Isolation and molecular characterization of *Bacillus cereus* from cow's raw milk

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DECLARATION

I, Zinathi Lukanji declare that this dissertation submitted to the University of Fort Hare for the degree of Masters of Science in Microbiology in the Faculty of Science and Agriculture, School of Biological and Environmental Sciences, and the work contained herein is my original work and has not been submitted at any other University in partial or entirety for the award of any degree.

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DEDICATION

I dedicate my dissertation work to my wonderful daughter, Lusanele Lukanji whom I could not be with during the course of this research study.

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ABSTRACT

Bacillus cereus is a group of ubiquitous facultative anaerobic spore forming Gram-positive rods commonly found in soil. It has been detected and implicated in several contaminated food products and raw milk in dairy farms and it causes foodborne gastroenteritis by producing several toxins. This study is aimed at characterizing virulence determinants of *B. cereus* from cow's raw milk. A total of 400 raw milk samples were collected in Fort Hare Dairy Trust and Middledrift Dairy Farm; and cultured on Polymyxin pyruvate Egg-Yolk Mannitol Bromothymol Agar (PEMBA) for 48 hours at 37°C. DNA was isolated from the isolates and 16S rDNA was amplified and sent to Central Analytical Laboratory for sequencing. The *gyrB* gene of *B. cereus* was also used to confirm the identity of the isolates. Antibiotic susceptibility profiles of the isolates together with virulence genes were investigated. Multilocus Sequence typing was used to investigate the genetic relatedness of some selected isolates. Furthermore, spores of the isolates were produced, harvested and their concentrations determined. All (100%) of the isolates were identified as having a 96-99% similarity to *B. cereus*, *B. thuringiensis* and *B. anthracis* using sequencing; while *gyrB* gene was observed in all (100%) of the isolates. Three virulence genes *nheA, nheB, nheC* encoding for non haemolysin enterotoxin were amplified in all (100%) the isolates. All (100%) of the isolates were susceptible to doxycycline, gentamycin, tetracycline, ciprofloxacin, chloramphenicol and streptomycin. Resistance to rifampicin and penicillin G was predominant with equal rate of 100%, while susceptibility to erythromycin, clindamycin and doxycycline ranged from 60% to 100%. The selected isolates were related and are descendants of the same ancestor. All (100%) the isolates produced spores. The *B. cereus* isolates contain virulence genes, has multiple antibiotic drug resistance and produce spores, which poses a health risk to the public and cannot be used as probiotics

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ABBREVIATIONS

ATCC American Type Culture Collection

CDC Center for Disease Control and Prevention

CFU Colony forming unit

DNA Deoxyribonucleic acid

EDTA ethylenediamine tetraacetic

EFSA European Food Safety Authority

glpF Glycerol uptake facilitator protein

gmk Guanylate kinase, putative

ilvD Dihydoxy-acid dehydratase

LAB Lactic acid bacteria

MLST Multi Locus Sequence Typing

ncbi National Center for Biotechnology Information

pta Phosphate acetyltransferase

pur Phosphoribosylaminoimidazole carboxamide

pycA Pyruvate carboxylase

rRNA ribosomal ribonucleic acid

sp	species
tpi	Triosephosphate isomerase
TAE	Tris-acetate-EDTA
UHT	Ultra high temperature
UK	United Kingdom
US	United State
UV	Ultra violet
ºC	degrees Celsius
β	Beta

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CHAPTER ONE

1.1 INTRODUCTION

1.1.1 MICROORGANISMS IN MILK

Raw milk from cows (i.e. unpasteurized) contains nutrients required to fulfill the nutritional requirements of a growing calf (Sargeant *et al*., 2001). The nutrients in the milk also serve as an attractive medium for bacterial growth; with microflora present in the milk determine milk quality. Generally, milk from a healthy cow is sterile inside the mammary gland. However, various bacteria might occur in the milk immediately after or during milking due to contact with contaminated sources such as air, soil, feaces and grass (Gundogan and Avci, 2014). These bacteria includes: *Lactococcus lactis*, *Micrococcus* spp., *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus uberis*, *Streptococcus thermophiles*, *Corynbacterium bovis*, *Microbacterium lacticum*, *Bacillus spp*., *Clostridium tyrobutyricum*, *Paenibacillus spp*., *Escherichia coli, and Mycobacterium tuberculosis* (Gundogan and Avci, 2014).

Bacilli are ubiquitous aerobic endospore-forming Gram positive rod shaped bacteria. They are often found in vegetables, dust and decaying organic matter (Bottene, 2010). Infections caused by *Bacillus* organisms are; ocular infections, endocarditis, bacteremia and septicemia, pneumonia, meningitis and muscular skeletal infection (Bottene, 2010). Antibiotics usually used to treat infections caused by *Bacillus* species include vancomycin, clindomycin, ciprofloxacin and gentamicin. However, most *Bacillus* strains are resistant to broad spectrum cephalosporins, ticarcillin-clavulanate, penicillin and ampicillin (Fernandes *et al*., 2014).

The *Bacillus spp*. group encompassing; *Bacillus cereus* (an opportunistic human pathogen); *Bacillus thuringiensis* (an entomopathogen); *Bacillus anthracis*, (the etiologic agent of anthrax) *Bacillus mycoides* and *Bacillus pseudomycoides* (both characterized by rhizoidal growth on agar plates) and *Bacillus weihenstephanenuchsis* (Bartoszewicz *et al*., 2008), *Bacillus pumilus*, *Bacillus sphaericus*, *Bacillus amyloliquefaciens*, *Bacillus subtilis* and *Bacillus circulans* are the most important species contaminating raw milk (De Janghe *et al*., 2008). It is very difficult to destroy *Bacillus* spores by conventional heating processes such as pasteurization once they have contaminated the milk (Wijnands *et al*., 2009).

Soiling of the udder and teats is one of the most important factors in the contamination of raw milk by *Bacillus* species (Banyko *et al*., 2009). When animals consume feed contaminated with spore-forming bacteria, large quantities of these bacteria may be present in their faeces which in turn can contaminate the udder and teats (Scheldeman *et al*., 2005). In addition, inadequately cleaned milking equipment, pipelines and farm bulk tanks may also be important sources of contamination. *Bacillus* species are known in the dairy industry for their harmful effects concerning food safety and product quality. These harmful effects include production of toxins, production of spoilage enzymes and possible interference with cheese making (Ahmed *et al*., 1995).

Bacillus cereus produces several types of enterotoxins that cause food poisoning resulting in diarrhea and emesis. The emetic syndrome is caused by a peptide toxin called cereulide while the diarrheal poisoning is caused by heat-labile enterotoxins: hemolysin BL (HBL), non heamolytic

(NHE) and cytotoxin CytK, produced during vegetative growth of *B. cereus* in the small intestine (Ehling-Schulz *et al*., 2006). Food poisoning caused by diarrheal toxin is characterized by abdominal cramps, profuse watery diarrhea and rectal tenesmus. The food poisoning caused by emetic toxin is identified by nausea, vomiting and malaise (Ahmed *et al*., 1995). Hemolysin BL and non heamolytic toxin complexes are organized in operons and the corresponding genes of the enterotoxin complex NHE has been shown to be transcribed together. Immunological assays are commercially available for the detection of NHE and HBL and monoclonal antibodies targeting these enterotoxin complexes have been generated but no such tools are yet available for CytK or cereulide (Ehling-Schulz *et al*., 2006).

1.1.2 *BACILLUS* SPECIES AS PROBIOTICS

Liu *et al* (2010) defines probiotics as viable cell preparations that have beneficial effect on the health of a host by improving its intestinal balance via improved feed value, enzymatic contribution to digestion, inhibition of pathogenic microorganisms, antimutagenic and anticarcinogenic activities, growth promoting factors and an increased immune response. The Nobel Prize winner Eli Metchnikoff was the first person to observe the beneficial properties conferred by some bacteria. In the early 20^{th} century, Metchnikoff discovered that "healthy bacteria" especially lactic acid bacteria (LAB) can have a positive influence on digestion and the immune system (Behnsen *et al*., 2013). Gram positive microorganisms are the most utilized probiotics to date, although the use of Gram negative microbes has also been reported. Strains of the genera *Bifidobacterium* and *Lactobacillus* are the main species used as treatment of intestinal dysfunctions (Patel *et al*., 2009). *Lactobacillus* has been extensively used in the food industry for

many years because they are able to convert sugars (including lactose) and other carbohydrates into lactic acid (Patel *et al*., 2009). Probiotics are often recommended by doctors and nutritionist to re-establish the gut flora when it gets disturbed after a course of antibiotics as part of the treatment for gut related candidiasis. There are claims that probiotics strengthen the immune system to combat allergies, excessive alcohol intake, stress, exposure to toxic substances and other diseases. But a scientific study supporting these claims is yet to be reported (Patel *at al*., 2009).

Less known genus such as certain spore-forming *Bacillus* species are being used as probiotics, these *Bacillus* species include *Bacillus clausii*, *Bacillus subtilis*, *Bacillus pumilus*, *Bacillus coagulans* and *Bacillus cereus* (Patel *et al*., 2009). Table 1 highlights some commercial probiotic products containing *Bacillus* spores. Since majority of food poisoning cases involves *B. cereus*, application of these *Bacillus* species as probiotics raises safety concerns. Therefore the use of these bacteria in preparations for human consumption requires development of strict standards for safety control and progress in legislation concerning this issue has been made in the USA, Canada, and Europe (EFSA, 2005).

Table 1: Commercial probiotic products containing *Bacillus* spores

Product	**Target**	**Organism**	**Manufacturer**
Bactisubtilis	Human	Capsule containing $1x10^9$ spores of *B. cereus* strain IP 5832 (ATCC 14893)	Marion Merrell Dow Laboratories
BaoZyme-aqua	Aquaculture shrimps	*B. subtilis* strains Wu-S and Wu-T at 10^8CFU g^{-1}	Sino-Aqua Corp Kaohsiung, Taiwan
Biosporin	Human	Mixture of two strains of *B. subtilis* 2335 and *B. licheniformis* 2336 (ratio is 3:1)	Biofarm, Dniepropetrovsk, Ukraine
Toyocerin	Calves, poultry, rabbits and swine.	*B. cereus* var *toyoi* (NCIMB-4011/ CNCM-1012) at a minimum concentration of $1x10^{10}$ CFU g^{-1} mixed with maize flour (4% by weight) and calcium carbonate (90% by weight).	Asahi Vet S.A., Tokyo (Head Off.), Japan
Promarine	Aquaculture-shrimps	Carries four strains of *B. subtilis*	Sino-Aqua company Kaohsiung, Taiwan
Subtyl	Human	Capsule carrying 10^6-10^7 spores of *B. cereus* species termed *B. cereus* var *vietnami*	Mekophar, Pharmaceutical Factory No. 24, Ho Chi Minh City, Vietnam
Enterogermina	Human	Mixture of four different strains of *B. clausii*. Vial (5ml) carrying $1x10^6$ spores of *B. clausii*	Sanoti Winthrop SpA, Milan, Italy
Biovicerin	Human	*B. cereus* strain GM suspension of 10^6 spores ml^{-1}	Geyer Medicamentos S. A. Porto Alegre, RS, Brazil
BioGrow	Poultry, calves and swine	Mixture of spores of *B. licheniformis* ($1.6x10^9$CFU g^{-1}) and *B. subtilis*	Provita Eurotech Ltd., Omagh, Northern Ireland, UK

(Patel et al., 2009)

1.1.3 PROPERTIES OF PROBIOTICS

Various characteristics and properties have been identified as crucial for a probiotic to be effective. Probiotic supplements should have an assured shelf life so that they will contain enough numbers of viable organisms at the time of ingestion. An effective probiotic agent must continue to keep sufficient viable microorganisms that can survive the host's digestive process, establish control in the gut, and produce a beneficial response in the host without pathogenic or toxic adverse effects. Colonization is inhibited by the acidic environment of the stomach and by the effects of bile in the duodenum. If enough viable organisms do not survive gut transit, the probiotic will not be able to stimulate a beneficial response in the host. A probiotic should also be able to adapt to healthy intestinal flora and not displace the native bacteria already present (Young *et al*., 2003). Other criteria for selection of probiotic strains include the organism's ability to adhere to the intestinal lining, reproduce, and produce antimicrobial substances. All of these factors enhance colonization resistance, which is a normal process present in the intestinal tract to prevent harmful microbes from establishing residence. Finally, an ideal probiotic will stimulate a mucosal and possibly a systemic immune response, producing a specific health benefit to the host. Bacterial fragments and genetically engineered bacteria are being investigated as possible alternatives to traditional probiotic agents (Patel *et al*., 2009).

1.2 PROBLEM STATEMENT

B. cereus occurs ubiquitously in the soil and in many raw and processed foods such as rice, milk and dairy products, spices, and vegetables. *B. cereus* has been recognized as an agent of food poisoning since 1955 (Horwood *et al*., 2004). Many strains of *B. cereus* are able to produce toxins and cause two types of food poisoning; emetic (which is characterized by nausea and vomiting) and diarrheal (which is manifested primarily by abdominal cramps and diarrhea) (Wijnands *et al*. 2009). Its spores are very hydrophobic and will attach to the surface of the pipelines in dairy plants where they can rapidly sporulate (Rukure, 1999). In addition to food poisoning, *B. cereus* causes a number of systemic and local infections in both immunologically compromised and immunocompetent individuals. Among those most commonly infected are neonates, intravenous drug abusers, patients sustaining traumatic or surgical wounds (Bottone, 2010). Most Eastern Cape inhabitants, especially in Alice, consume raw milk. Data about virulence determinants of *B. cereus* from raw milk is scanty especially in the Eastern Cape, South Africa. Hence more data concerning this pathogen isolated in Alice dairy farms has to be generated so as to help treat infections caused by *B. cereus* especially in the Eastern Cape.

1.3 AIM

The aim of this study is to characterize virulence determinants of *B. cereus* from cow's raw milk.

1.4 SPECIFIC OBJECTIVES

- To isolate and identify *B. cereus* from cow's raw milk.
- To evaluate virulence determinants in the isolates.
- To evaluate antibiogram characteristics in the isolates.
- To determine genetic relatedness of the isolates.
- To evaluate the ability of the isolates to produce spores.

CHAPTER TWO

2. LITERATURE REVIEW

2.1 WHAT IS *Bacillus cereus*?

Bacillus cereus is a large Gram positive rod that is motile by means of peritrichous flagella. The cell is typically 1-2µm in diameter by 3-5µm in length and form single endospores that maybe either centrally or paracentrally located without swelling of the sporangium. The organism sporulates freely on many media under well aerated conditions, although vegetative cells can also grow anaerobically (Gibbson *et al*., 1974). *B. cereus* is able to metabolize glucose, fructose and trehalose but not pentoses and many of the sugar alcohols. Certain strains of *B. cereus* can utilize sucrose, salicin, maltose, mannose, glycerol, m-inosol and lactose. A small percentage of *B. cereus* strains are urease positive; the majority actively hydrolyzes starch, casein, and gelatin (Gordon *et al*., 1977).

Bacillus cereus can survive as spores and vegetative cells. Both spore and vegetative forms of this species are frequently inhabitants of a wide range of environments, including soils and clays (particularly those associated with the rhizosphere), sediments, dust, natural waters, vegetation and many food types, notably cereals and cereal-derivatives, milk and dairy products, dried foods, spices, meat products and vegetables (Goepfert *et al*., 1972).

Growth and multiplication of vegetative cells typically occur within the temperature range of 10-50ºC (Gibbson *et al*., 1974) with the optimum between 28 and 35ºC (Gilbert, 1979). However,

psychrotrophic variants of *B. cereus* capable of growth and initiation of spoilage at temperatures as low as 5ºC have been isolated and identified in raw milk samples (Larkin *et al*., 1966). Johnson *et al*., (1983) determined that the germination of *B. cereus* spores in cooked rice and trypticase soy broth occurred between 5ºC and 50ºC, and was most rapid at 30ºC. Outgrowth and cell division occurred in cooked rice between 15ºC and 50ºC and generation times of 26-57min occur at optimum growth temperature near 30ºC (Johnson *et al*., 1983).

2.2 PATHOGENICITY OF *B. cereus*

Bacillus cereus is the causative agent of two distinct food poisoning syndromes: the diarrheal and emetic illnesses. The emetic food poisoning syndrome is mediated by a small temperature stable, preformed peptide called cereulide (Horwood *et al*., 2004). Cereulide is a cyclic dodecadepsipeptide and its small size and non-antigenicity makes it difficult to produce a reliable toxin detection method (Horwood *et al*., 2004). Cereulide is produced by a non-ribosomal peptide synthetase (NRPS) complex (Horwood *et al*., 2004). The purified cereulide causes swelling of mitochondria of Hep-2 cells (Agata *et al*., 1995). Agata *et al*. (1995) used an animal model to show that cereulide causes vomiting, potentially by binding to the 5-HT3 receptors in the stomach or small intestine to stimulate the vagus nerve and brain. The entire NRPS cluster has been characterized (Ehling-Schulz *et al*., 2006) resulting in a highly specific method for detection of cereulide producing *B. cereus* strains (Fricker *et al*., 2007). In the method, a 5´ nuclease (TaqMan) real-time polymerase chain reaction (pcr) for diagnostic purposes as well as a SYBR green I-based system, representing an interesting economic alternative for high through put screening purposes, was developed. The primers of both PCR

systems and the TaqMan probe anneal within an unusual insertion in the *cesA1* domain which encodes a novel type of α-ketoreductase that performs chiral reduction of α-ketoacyl- S-carrier proteins as opposed to the typical ketoreductases found in polyketide synthetases. This insertion has been shown to be highly specific for emetic *B. cereus* by hybridization studies and database analysis. The inclusivity and exclusivity of the TaqMan and SYBR green I assays were assessed using a test panel of 100 strains, including 23 emetic strains from different origins. Only the emetic *B. cereus* strains showed positive results in the above mentioned assays and all of the other strains tested negative (Ehling-Schulz *et al*., 2006).

Production of the emetic toxin has been shown to occur in skim milk within the temperature range of 12ºC–37°C, with more toxin produced at 12ºC and 15°C compared to higher temperatures (Finlay *et al*., 2000). The emetic toxin is highly resistant to environmental factors, showing stability from pH 2–11 and during heating to 100°C for 150 minutes (pH 8.7–10.6) (Jenson and Moir, 2003).

The diarrheal illness is caused by three types of enterotoxins which are haemolysin BL (HBL), non-haemolysin enterotoxin (NHE) and cytotoxin K. After consumption of food contaminated with spore-forming *B. cereus*, the spores pass the stomach, reach the small intestine where they germinate and grow. Also potentially some vegetative cells survive in the stomach and grow in the small intestine. During growth in the small intestine, enterotoxins are produced (Wijnands *et al*., 2009).

The diarrheal enterotoxins can be produced in the temperature range of 10–43°C, with an optimum of 32°C (Finlay *et al*., 2000). Production occurs between pH 5.5–10, with an optimum of pH 8 (Sutherland and Limond 1993). These enterotoxins are stable at pH 4–11 and inactivated by heating to 56°C for 5 minutes (Jenson and Moir, 2003). Maltodextrin is known to stimulate growth of *B. cereus* and to aid diarrheal enterotoxin production in reconstituted and stored infant milk formulae (Rowan and Anderson, 1997). It has also been shown that *B. cereus* produces more HBL and NHE under conditions of oxygen tension (low oxygen reduction potential) that simulate the anaerobic, highly reducing fermentative conditions encountered in the small intestine (Zigha *et al*., 2006). Up to 26% of *B. cereus* vegetative cells can survive conditions that simulate passage through the stomach. The survival rate of the vegetative cells is dependent on the strain type, phase of vegetative cell growth and the gastric pH (Wijnands *et al*., 2009). In contrast, spores of *B. cereus* are able to pass unaffected through the gastric barrier. The spores contain receptors that need triggering by certain low molecular weight substances to commence germination. These inducers may be present in the food as well as the intestinal epithelial cells. In the small intestine the spores germinate, grow and produce enterotoxins (Wijnands, 2008).

