 48 h. Purified colonies were grown on tryptic soy broth (Difco) and stored in 20% glycerol at -80°C.

### Morphological characterization

Colony and cell morphology were performed according to the standard protocols. Isolates were grown for 24-72 h at 50°C on the agar media described above. All the 65 isolates were examined using binocular microscope (BX100 Olympus) and characterised by Gram and spore staining (Moses et al., 2009).

### Detection of enzymes

Hydrolase production by thermotolerant bacterial isolates was screened by plating on starch (Khalil, 2011), carboxymethyl cellulose (Teather and Wood, 1982), skimmed milk (Zilda et al., 2012), pectin (Huang et al., 2012) and xylan (Gessesse and Gashe, 1997) agar plates for amylase, cellulose, protease, pectinase and xylanase activity respectively. All the assays were conducted at 55°C for 48 h.



**Figure 1.** The location of five hot springs in Eritrea. Maiwool and Akwar from Location 1 (Ghtelai area); Garbanabra and Gelti from Location 2 (Irafayle) and Elegeledi from Location 3 (Alid area).

#### Molecular characterization using partial 16S rRNA gene

Bacterial isolates were grown in Luria Broth medium (Tryptone, 10 g/L; yeast extract, 5 g/L; NaCl, 10 g/L; pH 7.0) at 55°C for 48 h. The cultures were centrifuged at 10,000 × *g* for 1 min, and the supernatant was removed and the pellet was retained. DNA was extracted as previously described (Sambrook and Russell, 2001) and was stored at -80°C until further analysis. Bacterial universal primers 8F (5'-AGRCTTTGATCCTGGCTCAG-3') and 1492R (5'-CGGCTACCTTGTTACGACTT-3') were used to amplify the 16S rDNA from genomic DNA (Heuer et al., 1997). Polymerase chain reaction (PCR) was performed in a thermocycler (PeQLab, VWR, Germany). Each reaction mixture (50 µl) contained; 25 µl of 10× PCR master mix (BIOLINE), 2.5 U of Taq DNA polymerase (BIOLINE), 0.2 µM of each primer and 25 ng of template DNA. The amplification was performed as follows. Initial denaturation for 5 min at 94°C, 30 cycles each of denaturation for 30 s at 94°C, annealing for 30 s at 55°C, primer extension for 1.5 min at 72°C and final extension for 10 min at 72°C. The PCR products were checked by gel electrophoresis using 1.2% (w/v) agarose gels stained with ethidium bromide (10 mg/l) and stored at -20°C.

Sequencing of PCR products of the 65 bacterial isolates was carried out by Macrogen, South Korea. Sequencing was conducted in one direction using the forward primer (27 F) as previously

described (Sanger et al., 1977). The BioEdit program was used to remove ambiguity and comparisons were done with the NCBI GenBank databases using Basic Local Alignment Tool (BLAST) algorithm (Altschul et al., 1990). Sequences were submitted to the GenBank database and were assigned the accession numbers KX549080- KX549103. The differences in the nucleotides were converted into distance matrices using Maximum Likelihood method (Saitou and Nei, 1987). A phylogenetic tree was constructed using MEGA 7 (Kumar et al., 2016).

#### Biochemical and physiological characterization of the selected isolates

Biochemical tests including indole production, motility test, catalase, oxidase, sugar fermentation test, casein hydrolysis test, Tween 20 hydrolysis test and gelatin liquefaction test were performed on the five isolates designated E5, M1, M13, G2 and G8 (Sneath et al., 1986). The ability to grow at different temperatures was evaluated by inoculating the isolates into LB agar medium (Difco) at 15, 20, 30, 40, 50, 60 and 70°C (Aanniz et al., 2015). The pH range for growth was determined by growing the isolates at 55°C in 10 ml LB broth (Difco) adjusted to pH 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13 using HCl or NaOH (Allan et al., 2005). Salt tolerance was determined by

**Table 1.** Bacterial count and number of isolates in the five hot springs.

Sampling site	cfu ml <sup>-1</sup>	Number of isolates	% of thermotolerant isolates
Maiwooi	1.1 x 10 <sup>4</sup>	16	24.6
Akwar	5.4 x 10 <sup>3</sup>	16	24.6
Garbanabra	1.1 x 10 <sup>5</sup>	21	32.3
Gelti	8.0 x 10 <sup>3</sup>	5	7.7
Elegedi	1.4 x 10 <sup>5</sup>	7	10.8

growing the cultures in 10 ml LB broth supplemented with NaCl to total concentrations of 1 to 13% (w/v) at 55°C (Nakamura and Swezey, 2017). Turbidities of both pH and salt tolerance series were determined using a dual-beam spectrophotometer (Versamax, Germany) set at 680 nm, at 24 h intervals.

#### Enzyme activities of the selected isolates

Direct quantification of enzyme activities by measuring the diameter of clearing zones on petri dishes was used to compare the five selected isolates. The enzyme activity assay included  $\alpha$ -amylase (Khalil, 2011), cellulase using cellulose powder and carboxymethyl cellulose (CMC) as substrates (Teather and Wood, 1982), caseinase (Adhikari et al., 2015), pectinases (Huang et al., 2012) and xylanases (Gessesse and Gashe, 1997). Incubation was done at 55°C for 48 h. The diameter of the clear zones was measured using a ruler. Multivariate analysis (MANOVA) was used to compare enzyme activity between the five selected isolates. Significance was tested at  $p = 0.05$ .

