Distribution, virulence and antimicrobial resistance profile of *Bacillus* species from the environment of four public hospitals in South Africa

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A dissertation submitted to the School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, for the degree of Master of Medical Science.

This is a dissertation by manuscript with an overall introduction and final summary.

This is to certify that the content of this dissertation is the original research work of Ms Zamile Nompumelelo Mbhele, supervised by:

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DECLARATION

I, Ms. Zamile Nompumelelo Mbhele, declare as follows:

1. That the work described in this dissertation has not been submitted to the University of KwaZulu-Natal or any other tertiary institution for purposes of obtaining an academic qualification, whether by myself or any other party.

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Zamile Nompumelelo Mbhele

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DEDICATIONS

This work is dedicated to Mbhele and Mvuna's family and my close friends for their support, understanding, and spiritual encouragement during my studies.

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Any omissions and shortcomings that may be identified in this piece of work remain the sole responsibility of the researcher. Z.N Mbhele

ABSTRACT

Hospital-acquired infections (HAIs) are counted as the most crucial global health crisis. The hospital environment may be colonized by opportunistic pathogens, which can lead to HAIs in hospitalized patients. As a result, microbial monitoring of the hospital environment is important in managing these pathogenic organisms. This research aimed to investigate the prevalence, antimicrobial resistance, virulence genes, and genetic diversity of *Bacillus* spp. on hospital surfaces in public hospitals in KwaZulu-Natal (KZN), South Africa (SA). A total of 777 swabs were collected from four different public hospitals classified as A (Central), B (Tertiary), C (Regional), and D (District), within three different wards (paediatric, general ward, and intensive care unit (ICU) and 11 touchable surfaces belonging to medical devices and well used equipment. Samples were assessed for the existence of *Bacillus* spp in these four public hospitals. *Bacillus* was isolated using the microbial plating method on a selective *Bacillus* medium, and the biochemical characteristics confirmed, including oxidase, catalase, motility, and triple sugar iron (TSI). Molecular confirmation was also performed using polymerase chain reaction (PCR) targeting the *MotB* gene for *Bacillus cereus* and 16s rRNA gene for *Bacillus subtilis*. The minimum inhibitory concentration (MIC) technique evaluated the antimicrobial resistance profile using the Clinical Laboratory Standards Institute (CLSI) guidelines. Molecular characterization was conducted for seven antimicrobial resistance genes and 11 virulence factors using PCR. Genetic relatedness between the isolates across the four hospitals was evaluated by enterobacterial repetitive intergenic consensus PCR (ERIC-PCR). A total of 777 samples were collected in the hospital environments, of which 135 were positive for *Bacillus* spp. Species identification revealed 4 % (6) as *Bacillus subtilis* and 96 % (129) as *B. cereus*. The overall prevalence of *Bacillus* spp. per hospital was 24 % (32/135) for Central (hospital A), 33 % (45/135) for Tertiary (hospital B), 27 % (36/135) for Regional (hospital C) and 16 % (22/135) for District hospital (hospital D). A statistically significant difference in *Bacillus* prevalence (p = 0.044) was found between tertiary and regional hospitals (p = 0.000).The prevalence among the wards was averaging 33 % (45/135), noting ICU with the highest prevalence of 35 % (47/135). In terms of the wards, no statistically significant differences were found (p = 0.133). The hospital and the wards had a strong correlation (r = 0.525, p = 0.000).The highest prevalence on frequently touched sites was a bed with 15 % (20/135), drip stands and sinks with 12 % (16/135) respectively, ward phones with 11 % (15/135), and nurses' tables with 10 % (14/135). Complete resistance was observed against