A crucial virulence factor required for causing the diarrheal symptoms is the ability of the vegetative cells and spores of *B. cereus* to adhere to the epithelial cell wall of the small intestine. The adhesion efficiency of spores and cells has been shown to be low, approximately 1% (Wijnands, 2008). The ability of the enterotoxins to act as tissue destructive proteins and damage the plasma membrane of the epithelial cells of the small intestine suggests a role for these enterotoxins in causing diarrhea (Senesi and Ghelardi, 2010). Beecher *et al*. (1995) showed HBL causes fluid accumulation in ligated rabbit ileal loops, implicating a role in diarrhea. However,

direct involvement of NHE and cytotoxin K in causing diarrhea is yet to be demonstrated (Senesi and Ghelardi, 2010). Diarrhoeal enterotoxins are unstable at low pH and are degraded by digestive enzymes, any enterotoxins pre-formed in food would be destroyed during passage through the stomach and so not cause illness if ingested (Jenson and Moir, 2003).

Efficient horizontal DNA transfer systems are present within the *B. cereus* group, enabling plasmids to be transferred among strains of different species of this group (*B. cereus*, *B. anthracis* and *B. thuringiensis*). The plasmids are known to be important determinants of virulence properties of *B. cereus* strains, since they contain genes responsible for virulence such as the *ces* gene cluster required for cereulide formation and emetic disease (Arnesen *et al*., 2008). Chromosomal DNA contains genes associated with the diarrheal disease, and is therefore present in all strains (Arnesen *et al*., 2008).

2.3 EPIDEMIOLOGY OF *B. CEREUS*

B. cereus is ubiquitously distributed in the natural environment which includes decaying organic matter, fresh and marine waters, vegetables and fomites, and the intestinal tract of invertebrates from which soil and food products may become contaminated leading to the transient colonization of the intestine (Bottone, 2010). Strains of *B. cereus* differ in their growth and survival characteristics. Strains isolated from food and humans can be classified as either mesophilic (grows well at 37ºC but do not grow below 10ºC) or psychotropic (grow well at refrigerator temperature but poorly at 37ºC). The reported maximum salt concentration tolerated

by *B. cereus* for growth is about 7.5%. *B. cereus* grows optimal in the presence of oxygen (Mols *et al*., 2009).

B. cereus food poisoning can be caused by either ingesting large numbers of bacterial cells and or spores in contaminated food (diarrheal type) or by ingesting food contaminated with pre-formed toxin (emetic type). Transmission of this disease results from consumption of contaminated foods, improper food handling/storage and improper cooling of cooked foodstuffs (Svensson *et al*., 2005).

Epidemiological evidence suggests that the majority of outbreaks worldwide due to *B. cereus* have been associated with concentrations in excess of 10^5 cfu/g in implicated foods (Louisiana Department of Health & Hospitals, 2013). Since the organism can be recovered from stool samples from healthy persons, the mere presence of *B. cereus* in feces or vomitus of ill persons is not definitive evidence for infection says the (Department of health and hospitals, 2013). Rare cases of both emetic and diarrheal illness have been reported involving 10^3 –10^5 cfu/g of *B. cereus* in food. These cases occurred in infants or aged and infirm individuals (Kramer and Gilbert, 1989; Becker *et al*., 1994). Laboratory studies on the formation of emetic toxin in boiled rice cultures support this finding, with greater than 10^6 cfu/g of *B. cereus* required for toxin production to occur (Finlay *et al*., 2002).

The bacterium has been found in postsurgical and traumatic wounds and can cause opportunistic infections especially in immunocompromised individuals; such as septicemia, meningitis and pneumonia. *B. cereus* has also been known to occasionally cause localized eye infections in humans (Schoeni and Wong, 2005).

B. cereus causes two types of foodborne gastroenteritis: emetic syndrome and diarrheal syndrome. The emetic syndrome is mediated by a highly stable, low molecular weight cyclic peptide toxin called cereulide (Svensson *et al*., 2005). This toxin can withstand high temperatures and exposure to trypsin, pepsin and pH extremes (CDC, 1994). The symptoms of nausea, vomiting and abdominal cramping occur within 1–5 hours of ingestion, with recovery usually within 6–24 hours. The majority of food poisoning cases have been associated with rice and rice dishes but other types of food such as vanilla slices, cooked vegetables, grilled chicken, soy bean curd, fried fish have also been implicated (Svensson *et al*., 2005).

Diarrheal illness characterized by an incubation period of 6-24 hours is mediated by a heat and acid labile enterotoxin that is sensitive to proteolytic enzymes (CDC, 1994). The illness usually lasts for 12–14 hours, although it can continue for several days. Symptoms are usually mild with abdominal cramps, watery diarrhea and nausea. Meat, milk, vegetables and fish have been the predominant food types associated with the diarrheal illness (CDC, 1994).

In most countries such as Australia and New Zealand, *B. cereus* related food poisoning is not notifiable hence incidence data is scanty. This is due to mild, short duration and self-limiting symptoms of food poisoning caused by the organism. Hall *et al*. (2005) estimated that *B. cereus* is accountable for 0.5% of foodborne illness caused by known pathogens in Australia. In 2011, in both Australia and New Zealand there was one foodborne *B. cereus* outbreak reported while in 2010 there was none reported (Lim et al., 2012). *B. cereus* was responsible for 5.4% and 32% of foodborne outbreaks in Nertherlands in 2006 and Norway in 2000 (Wijnand, 2008). This organism was estimated to have caused about 0.7% of foodborne illness caused by 31 major pathogens (Scallen *et al*., 2011). In 1994 Taiwan Department of Heatlth reported, out of 74 outbreaks of foodborne diseases caused by bacteria, 11 were caused by *B. cereus* (Kotiranta *et al*., 2000).

In Hungary, Finland, Bulgaria and Norway the diarrheal type of food poisonings have been reported more often than the emetic type, which was prevalent in Japan and in the United Kingdom between 1950 and 1985 (Kotiranta et al., 2000). Kotiranta *et al*. (2000) further said, "the percentages of foodborne illnesses caused by *B. cereus* differed from country to country, between 1973–1985, *B. cereus* caused 17.8% of the total bacterial food poisonings in Finland, 11.5% in the Netherlands, 0.8% in Scotland, 0.7% in England and Wales, 2.2% in Canada, 0.7% in Japan, and 15.0% (between 1960 and 1968) in Hungary". In the United States *B. cereus* is not a common cause of foodborne disease, the incidence being only 1.3% of the bacterial food poisoning cases reported between 1997 and 1982 (Kotiranta *et al*., 2000). Table 2 outlines some major outbreaks associated with *B. cereus*.

Table 2: Prevous major outbreaks associated with *B. cereus* (>50 cases and/or ≥1 fatality)

Year	No. of cases (fatalities)	Type of food	Syndrome type	Country	Comments about the food
2008	1 (1)	Spaghetti with tomato sauce	Emetic	Belgium	Food stored at room temperature for 5 days after preparation. *B. cereus* and cereulide isolated from pasta
2007	2 (1)	Asparagus sauce	Emetic	Australia	Prior to serving, the sauce was stored for 2 hours in a hot kitchen (up to 37°C), permitting *B. cereus* growth
2003	4 (1)	Pasta salad	Emetic	Belgium	Food stored for 3 days in fridge at 14°C, permitting *B. cereus* growth.
2000	173	Cake	Diarrheal	Italy	*B. cereus* isolated from food and rolling board. Rolling board likely source of contamination
1998	44 (3)	Vegetable puree	Diarrheal	France	Cytotoxin K produced by *B. cereus* involved
1991	139	Barbequed pork	Diarrheal	US	*B. cereus* spores from dried foods, slaughtered animals or worker hands likely source of contamination. Unrefrigerated storage of cooked pork for >18 hours permitted *B. cereus* growth
1989	55	Cornish game hens	Diarrheal	US	Inadequate thawing and cooking, cross- contamination from basting brush used before and after cooking, inadequate refrigeration

(Arnesen *et al*., 2008)

2.4 ANTIBIOTIC SENSITIVITY OF *B. CEREUS*

Generally, most *B. cereus* isolates are resistant to β-lactam antibiotics because they produce the enzyme β-lactamase. There are three different forms of β-lactamases that have been reported among different strains of *B. cereus*. Beta-lactamase I belong to the class A β-lactamases and is an extracellular penicillinase with a serine in the active site. Beta-lactamase II, a class B β-lactamase, is activated by binding Zn(II) and Co(II) ions. Beta-lactamase III of *B. cereus* 569 is a class A membrane bound lipoprotein also having a secreted form. In addition, *B. cereus* has shown resistance to third generation cephalosporins.

In a scenario where there is a suspected *B. cereus* infection case, empirical therapy may be necessary while awaiting the antibiotic susceptibility testing profile. Resistance of *B. cereus* to erythromycin, tetracycline and carbapenem has been reported and may complicate the selection of an empirical treatment choice (Bottone, 2010). Weber *et al*., (1988) conducted a study where 54 *B. cereus* strains isolated from blood cultures tested by microdilution showed susceptibility to imipenem, vancomycin, chloramphenicol, gentamicin, erythromycin, tetracycline and ciprofloxacin. Based on the results they obtained, the authors stated that "the drug of choice for *B. cereus* infections appears to be vancomycin" and that broad-spectrum cephalosporins and ticarcillin-clavulanate should be avoided in the empirical treatment of patients with suspected *B. cereus* infection.

A study conducted by Turnbull *et al*., (2004) concluded that resistance to penicillin, ampicillin, cephalosporins and trimethoprim is constant while susceptibility to clindamycin, erythromycin, chloramphenicol, vancomycin, the aminoglycosides and tetracycline is usually observed. Susceptibility to ciprofloxacin was uniform and it has been shown to be highly effective in the treatment of *B. cereus* wound infections.

2.5 SPORES OF *B. cereus*

Viktoria (2012) reported that all *Bacillus* species can form heat-stable endospores. *B. cereus* spores are an important factor in food-borne illness. The spores have no detectable metabolic activity and can survive in the absence of nutrients for many years. The first event in sporulation is an unequal division of the cytoplasm, resulting in large and small progeny each with the complete genome. After a series of morphological changes the mother cell lyses and releases the spore into the environment (Viktoria, 2012). An endospore is a dormant, tough and non-reproductive structure and the process of spore formation requires about 6 hours (Henriques and Moran, 2007). The primary function of endospores is to ensure the survival of the bacterium through periods of environmental stress. The spores are highly resistant to heat, drying, toxic chemicals, UV radiation, gamma radiation and other adverse environmental factors. *Bacillus* spores are among the life forms most resistant to inactivation, with examples of spores being revived from amber 25-40 million years old or from brine inclusions dated at 250 million years (Henriques and Moran, 2007).

B. cereus sensu *stricto* spores have a more hydrophobic surface than any other *Bacillus* spp. spores. Therefore, they adhere to surfaces such as steel and plastics and are difficult to remove during cleaning (Granum, 2007). A study conducted by Granum in 2007 revealed that the spores can adhere to Caco-2 cells in culture, indicating that they may adhere to the intestinal epithelium. The spores of some *B. cereus* strains are more resistant to heat than those of other mesophilic *Bacillus* spp. such as *B. subtilis* and *B. licheniformis* (Granum, 2007). *B. cereus* spores are capable of surviving most procedures applied in the cooking of food. Carlin et al. (2006) investigated the heat tolerance of the spores of 17 cereulide producing strains and 83 cereulide nonproducing strains of *B. cereus* and reported that the spores of the emetic strains were many fold more heat resistant than those of the non producers. The spores of the strains producing emetic toxin exhibited higher D-values ($P < 0.001$) at 90 °C, as well as higher survival rates after 120 min of heating at 90 °C ($P < 0.001$), than did those of the non emetic strains (Carlin *et al*., 2006). These experimental facts show that emetic *B. cereus* spores in food are very difficult to destroy. Since the spores are metabolically dormant, they must return to active growth, which they do through the process of germination.

Germination consists of a series of degradative events, during which the various permeability barriers responsible for a significant degree of endospore resistance properties are broken down. These events result in rehydration of the core, facilitating entry of molecules from the external environment (Henriques and Moran, 2007). The major germinant of *B. cereus* spores is inosine, glycine and other neutral L-amino acids and purine ribosides also induce germination. L-alanine is the most effective amino acid stimulating germination (Broussolle *et al*., 2008).

2.6 *B. CEREUS* IN NON GASTROINTESTINAL INFECTIONS

2.6.1 ENDOPHTHALMITIS

Endophthalmitis is a vision-threatening eye infection resulting from traumatic or systemic microbial infection of the interior of the eye. The outcome of the infection varies with the microbial agent involved and the rapidity of and response to treatment (Callegan *et al*., 1999). Endophthalmitis caused by *B. cereus* is a devastating malignant eye infection because of the rapidity with which the infection progresses and the bacterium's elaboration of a multitude of extracellular tissue destructive virulence factors. *B. cereus* endophthalmitis can be divided into two categories: exogenous, attributable to globe-penetrating eye trauma, and endogenous, originating through the hematogenous seeding of the posterior segment of the eye from a distant site or through direct intravenous acquisition through blood transfusion, indwelling devices, or contaminated needles or injection paraphernalia or illicit drugs or by iatrogenic administration of medications such as B vitamins and insulin (Callegan *et al*., 1999).

Bouza *et al*. (1979) reported a case of severe sup purative endogenous panophthalmitis caused by *B. cereus* in a 43-year-old man, which resulted from the intravenous administration of B vitamins obtained from three multidose vitamin and mineral containing vials which when cultured grew pure cultures of *B. cereus*. The patient received twice-weekly intravenous injections by his private physician for several weeks. The last injection was administered less than 24 hours prior to the onset of the patient's ocular symptoms, which consisted of a 12 hour history of pain, swelling and severe loss of vision in the right eye (Bouza *et al*., 1979).

A review by Bottone (2010) reported a case where a 45 year old patient with a history of insulin dependent diabetes mellitus presented with redness and worsening pain in his left eye 3 days post cataract surgery. A rapid diagnosis of endophthalmitis was made by Gram staining of vitreous fluid, which showed numerous Gram positive bacilli. Wet preparation of the vitreous fluid showed motile bacilli. Despite the administration of intravitreal and systemic vancomycin and ceftazidime on the day of admission, the infection progressed, requiring the enucleation of the eye on the same day. Cultures of vitreous fluid and blood grew *B. cereus* (Bottene, 2010).

2.6.2 OSTEOMYELITIS

Bone infections by *B. cereus* are somewhat rare, and as of 1994, only nine cases were reported by (Bottene, 2010). Most of the cases were not reported individually but, rather, were included as part of reviews of the spectrum of *B. cereus* infections. *B. cereus* osteomyelitis in these patients was associated with intravenous drug addiction and surgical trauma. Infections were either mono-bacterial or mixed with another co-pathogen such as *Staphylococcus aureus*. In no instance was a case of *B. cereus* osteomyelitis documented in the absence of one or more risk factors, e.g., in addition to alcohol abuse or sickle cell-thalassemia disease (Bottene, 2010).

2.6.3 CENTRAL NERVOUS SYSTEM INFECTIONS

The pathogenesis of *B. cereus* central nervous system infection in most cases is obscure, although several risk factors are worthy of consideration (Guar *et al*., 2001). A substantial number of patients developed necrotizing brain lesions following intrathecal induction

chemotherapy. Presumably, in addition to promoting neutropenia, this procedure could introduce ubiquitous *B. cereus* spores from a multitude of environmental sources and fomites. Other routes of acquisition include bacteremia from a distal site and infected central venous catheters and other catheters used for the periodic administration of remission induction chemotherapy (Bottene, 2010). Central nervous system invasion by *B. cereus* includes meningitis, meningoencephalitis, subarachnoid hemorrhage and brain abscesses occurring in pediatric and adult patients generally in the setting of immunosuppression due to leukemia and other malignancies.

A patient who was diagnosed with T cell acute lymphocytic leukemia through the examination of a bone marrow biopsy specimen complained of bruising and bleeding of the gums and nose and a 2 week history of watery non bloody diarrhea. Seven days later, the patient developed chills, which was followed by a febrile episode 2 days later. Blood cultures were obtained, which grew *B. cereus* bacteria (Bottone, 2010).

2.7. ANALYTICAL METHODS USED FOR SPECIES AND SUBSPECIES CLASSIFICATION OF THE *B. CEREUS* GROUP

2.7.1. PHENOTYPE

Several studies have been performed to try to classify the *B. cereus* group using phenotypic characteristics. The phenotypic characteristics examined have included colony morphology, cell morphology, including spore shape and position. Physiological characteristics: degradation of various materials such as chitin, lecithin, casein and gelatin has also been investigated. Other

tests investigated have included antibiotic susceptibility, acid production from sugars and single carbon sources for energy and growth such as acetate, lactate, citrate or malonate. The ability to grow in different sodium chloride concentrations, at varying temperatures, or anaerobically, have also been studied (Priest *et al.,* 1988).

2.7.2. DNA HYBRIDIZATION

Phenotypic characterization of identifying bacteria is at times inconclusive. Brenner *et al*. (2001) describe DNA: DNA hybridization as a useful tool to determine relatedness among bacteria (Brenner *et al*., 2001). DNA hybridization is a technique that is based upon the ability of a native double stranded DNA to reversibly denature (at high temperature) into its two complementary single strands. Denatured DNA will remain as single strands when it is quickly cooled to room temperature after denaturation. If it is then placed at a temperature between 25 and 30ºC below its denaturation point, the complementary strains will reassociate to again form a double stranded molecule that is extremely similar, if not identical, to native double stranded DNA. Denatured DNA from a given bacterium can be incubated with denatured DNA (or RNA) from other bacteria and will form heteroduplexes with any complementary sequences present in the heterologous strand DNA hybridization (Barker, 2006).

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2.7.3. rRNA METHODS – SEQUENCING AND RIBOTYPING

2.7.3.1. ANALYSIS OF DNA SEQUENCES CODING FOR rRNA GENES

Ribosomal RNA genes are highly conserved and widely known as a powerful molecular chronometer (Ash *et al*., 1991). Sequencing of genes encoding for 16S and 23S ribosomal RNA is a recognized tool for classifying prokaryotes and identifying species (Barker, 2006). A study conducted by Ash *et al*., (1991) reported that 16S rRNA and 23S rRNA sequences of *B. anthracis* (Sterne strain) and *B. cereus* emetic toxin producing strain NCTC 11143 are identical and different from the *B. cereus* NCDO 1771 type strain in only a single nucleotide. However, the type strains of *B. mycoides* and *B. thuringiensis* differed from each other and from *B. anthracis* and *B. cereus* strains by four to nine nucleotides (Ash *et al*., 1991).

The 16S–23S rDNA intergenic transcribed spacers (ITS) are the most variable regions of the ribosomal operon (Barker, 2006). Harrell *et al*., (1995) sequenced this region and differentiated between strains of *B. anthracis* and *B. cereus* although the difference was only 1 nucleotide. The two *B. anthracis* tested could not be differentiated as they had identical sequences. However, there were 12 nucleotide differences and 1 deletion between *B. anthracis* and *B. mycoides* (Harrel *et al*., 1995).