## RESULTS

### Morphological and cellular characterization of isolates

Based on colony morphology, 65 thermotolerant bacterial isolates were obtained from the five hot springs in Eritrea. The highest number (21) of the isolates was obtained from Garbanabra while the lowest (5) was from Gelti. The colony forming units of thermotolerant bacteria that grew at 55°C varied from  $5.4 \times 10^3$  in Akwar to  $1.4 \times 10^4$  cfu ml<sup>-1</sup> in Elegedi (Table 1).

The number of isolates obtained from Akwar and Maiwooi were almost twice those from Gelti and Elegedi. The 65 isolates were Gram positive, rod-shaped, and endospore forming (Table 2). The spores observed were terminal as shown in Figure 2.

### Hydrolase activity

A positive result for hydrolase activity was indicated by the clear zone formed around the colony (Figure 2). Out of the 65 isolates; 19 isolates produced a positive reaction for amylases, 36 for carboxymethyl cellulases, eight for proteases, 10 for xylanases and eleven for pectinases (Table 2). More than half (36 out of 65) were able to produce carboxymethyl cellulases. Six isolates

which showed CMCase activity were from the genus *Bacillus*, while seven belonged to *Brevibacillus*. The five selected isolates; E5, G2, G8, M1 and M13 showed CMCase activity. However, only E5, G2 and M1 showed positive activity when cellulose powder was used as a substrate.

### 16S rRNA analysis

Genomic DNA was extracted from all the 65 thermotolerant isolates. 16S rRNA gene amplification with bacterial specific primers yielded an amplification product of approximately 1500 bp. From the 65 amplified PCR products sent for sequencing, only 24 appeared to be unambiguous and were considered for phylogenetic analysis. The 24 isolates were submitted to the NCBI database and were assigned accession numbers (KX549080-KX549102).

Sequence comparison with the Genbank and EZ-taxon databases using BLAST pairwise alignment was done and the affiliations of the 24 isolates to the closest reference strain were determined. The identification of the 24 isolates based on the sequence comparison with the Genbank, NCBI and reference strains belonging to genera *Bacillus* and *Brevibacillus* is shown in Table 3. BLAST analysis of the partial sequences showed that 19 out of the 24 isolates showed high similarity (> 99%) to reference strains of the genera *Bacillus* and *Brevibacillus* available in the Genbank and EZ-taxon databases. Five isolates (E5, G2, G8, M1 and M13) showed moderate sequence similarity (97.2 - 99%) to reference strains. Isolates G8, G9, M9 and M12 showed similarity (99.0 - 99.6%) to *Bacillus aerius* strain 24K (AJ831843) described by Shivaji et al. (2006). Four isolates (E5, G4, G5 and M5) were shown to affiliate (98.0 - 99.7%) with *Bacillus sonorensis* strain NRBC AYTNO1000016 (Palmisano et al., 2001). M13 was the only isolate which affiliated with *Bacillus licheniformis* strain 9945A (Gwinn and Thorne, 1964). The other 15 isolates (G1, G2, G3, G6, G11, G12, G14, G18, G19, G20, M1, M3, M10, M11 and M14) showed 98.1 to 100% similarity to *Brevibacillus borstelensis* strain NRRL (Shida et al., 1996).

The five isolates that showed moderate similarity are indicated in bold letters. Out of 13 isolates sequenced from Garbanabra, nine affiliated with the genus *Brevibacillus* while the other five belonged to *Bacillus*. In

**Table 2.** Morphological and biochemical characteristics of bacterial isolates from hot springs in Eritrea that produced extracellular enzymes.