ampicillin (100 %; 135/135), and high resistance against ciprofloxacin (99 %; 134/135), amoxicillin (97 %; 131/135), tetracycline (82 %; 112/135), cefotaxime (51 %; 69/135), and erythromycin (43 %; 59/135). Lower resistance was observed against meropenem (20 %; 27/135) and imipenem (25 %; 26/135). A total of 43 different antibiograms were detected, with 97 % (132/135) of the isolates found to be multidrug resistance (MDR). No statistically significant difference was observed between the antibiotics tested ($p \geq 0.05$). The observed resistance genes were *ermB* (56 %; 33/59) (conferring erythromycin resistance), tetracycline resistance-conferring genes were *tetM* (5 %; 5/112) and *tetK* (4 %; 4/112). No *tetA, tetB, tet39,* and the *blm* gene (beta-lactamase resistance-conferring gene) were detected. Different toxins produced by *Bacillus* are associated with the pathogenicity of *Bacillus* species. Prevalence of the virulence enterotoxin genes associated with diarrhea prevailed in 88 % (99/135) of *hblD*, 77 % (104/135) in *hblA* and *CytK* respectively, *nheC* 67 % (90/135), *nheB* 65 % (88/135), *nheA* 64 % (89/135), *hblC* 55 % (74/135), *bceT* 44 % (59/135), *hlyII* 37 % (50/135), and finally *EntFM* 27 % (37/135*)* of the isolates housed the gene. No cereulide (ces gene) causing emetic syndrome was detected. Typing using ERIC-PCR noted type clusters composed of isolates from different wards, environmental sites, and equipment and showing high genetic diversity, indicating no common infection sources. This study revealed a moderate prevalence of *Bacillus* spp. collected from the environment of the four public hospitals. High resistance was observed for some antibiotics that are usually effective against *Bacillus;* this may serve as a potential risk for effective treatment of *Bacillus* infections. Most isolates harboured virulence factors that cause diarrheal syndrome rather than those causing the emetic syndrome. These findings highlight the need for microbial surveillance of hospital environments to inform and improve current intervention programs for cleaning methods in public hospitals to reduce environmental contamination and transmission of pathogenic *Bacillus* spp. infections associated with HAI's in hospitalised patients.

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LIST OF ABBREVIATIONS AND ACRONYMS

α Alpha

ATCC	America type of culture collection
AMX	Amoxicillin
AMP	Ampicillin
AFLP	Amplified fragment length polymorphism
API	Analytical profile index
ALO	Anthrolysin-O
AMR	Antimicrobial resistance
BCM	*Bacillus* chromogenic medium
Bp	Base pairs
β	Beta
BREC	Biomedical research ethics committee
BSI	Bloodstream infection
Blm	Metallo-beta-lactamase type 2
CTX	Cefotaxime
CLABSIs	Central Line-Associated Bloodstream Infections
CNS	Central nervous system
CDC	Centre for Diseases Control and Prevention
CDS	Coding sequences
CLO	Cereolysin O
Ces	Cereulide
χ^2	Chi-squared
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standard Institute
CaCo2	continuous line of heterogeneous human epithelial colorectal adenocarcinoma cells
C	Cytosine
CytK	Cytotoxin K
°C	Degree Celsius
DNA	Deoxy-ribonucleic acid
ERIC-PCR	Enterobacterial repetitive intergenic consensus PCR
EntFM	Enterotoxin FM
BceT	Enterotoxin T
ELISA	Enzyme-linked immunosorbent assay
Emrb	Erythromycin
γ	Gamma
GI	Gastrointestinal
GW	General ward
g	Grams
>	Greater than
G	Guanine
HRKM	Health Research and Knowledge Management Database
HSCT	Hematopoietic stem cell transplantation