2.7.3.2. RIBOTYPING

Ribotyping is a method of characterizing bacterial strains by digesting chromosomal DNA with restriction enzymes and transferring the digested DNA to a membrane by Southern blotting. The membrane bound DNA is then hybridized to a prokaryotic 16S rRNA gene probe. Ribotype patterns are produced which reflect the distribution of restriction enzyme sites within and between the 16S rRNA operons (Barker, 2006).

The ribotying technique was used by Priest *et al*., (1994) to compare patterns from *B. anthracis, B. cereus, B. thuringiensis* (43 strains from ten serovars) and *B. mycoides*. The ribotype patterns from the four *B. anthracis* strains were found to comprise a distinct group and RFLPs for *B. thuringiensis* strains correlated well with their serotype while the three *B. cereus* strains were dispersed on the resultant phylogenetic tree (Priest *et al*., 1994).

2.7.4. RANDOM AMPLIFIED POLYMORPHIC DNA (RAPDS)

The RAPD technique makes use of a single short primer in a PCR reaction to amplify sections of DNA. After electrophoresis, band patterns are observed and strains producing identical bands are likely to have identical or very similar genomes (Baker, 2006).

Daffonchio *et al*., (1999) made use of this method, where 101 strains from the genus *Bacillus*, including 61 strains from the *B. cereus* group were characterized. Daffonchio *et al*., (1999) amplified a characteristic band of 850 bp from all strains of the *B. cereus* group but not in strains from other *Bacillus* species. Southern hybridisaton with a digoxigenin (DIG) labeled probe made with DNA cut from the 850-bp RAPD band hybridized only with *B. cereus* group strains. The 850-bp fragment was amplified by PCR and products were obtained from *B. cereus* group strains, including *B. anthracis* strains. The DNA was digested with the restriction enzyme *Alu*I. A unique restriction profile was found in the *B. anthracis* strains separating *B. anthracis* from the other *B. cereus* group species (Daffonchio *et al*., 1999).

2.7.5. TYPING METHODS USING RESTRICTION ENZYME DIGESTS

2.7.5.1. PULSED FIELD GEL ELECTROPHORESIS (PFGE)

PFGE is a widely used highly discriminatory molecular technique based on the comparison of the restriction-digested genomic DNA fragment patterns (Otlewska *et al*., 2013). Carlson *et al*. (1996) carried out PFGE to try to assess the genomic variation between *B. cereus* and *B. thuringiensis* strains. Carlson *et al*., (1996) found the chromosome map of 4.3 Mb to be similar to a revised map of the chromosome of the *B. cereus* type strain ATCC 14579, except that the *B. thuringiensis* subsp. *canadensis* HD224 chromosome lacked a *NotI* site and had two additional *AscI* sites (Carlson *et al*., 1996). A PFGE study to analyze a pseudo-outbreak of *B. cereus* in a pediatric unit was performed by Lui *et al*., (1997). Different restriction endonucleases were tested, and *Sma*I was found to give the best result for PFGE. Among the 26 clinical isolates of *B. cereus* and the type strain of the species, 15 distinct PFGE patterns were distinguished. PFGE

after DNA macrorestriction with *Sma*I could clearly differentiate between the epidemiologically related isolates and the unrelated isolates (Liu *et al*., 1997).

2.7.6. TYPING METHODS USING DNA SEQUENCES OF PROTEIN CODING GENES

Molecular typing methods depend on comparing DNA fragments from a single locus or multilocus. This approach is useful for studying epidemic outbreaks in a hospital or other community but to investigate the long-term evolution of bacterial populations, a multilocus method is required. Genes that control essential cell metabolism are very stable and diversify slowly (Yamada *et al*., 1999).

2.7.6.1. SINGLE GENE PHYLOGENETIC TYPING – *gyrB*

The *gyrB* gene encodes the subunit B protein of DNA gyrase, a type II DNA topoisomerase which plays an essential role in DNA replication and is distributed universally among bacterial species. The rate of molecular evolution deduced from *gyrB* gene sequences is faster than that deduced from 16S rRNA gene sequences. *GyrB* gene sequences have been used in phylogenetic studies results from these studies have indicated that *gyrB* is a suitable phylogenetic marker for the study of phylogenetic and taxonomic relationships at the species level. Yamada *et al*., (1999) used the technique of PCR amplification of *gyrB* to test boiled rice for *B. cereus* contamination. Boiled rice was artificially contaminated with *B. cereus*, homogenized and incubated for 15 h in Nutrient Broth. PCR amplification was performed directly on the broth, without DNA extraction indicating the convenience of this approach (Yamanda *et al*., 1999). Sequence analysis of *gyrB*

has also been used to compare 18 strains of *B. cereus,* five *B. thuringiensis* and reference strains of *B. anthracis, B. mycoides, B. pseudomycoides* and *B. weihenstephanensis.* Additionally, seven environmental isolates from spacecraft associated surfaces were included. The phylogenetic tree generated from a 1.2-kb sequence of the *gyrB* gene however produced four distinct groups. Group 1 included *B. anthracis* and 10 *B. cereus* strains while Group 2 was made up of 7 *B. cereus* and 1 *B. thuringiensis* strain. Group 3 was solely *B. thuringiensis* and group 4 was a single *B. mycoides*. DNA:DNA reassociation values from hybridization analyses recovered the same four groups with the exception of the *B. thuringiensis* group. *GyrB* sequence analysis and DNA hybridization both discriminated strains below species level (La Duc *et al.,* 2004).

2.7.6.2. MULTILOCUS SEQUENCE TYPING (MLST)

Multilocus sequence typing (MLST) is an unambiguous procedure for characterizing isolates of bacterial species using the sequences of internal fragments of (usually) seven house-keeping genes. Approximately 450-500 bp internal fragments of each gene are used, as these can be accurately sequenced on both strands using an automated DNA sequencer. For each house-keeping gene, the different sequences present within a bacterial species are assigned as distinct alleles and for each isolate, the alleles at each of the seven loci define the allelic profile or sequence type (ST). Housekeeping genes are generally chosen for MLST as they code for essential proteins and would be present in all strains without insertions or deletions (which would make alignments difficult) (Hoffmaster *et al*., 2008).

The steps required for MLST are as follows: -

(1). Select about seven ‘housekeeping’ genes, (2). sequence the gene fragments on both strands, (3). compare sequences of each fragment with known alleles, (4). give each allele for each locus a number e.g. *glp*-5, *gmk*-4, etc., (5). the seven alleles define the ST, (6). each ST now has a unique code, e.g. ST 32 (5, 4, 3, 4, 15, 6, 16), (7). add the data to the MLST database.

An MLST study consisting of on **77** *Bacillus* strains isolated from various hosts (humans, animals) and locations (food and soil) was conducted. The number of alleles at individual loci ranged from 25-40, and a total of 53 STs were identified. Analysis of the sequence data showed that the population structure of the *B. cereus* group is weakly clonal. In particular, all five *B. anthracis* isolates analyzed had the same ST (Helgason *et al*., 2003).

CHAPTER THREE

3. MATERIALS AND METHODS

3.1 STUDY SITES

A total of 400 raw milk samples were collected in Fort Hare Dairy Trust (FHDT) (200) and Middledrift Dairy Farm (MDF) (200) from lactating cows according to the recommendations of the National Mastitis Council (1987) methods. Briefly, teats were thoroughly washed, dried with clean towel, and sprayed with 70% ethanol. The first few jets of milk were discarded, and 10 ml of milk samples from each quarter was collected in a sterile McCartney bottle. All samples were kept at 4°C and transported immediately to the laboratory for bacteriological examination. Physical examinations were conducted on all lactating cows for evidence and signs of clinical mastitis. Cows were categorized as clinical if they exhibited clinical features of mastitis, or sub-clinical if no apparent signs were present but they had a positive bacterial isolation and a positive California mastitis test. The FHDT is located in Alice, a small town situated 20 kilometers to the east of Fort Beaufort and the MDF is located in Middledrift, a small town situated 90 kilometers North West of East London in the Eastern Cape of South Africa. These commercial dairy farms are situated in Nkonkobe Municipality in Amathole District. The FHDT has 800 cows which produce approximately 3.6 million liters of milk annually while MDF has 600 cows with the production of 1.2 million liters of milk annually. Samples were transported to the University of Fort Hare (UFH) microbiology laboratory in an ice packed cold box. Upon arrival to the Laboratory, samples were analysed immediately.

3.2 ISOLATION AND CULTURING

Approximately 10 µl of the sample was spread plated on Polymyxin pyruvate Egg-Yolk Mannitol Bromothymol Agar (PEMBA) and incubated at 37ºC for 48 hours. Shapely blue colonies with white margin were streaked on the same media to get pure isolates.

3.3 GRAM STAINING

A smear was prepared on a clean glass slide and then heat fixed by carefully passing the slide through a Bunsen burner. The slide was flooded with crystal violet for one minute and gently rinsed in slowly running tap water and drained against a paper towel. Gram‘s iodine was poured on the smear for one minute. When one minute had elapsed, the slide was washed with 95% alcohol for 30 seconds and then rinsed with a gentle stream of water and drained carefully. Then smear was counterstained with safranin for 30 seconds and washed, drained and blot dried. Then slide was examined under oil at X1000 magnification (Bergey *et al*., 1994).

3.4 CATALASE TEST

The catalase test was performed by flooding the growth of the bacteria in question from nutrient agar plate with 1.0 ml of 3% hydrogen peroxide and observation for effervescent (formation of small bubbles) which indicates a positive test was noted. (Bergey *et al*., 1994).

Table 3: Primers used in the study

Target gene	Primer sequence 5´-3´	Reference
16S rDNA	PA Forward - AGAGTTTGATCCTGGCT PE Reverse - CCTTCAATTCCTTTGAGTTT	Ahaotu et al., 2013
gyrB	BC Forward - GTTTCTGGTGGTTTACATGG BC Reverse - TTTTGAGCGATTTAAATGC	Manzano et al., 2003
hblA[1]	HBLA Forward - GTGCAGATGTTGATGCCGAT HBLA Reverse - ATGCCACTGCGTGGACATAT	Hansen et al., 2001
hblC[1]	L_2 Forward - AATCAAGAGCTGTCACGAAT L_2 Reverse - CACCAATTGACCATGCTAAT	Hansen et al., 2001
hblD[1]	L_1 Forward - AATGGTCATCGGAACTCTAT L_1 Reverse - CTCGCTGTTCTGCTGTTAAT	Hansen et al., 2001
nheA[2]	nheA Forward - TACGCTAAGGAGGGGCA nheA Reverse - GTTTTTATTGCTTCATCGGCT	Hansen et al., 2001
nheB[2]	nheB Forward - CTATCAGCACTTATGGCAG nheB Reverse - ACTCCTAGCGGTGTTCC	Hansen et al., 2001
nheC[2]	nheC Forward - CGGTAGTGATTGCTGGG nheC Reverse - CAGCATTCGTACTTGCCAA	Hansen et al., 2001
gmk[3]	GMK Forward - ATTTAAGTGAGGAAGGGTAGG GMK Reverse - GCAATGTTCACCAACCACAA	pubmlst.org/bcereus/info/primers.shtml
pta[3]	PTA Forward - GCAGAGCGTTTAGCAAAAGA PTA Reverse - TGCAATGCGAGTTGCTTCTA	pubmlst.org/bcereus/info/primers.shtml
pur[3]	PUR Forward - CTGCTGCGAAAAATCACAAA PUR Reverse - CTCACGATTCGCTGCAATAA	pubmlst.org/bcereus/info/primers.shtml
ilvD[3]	ILV Forward - CGGGGCAAACATTAAGAGAA ILV Reverse - GGTTCTGGTCGTTTCCATTC	pubmlst.org/bcereus/info/primers.shtml
tpi[3]	TPI Forward - GCCCAGTAGCACTTAGCGAC TPI Reverse - CCGAAACCGTCAAGAATGAT	pubmlst.org/bcereus/info/primers.shtml
pycA[3]	PYC Forward - GCGTTAGGTGGAAACGAAAG PYC Reverse - CGCGTCCAAGTTTATGGAAT	pubmlst.org/bcereus/info/primers.shtml
glpF[3]	GLP Forward - GCGTTTGTGCTGGTGTAAGT GLP Reverse - CTGCAATCGGAAGGAAGAAG	pubmlst.org/bcereus/info/primers.shtml

[1]haemolysin virulence gene; [2]non haemolytic virulence gene; [3]housekeeping genes

3.5 EXTRACTION OF DNA

DNA was extracted using the boiling method by Torres *et al*., (2003) with modification. A colony was picked and suspended in 200µl of DNA extraction solution from seegene and boiled on Dri-Block DB.2A (Techne, Johannesburg, South Africa) for 15 minutes at 100ºC then centrifuged at 13500 rpm for 10 minutes (Thermo Fisher Scientific, Schwerte, Germany). The supernatant was transferred on a sterile eppendorf tube and the pellet was discarded.

3.6 POLYMERASE CHAIN REACTION

3.6 1 SCREENING FOR THE 16S rDNA GENE

A template of 5 µl DNA was amplified in 25 µl reaction mixture consisting of 12 µl of PCR master mix (containing 0.05 U/µL of *Taq* DNA polymerase, reaction buffer, 4 mM $MgCl_2$, and 0.4 mM of each dNTP), 1 µl of forward and reverse primers (Table 3) each and nuclease free water 6 µl. The PCR conditions consisted of 35 cycles of denaturation at 94ºC for 1 minute, annealing at 55ºC for 1 minute, extension at 72ºc for 1 minute. An initial denaturation was performed at 95ºC for 5 min and after completion of 35 cycles final extension was carried out at 72ºC for 7 minutes. The PCR products were analyzed in 1.8% agarose gel prepared in 0.5 X TAE buffer containing ethidium bromide as follows: about 5 µl of the PCR products was mixed with 2 µl of 6X gel loading dye and loaded onto gel submerged in 0.5X TAE buffer. A 100 bp DNA ladder was used to determine the approximate molecular weight of PCR products. A constant voltage of 100 V/cm was applied for 50 minutes. The amplicons were viewed using the

UV trans-illuminator (Uvitec, Cambridge, United Kingdom). The gel was photographed by an UV documentation system (Uvitec, Cambridge, United Kingdom).

3.6.2 DETECTION OF THE *gyrB* GENE FROM THE ISOLATES

B. cereus ATCC 10876 was used as a reference strain. Amplification of the *gyrB* gene was carried out in 25 µl of reaction mixture containing 5 µl of DNA template, 12 µl of PCR Master Mix (containing 0.05 U/µL of *Taq* DNA polymerase, reaction buffer, 4 mM $MgCl_2$, and 0.4 mM of each dNTP), 1 µl of forward primer, 1µl of reverse primers (Table 3) and 6 µl of nuclease free water. Amplification consisted of 35 PCR cycles in a thermocycler. The cycling programme was: initial denaturation at 95ºC for 5 minutes followed by 35 cycles of denaturation at 95ºC for 1 minute, annealing at 57ºC for 1 minute, extension at 72ºC for 1 minute. The PCR was ended with a final extension at 72ºC for 7 minutes and the amplified products were cooled at 4ºC. The PCR products were analyzed in 1.8% agarose gel prepared in 0.5 X TAE buffer containing ethidium bromide. The analaysis was as follows: 5 µl of PCR products were mixed with 2 µl of 6X gel loading dye and loaded onto gel submerged in 0.5X TAE buffer. Electrophoresis was carried out in a submarine gel electrophoresis system at 100 V for 1 hour. The gel was photographed by an UV transilluminator (Uvitec, Cambridge, United Kingdom).

3.6.3 SCREENING FOR THE *hbl* AND *nhe* ENTEROTOXIN GENES

The PCR reaction (25µl) included 5 µl of DNA template, 12 µl of PCR Master Mix (containing 0.05 U/µL of *Taq* DNA polymerase, reaction buffer, 4 mM $MgCl_2$, and 0.4 mM of each dNTP),

1 µl of forward primer, 1 µl of reverse primers (Table 3) and 6 µl of nuclease free water. The PCR conditions were: initial denaturation at 95ºC for 5 minutes followed by 30 cycles of denaturation at 94ºC for 1 minute, annealing at (depending on the primer pair used) for 45 seconds, extension at 72ºC for 2 minutes. A final extension step was carried out at 72ºC for 7 minutes. The annealing temperatures of the primer pairs were as follows: HBLA forward/HBLA reverse 55ºC, L_1 forward/L_1 reverse 51ºC, L_2 forward/L_2 reverse 51ºC, nheA forward/nheA reverse 51ºC, nheB forward/nheB reverse 53ºC, nheC forward/nheC reverse 53ºC. DNA from *B. cereus* ATCC 10876 was used as a positive control. The PCR products were analyzed by agarose gel electrophoresis as previously described

3.6.4 SCREENING FOR THE HOUSEKEEPING GENES

Amplification of the housekeeping genes was carried out in a 25 µl of reaction mixture as previously described. The PCR cycling conditions consisted of an initial denaturation at 95ºC for 5 min followed by 34 cycles of denaturation at 94ºC for 1 minute, annealing at (depending on the primer set used) for 1 minute, extension at 72ºC for 1 min. The PCR was ended with a final extension at 72ºC for 7 minutes. The annealing temperatures of the primer pairs were as follows: GMK forward/GMK reverse 56ºC, PTA forward/PTA reverse 56ºC , PUR forward/PUR reverse 56ºC, ILV forward/ILV reverse 58ºC, TPI forward/TPI reverse 58ºC, PYC forward/PYC reverse 57ºC, GLP forward/GLP reverse 59ºC. The PCR products were analyzed by agarose gel electrophoresis as previously described.

3.7 SEQUENCING

The amplicons of the 16S rDNA gene and some selected housekeeping genes were sent to the Central Analytical Laboratory at Stellenbosch University in Cape Town for sequencing. The online ncbi software was used to blast the 16S rDNA sequences.

3.8 DATA ANALYSIS

The alignment of 16S rDNA was done using MEGA 6 software (Tamura *et al*., 2013). The house keeping genes sequences were cleaned and queried to the MLST database http;//www.pubmlst.org/bcereus. These were then assigned allele numbers based on those already described in the *B. cereus* MLST database. Isolates were then assigned ST on the basis of the combination of the seven alleles. A phylogenetic tree was constructed based on the multiple alignments of the concatenated sequences of the MLST genes using (Tamura *et al*., 2013).

3.9 ANTIBIOGRAM

Cultures of the isolates were grown on nutrient agar plates overnight at 37°C. Approximately five colonies were emulsified in 2 ml of sterile saline and the turbidity was adjusted to a 0.5 McFarland standard. Sterile swabs dipped into this suspension and squeezed against the side of the suspension tube to remove excess fluid were streaked across predried Mueller Hinton Agar plates, three times for each plate, with the plate rotated approximately 90° between each streaking. After approximately 10 to 15 minutes, to allow absorption of excess moisture into the agar, antibiotics were placed on the agar plate using a dispenser. The plates were incubated at 37ºC for 18 hours. (Hudzicki, 2013). The diameter of the inhibition zone was determined according to the CLSI guidelines for aerobically grown bacteria. Twelve antibiotics used were chosen: tetracycline (30µg), rifampicin (5µg), vancomycin (30µg), doxycycline (30µg), ampicillin (10µg), erythromycin (15µg), ciprofloxacin (5µg), clindamycin (12µg), chloramphenicol (30µg), gentamycin (10µg), streptpmycin (10µg) and penicillin (10µg) (Oxoid, ThermoScientific, United Kingdom).