Isolate	Colony characterization				Cell characterization			Extracellular enzymes detected					
	Colour	Shape	Elevation	Size	Shape	Gram reaction	Presence of endospores	Amylase	CMCase	Xylanase	Pectinase	Protease <sup>1</sup>	Protease <sup>2</sup>
A1	Brown	Curled	Flat	Medium	Rod	+	+	+	-	-	-	-	-
A2	Brown	Round	Flat	Medium	Rod	+	+	+	+	+	-	+	+
A3	Cream	Curled	Flat	Medium	Rod	+	+	-	-	-	-	-	+
A4	Cream	Irregular	Flat	Big	Rod	+	+	-	-	-	-	-	+
A5	Cream	Round	Flat	Medium	Rod	+	+	+	+	+	+	-	+
A6	Cream	Irregular	Flat	Big	Rod	+	+	-	+	-	-	-	+
A7	White	Round	Flat	Medium	Rod	+	+	-	-	-	-	-	+
A8	Brown	Curled	Raised	Medium	Rod	+	+	-	-	-	-	-	-
A9	Yellow	Round	Flat	Medium	Rod	+	+	-	+	-	-	+	+
A10	Cream	Round	Flat	Medium	Rod	+	+	-	+	+	+	-	-
A11	Cream	Round	Flat	Medium	Rod	+	+	-	-	-	-	-	+
A12	Cream	Irregular	Flat	Medium	Rod	+	+	-	+	-	-	-	+
A13	Cream	Irregular	Flat	Big	Rod	+	+	-	+	-	-	-	+
A14	Watery gray	Round	Flat	Big	Rod	+	+	-	-	-	-	-	+
A15	Dark brown	Round	Flat	Small	Rod	+	+	-	-	-	-	-	+
A16	Light brown	Curly	Raised	Medium	Rod	+	+	-	-	-	-	-	+
E1	Brown	Round	Flat	Small	Rod	+	+	-	+	-	-	-	+
E2	Brown	Wrinkled	Flat	Big	Rod	+	+	-	-	-	-	-	+
E3	Cream	Waivy	Flat	Medium	Rod	+	+	-	+	-	-	-	+
E4	Cream	Round	Flat	Medium	Rod	+	+	-	+	-	-	+	+
E5	White	Irregular	Flat	Medium	Rod	+	+	+	+	+	+	-	+
E6	Cream	Concentric	Flat	Medium	Rod	+	+	+	+	-	-	-	+
E7	Cream	Round	Flat	Medium	Rod	+	+	-	-	-	-	-	-
G1	Cream	Round	Flat	Medium	Rod	+	+	-	-	-	-	-	-
G2	Watery brown	White	Flat	Medium	Rod	+	+	-	+	-	-	+	+
G3	Brown	Wrinkled	Flat	Big	Rod	+	+	-	-	-	-	-	-
G4	Yellow	Contoured	Flat	Medium	Rod	+	+	-	-	-	-	-	-
G5	Brown	Wrinkled	Flat	Big	Rod	+	+	+	+	-	-	-	+
G6	Yellow	Contoured	Flat	Medium	Rod	+	+	-	+	-	-	-	+
G7	Shiny cream	Shell-like	Flat	Medium	Rod	+	+	-	+	-	-	-	+
G8	Cream	Round	Flat	Medium	Rod	+	+	+	+	+	+	-	+
G9	Brown	Curled	Flat	Medium	Rod	+	+	+	-	-	-	-	-
G10	Cream	Concentric	Flat	Medium	Rod	+	+	-	-	-	-	-	-
G11	White	Filamentous	Flat	Medium	Rod	+	+	-	+	-	-	-	+
G12	White	Filamentous	Flat	Medium	Rod	+	+	-	-	-	-	-	-

Table 2. Contd.

Isolate	Colony characterization				Cell characterization			Extracellular enzymes detected					
	Colour	Shape	Elevation	Size	Shape	Gram reaction	Presence of endospores	Amylase	CMCase	Xylanase	Pectinase	Protease <sup>1</sup>	Protease <sup>2</sup>
G13	Brown	Wrinkled	Flat	Big	Rod	+	+	+	+	-	+	-	+
G14	Shiny cream	Shell-like	Flat	Medium	Rod	+	+	-	-	+	-	-	+
G15	Shiny cream	Shell-like	Flat	Medium	Rod	+	+	-	+	-	+	+	+
G16	Brown	Wrinkled	Flat	Big	Rod	+	+	-	+	-	-	-	+
G17	Shiny cream	Shell-like	Flat	Medium	Rod	+	+	+	+	-	+	+	+
G18	Light brown	Curly	Raised	Medium	Rod	+	+	-	-	-	-	-	-
G19	White	Contoured	Flat	Medium	Rod	+	+	-	-	-	-	-	-
G20	White	Filamentous	Flat	Medium	Rod	+	+	-	+	-	-	-	+
G21	Brown	Round	Flat	Small	Rod	+	+	-	+	-	+	-	+
J1	Yellow	Contoured	Flat	Medium	Rod	+	+	-	-	+	-	-	+
J2	Brown	Wrinkled	Flat	Big	Rod	+	+	-	+	-	-	+	+
J3	Shiny cream	Shell-like	Flat	Medium	Rod	+	+	-	-	-	-	-	-
J4	Brown	Curled	Flat	Medium	Rod	+	+	-	-	-	-	+	+
J5	Brown	Wrinkled	Flat	Big	Rod	+	+	-	-	-	-	-	-
M1	Cream	Round	Flat	Medium	Rod	+	+	-	+	-	-	-	+
M2	Cream	Round	Flat	Small	Rod	+	+	-	+	-	-	-	+
M3	White	Filamentous	Flat	Big	Rod	+	+	-	+	-	-	-	+
M4	White	Wrinkled	Flat	Small	Rod	+	+	-	-	-	-	-	-
M5	Yellow	Round	Flat	Medium	Rod	+	+	+	+	-	-	-	+
M6	Cream	Round	Flat	Medium	Rod	+	+	-	+	-	-	-	+
M7	Brown	Curled	Flat	Medium	Rod	+	+	-	-	-	-	-	-
M8	Brown	Round	Flat	Medium	Rod	+	+	+	+	+	+	-	-
M9	Watery brown	White	Flat	Medium	Rod	+	+	+	+	-	-	-	+
M10	Cream	Round	Flat	Medium	Rod	+	+	+	+	+	-	-	+
M11	Yellow	Contoured	Flat	Medium	Rod	+	+	+	-	-	-	-	-
M12	White	Filamentous	Flat	Big	Rod	+	+	-	-	-	-	-	-
M13	Brown	Round	Flat	Small	Rod	+	+	+	+	+	+	-	+
M14	Faint brown	Round	Flat	Big	Rod	+	+	+	+	-	-	-	+
M15	Cream	Round	Flat	Medium	Rod	+	+	+	-	-	-	-	-
M16	White	Contoured	Flat	Small	Rod	+	+	+	+	-	-	-	+

<sup>1</sup>Protease activity was done using <sup>1</sup>skimmed milk and <sup>2</sup>casein as substrates. + indicates positive activity while - signifies negative reaction or no observable activity.