HBL	Hemolysin enterotoxin
HGT	Horizontal gene transfer
HAI	Hospital-acquired infection
HRS	Hours
HIV	Human immunodeficiency virus
H2O2	Hydrogen peroxide
IMI	Imipenem
i.e.	In other words
IPC	Infection, prevention and control
ICU	Intensive care unit
kDa	Kilo Dalton
KZN	KwaZulu-Natal
LDH	Lactate dehydrogenase
MLSB	Macrolide, linconsamide and streptogramin B
MEYP/MYP	Mannitol egg yolk polymyxin agar
Mb	Megabyte
MER	Meropenem
NHSN	National Healthcare Safety Network
µ	Micro
µg	Microgram
µL	Microlitre
mL	Millimetre
MIC	Minimum inhibitory concentration
MLEE	Multi-locus enzyme electrophoresis
MLST	Multi-locus sequencing typing
Viz	Namely
NTC	No template control
NRPS	Non-ribosomal peptides cereulide synthetase
NI	Nosocomial infections
PAED	Paediatrics
%	Percent
PPE	Personal protective equipment
PI-PLC	Phosphatidylcholine specific phospholipase
PC-PLC	Phosphatidylcholine preferring phospholipase
PI	Phosphatidylinositol
plcR	Phospholipase-c-regulator
PCR	Polymerase chain reaction
PEMBA	Polymyxin egg yolk mannitol bromothyl blue agar
HlyII	Potentially enterotoxin hemolysin
PFGE	Pulsed-field gel electrophoresis
RAPD	Random amplification of polymorphic DNA
REP-PCR	Repetitive element sequence-based PCR
RE	Restriction enzyme/endonuclease

RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
ST	Sequence type
SA	South Africa
SMase	Sphingomyelinase
SPSS	Statistical package for social sciences
SSI	Surgical and soft tissue infections
TET	Tetracycline
TLO	Thuriolysin-O
TRREE	Training and resources in research evaluation
TSI	Triple sugar iron
TSB	Tryptone soy broth
USA	United States of America
UKZN	University of KwaZulu-Natal
UPGMA	Unweighted pair group with arithmetic average
UTI	Urinary tract infections
WHO	World Health Organization

LIST OF APPENDICES

CHAPTER 1: **INTRODUCTION AND LITERATURE REVIEW**

1.1 Introduction

Hospital environmental surfaces are reservoirs for pathogens and play a vital role in hospital acquired infections (HAIs) (Dancer, 2004; O'Orman & Humphreys, 2012; Page *et al.,* 2009; Palza *et al.,* 2018; Suleyman *et al.,* 2018). Nosocomial infections (NIs) are used to define HAIs (Khan *et al.,* 2015). It alludes to infectious illnesses acquired by patients after 48 hours of hospitalization that were not present upon hospitalization and unrelated to the health issue for which the patient was admitted (Lalami *et al.,* 2016; Mohammed & El Seifi, 2014). These HAIs can be caused by microbial, fungal, and viral microorganisms (Khan *et al.,* 2015). Bacteria cause approximately 90% of infections, while protozoans, fungi, viruses, and mycobacteria play a smaller role in comparison to bacterial infections (Gatermann *et al.,* 2005; Khan *et al.,* 2015). Multidrug-resistant (MDR), organisms are defined as isolates showing resistance to more than one antibiotic originating from three different antibiotic classes (Bassetti *et al.,* 2013). Resistance to antibiotics to HAI-causing bacteria can lead to the rise in HAI-related death and morbidity in hospitalized individuals (Kaier *et al.,* 2020).

Infection occurs in immunocompromised patients in health care facilities, nursing homes, recovery homes, outpatient facilities, and clinical settings. Medical care staff can spread disease, notwithstanding tainted protective gear, bedclothes, or air beads (Kunwar *et al.,* 2019). Human beings are an essential primary source of spread through human exercises like sniffling, talking, and laughing (Kunwar *et al.,* 2019). The disease can begin from the exterior environment, contact with another infected patient, or a contaminated health care worker. In a few cases, the source of the infection is undetermined. In some cases, the microorganism is derived from the patient's own skin microbiota and becomes opportunistic following surgery or other procedures that compromise the protective skin barrier (Kunwar *et al.,* 2019; Srikath *et al.,* 2008).