3.10 PRODUCTION OF *B. cereus* SPORES

An overnight colony of the isolate was inoculated in Luria Bertani broth and incubated at 30ºC shaking 150 rpm for overnight. Precultures of the isolates were diluted (1:200) in 25ml of fresh minimal sporulation medium in 100ml flasks and incubated at 24°C shaking (150 rpm) for 2–3 days. Sporulation was monitored by phase contrast microscopy. Once more than 80% of cells were free spores, spores were harvested and resuspended in precooled phosphate washing buffer

by vigorous vortexing. After 6 washing steps (7min., 4000 rpm at 4°C), the spore suspension was stored at 4°C. For the determination of the spore concentration, suspensions were heated to 80°C for 15 minutes to kill residual vegetative cells. Serial dilutions of heat-treated and untreated samples were plated on Luria Bertani agar plates. After incubation at 30°C over night, CFU were counted (Viktoria, 2012).

3.11 STATISTICAL ANALYSIS

Mean and standard deviation of antibiotic profiles of isolates from FHDT and MDF was calculated using statistical analysis programme SPSS 22.

CHAPTER FOUR

4. RESULTS

All twenty five isolates were characterized as Gram positive, spore-forming (appendix A), catalase (appendix B) and oxidase positive (appendix C) rod shaped bacteria.

When the isolates were screened for the 16S rDNA gene, 100% of the isolates amplified the gene and gave the expected fragment size of 940 bp (Fig. 1).

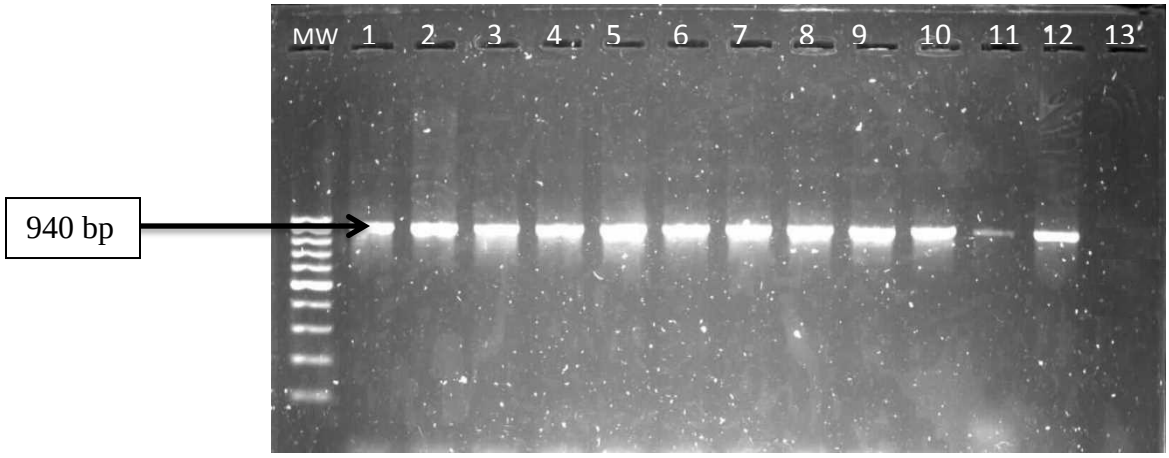

Fig 1: PCR analysis of the 16S rDNA gene. Lanes: MW-DNA ladder (100bp); 1-2104$_{M}$; 2-8100$_{F}$; 3-2104$_{M}$; 4-0025$_{M\ Y.C}$; 5-10040$_{F}$; 6-0055$_{M}$; 7-2598$_{M}$; 8-8100$_{F\ Y.C}$; 9-1063$_{F}$; 10-1002$_{F}$; 11-9010$_{F\ Y.C}$;12-2179$_{M}$; 13-Negative control

The 16S rDNA gene sequences showed 96-99% identity for *B. cereus, B. thuringiensis* and *B. anthracis* for all isolates. Their alignments are in appendix D and F.

Screening for the *gyrB* gene of *B. cereus*

The *gyrB* gene was amplified by PCR from isolates and reference strain *B. cereus* ATCC 10876, which resulted in the expected fragment size of 374 bp (Fig. 2).

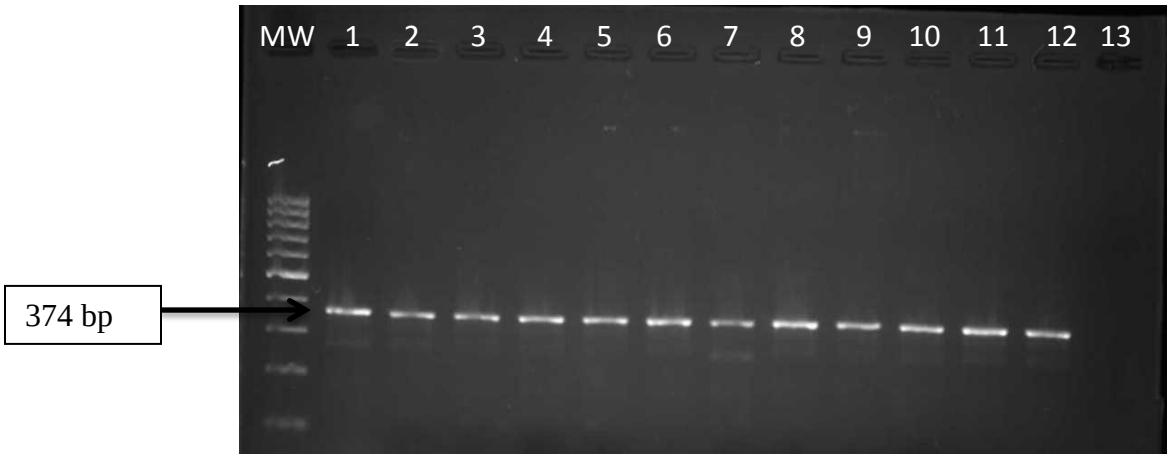

Fig 2: PCR analysis of the *gyrB* gene of *B. cereus* of the isolates. Lanes: MW-DNA ladder (100 bp); 1-*B. cereus* ATCC10876; 2-8100_{F}; 3-$0083_{M\ YC}$; 4-7001_{M}; 5-$1237_{F\ YC}$; 6-10122_{F}; 7-$9010_{F\ YC}$; 8-1063_{F}; 9-2374_{M}; 10-2104_{M}; 11-9042_{M}; 12-10040_{F}; 13-Negative control.

Screening for the *hbl* and *nhe* enterotoxin genes

None of the isolates amplified the genes, *hblA*, *hblC* and *hblD*. However the reference strain was positive for all the genes.

When the isolates were screened for the *nheA* gene, 100% of the isolates with the reference strain amplified the gene, which resulted in the expected fragment size of 499 bp (Fig. 3).

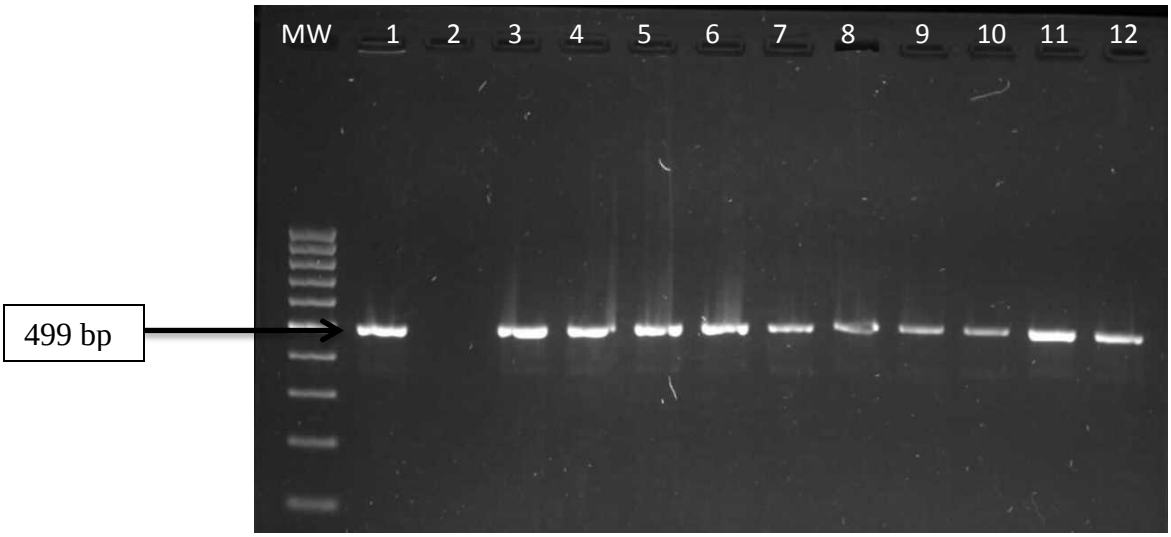

Fig 3: PCR analysis of the *nheA* gene. Lanes: MW-DNA ladder (100 bp); 1- *B. cereus* ATCC10876; 2- Negative control; 3-$0083_{M\ YC}$; 4-7001_{M}; 5-$1237_{F\ YC}$; 6-10122_{F}; 7-$9010_{F\ YC}$; 8-1063_{F}; 9-2374_{M}; 10-2104_{M}; 11-9042_{M}; 12-10040_{F}

All the isolates with the reference strain were positive for the *nheB* gene which resulted in the expected fragment size of 769 bp (Fig. 4).

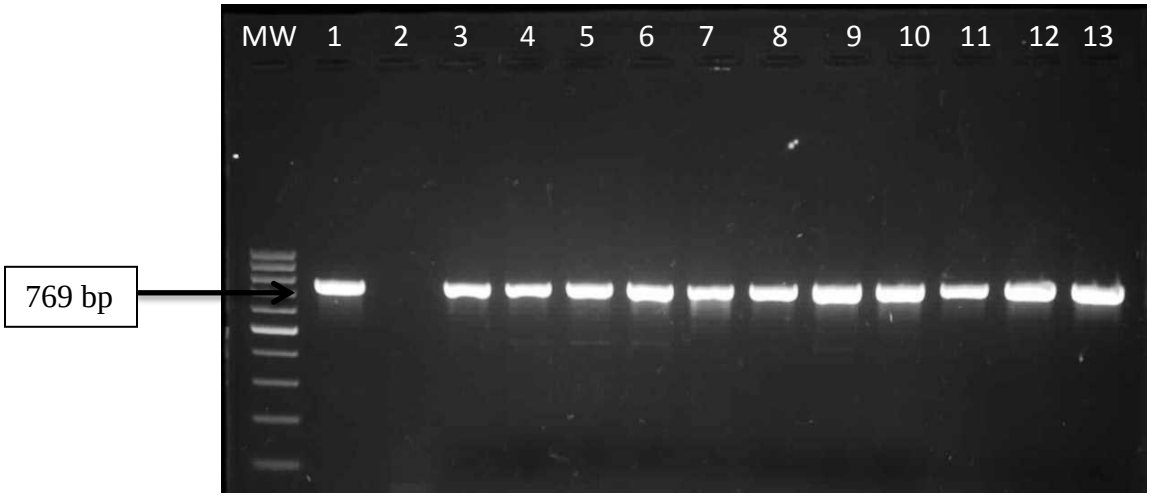

Fig 4: Gel electrophoresis picture of the PCR analysis of the *nheB* gene. Lanes: MW-DNA ladder (100 bp); 1-*B. cereus* ATCC10876; 2- Negative control; 3-$0083_{M\ YC}$; 4-7001_{M}; 5-$1237_{F\ YC}$; 6-10122_{F}; 7-$9010_{F\ YC}$; 8-1063_{F}; 9-2374_{M}; 10-2104_{M}; 11-9042_{M}; 12-10040_{F}; 13- 8100_{F}

Amplification of the *nheC* gene was observed in 100% of the isolates including the reference strain which resulted in the fragment size of 581 bp (Fig. 5).

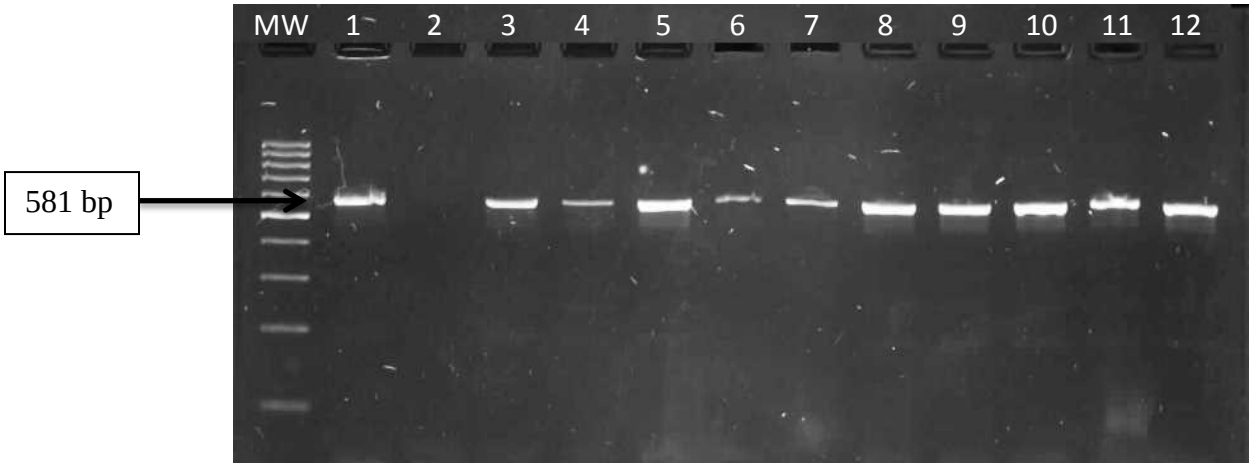

Fig 5: PCR analysis of the *nheC* gene. Lanes: MW-DNA ladder (100 bp); 1-*B. cereus* ATCC10876; 2- Negative control; 3-$0083_{M\ YC}$; 4-7001_{M}; 5-$1237_{F\ YC}$; 6-10122_{F}; 7-$9010_{F\ YC}$; 8-1063_{F}; 9-2374_{M}; 10-2104_{M}; 11-9042_{M}; 12-10040_{F}

Antibiotic susceptibility profiles

Hundred percent of the isolates were susceptible to doxycycline, gentamycin, tetracycline, ciprofloxacin, chloramphenicol and streptomycin. Multidrug-resistance pattern was observed in 100% of the isolates to at least two or more antibiotics. Hundred percent of the isolates showed resistance to rifampicin and penicillin G while 12%, 56%, and 64% were resistant to clindamycin, vancomycin and ampicillin resepctively. Susceptibility to erythromycin, clindamycin and doxycycline was observed for 60%, 64% and 100% of the isolates respectively. Table 4 shows various degrees of susceptibility patterns of the isolates against the antibiotics tested.

Table 4: Antimicrobial susceptibility profiles of the isolates

Antimicrobial agent	**Concentration (µg)**		**Number of isolates (%)**	
		Resistant	Intermediate	Susceptible
Clindamycin	2	3 (12)	6 (24)	16 (64)
Doxycycline	30	0	0	25 (100)
Erythromycin	15	0	10 (40)	15 (60)
Gentamycin	10	0	0	25 (100)
Tetracycline	30	0	0	25 (100)
Vancomycin	30	14 (56)	0	11 (44)
Ciprofloxacin	5	0	0	25 (100)
Rifampicin	5	25 (100)	0	0
Ampicillin	10	16 (64)	0	9 (36)
Chloramphenicol	30	0	0	25 (100)
Streptomycin	10	0	0	25 (100)
Penicillin G	10	25 (100)	0	0

The percentage antibiotic resistance was compared between the two farms and represented in bar graphs below (Fig. 6 and 7).

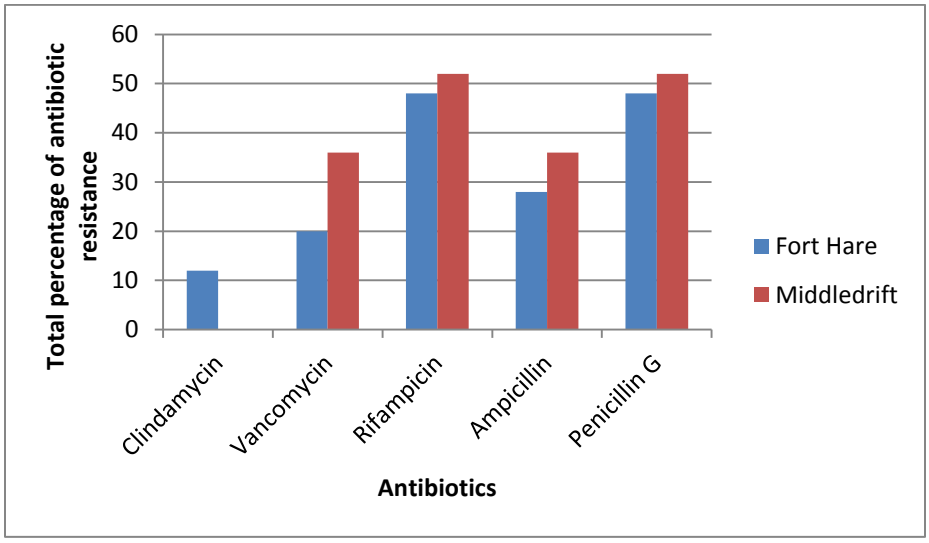

Figure 6: Bar graph of total percentage of antibiotic resistance between the two farms.

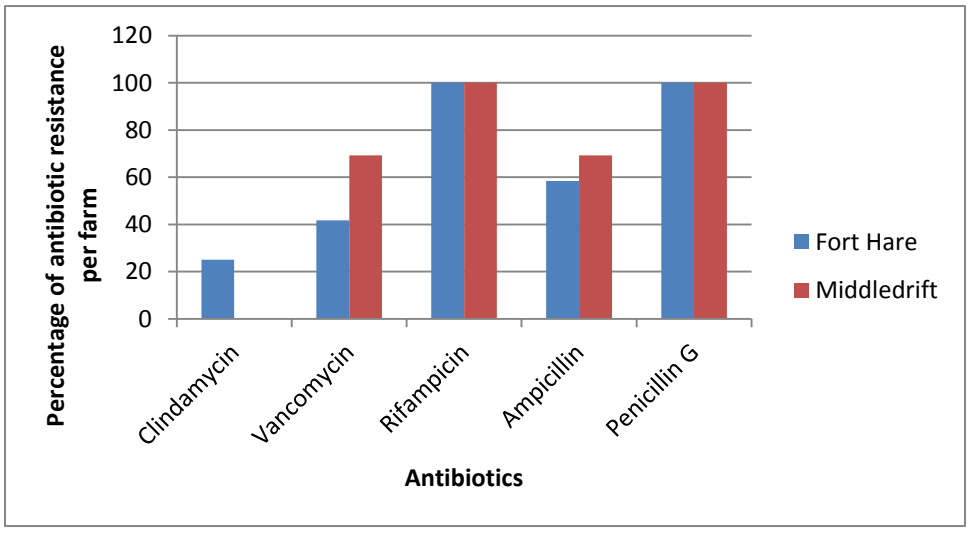

Figure 7: Bar graph of antibiotic resistance percentage per farm.

Mean and standard deviation of antibiotic profiles of isolates from both dairy farms were calculated using SPSS 22 and shown in Table 5. The standard deviation ranged from 0.000 to 1.02986 and mean ranged from 1.000 to 3.0000.

Table 5: Standard deviation and mean of antibiotic profiles of isolates from two farms.