Maiwooi, five out of nine isolates had shown similarity (98.1 to 100%) to *Brevibacillus*.

The phylogenetic tree of the 16S rRNA partial sequences of the 24 isolates revealed two major

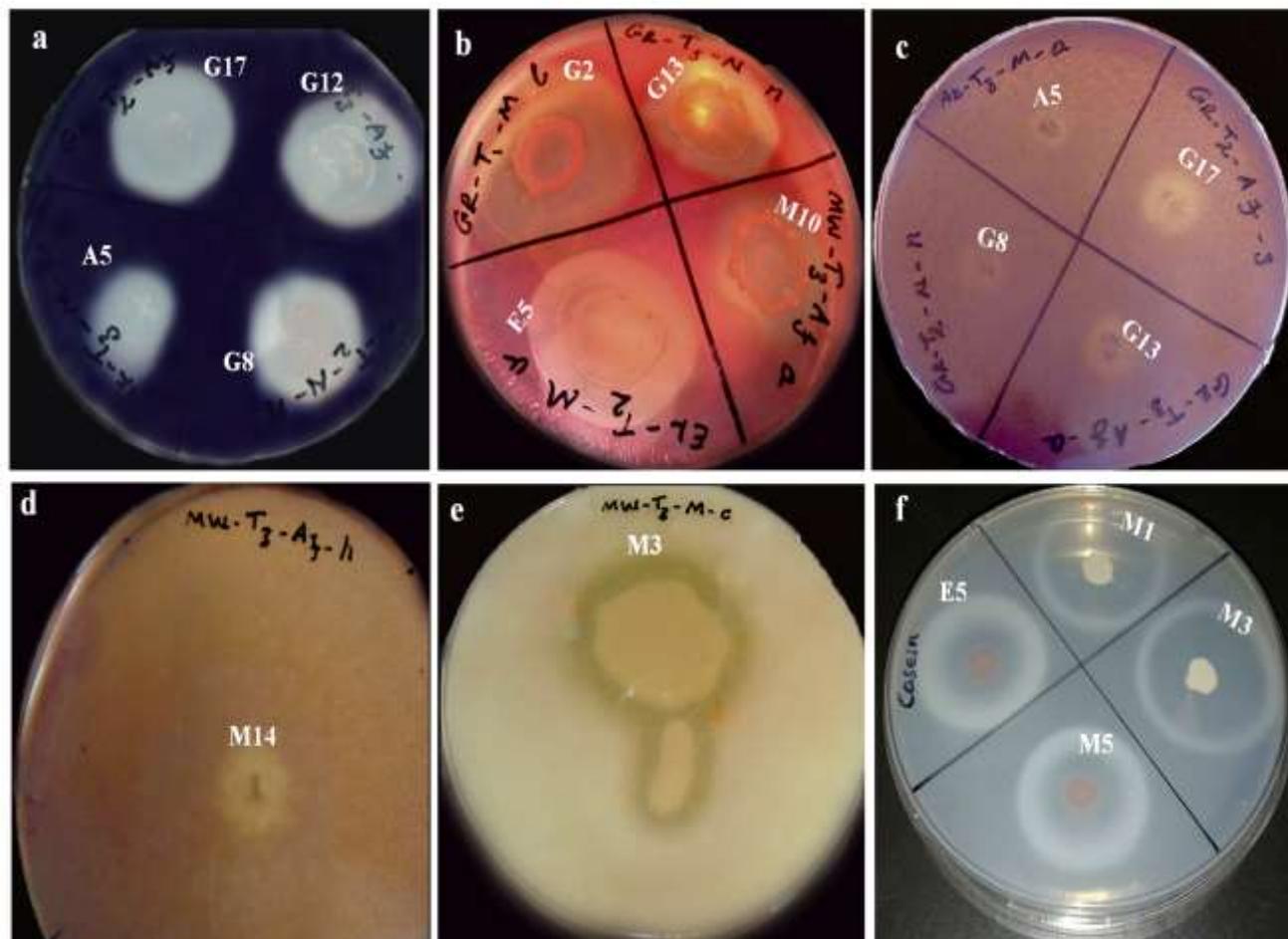
clusters (Figure 3). One cluster containing strains belonging to the genus *Bacillus* and the other



**Figure 2.** Spores (green) and vegetative cells (pink) of the isolate E5 obtained from the Elegedi hot spring in Eritrea. Ellipsoidal spores lie terminally as shown by the arrows.

**Table 3.** Affiliation of partial sequences of 24 bacterial isolates from hot springs in Eritrea with the 16S rDNA gene sequences in the GenBank.