The United States of America (USA) National Healthcare Safety Network, together with the Centre for Disease Control and Prevention (CDC), has categorized HAIs sites into thirteen forms with fifty nosocomial infectious disease sites, biologically based and clinical principles (Khan *et al.,* 2015). The common areas include gastroenteritis, meningitis, respiratory infections, surgical and soft tissue infections (SSIs), bladder-related diseases, pneumonia, and central line-associated bloodstream infections (CLABSIs) (Khan *et al.,* 2015;

Raka *et al.,* 2006). Several reasons influence the transmission of these pathogens: bacterial viability, contamination frequency, and bacterial load of handheld touched or environmental surfaces (Boyce, 2007; Phoon *et al.,* 2018). The most frequent forms of bacterial spread in hospital environments are infected hands, or through gloves by medical workers, invasive procedures (such as laparoscopy) conducted, wading microbe-contaminated water on clean surfaces, droplets from respiratory infections, and contact with unsterilized hospital surfaces or equipment (Phoon *et al.,* 2018; Weinstein & Hota, 2004).

All these reservoirs in the healthcare setting raise the risk of nosocomial infection (Phoon *et al.,* 2018). As a result of the frequent occurrences, not only do these infections increase hospitalization and medical costs; more antibiotics are utilized, increasing the probability of antimicrobial resistance resulting in elevated morbidity and mortality (Dramowski *et al.,* 2016; Khan *et al.,* 2015; Nero *et al.,* 2012). The emergence of multidrug resistance (MDR) microbes has become a global epidemic, putting strains on medical health facilities, especially in patients in intensive care units (ICU). As a result, one in ten patients admitted to a healthcare facility in developing countries has a high possibility of acquiring HAIs (Revelas, 2012). Acquisition of antimicrobial resistance of clinically essential microorganisms in healthcare settings is associated with misuse and non-essential prescriptions of antimicrobial drugs (Almagor *et al.,* 2018). The patients' extended hospitalization has a secondary indirect cost to the patient concerning the possible loss of income and failure in providing for their families, as patients are often breadwinners (Liu *et al.,* 2019). Past studies directed in North America and Europe uncovered that five to a modest amount of all hospitalizations prompted the advancement of HAIs, while Asia, Latin America, and Sub-Saharan Africa revealed over 40 % of hospitalizations with HAIs (Bereket *et al.,* 2012; Khan *et al.,* 2017).

The incidence of HAIs may be reduced by enforcing strict infection and control initiatives in hospitals. A traditional multidisciplinary approach is essential for developing local guidelines that can provide day-to-day advice, monitor the use of and manage control measures for broad-spectrum antimicrobial agents (Mehta *et al.,* 2014). Infection prevention and control (IPC) is a program inside healthcare facilities to diminish the spread of contamination causing infection among healthcare workers and staff (Popescu, 2019). The most critical IPC measures are surveillance, isolation, handwashing, disinfection, and sterilization (Popescu, 2019). Unfortunately, in low, middle-income countries, most healthcare

facilities do not effectively implement IPC measures due to insufficient staff members, overcrowding, and funding (Manchanda *et al.,* 2018). Lack of allocation of assets, inadequate framework, improper utilization of antimicrobials, and deficiently trained staff are critical limitations for successful IPC strategies (Manchanda *et al.,* 2018; Allegranzi *et al.,* 2011).

Microbes account for approximately 90 % of contaminations, while protozoans, organisms, and mycobacteria contribute less (Khan *et al.,* 2015). The bacteria species mainly involved in HAIs involve Enterobacterales members (*Klebsiella pneumoniae, Proteus mirabilis, Serratia marcescens,* and *Escherichia coli*), *Streptococcus* spp., *Enterococcus* sp, *Pseudomonas aeruginosa, Acinetobacter* spp., *Staphylococcus aureus* including coagulasenegative staphylococci, *Bacillus cereus,* and *Legionella* (Dancer, 2014; Gatermann *et al.,* 2005). The prolonged existence of these microbes within the environment can act as a transmission and dispersal source within the healthcare facility environment and surfaces (Esteves *et al.,* 2016).