Site		**N**	**Minimum**	**Maximum**	**Mean**	**Std. Deviation**
Fort Hare	Tetracycline	12	1.00	1.00	1.0000	.00000
	Rifampicin	12	3.00	3.00	3.0000	.00000
	Vancomycin	12	1.00	3.00	1.8333	1.02986
	Doxycycline	12	1.00	1.00	1.0000	.00000
	Ampicillin	12	1.00	3.00	2.1667	1.02986
	Erythromycin	12	1.00	2.00	1.4167	.51493
	Ciprofloxacin	12	1.00	1.00	1.0000	.00000
	Clindamycin	12	1.00	3.00	1.6667	.88763
	Chloramphenicol	12	1.00	2.00	1.1667	.38925
	Gentamicin	12	1.00	1.00	1.0000	.00000
	Streptomycin	12	1.00	1.00	1.0000	.00000
	Penicillin_G	12	3.00	3.00	3.0000	.00000
Middledrift	Tetracycline	13	1.00	1.00	1.0000	.00000
	Rifampicin	13	3.00	3.00	3.0000	.00000
	Vancomycin	13	1.00	3.00	2.3846	.96077
	Doxycycline	13	1.00	1.00	1.0000	.00000
	Ampicillin	13	1.00	3.00	2.3846	.96077
	Erythromycin	13	1.00	2.00	1.3077	.48038
	Ciprofloxacin	13	1.00	1.00	1.0000	.00000
	Clindamycin	13	1.00	2.00	1.3077	.48038
	Gentamicin	13	1.00	1.00	1.0000	.00000
	Streptomycin	13	1.00	1.00	1.0000	.00000
	Penicillin_G	13	3.00	3.00	3.0000	.00000

Determining genetic relatedness of the isolates using MLST

When the isolates were screened for the housekeeping genes, some isolates did amplify all the genes whereas isolates 4, 5, 6 and 12 did not amplify the *ilvD* gene. Table 6 shows the isolates that did and did not amplify the housekeeping genes with their STs.

Table 6: STs with the housekeeping genes of the isolates

Isolate	**ST**	***glp***	***gmk***	***ilvD***	***pta***	***pur***	***pyc***	***tpi***
2	32	P	P	P	P	P	P	P
4	165	P	P	A	P	P	P	P
5	165	P	P	A	P	P	P	P
6	165	P	P	A	P	P	P	P
7	32	P	P	P	P	P	P	P
12	165	P	P	A	P	P	P	P

ST- Sequence type; isolates 2- 2265_{M}, 4-2079_{M}, 5-7017_{F}, 6-2529_{M}, 7-$8100_{F\ YC}$, 12-2179_{M}; P- presence of the gene in the isolate; A- absence of the gene in the isolate.

The alleles ranged from *gmk* 44 to *pyc* 3 for ST165 and ST32 was frequent. Table 7 shows the isolates with their allele numbers and STs.

Table 7: Allelic profiles and STs of isolates obtained from (pubmlst.org/cgi-bin/submissions/submit.pl?file=ba-isolates.xml&page=alleles) after sequence submission.

Isolate	**ST**	***glp***	***gmk***	***ilvD***	***pta***	***pur***	***pyc***	***tpi***
2	32	5	4	3	4	15	6	16
4	165	3	44	-	5	16	3	4
5	165	3	44	-	5	16	3	4
6	165	3	44	-	5	16	3	4
7	32	5	4	3	4	15	6	16
12	165	3	44	-	5	16	3	4

ST- Sequence type; isolates 2- 2265_{M}, 4-2079_{M}, 5-7017_{F}, 6-2529_{M}, 7-$8100_{F\ YC}$, 12-2179_{M}

A dendogram of the genetic relatedness of selected isolates using concatenated sequences from seven housekeeping alleles were constructed (Fig. 8). The dendogram has two clusters: cluster I which has the genes *tpi*, *gmk*, *pyc* and *ilvD*; cluster II which is made up of the genes *pta*, *pur* and *glp*.

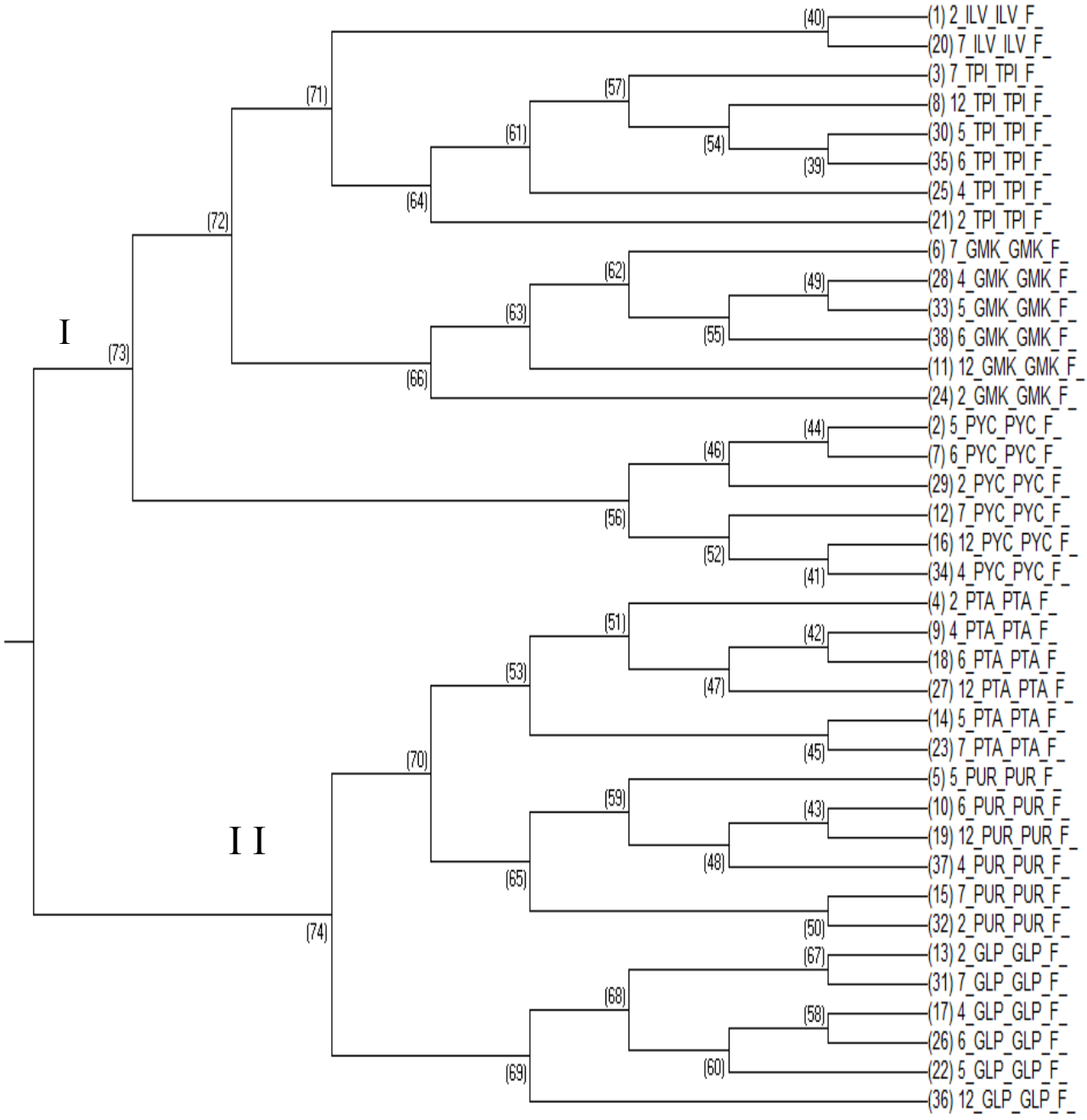

Fig 8: Relationships of selected isolates of this study using concatenated sequences from seven housekeeping alleles.

Production and isolation of spores from isolates

The spores were produced and their formation was monitored by phase contrast microscopy (appendix J). When the spores were inoculated to a nutrient rich media, they geminated and vegetative cells grew. Concentration of geminated spores for the control was higher than that of the heat treated samples (appendix K). The colony forming unit of the isolate that was not heat treated was 1 610 000 CFU/ml and that of the heat treated isolate was 10 000 CFU/ml.

CHAPTER FIVE

5. 1 DISCUSSION

Bacillus cereus is a Gram positive spore forming rod that is ubiquitously distributed in the natural environment which includes decaying organic matter, fresh and marine waters, vegetables and formites (Bottene 2010). The occurrence of *B. cereus* in food is a cause for concern for human health. This pathogen has been found to be responsible for spoilage of milk and other food products and is a major food borne pathogen which causes two different types of food poisoning (Das *et al*., 2009). It has also been isolated from mastitic cows (Oliver *et al*., 2005).

In the present study we found 100% of our isolates were Gram, catalase, oxidase positive, spore forming, rod shaped bacteria. These results were in agreement with the results of the study by Kotiranta *et al*., (2000); Ouoba *et al*., (2007); Catia *et* a*l*., (2008). The isolates were identified as *B. cereus/B. anthracis/B. thuringiensis* (96-99% similarity to GenBank sequences) based on 16S rDNA sequencing. The same observation was made by Ahaotu *et al*., (2013). Less than 1% variation was observed when the 16S rDNA nucleotides sequence of *B. cereus*, *B anthracis* and *B thuringiencies*. However, the recommended conservative criterion for dermacation of species is a cut-off of 3% divergence (Helgason *et al*., 2004). Therefore, the analysis of the 16S rDNA sequence of this gene might be used to characterize a strain as belonging to *B. cereus* group without being able to identify the species. The use of *gyr*B gene to differentiate the group was proposed by Jensen *et al*. (2005). However, detection and differentiation of *B. cereus* from *B.*

cereus group was enabled by specific primers targeting the gyrase, sphingomyelinase and *groEL* gene (Manzano *et al*., 2003; Chang *et al*., 2003).

The genes *hblA, hblC* and *hblD* encode the three component haemolysin BL enterotoxin (Fricker *et al*., 2007). However, the isolates in our study did not habour any *hbl* genes. It is not surprising since these genes are found only in 50% of *B. cereus* (Thorsen *et al*., 2011). Similar results were reported by Ahaotu *et al*. (2013) where they observed only one *B. cereus* A1 isolate positive for the presence of all three *hbl* genes. However Thorsen *et al*. (2011) reported that 55% of the *B. cereus* isolates haboured all the three genes encoding haemolysin BL enterotoxin. We found 100% of our isolates including the reference strain *B. cereus* ATCC 10876 habouring all the three genes (*nheA, nheB,* and *nheC*). These results were in agreement with Ahaotu *et al*. (2013) were he reported 100% of the tested *B. cereus* isolates possessed all the three genes. The percentage of isolates habouring all three genes in our study is greater than that observed by Hansen and Hendriksen (2001) 50% and Thorsen *et al*. (2011) 48% using the same primers. Occurrence of non-haemolytic enterotoxin genes seems to be more frequent than haemolysin BL enterotoxin genes in *B. cereus* strains (Ahaotu *et al*., 2013), a situation similar to our own.

In addition to virulence genes, the resistance of microorganisms to antibiotics further bolsters their virulence characteristics. Resistance to antibiotics by microorganisms poses a serious public health problem. The isolation of a large number of multi-antibiotic resistant *B. cereus* strains from food is worrying because antibiotics are a common therapeutic measure adopted to combat acute necrotising gastritis caused by *B. cereus*, mainly in immunocompromised patients (Fernandes *et al*., 2014).

A prevalent attribute of antibiotic resistant bacteria is an increased abundance of β-lactamases. This can be the result of genetic engineering, or it can be caused by the selection of resistant variants in the presence of antibiotics (Fenselau *et al*., 2008). In the present study the isolates exhibited various degrees of susceptibility patterns against the antibiotics tested. Isolates were resistant against β-lactam antibiotics including penicillin (100%), ampicillin (64%), and rifampicin (100%). These results were consistent with the study of Fernandes *et al*. (2014) where they reported 100% of *B. cereus* isolates were resistant to penicillin and ampicillin. Beta lactamase genes are found in the wild type genomes of many bacteria, including *Bacillus* species. These chromosomal β-lactamases do not generally provide effective antibiotic resistance in wild-type bacilli, however under antibiotic selection pressure, a number of strains show increased resistance suggesting mutation-induced up-regulation of β-lactamase expression (Fenselau *et al*., 2008).

In this study, it was observed that all isolates were susceptible to gentamicin, tetracycline, chloramphenicol, ciprofloxacin and doxycycline whereas only 56% were susceptible to erythromycin. Thereby suggesting that these aforementioned antibiotics can be used to treat infections caused by *Bacillus spp*. The results found in our study were similar to those reported by Gundogan and Avci (2014) where they detected 100% of *B. cereus* strains were susceptible to gentamicin, tetracycline, chloramphenicol, ciprofloxacin and erythromycin. Also Fernandese *et al*. (2014) reported 100% of the tested *B. cereus* isolates were susceptibility to gentamicin; tetracycline and 90% were susceptible to erythromycin.

When antibiotic resistance was compared between isolates from FHDT and MDF, isolates from MDF had the highest antibiotic resistance percentages. However they were not resistant to clindamycin. Fifty two percent of isolates from MDF were resistant to penicillin G and rifampicin whereas 36% of isolates from FHDT were resistant to these antibiotics. Sixty two percent of MDF isolates were resistant to ampicillin and vancomycin as compared to the 28% and 20% isolates from FHDT for these antibiotics respectively. Comparison of antibiotic resistance percentage for each farm was: hundred percent of isolates from FHDT were resistant to rifampicin and penicillin G and the same percentage was observed for MDF isolates. Sixty nine percent of isolates from MDF were resistant to vancomycin and ampicillin whereas 58%, 42% and 25% of isolates from FHDT were resistant to ampicillin, vancomycin and clindamycin respectively.

Isolates from FHDT had the same antibiotic susceptibility profiles to tetracycline, doxycycline, ciprofloxacin, gentamicin, penicillin, rifampicin and streptomycin and their standard deviation was zero, meaning there was not variation in their profiles. For vancomycin, ampicillin, erythromycin and clindamycin their standard deviation ranged from 0.51493 to 1.02986, meaning there was variation in their pattern (some isolates were susceptible, intermediate and resistant). MDF isolates had the same antibiotic profiles because their standard deviation was zero. All isolates were susceptible to these antibiotics: tetracycline, doxycycline, ciprofloxacin, gentamicin, streptomycin, and chloramphenicol; all isolates were resistant to these antibiotics: penicillin and rifampicin. Standard deviation for vancomycin, ampicillin, erythromycin and clindamycin ranged from 0.48038 to 0.96077, meaning the isolates had variation in those antibiotic profiles.

Multilocus sequence typing is based on sequencing a number of essential or housekeeping genes spread around the bacterial chromosome and is routinely used in many laboratories (Helgason *et al*., 2004). The isolates were clustered according to each MLST genes and were descendants of a common ancestor. Isolates 5 and 7 from FHDT samples were closely related to each other only in cluster II for the *pta* gene. Isolates 7 and 2 were closely related in gene clusters *pyc*, *ilvD*, *glp* and *pur* and have the same ST32 yet are from different dairy farms. This could be as a result of movement of cows from these two dairy farms. Although isolates 4, 5, 6 and 12 had ST165 not all of them were closely related to each other. Isolates 4 and 6 were closely related in gene clusters *pta* and *glp*; 5 and 6 in *tpi* and *pyc*. Isolates with ST165 have been found in Japan (Vassileva *et al*., 2007) and Brazil (Didelot *et al*., 2008). Sequence type 165 is a variant of ST26 with an allelic profile *glp* 3, *gmk* 2, *ilvD* 31, *pta* 5, *pur* 16, *pyc* 3, *tpi* 4 and ST164 with an allelic profile *glp* 3, *gmk* 2, *ilvD* 63, *pta* 5, *pur* 36, *pyc* 3, *tpi* 4 (Vassileva *et al*., 2007). Vassileva *et al*. (2007) reported that strains of *B. cereus* with ST26, ST164 and ST165 produce emetic toxin, cereulide which cause emetic food poisoning. Isolates with ST165 in our study could produce the cereulide toxin, although this toxin was not investigated in this study. Isolates 2 and 7 had the same ST32. *B. cereus* with ST32 was reported by Hansen *et al*. (2011), where it was isolated in United States of America from cerebrospinal fluid of a patient suffering from meningitis (Guar *et al*., 2001). Also, *B. cereus* ATCC 10987 from Canada had ST32 (Hansen *et al*., 2011).

The isolates were able to produce spores. The concentration of germinated spores for the control were lower than that of the heat treated samples when they were spread plated in a nutrient rich medium. Similar observations were made by Brasil-SP team wiki (2014).

5.2 CONCLUSION

B. cereus was isolated from cow's raw milk from both FHDT and MDF. All *B. cereus* isolates haboured the three non heamolytic enterotoxin genes and were able to produce spores that can germinate and produce toxins in the small intestine. This poses a serious public health risk as people surrounding the two dairy farms buy this raw milk and consumes it. The multi antibiotic drug resistance observed for most isolates makes it a challenge to treat infections caused by *B. cereus*. The *B. cereus* isolates from both dairy farms were related and some closely related.