Isolate	Sampling site	Accession number	Affiliated to	Closest match in BLAST	Length (bp)	%	Reference
<b>E5</b>	Elegedi	KX549080	AYTN0100001	<i>Bacillus sonorensis</i> NBRC	270	98.0	Palmisano et al. (2001)
G1	Garbanabra	KX549081	D78456	<i>Brevibacillus borstelensis</i> NRRL	390	100	Shida et al. (1996)
<b>G2</b>	Garbanabra	KX549082	D78456	<i>Brevibacillus borstelensis</i> NRRL	317	97.2	Shida et al. (1996)
G3	Garbanabra	KX549083	D78456	<i>Brevibacillus borstelensis</i> NRRL	566	100	Shida et al. (1996)
G4	Garbanabra	KX549084	AYTN0100001	<i>Bacillus sonorensis</i> NBRC	565	99.5	Palmisano et al. (2001)
G5	Garbanabra	KX549085	AYTN0100001	<i>Bacillus sonorensis</i> NBRC	560	99.7	Palmisano et al. (2001)
G6	Garbanabra	KX549086	D78456	<i>Brevibacillus borstelensis</i> NRRL	577	100	Shida et al. (1996)
<b>G8</b>	Garbanabra	KX549087	AJ831843	<i>Bacillus aerius</i> 24K	510	99.0	Shivaji et al. (2006)
G9	Garbanabra	KX549088	AJ831843	<i>Bacillus aerius</i> 24K	430	99.5	Shivaji et al. (2006)
G11	Garbanabra	KX549089	D78456	<i>Brevibacillus borstelensis</i> NRRL	498	100	Shida et al. (1996)
G12	Garbanabra	KX549090	D78456	<i>Brevibacillus borstelensis</i> NRRL	519	100	Shida et al. (1996)
G14	Garbanabra	KX549091	D78456	<i>Brevibacillus borstelensis</i> NRRL	392	99.5	Shida et al. (1996)
G18	Garbanabra	KX549092	D78456	<i>Brevibacillus borstelensis</i> NRRL	562	100	Shida et al. (1996)
G19	Garbanabra	KX549093	D78456	<i>Brevibacillus borstelensis</i> NRRL	513	100	Shida et al. (1996)
G20	Garbanabra	KX549094	D78456	<i>Brevibacillus borstelensis</i> NRRL	420	100	Shida et al. (1996)
<b>M1</b>	Maiwooi	KX549095	D78456	<i>Brevibacillus borstelensis</i> NRRL	440	98.1	Shida et al. (1996)
M3	Maiwooi	KX549096	D78456	<i>Brevibacillus borstelensis</i> NRRL	475	100	Shida et al. (1996)
M5	Maiwooi	KX549097	AYTN01000016	<i>Bacillus sonorensis</i> NBRC	349	99.7	(Palmisano et al., 2001)
M9	Maiwooi	KX549098	AJ831843	<i>Bacillus aerius</i> 24K	367	99.3	Shivaji et al. (2006)
M10	Maiwooi	KX549099	D78456	<i>Brevibacillus borstelensis</i> NRRL	497	100	Shida et al. (1996)
M11	Maiwooi	KX549100	D78456	<i>Brevibacillus borstelensis</i> NRRL	466	100	Shida et al. (1996)
M12	Maiwooi	KX549101	AJ831843	<i>Bacillus aerius</i> 24K	442	99.6	Shivaji et al. (2006)
<b>M13</b>	Maiwooi	KX549102	766760	<i>Bacillus licheniformis</i> 9945A	567	98.7	Gwinn and Thorne (1964)
M14	Maiwooi	KX549103	D78456	<i>Brevibacillus borstelensis</i> NRRL	530	100	Shida et al. (1996)



**Figure 3.** Culture plates showing positive hydrolase activities from the selected prominent bacterial isolates obtained from hot springs in Eritrea. The enzyme activity assay included amylase (a), cellulase (b), pectinase (c), xylanase (d) as well as protease using skimmed milk (e) and casein (f) activities. Clear zones indicate positive hydrolase activity.

containing strains affiliated with *Brevibacillus* with bootstrap values of 92 and 94 respectively.

#### Characterization of five bacterial isolates with moderate similarity

The five isolates (E5, G2, G8, M1 and M13) that showed moderate similarities (97.2 - 99.0%) with strains in Genbank and EZ-taxon databases were further characterized. The partial sequences of E5, G8 and M13 revealed similarity with *Bacillus* (98.0 - 99%) while G2 and M1 affiliated with the genus *Brevibacillus* (97.2 - 98.1%). The five isolates were Gram positive endospore forming rods. The growth of E5 was observed to occur between 20 - 60°C (Table 4).

E5 grew at a temperature range of 20 - 60°C. The others; G2, G8, M1 and M13 grew at a temperature range between 15 and 60°C. The isolates E5, G8 and M13 formed brown colonies on casein agar plates, whereas

G2 and M1 formed white colonies. Isolate E5 grew between pH 5 and pH 11, G2 and M13 between pH 6 and 11, G8 between pH 6 - 10 and M13 between pH 7 and 13. Isolates E5, G2 and G8 tolerate up to 11.6% NaCl while M17 and M13 tolerated up to 10% NaCl. The five selected isolates were indole negative and showed no motility at 55°C. All except E5 were catalase positive. All the five isolates produced acid from glucose, xylose and mannitol. E5, G8 and M13, whose sequence affiliated with the genus *Bacillus*, also produced acid from galactose, inositol as well as melibiose and were shown to hydrolyze Tween 20.

#### Enzyme activity assay

Halo size, a semi-quantitative method, indicates the efficiency of a colony in producing particular enzyme (Scorsetti et al., 2012). The agar diffusion method was employed to quantify enzyme activities by measuring the

**Table 4.** Characteristics of bacterial isolates from Eritrean hot springs with moderate (97.2-99%) similarity to *Bacillus* and *Brevibacillus*.