Of the 34 species of *Bacillus*, the two most notable are anthrax's causative agent, *B. anthracis,* and *B. cereus* causing food poisoning (Bhandari *et al.,* 2013; Farrar & Reboli, 2006; Logan, 2012). However, in clinical settings, *Bacillus* species have been mainly considered as environmental contaminants. *Bacillus* infections are challenging to treat due to their MDR profiles, lack of updated antibiotic profile records, and shortage of new antimicrobial agents to treat infections, which leads to delays in therapy compromising clinical outcomes (Glasset *et al.,* 2018). Few cases have reported on HAIs related to *Bacillus* outbreaks, especially in South Africa. *Bacillus* spp. has been recognized as the causative agent of microbial diseases like pneumonia, bacteraemia, ocular infections, and soft tissue infections in the hospital environment; it is responsible for roughly 10 % of patient deaths (Bottone, 2010; Glasset *et al.,* 2018; Hansford *et al.,* 2014; Ikeda *et al.,* 2015; Rishi *et al.,* 2013; Saito *et al.,* 2016).

1.2 The genus *Bacillus*

The genus *Bacillus* contains diverse species ubiquitous in the environment and includes both harmless and pathogenic species (Messelhäußer & Ehling-Schulz, 2018). *Bacillus* is classified into two main groups according to their physiological attributes, gastrointestinal diseases, and nongastrointestinal infections. These diseases are induced by various toxins produced by bacteria (Gao *et al.,* 2018).

1.2.1 *Bacillus* bacteria

Bacillus is a member of the phylum Firmicutes, an incredibly diverse group of bacteria (Vos et al., 2011). First described in 1872, this genus now contains 293 named species/subspecies (Patel & Gupta, 2020). *Bacillus* species are Gram-positive aerobic or facultatively anaerobe (Messelhäußer & Ehling-Schulz, 2018), although some appear Gram variable in the bacterial growth stage (Fritze, 2004; Lee et al., 2012). It is chemoheterotrophic, usually motile with peritrichous flagella except for *B. anthracis* and *B. mycoides*, oxidase positive, and catalase-positive (Amin et al., 2015; Waites et al., 2008). *Bacillus* has an optimum growth temperature of 28 ºC to 35 ºC except for *B. stearothermophilus* that usually grows at 65 ºC with a minimum growth temperature of 4 ºC. *Bacillus* species usually grow at pH 4.9 to 9.3 (Schneider et al., 2015). Still, in food, pH's inhibitory effect is reduced, evidenced by limited growth on meat at pH 4.35 (Bintsis, 2017). Also, it has a 7.5 % salt tolerance (Lattuada & McClain, 1998). *Bacillus* species have been found all over the environment and have been confirmed to colonize the human gastrointestinal tract to an abundance of 10^4 spores/g of faeces (Bernardeau et al., 2017; Hong et al., 2009). *Bacillus* is commonly found in soil as it contains favourable physiological characteristics allowing *Bacillus* to thrive. *Bacillus* spp. can compete with other bacteria for nutrients giving it the ability to develop highly resilient spores; this also production of metabolites prevents other microbes from growing or is toxic to other bacteria (Amin et al., 2015).

1. 2.2 *Bacillus* spores

Bacillus species are rod-shaped and yield endospores resistant to unfavourable external conditions (Němečková *et al.*, 2012; Vos *et al.*, 2011). The development of single endospores in the vegetative state is a trait of *Bacillus* species. Its spores may appear as oval or cylindrical, subterminally, or terminally (Weber *et al.*, 2017). It usually presents *B. subtilis, B. cereus, B. licheliforms, B. megatereum*, and *B. pumilis* (Weber *et al.*, 2017).