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APPENDICE

Appendix A

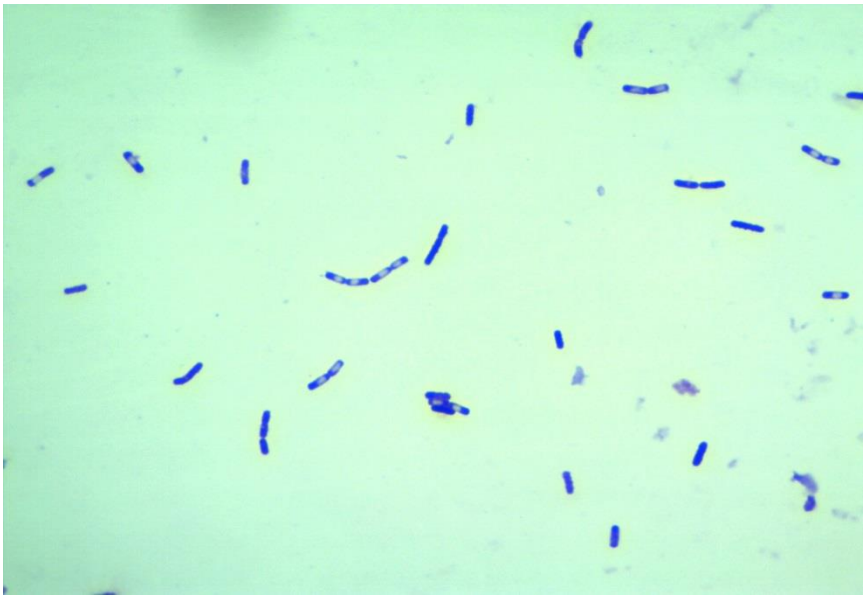


Fig 9: Image of an isolate that is Gram positive rods with spores centrally located

Appendix B

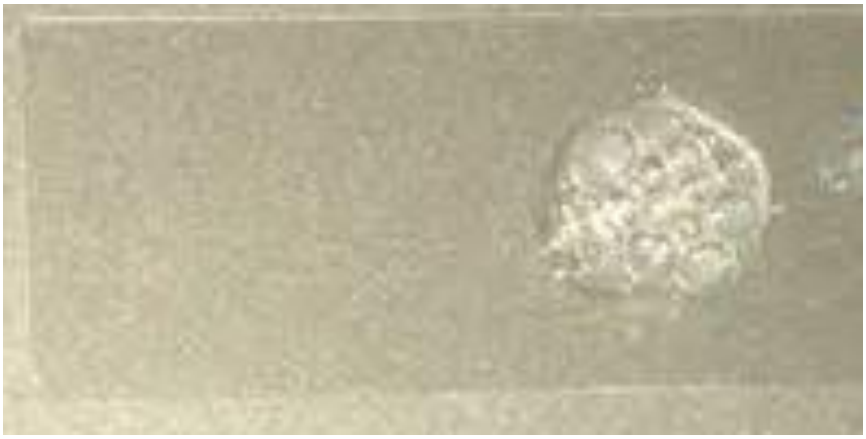


Fig 10: Image of a catalase positive isolate

Appendix C

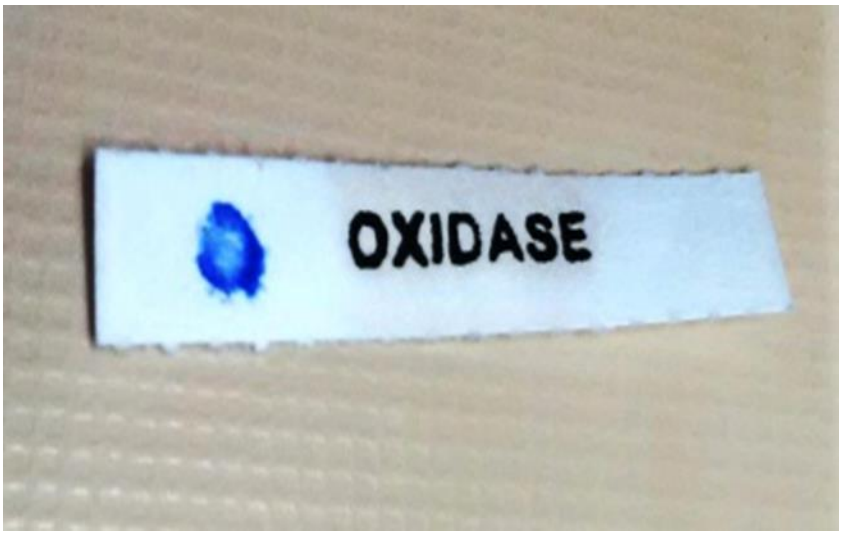
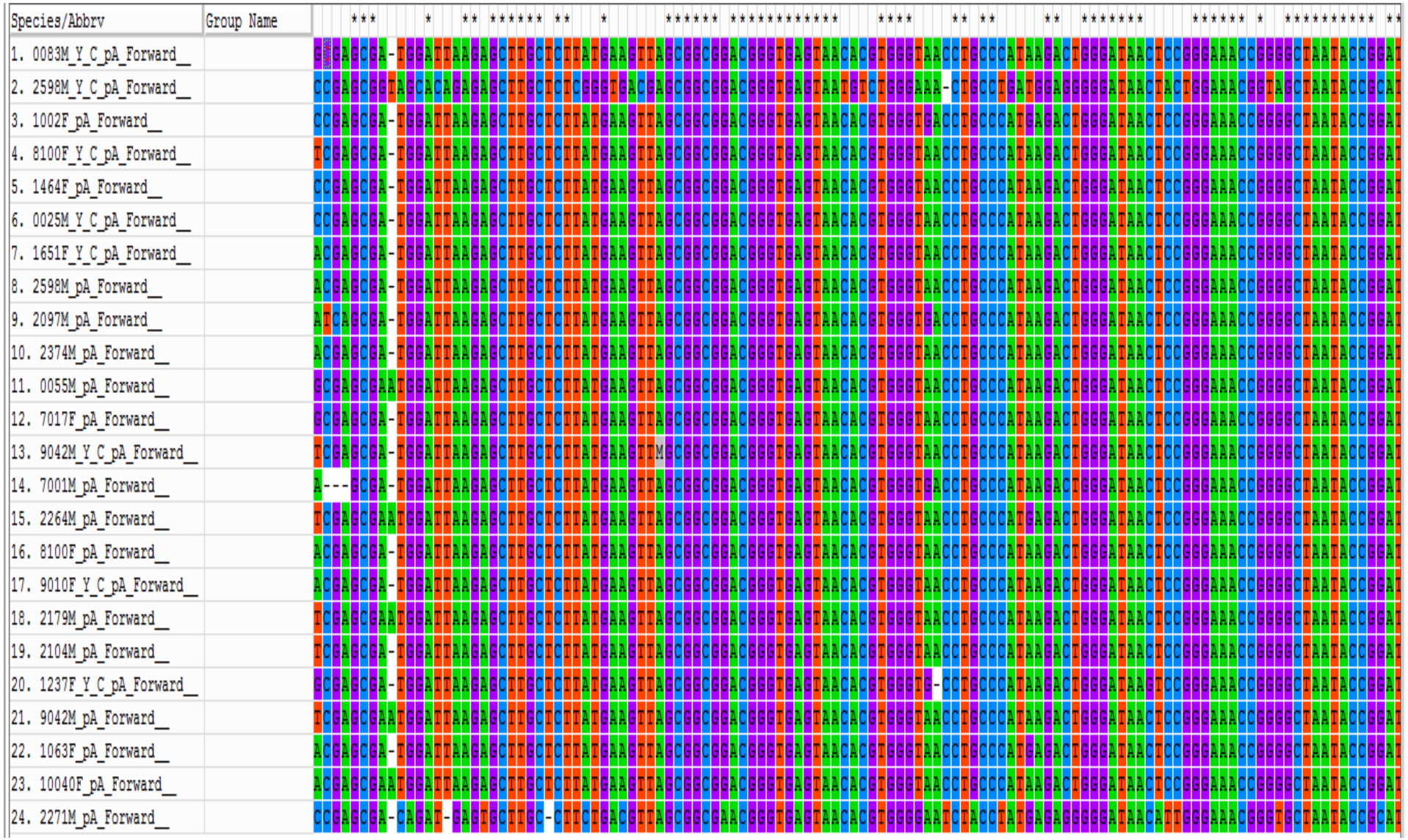


Fig 11: image of an oxidase positive isolate

Appendix D


Species/Abbrv
Group Name
1. 0083M_Y_C_pA_Forward_
2. 2598M_Y_C_pA_Forward_
3. 1002F_pA_Forward_
4. 8100F_Y_C_pA_Forward_
5. 1464F_pA_Forward_
6. 0025M_Y_C_pA_Forward_
7. 1651F_Y_C_pA_Forward_
8. 2598M_pA_Forward_
9. 2097M_pA_Forward_
10. 2374M_pA_Forward_
11. 0055M_pA_Forward_
12. 7017F_pA_Forward_
13. 9042M_Y_C_pA_Forward_
14. 7001M_pA_Forward_
15. 2264M_pA_Forward_
16. 8100F_pA_Forward_
17. 9010F_Y_C_pA_Forward_
18. 2179M_pA_Forward_
19. 2104M_pA_Forward_
20. 1237F_Y_C_pA_Forward_
21. 9042M_pA_Forward_
22. 1063F_pA_Forward_
23. 10040F_pA_Forward_
24. 2271M_pA_Forward_


Species/Abbrv
Group Name
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2. 2598M_Y_C_pA_Forward_
3. 1002F_pA_Forward_
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5. 1464F_pA_Forward_
6. 0025M_Y_C_pA_Forward_
7. 1651F_Y_C_pA_Forward_
8. 2598M_pA_Forward_
9. 2097M_pA_Forward_
10. 2374M_pA_Forward_
11. 0055M_pA_Forward_
12. 7017F_pA_Forward_
13. 9042M_Y_C_pA_Forward_
14. 7001M_pA_Forward_
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16. 8100F_pA_Forward_
17. 9010F_Y_C_pA_Forward_
18. 2179M_pA_Forward_
19. 2104M_pA_Forward_
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10. 2374M_pA_Forward_
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13. 9042M_Y_C_pA_Forward_
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19. 2104M_pA_Forward_
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Species/Abbrv
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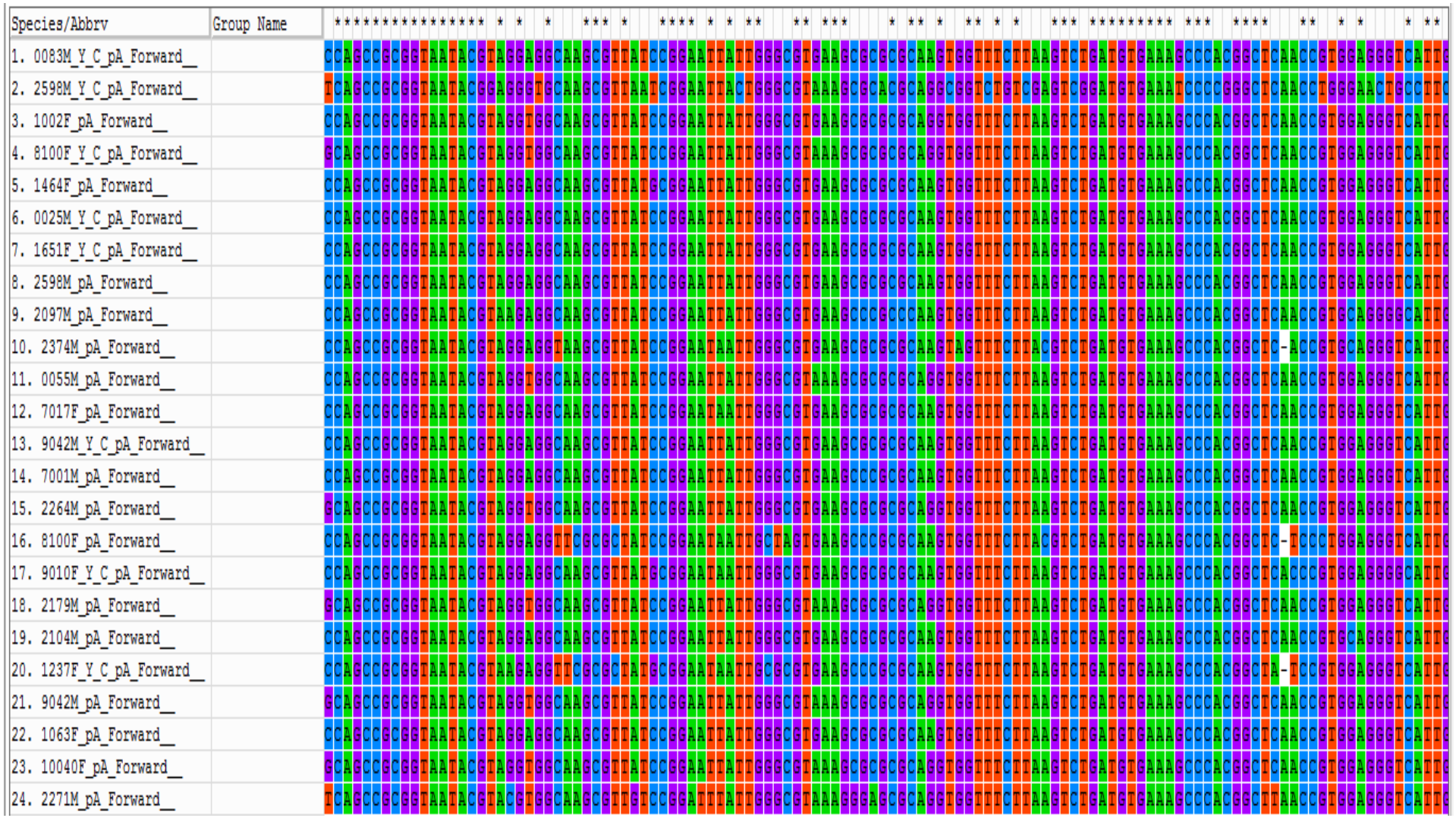
5. 1464F_pA_Forward_
6. 0025M_Y_C_pA_Forward_
7. 1651F_Y_C_pA_Forward_
8. 2598M_pA_Forward_
9. 2097M_pA_Forward_
10. 2374M_pA_Forward_
11. 0055M_pA_Forward_
12. 7017F_pA_Forward_
13. 9042M_Y_C_pA_Forward_
14. 7001M_pA_Forward_
15. 2264M_pA_Forward_
16. 8100F_pA_Forward_
17. 9010F_Y_C_pA_Forward_
18. 2179M_pA_Forward_
19. 2104M_pA_Forward_
20. 1237F_Y_C_pA_Forward_
21. 9042M_pA_Forward_
22. 1063F_pA_Forward_
23. 10040F_pA_Forward_
24. 2271M_pA_Forward_


Species/Abbrv
Group Name
1. 0083M_Y_C_pA_Forward__
2. 2598M_Y_C_pA_Forward__
3. 1002F_pA_Forward__
4. 8100F_Y_C_pA_Forward__
5. 1464F_pA_Forward__
6. 0025M_Y_C_pA_Forward__
7. 1651F_Y_C_pA_Forward__
8. 2598M_pA_Forward__
9. 2097M_pA_Forward__
10. 2374M_pA_Forward__
11. 0055M_pA_Forward__
12. 7017F_pA_Forward__
13. 9042M_Y_C_pA_Forward__
14. 7001M_pA_Forward__
15. 2264M_pA_Forward__
16. 8100F_pA_Forward__
17. 9010F_Y_C_pA_Forward__
18. 2179M_pA_Forward__
19. 2104M_pA_Forward__
20. 1237F_Y_C_pA_Forward__
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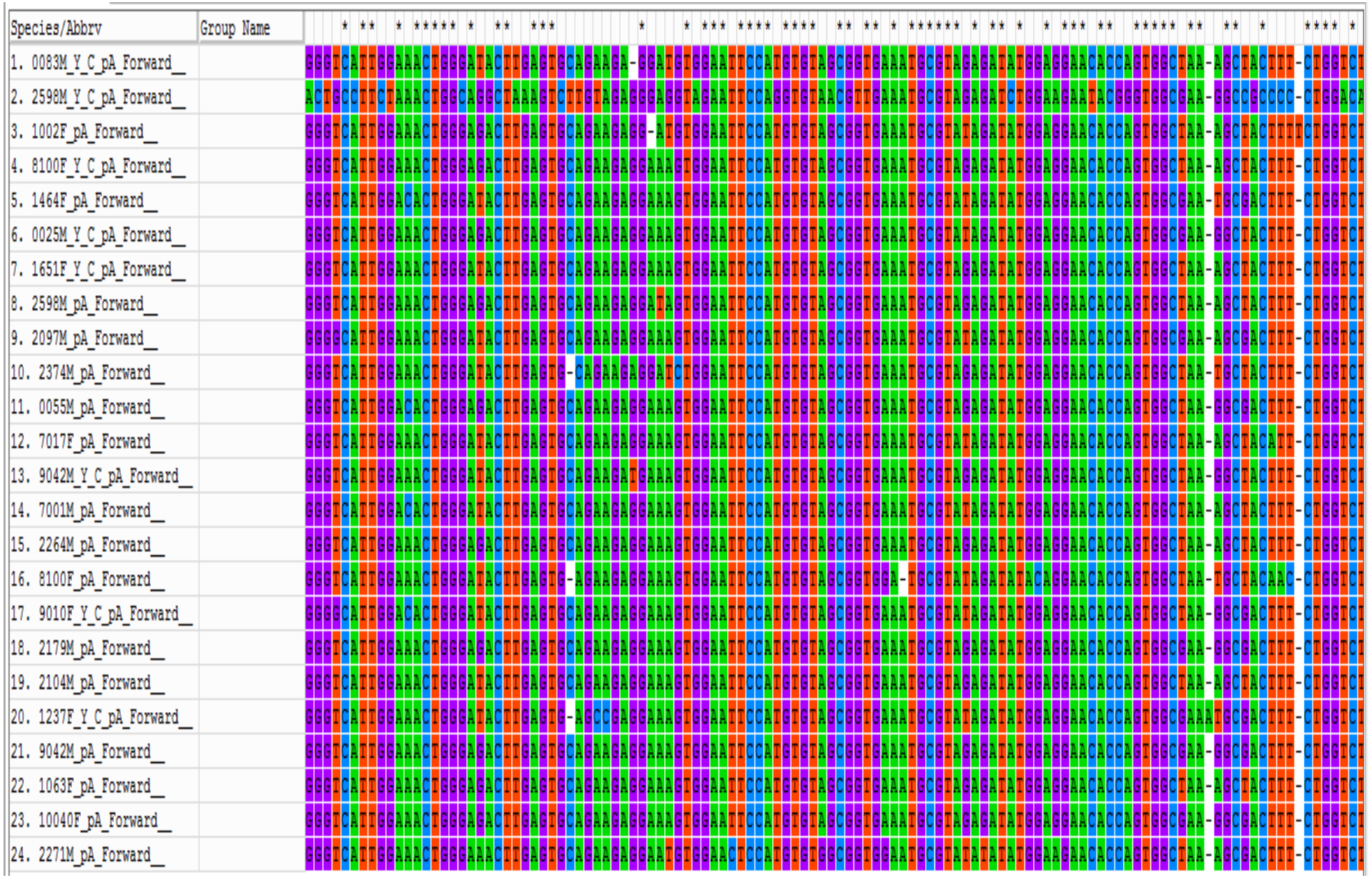
22. 1063F_pA_Forward__
23. 10040F_pA_Forward__
24. 2271M_pA_Forward__