Characteristic	Isolate					Reference strains*			
	E5	G2	G8	M1	M13	1	2	3	4
Growth temperature (°C) range	20-60	15-60	15-60	15-60	15-60	10-40	15-45	8-40	15-50
Growth pH range	5-11	6-11	6-10	7-13	6-11	5.5-9	5.5-10	6-9	NA
Growth in the presence of NaCl									
1%	+	+	+	+	+	+	+	+	+
4%	+	+	+	+	+	+	+	+	-
7%	+	+	+	-	+	+	+	+	-
10%	+	+	+	-	+	+	+	+	-
13%	+	+	+	-	-	-	-	-	NA
16%	-	-	-	-	-	-	-	-	NA
Hydrolysis of									
Casein	+	+	+	+	+	+	NA	NA	+
Starch	+	-	+	-	+	+	NA	NA	-
CMC	+	+	+	+	+	NA	NA	NA	NA
Cellulose	+	+	-	+	-	NA	NA	NA	NA
Pectin	+	-	+	-	-	NA	NA	NA	NA
Xylan	-	-	+	-	+	NA	NA	NA	NA
Tween 20	+	-	+	+	-	NA	NA	NA	NA
Catalase test	-	+	+	+	+	+	+	+	NA
Indole test	-	-	-	-	-	-	-	-	NA
Motility test at 55 °C	-	-	-	-	-	NA	NA	NA	NA
Acid from									
Glucose	+	+	+	+	+	+	+	+	+
Inositol	+	-	-	-	+	NA	NA	NA	NA
Xylose	+	+	+	+	+	+	+	+	NA
Melbiose	-	-	+	-	+	+	+	+	NA
Mannitol	+	+	+	+	+	+	+	+	-
Galactose	+	-	+	-	+	+	+	+	+

\*Reference strains: 1, *Bacillus licheniformis* MTCC 429; 2, *Bacillus sonorensis* DSM 13779; 3, *Bacillus aerius* 24K; 4, *Brevibacillus borstelensis* LMG 16009. Data on strains 1, 2 and 3 was obtained from Shivaji et al. (2006), strain 4 from Allan et al. (2005). +, positive; -, negative; NA, no data available.

diameter of the zone of clearance (Figure 4). E5, G8 and M13, affiliated with the genus *Bacillus*, showed amylase activity while G2 and M1, belonging to the genus *Brevibacillus*, exhibited no amylase activity. Isolates G8 and M13 showed significantly higher amylase activity ( $p < 0.05$ ) than the other three isolates.

All the five isolates, E5, G2, G8, M1 and M13, were able to produce extracellular CMCase. Isolate G2 recorded significantly higher activity ( $p < 0.05$ ) while isolate E5 recorded the least activity. There was no significant difference in caseinase activity between five isolates ( $p < 0.05$ ). All the five isolates showed positive caseinase activity. Cellulase activity was recorded for isolates E5, G2 and M1, while G8 and M13 showed no activity. G8 and M13 were the only isolates that showed xylanase activity. Pectinase activity was only registered by isolates E5 and G8. Caseinase activity was significantly the highest compared to other substrates ( $p < 0.05$ ). Amylase and CMCase activities were also

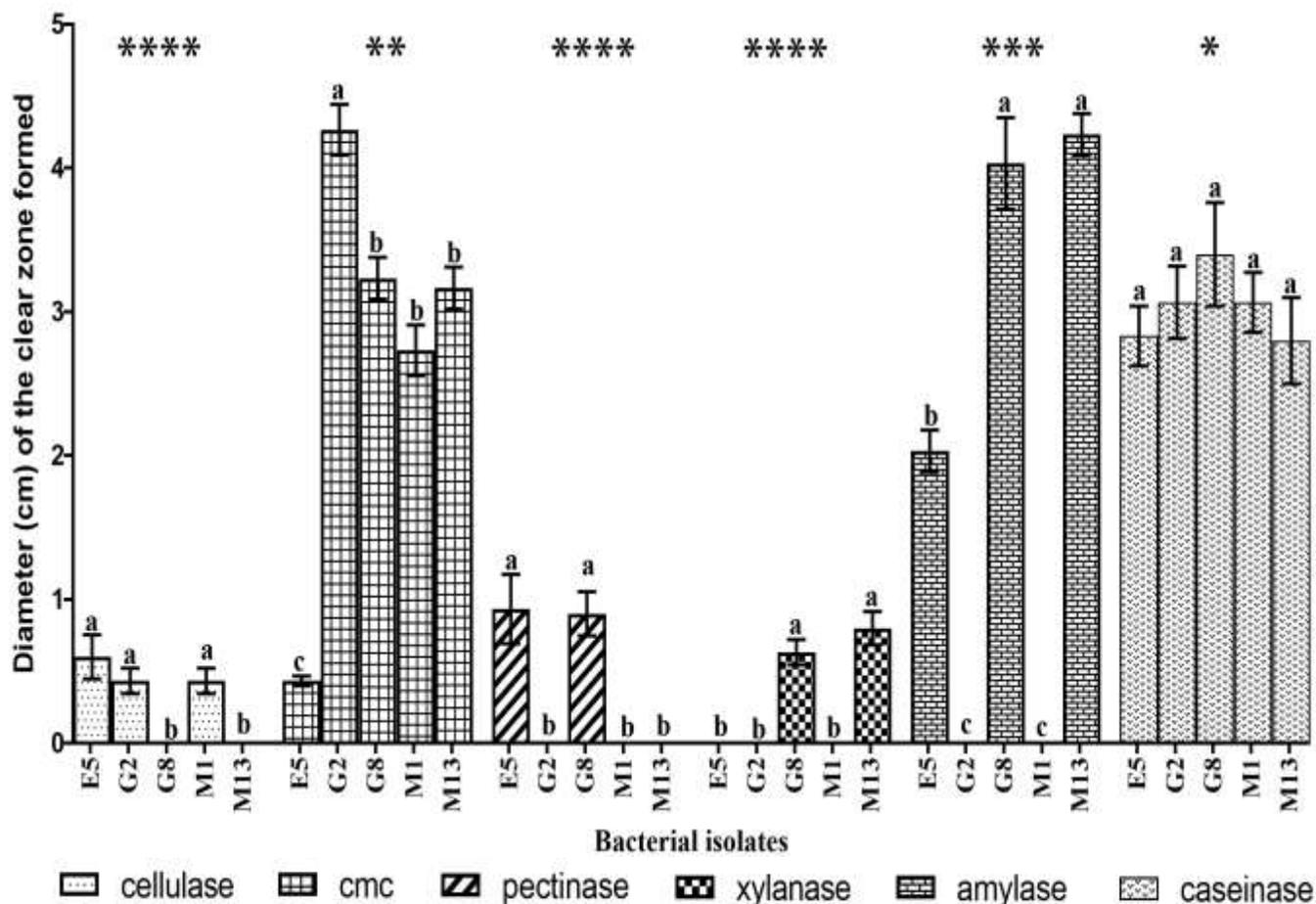
significantly higher ( $p < 0.05$ ) than cellulase, pectinase and xylanase activities.