As a result, these microbes can withstand extreme conditions even more so than nonsporulating microbial entero-pathogens (Swick *et al.*, 2016). They can also spread to a variety of environments, including water and raw and unprocessed food (From *et al.*, 2005).

Spores created by *B. cereus* have extremities or potentially pili and are more hydrophobic than other *Bacillus* spores. These properties empower the spores to stick to a wide range of surfaces and oppose expulsion during cleaning and sterilization (Bintsis, 2017; Stalheim & Granum, 2001).

Bacillus spore-forming microorganisms are causative agents during food and drug manufacturing processes because their spores can survive production pasteurization to form biofilms in stainless steel pipes and equipment (Gopal *et al.*, 2015). Spores of *B. cereus* in the food industry are especially problematic since spores can be entirely immune to decontamination procedures as well as gamma rays. (Bottone, 2010).

1.2.3 *Bacillus* taxonomy and species

B. subtilis was first identified as the hay/grass-associated bacterium *Vibrio subtilis* by the German scientist Christian Gottfried Ehrenberg in 1835 (Reviewed by Rasmussen *et al*., 2009). A German botanist, Ferdinand Cohn, renamed the organism *Bacillus subtilis* in 1872, after its ability to produce endospores that were round, oval, or cylindrical (Amaresan *et al.*, 2020). Later, Cohn and Robert Koch, a German physician, also made some other discoveries about *Bacillus* spores, such as its resistance to heat and development cycle of spore formation, vegetative cell to spore studying *B. subtilis* and *B. anthracis,* respectively (Gould, 2006; Keynan & Sandler. 1983; Slepecky & Hemphill, 2006). Smith *et al.* (1952) published the first comprehensive study on *Bacillus* based on a comparative study of 1134 strains representing 179 different species; from this study, 19 species types were identified (Logan *et al.,* 2015; Smith *et al.,* 1952).

Since then, *Bacillus* spp. are classified into two types, i.e., *B. cereus* and *B. subtilis*. The *B. cereus* group divided into eight subspecies, namely *B. anthracis*, *B. cereus* (common human pathogen), and the known entomologic pathogens *B. mycoides*, *B. weihenstephanensis*, *B. thuringiensis*, *B. cytotoxicus*, *B. toyonensis,* and *B. pseudomycoides* (Kim *et al.,* 2005). Amongst the genus *Bacillus,* the *B. cereus* group is known for being the most taxonomically ambiguous group. *Bacillus subtilis*, *B. licheniformis*, *B. pumilis*, and *B. mojavensis* are subspecies of the *B. subtillis* group (Němečková *et al.,* 2012; Osman *et al.,* 2018).