Species/Abbrv
Group Name
1. 0083M_Y_C_pA_Forward_
2. 2598M_Y_C_pA_Forward_
3. 1002F_pA_Forward_
4. 8100F_Y_C_pA_Forward_
5. 1464F_pA_Forward_
6. 0025M_Y_C_pA_Forward_
7. 1651F_Y_C_pA_Forward_
8. 2598M_pA_Forward_
9. 2097M_pA_Forward_
10. 2374M_pA_Forward_
11. 0055M_pA_Forward_
12. 7017F_pA_Forward_
13. 9042M_Y_C_pA_Forward_
14. 7001M_pA_Forward_
15. 2264M_pA_Forward_
16. 8100F_pA_Forward_
17. 9010F_Y_C_pA_Forward_
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19. 2104M_pA_Forward_
20. 1237F_Y_C_pA_Forward_
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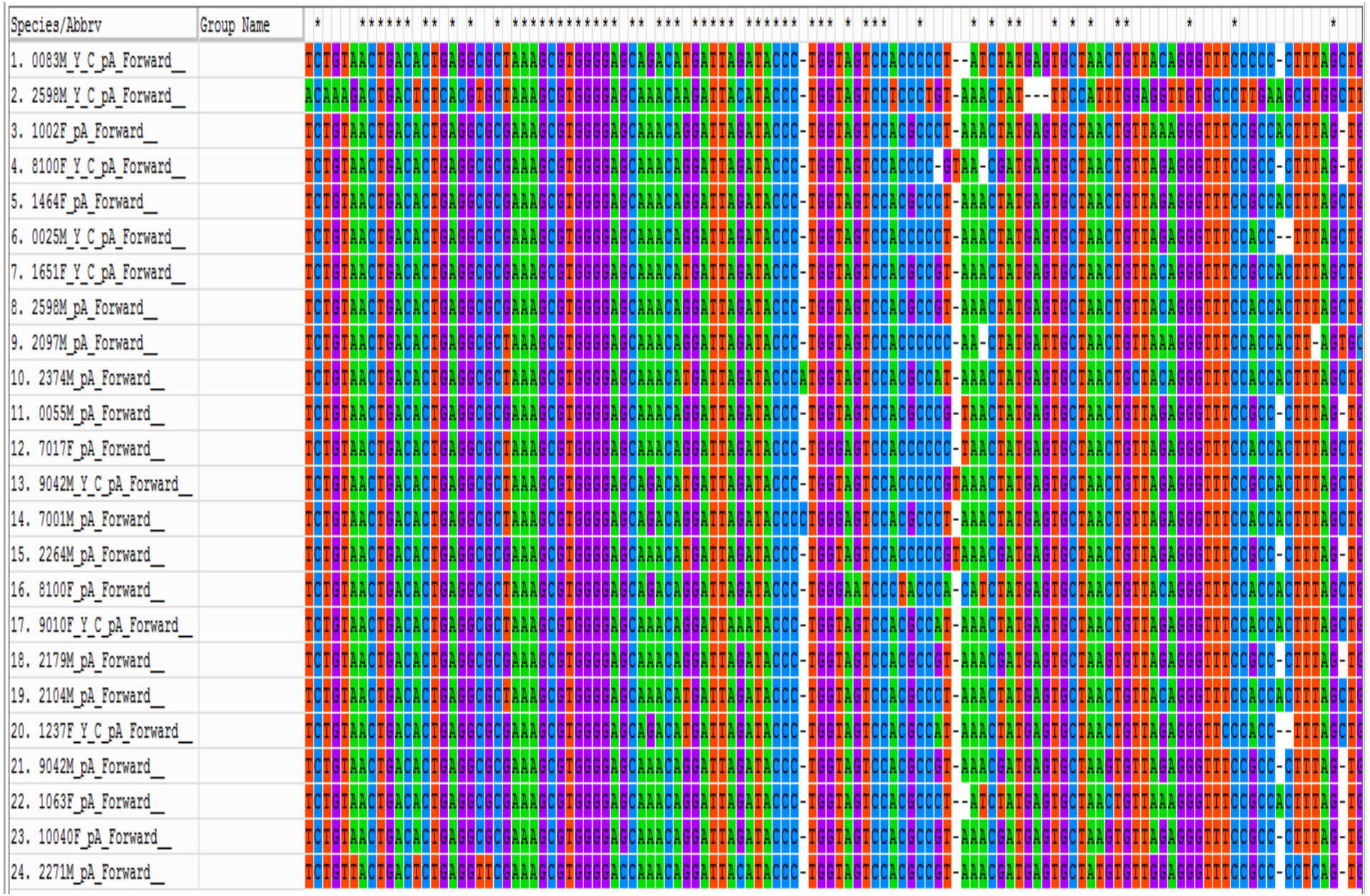
22. 1063F_pA_Forward_
23. 10040F_pA_Forward_
24. 2271M_pA_Forward_


Species/Abbrv
Group Name
1. 0083M_Y_C_pA_Forward_
2. 2598M_Y_C_pA_Forward_
3. 1002F_pA_Forward_
4. 8100F_Y_C_pA_Forward_
5. 1464F_pA_Forward_
6. 0025M_Y_C_pA_Forward_
7. 1651F_Y_C_pA_Forward_
8. 2598M_pA_Forward_
9. 2097M_pA_Forward_
10. 2374M_pA_Forward_
11. 0055M_pA_Forward_
12. 7017F_pA_Forward_
13. 9042M_Y_C_pA_Forward_
14. 7001M_pA_Forward_
15. 2264M_pA_Forward_
16. 8100F_pA_Forward_
17. 9010F_Y_C_pA_Forward_
18. 2179M_pA_Forward_
19. 2104M_pA_Forward_
20. 1237F_Y_C_pA_Forward_
21. 9042M_pA_Forward_
22. 1063F_pA_Forward_
23. 10040F_pA_Forward_
24. 2271M_pA_Forward_

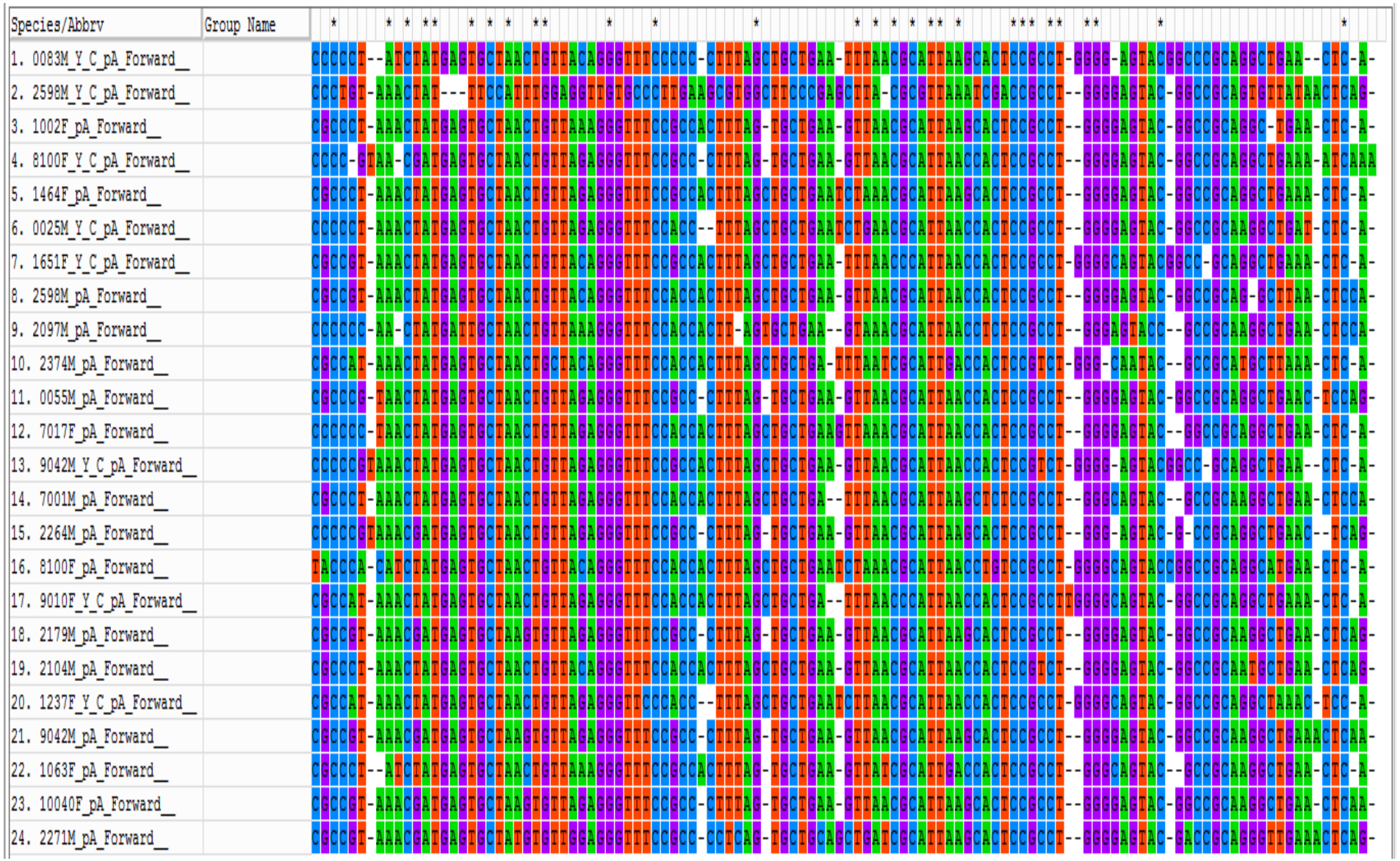
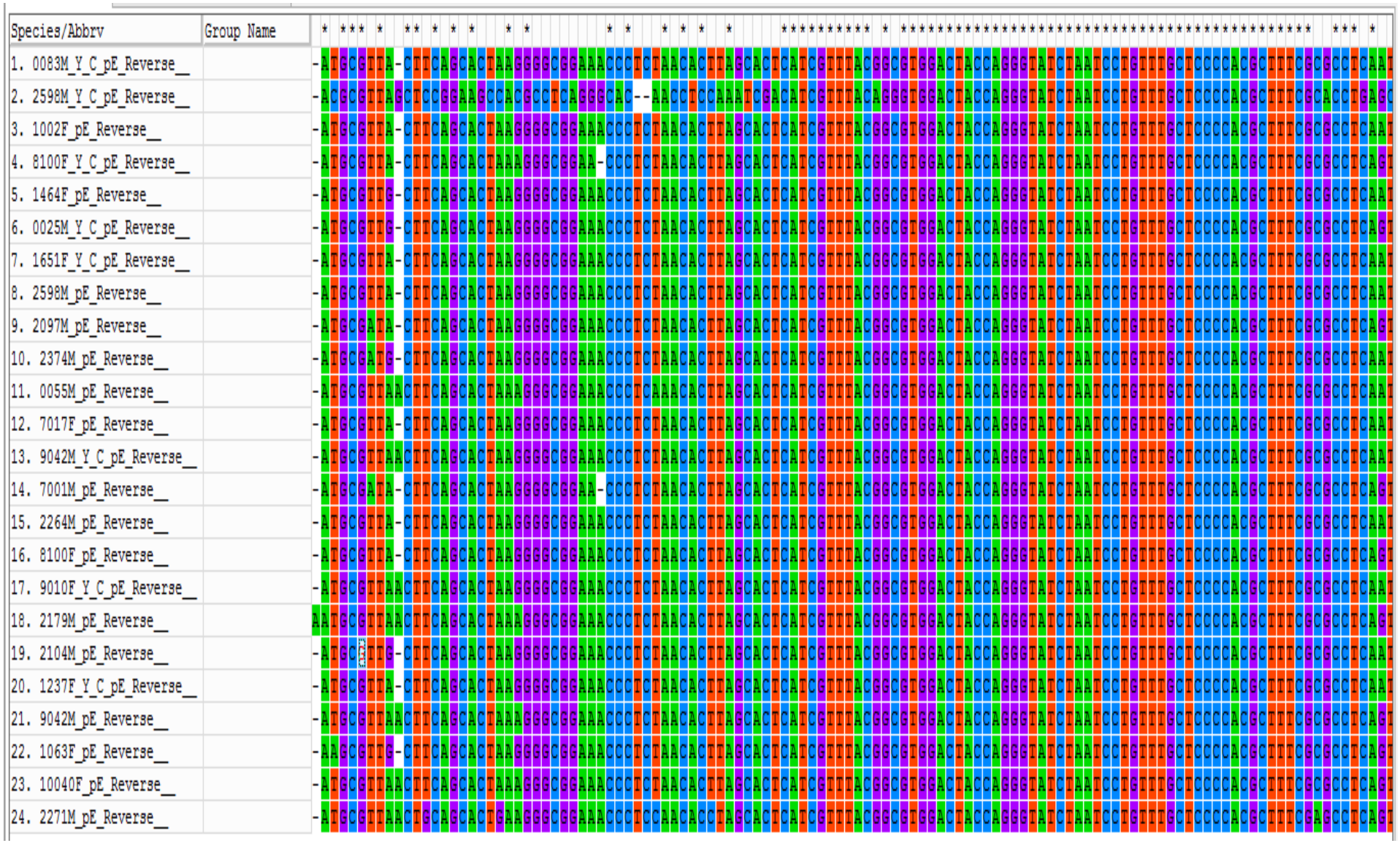


Fig 12: Alignment of 16S rDNA using the forward primer

Appendix E


Species/Abbrv
Group Name
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2. 2598M_Y_C_pE_Reverse__
3. 1002F_pE_Reverse__
4. 8100F_Y_C_pE_Reverse__
5. 1464F_pE_Reverse__
6. 0025M_Y_C_pE_Reverse__
7. 1651F_Y_C_pE_Reverse__
8. 2598M_pE_Reverse__
9. 2097M_pE_Reverse__
10. 2374M_pE_Reverse__
11. 0055M_pE_Reverse__
12. 7017F_pE_Reverse__
13. 9042M_Y_C_pE_Reverse__
14. 7001M_pE_Reverse__
15. 2264M_pE_Reverse__
16. 8100F_pE_Reverse__
17. 9010F_Y_C_pE_Reverse__
18. 2179M_pE_Reverse__
19. 2104M_pE_Reverse__
20. 1237F_Y_C_pE_Reverse__
21. 9042M_pE_Reverse__
22. 1063F_pE_Reverse__
23. 10040F_pE_Reverse__
24. 2271M_pE_Reverse__


Species/Abbrv
Group Name
1. 0083M_Y_C_pE_Reverse__
2. 2598M_Y_C_pE_Reverse__
3. 1002F_pE_Reverse__
4. 8100F_Y_C_pE_Reverse__
5. 1464F_pE_Reverse__
6. 0025M_Y_C_pE_Reverse__
7. 1651F_Y_C_pE_Reverse__
8. 2598M_pE_Reverse__
9. 2097M_pE_Reverse__
10. 2374M_pE_Reverse__
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12. 7017F_pE_Reverse__
13. 9042M_Y_C_pE_Reverse__
14. 7001M_pE_Reverse__
15. 2264M_pE_Reverse__
16. 8100F_pE_Reverse__
17. 9010F_Y_C_pE_Reverse__
18. 2179M_pE_Reverse__
19. 2104M_pE_Reverse__
20. 1237F_Y_C_pE_Reverse__
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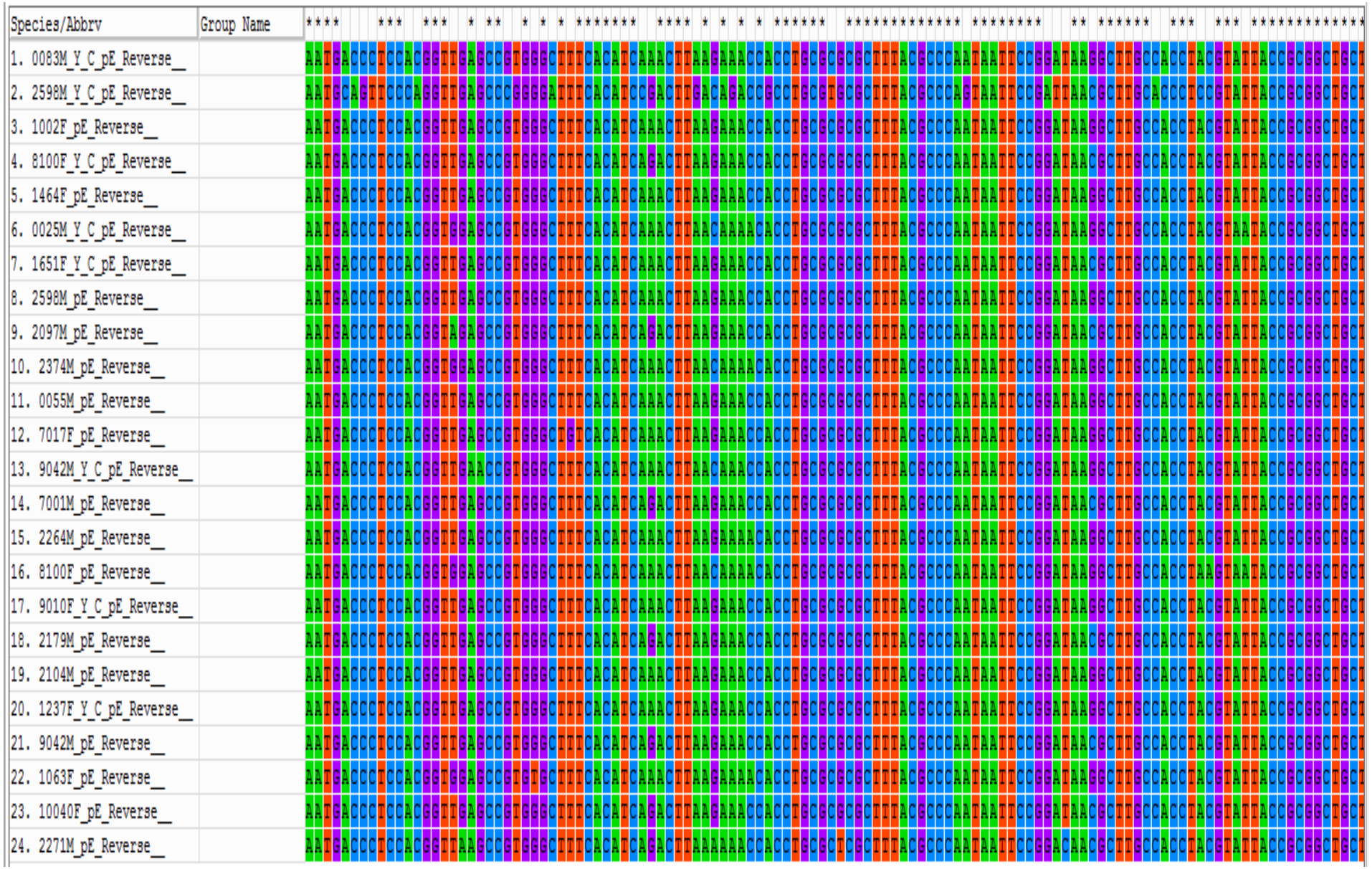
22. 1063F_pE_Reverse__
23. 10040F_pE_Reverse__
24. 2271M_pE_Reverse__


Species/Abbrv
Group Name
1. 0083M_Y_C_pE_Reverse__
2. 2598M_Y_C_pE_Reverse__
3. 1002F_pE_Reverse__
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5. 1464F_pE_Reverse__
6. 0025M_Y_C_pE_Reverse__
7. 1651F_Y_C_pE_Reverse__
8. 2598M_pE_Reverse__
9. 2097M_pE_Reverse__
10. 2374M_pE_Reverse__
11. 0055M_pE_Reverse__
12. 7017F_pE_Reverse__
13. 9042M_Y_C_pE_Reverse__
14. 7001M_pE_Reverse__
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17. 9010F_Y_C_pE_Reverse__
18. 2179M_pE_Reverse__
19. 2104M_pE_Reverse__
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21. 9042M_pE_Reverse__
22. 1063F_pE_Reverse__
23. 10040F_pE_Reverse__
24. 2271M_pE_Reverse__


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2. 2598M_Y_C_pE_Reverse__
3. 1002F_pE_Reverse__
4. 8100F_Y_C_pE_Reverse__
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7. 1651F_Y_C_pE_Reverse__
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9. 2097M_pE_Reverse__
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21. 9042M_pE_Reverse__
22. 1063F_pE_Reverse__
23. 10040F_pE_Reverse__
24. 2271M_pE_Reverse__