The hydrolase activities, tolerance to high temperature, pH range and high NaCl concentrations were used to rank the effectiveness of the five isolates (Table 5). From the five selected isolates E5 had the highest score (7) followed by G8 (6). M13 had the lowest score of 3.

A score of one (1) was assigned to the positive reactions of the six hydrolase activities. Temperature tolerance of the five isolates were similar and hence was not considered in the ranking. A score of one (1) was assigned for those isolates that grew at pH 5 or 13. An isolate that grew above 10% NaCl was also given a score of one (1).

## DISCUSSION

A total of 65 thermotolerant bacterial isolates were



**Figure 4.** Ability of the five selected bacterial isolates, obtained from the hot springs in Eritrea, to produce extracellular enzymes. Bars (isolates) in each tested enzyme activity with the same alphabet letter represent isolate means with no significant difference  $p = 0.05$  level of confidence. The tested enzyme activity with grand means that are not significantly different have the same number of asterisk(s). Values in the figure are means of three replicates with standard deviation.

**Table 5.** Ranking of five selected bacterial isolates obtained from five hot springs in Eritrea.

Rank	Isolate	Hydrolase activity	pH tolerance	NaCl tolerance	Total score
1	E5	5	1	1	7
2	G8	5	0	1	6
3	M1	3	1	0	4
3	G2	3	0	1	4
4	M13	3	0	0	3

isolated from five hot springs in Eritrea. Thermotolerant bacteria were present in all samples analyzed. The cfu counts per ml ranged from  $5.4 \times 10^3$  in Akwar to  $1.4 \times 10^5$  cfu/ml in Egededi. Relatively higher cfu counts were retrieved from Egededi, a boiling hot spring than the other hot springs. The total counts in this study were higher than 50-5000 cfu/ml recorded in Moroccan hot springs (Aanniz et al., 2015) and 170-1330 cfu/ml recorded in the geothermal springs in Saudi Arabia (Khiyami et al.,

2012).

The sequences for 24 isolates were without ambiguities. All the 24 isolated microbes belonged to the domain bacteria, phylum *Firmicutes*, class *Bacilli*, order *Bacillales*, within two different families: *Bacillaceae* and *Paenibacillaceae*. Among these were; *B. sonorensis*, *B. licheniformis*, *B. aerius* and *B. borstelensis*. Bacilli were previously isolated from hot springs in Saudi Arabia using the same culture medium (Khalil, 2011). This indicates

that the enrichment culture medium used in this study is suitable for cultivation of the bacilli group. The other reason, which cannot be ruled out, is that *Bacillus* species are spore-forming ubiquitous bacteria in thermal hot springs. They were observed to be the predominant groups isolated from hot springs in Indonesia using other enrichment medium such as spring water enriched with nutrient broth (Yohandini, 2015). *Bacillus* have also been isolated using nutrient broth supplemented with 1% Tween or Olive oil from geothermal springs in Armenia (Shahinyan et al., 2015), Castenholz TYE medium from hot springs in India (Verma et al., 2014), Tryptone Soy Agar (TSY) from hot springs in Morocco (Aanniz et al., 2015) and nutrient agar from hot springs in Fiji (Narayan et al., 2008). The genus *Bacillus* and related genera are reported to be widely distributed in nature. It includes thermophilic, psychrophilic, acidophilic, alkaliphilic and halophilic bacteria that utilize a wide range of carbon sources for heterotrophic growth or are the autotrophs (Panda et al., 2014). In the present study, the isolates were recovered from five hot springs in Eritrea with different temperature regimes (49 - 100°C), as well as different sodium concentration levels ranging from 0.06 to 3640 mg/lit at near neutral pH.