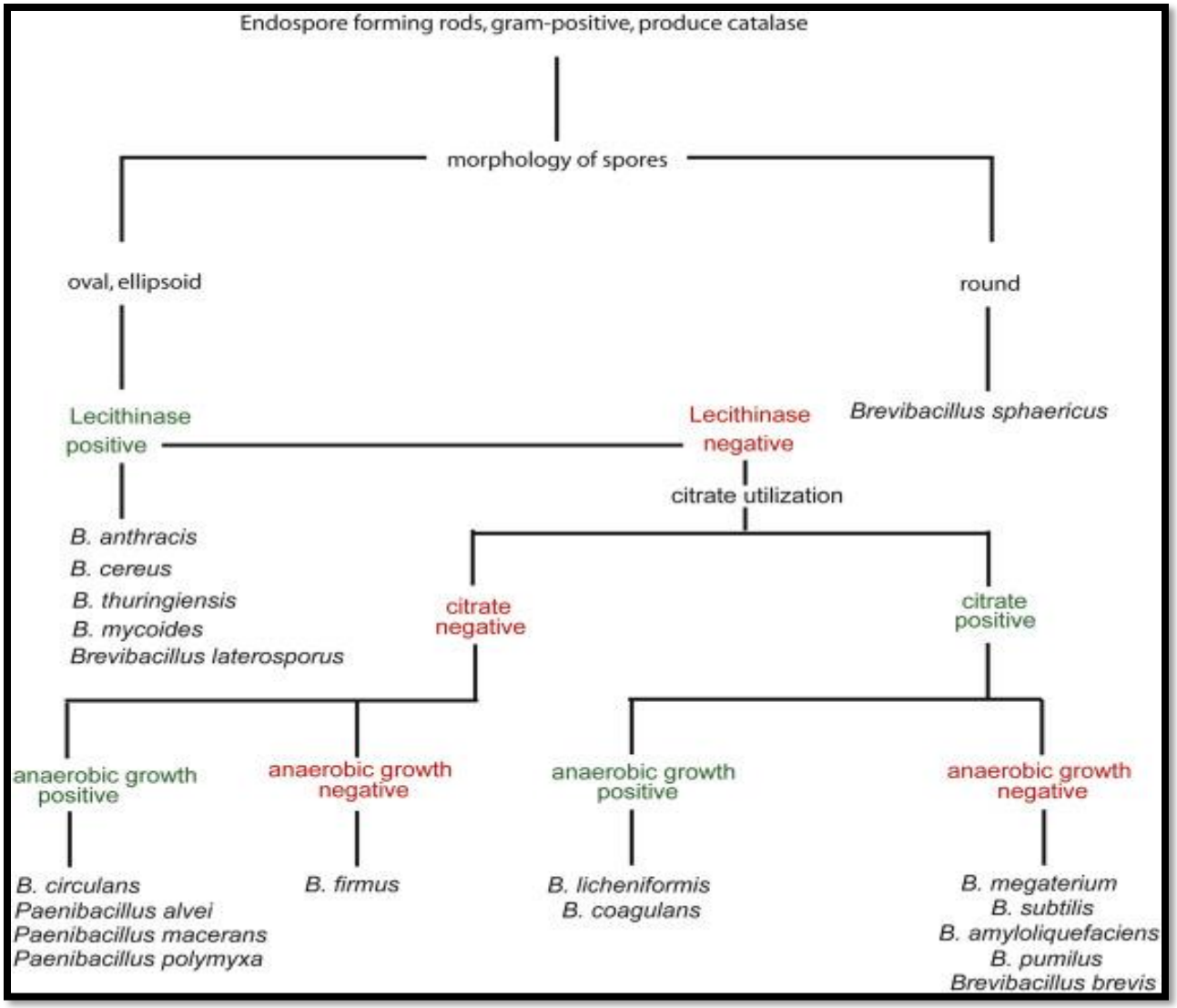

Figure 1.1: Differentiation of genus *Bacillus* by their morphologic and phenotypic features (Amarasen *et al.*, 2020).

1.2.4 *Bacillus* genome composition

Bacillus species are much more diverse than other bacterial strains, as evidenced by the wide range of DNA base ratios (32 % to 69 % guanine (G) + cytosine (C) content) between species, which is far broader than the usually considered ratio reasonable for a genus (Logan *et al.*, 2015). *B. subtilis'* genome consists of 4 214 810 base pairs, which are made up of 4 100 protein coding genes and 18 amino acid permeases (Logan *et al.*, 2015; Selvaraj *et al.*, 2014; Wipat & Harwood, 1999). *Bacillus anthracis* has 5 508 coding sequences (CDSs) and 5 227 293 bp (a total of 5 838 CDSs and 5 503 799 bp including the virulence plasmids), while *Bacillus cereus* carries 5 366 CDSs and 5 426 909 bp (Logan *et al.*, 2015). The genetic map of *B. cereus* revealed a chromosome that varies between 2.4 Mb to more than 5,5 Mb (Leornand et al.,

1998). Furthermore, the *B. cereus* genome has a distinct structure that consists of one stable entity containing essential genes and another, less stable region that could be subject to rearrangements such as insertions, deletions, and inversions. Furthermore, in the case of the *B. cereus* with the smaller chromosome, the pyruvate dehydrogenase gene was carried by one stable extrachromosomal element, indicating that housekeeping genes may exist outside the main chromosome (Léonard *et al.*,1998). This genome has a large portion that is easily mobilized into other genetic elements. These are subjected to frequent relocations between episomal elements and chromosomes (Carlson & Kolsto, 1994).