Species/Abbrv
Group Name
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2. 2598M_Y_C_pE_Reverse__
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4. 8100F_Y_C_pE_Reverse__
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7. 1651F_Y_C_pE_Reverse__
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9. 2097M_pE_Reverse__
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12. 7017F_pE_Reverse__
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14. 7001M_pE_Reverse__
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16. 8100F_pE_Reverse__
17. 9010F_Y_C_pE_Reverse__
18. 2179M_pE_Reverse__
19. 2104M_pE_Reverse__
20. 1237F_Y_C_pE_Reverse__
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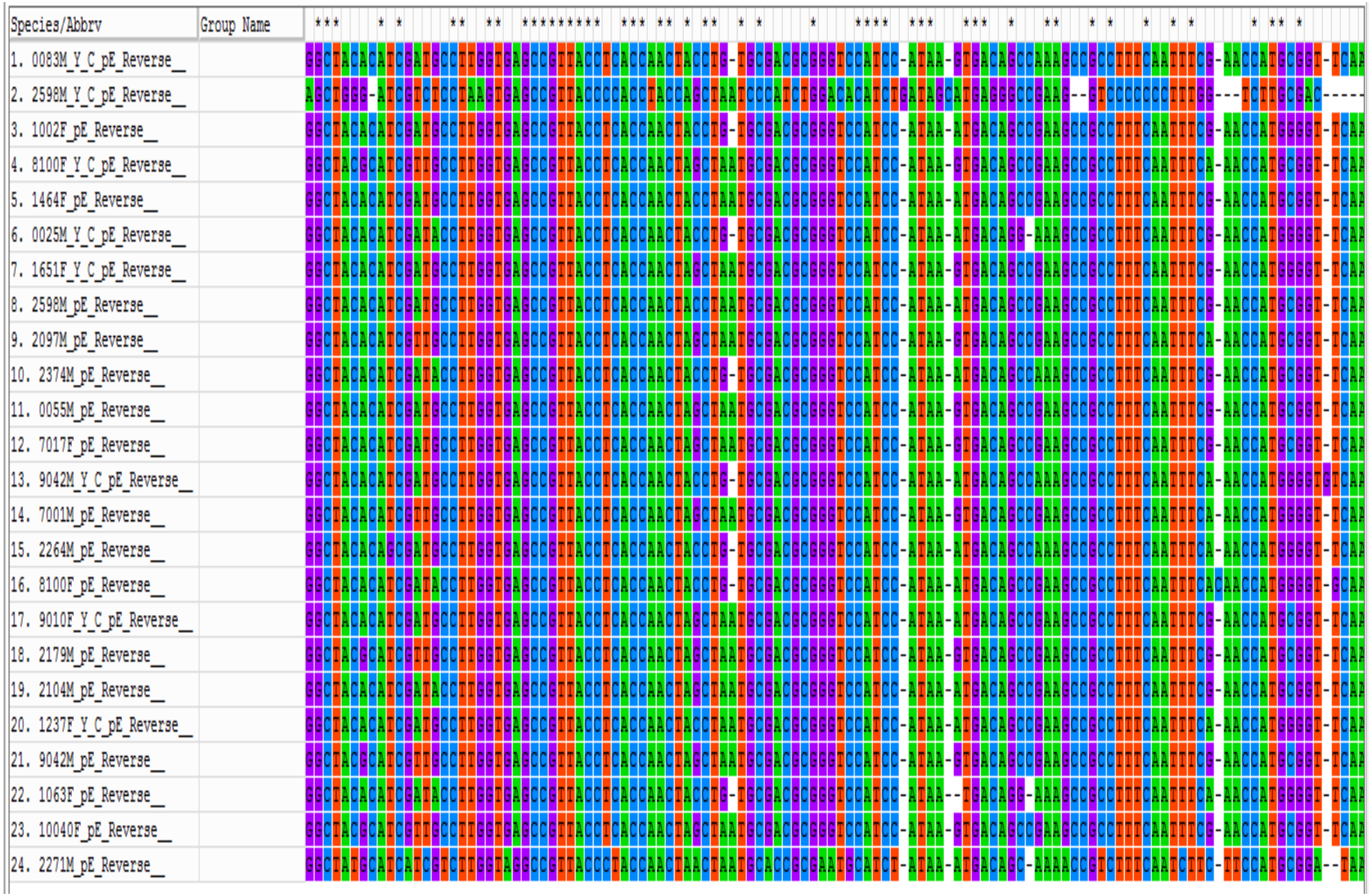
Species/Abbrv
Group Name
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2. 2598M_Y_C_pE_Reverse__
3. 1002F_pE_Reverse__
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6. 0025M_Y_C_pE_Reverse__
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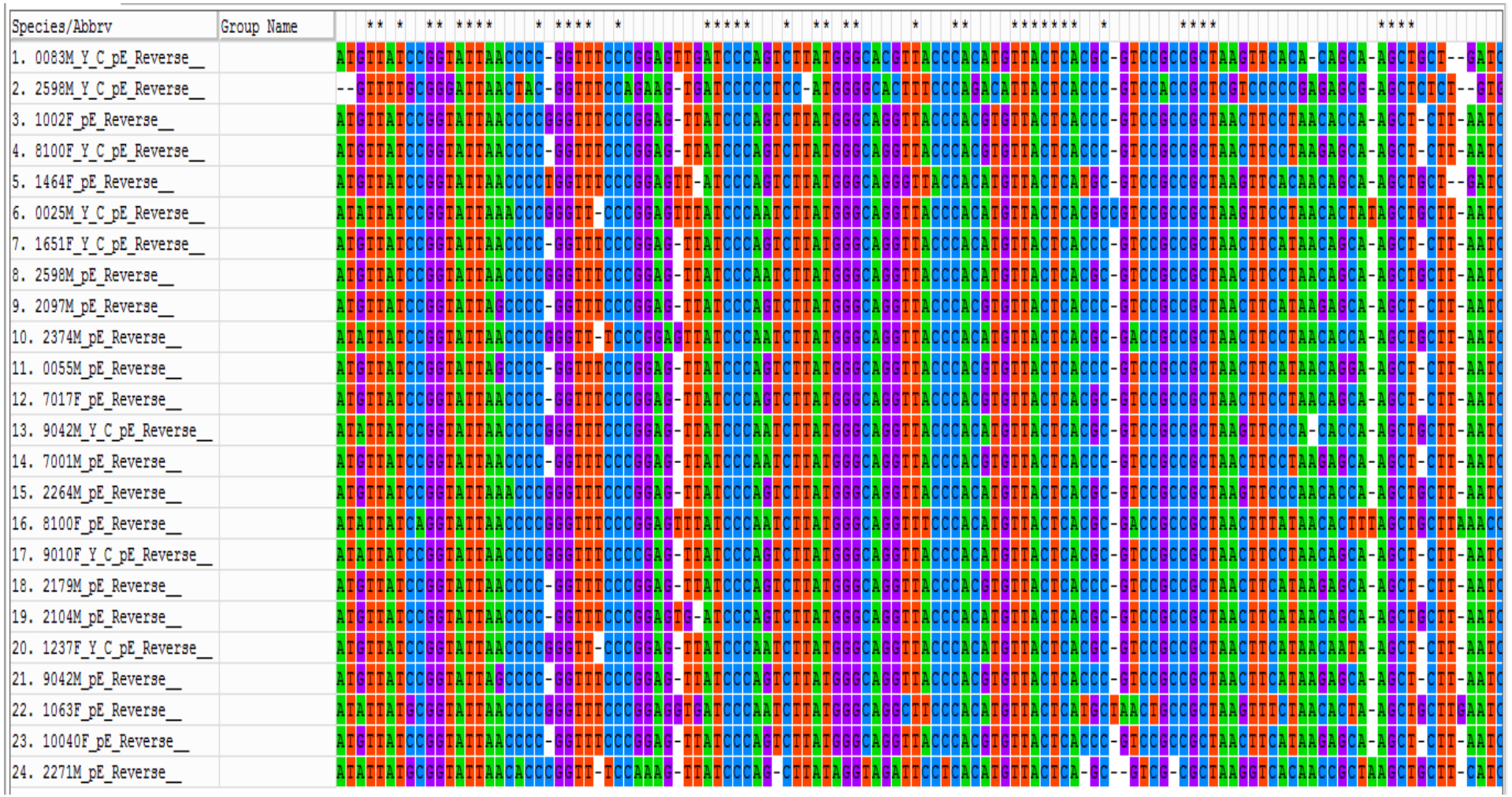
24. 2271M_pE_Reverse__


Species/Abbrv
Group Name
1. 0083M_Y_C_pE_Reverse__
2. 2598M_Y_C_pE_Reverse__
3. 1002F_pE_Reverse__
4. 8100F_Y_C_pE_Reverse__
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12. 7017F_pE_Reverse__
13. 9042M_Y_C_pE_Reverse__
14. 7001M_pE_Reverse__
15. 2264M_pE_Reverse__
16. 8100F_pE_Reverse__
17. 9010F_Y_C_pE_Reverse__
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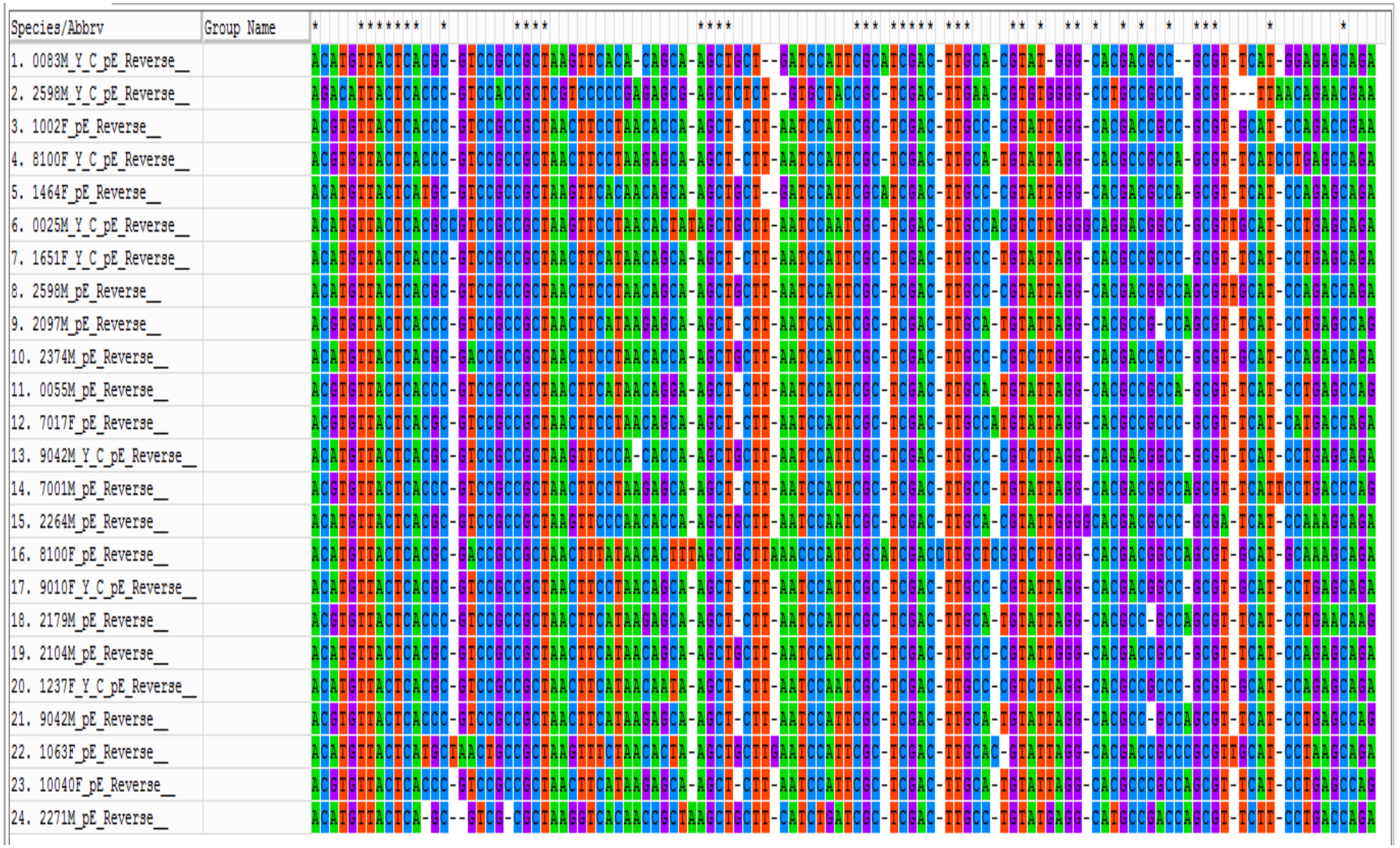

Fig 13: Alignment of 16S rDNA using the reverse primer

Appendix F

Table 8: Diameters of zones of inhibition of the isolates

Isolates	**T 30µg**	**RP 5µg**	**VA 30µg**	**DXT 30µg**	**AP 10µg**	**E 15µg**	**CIP 5µg**	**CD 2µg**	**C 30µg**	**GM 10µg**	**S 10µg**	**PG 10µg**
2179_{M}	20	12	17	21	9	21	25	15	24	20	19	8
2079_{M}	23	13	18	19	10	25	28	20	25	23	25	8
2598_{M}	20	12	17	21	10	20	24	17	20	19	19	7
1002_{F}	23	13	18	24	9	25	27	20	25	23	23	8
8100_{F}	22	13	18	21	10	23	26	19	25	22	16	8
$1464_{F\ YC}$	22	13	16	22	24	22	25	13	24	21	21	25
$0083_{M\ YC}$	24	13	17	24	11	23	28	21	25	22	22	9
10040_{F}	20	12	16	22	22	20	24	13	24	20	15	23
10122_{F}	24	14	18	25	26	16	25	16	26	20	23	26

CIsolates	T 30µg	RP 5µg	VA 30µg	DXT 30µg	AP 10µg	E 15µg	CIP 5µg	CD 2µg	C 30µg	GM 10µg	S 10µg	PG 10µg
$1237_{F\ YC}$	24	13	18	25	11	25	28	25	22	22	20	14
2104_{M}	24	15	19	24	10	25	26	22	25	23	20	10
7017_{F}	24	15	19	24	9	25	22	20	26	23	23	11
$8100_{F\ YC}$	23	13	17	23	10	19	25	23	20	19	18	8
$1651_{F\ YC}$	24	14	18	24	11	23	27	20	24	23	22	10
2374_{M}	24	12	17	24	25	23	25	15	25	23	21	25
$9010_{F\ YC}$	25	13	18	24	27	24	23	16	24	22	23	26
9042_{M}	23	13	18	24	11	25	28	21	26	22	22	10
$0025_{M\ YC}$	23	13	18	24	10	24	28	22	25	22	21	8
0055_{M}	23	13	17	25	24	20	24	19	23	21	20	22
2264_{M}	20	12	16	20	10	22	25	20	20	17	19	8

Isolates	T 30µg	RP 5µg	VA 30µg	DXT 30µg	AP 10µg	E 15µg	CIP 5µg	CD 2µg	C 30µg	GM 10µg	S 10µg	PG 10µg
2529_{M}	22	12	13	22	10	25	28	23	25	24	27	8
$9042_{M\ YC}$	23	12	16	23	24	21	25	15	21	21	21	23
9010_{F}	22	11	16	21	10	21	24	21	18	18	15	8
1063_{F}	22	12	17	23	24	24	24	14	24	22	21	23
7001_{M}	24	12	13	23	20	22	25	15	22	22	21	23

T-Tetracycline, RP-Rifampicin, VA-Vancomycin, DXT-Doxycycline, AP-Ampicillin, E-Erythromycin, CIP-Ciprofloxacin, CD-Clindamycin, C-Chloramphenicol, GM-Gentamicin, S-Streptomycin and PG-Penicillin. Zones of inhibition were measured in millimeters

Appendix G

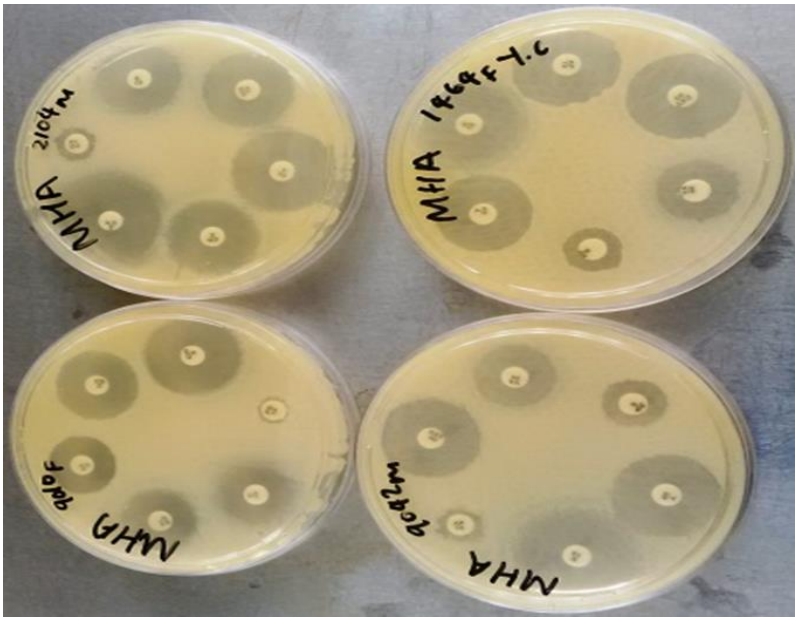

Fig 14: image of Mueller Hinton agar with antibiotic disks

Appendix H

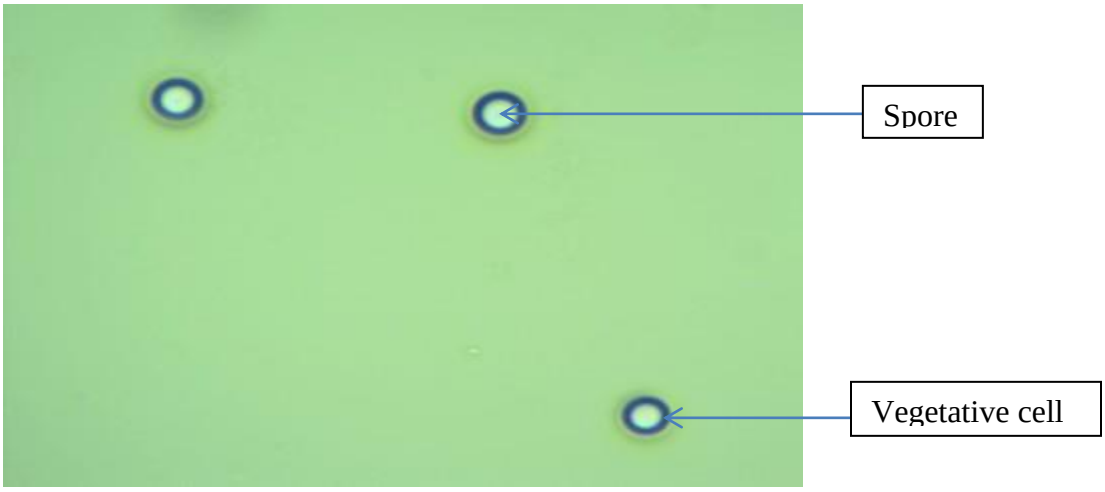

Fig 15: Phase contrast micrograph of sporulated isolate culture after3 days incubation

Appendix K

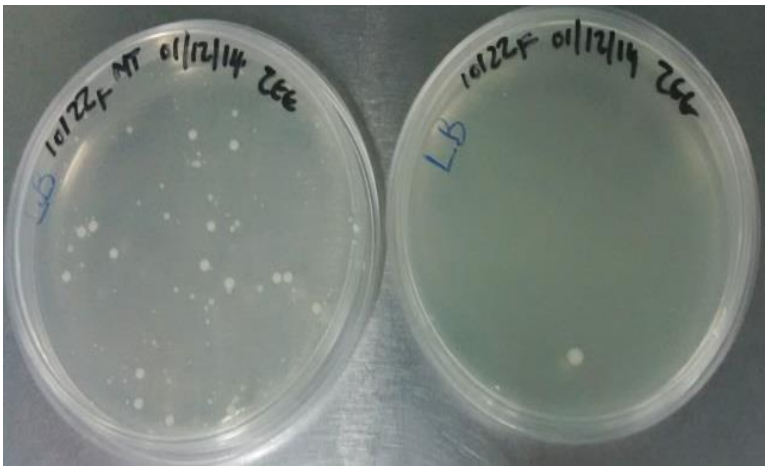

Fig 14: Concentration of germinated spores for the control (left) and heat-treated (right) samples