The sequences of the 24 isolates were shown to form two clusters on the phylogenetic tree. One cluster included the genus *Bacillus* and nine other isolates. Included in the other cluster are isolates represented in the genus *Brevibacillus* and the 14 other isolates. All the 14 isolates showed similarity to *B. borstelensis* species. *Brevibacillus* and *Bacillus* are known to co-inhabit in diverse environments including rocks, dust, aquatic environments, guts of various insects and animals (Nicholson, 2002).

The thermotolerant bacteria isolated from the Eritrean hot springs, in the present study, were shown to produce hydrolytic enzymes such as amylases, cellulases, proteases, pectinases and xylanases at 55°C. Plate assays revealed that 19 of the isolates were amylase producers and 45 were protease producers. The increased stability of thermophilic enzymes at high temperature, chemical denaturants and pH changes makes them suitable for harsh industrial processes. Higher reaction rates and process yields of enzymatic reactions are achieved at high temperatures because of the decrease in viscosity, the increase in the diffusion coefficient of substrates as well as an increase in the solubility of substrates and products (Haki and Rakshit, 2003). Screening of microorganisms for amylase production allows for the discovery of novel amylases required for specific industrial applications. Thermostability is the most important property of an effective amylase, because liquefaction and saccharification of starch are performed at high temperature. In the present study, out of 65 isolates from the hot springs in Eritrea, 19 were positive for amylase production. Among the amylase positive isolates, seven

were affiliated to *Bacillus* based on their partial 16S rRNA gene sequence similarity and three to *Brevibacillus*. Isolates E5, G8 and M13 which tested positive for amylase activity possessed thermostability and halotolerability. E5 was also able to grow at slightly acidic levels (pH 5). The three of them were also observed to thrive at pH 11. This suggests that the enzymes obtained from these isolates are promising in their application in starch, detergent and textile industries.

Proteases have a long history of application in the food and detergent industries. They are also used in the leather industry for dehairing and bating of hides as a substitute for toxic chemicals. The use of the *Bacillus* species for protease production offers several advantageous like significant activity, stability, substrate specificity, short period of fermentation, mere downstream purification and low cost production (Aqel et al., 2012). In the present study, skimmed milk as well as casein were used as substrates to assess protease activity of the isolates. Eight of the isolates in the present study showed positive protease activity in media supplemented with skimmed milk as a substrate while 45 isolates showed positive activity using casein. The five possible novel isolates exhibited protease activity in medium supplemented with casein as a substrate.

Xylanase and pectinase screening of the isolates was also observed using xylan and pectin as a carbon source. Ten isolates were xylanase positive while those that showed positive pectinase activity were eleven. Isolates E5, G8, M9 and M13 belonging to the genus *Bacillus* were among those that showed positive xylanase and pectinase activities. E5 was shown to grow at a temperature of 60°C and slightly acidic pH of 5. Therefore, its xylanase and pectinase may have potential uses in industries such as detergent, food, pharmaceutical, leather, agriculture, kraft pulp prebleaching process and molecular biology reagents.

Physiological and biochemical characterization of the five isolates revealed some differences from strains in the Genbank and EZ-taxon databases. Isolate E5 was shown to grow at a maximum temperature of 60°C and 5-11 pH range, while *B. sonorensis* DSM 13779 which had shown 98% BLAST similarity with E5 did not show growth above 45°C (Shivaji et al., 2006). Notably, E5 isolated from the boiling hot spring with a temperature of 100°C did not grow in cultures above 60°C. *Bacillus* spp. are known to form spores and become dormant during extreme environmental conditions. This could explain why E5 did not grow above 60°C while it was isolated from the boiling hot spring. The other four isolates (G2, G8, M1 and M13), like E5, were shown to grow at 60°C. Isolate E5 was able to grow in the presence of 13% NaCl while *B. sonorensis* DSM 13779 was not able to grow above 10% NaCl (Shivaji et al., 2006). Isolates G2 and M1 showed moderate similarities (97.2 and 98.1, respectively) with strain *B. borstelensis* LMG. The maximum growth temperature (60°C) of the isolates G2

and M1 was 10°C higher than the *B. borstelensis* LMG 16009 (Allan et al., 2005). The isolates G2 and M1, unlike *B. borstelensis* LMG 16009, were shown to produce acid from mannitol. These observations could indicate the potential that these isolates could be novel.

## Conclusion

To the best of our knowledge, the present study investigated for the first time thermotolerant bacteria that produce enzymes from five hot springs in Eritrea using culture methods. Most of the isolates were thermotolerant and showed positive hydrolase activities. The sequences of the 24 isolates showed similarity with *Bacillus* and *Brevibacillus* from the phylum *Firmicutes*. Five isolates (E5, G2, G8, M1 and M13) showed moderate similarities with strains in Genbank and EZ-taxon databases. Moreover, the physiological and biochemical behavior of these isolates was not similar to the strains of the same species. When the five isolates were ranked based on hydrolase activities, tolerance to high temperature, acidic or alkaline pH levels and high NaCl concentrations, E5 was observed to be the most effective followed by G8, G2, M1 and M13. Taken together, the 16S rDNA data, physiological and biochemical characteristics provide possible evidence for the novel nature of the five isolates.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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