1.3 *Bacillus* infections

Bacillus cereus is the dominant species in this genus that generates two main types of gastrointestinal infections: diarrhoea and emetic syndrome. The emetic disease (vomit and nausea) is induced by various emetic toxins produced by cells during its growth in food products identified as cereulides (Ehling-Schulz *et al.,* 2006; Gao *et al.,* 2018). Products susceptible to *B. cereus* contaminants include pasta, meat, rice, flavours, vegetable, processed food; spores activated during the cooking and pasteurization in inadequate temperatures (Tallent *et al.,* 2015). Due to its character-producing spores, psychotropic strains can endure refrigeration temperatures (Gopal *et al.,* 2015). The Royal Hospital in Oman revealed a hospital-acquired outbreak of *B. cereus* in 2008, with 58 incidents of *B. cereus* food poisoning gastroenteritis caused by a violation of basic hygiene standards in the kitchen. The outbreak was due to the consumed meal of eggs, vegetables, milk dessert, chicken, macaroni, oats, and foul (beans) masala (Al-Abri *et al.,* 2011). Diarrhoeal syndrome, contrarily, is triggered by known heat-labile diarrhoeal enterotoxins produced in the small intestine during *B. cereus* vegetative growth (Ehling-Schulz *et al.,* 2006; Gao *et al.,* 2018). These infections are diverse and tend to occur in immune-compromised people and are associated with implanted medical devices and surgical operations (Glasset *et al.,* 2018; Saito *et al.,* 2016). *Bacillus* species are also capable of other severe infections known as nongastrointestinal diseases, such as bacteremia, meningitis, septicaemia, and endocarditis; some toxicity-inducing elements still undetermined (Glasset *et al.,* 2018; Ikeda *et al.,* 2015).

1.3.1 Gastrointestinal disease

Bacillus cereus is a dominant pathogen known for instigating different gastrointestinal (GI) diseases caused by two different toxins (see Table 1-1). The emetic illnesses are caused by toxins formed in food contaminated with *B. cereus*. These toxins cause emetic illnesses. The diarrheal type is caused by various enterotoxins produced by microorganisms in the intestines. (Table 1-1). Cereulide is a small, heat sustainable depsipeptide that causes emetic diseases in humans (Owusu-Kwarteng *et al.,* 2017; Messelhauser *et al.,* 2014). This factor is effectively established in the food-matrix, and extreme food poisoning can lead to encephalopathy and liver-related diseases (Ehling-Schulz *et al.,* 2019; Messelhäußer & Ehling-Schulz, 2018). The prevalence of these two syndromes has increased, with *B. cereus* reported as the second most common food-borne outbreak bacterium in France between 2007 to 2014 by Glasset *et al.* (2016) and was classified as third in China (Mao *et al.,* 2010) and Europe after *S. aureus* (Glasset *et al.,* 2018).

The gastrointestinal illness usually affects the neonates, immunocompromised hosts, as well as critically ill patients (Bottone, 2010). *Bacillus* spp. reported clinical outbreaks are rare; sources identified attributed to contaminated hospital environments (Logan, 2012). Cheng *et al.* (2017) reported a recurring infection of *Bacillus* bacteremia linked with infected linen in Queen Mary Hospital, Hong Kong, in the specialized hospital locations (acute emergency, hematopoietic stem cell transplantation (HSCT), and comprehensive services of oncology. A total of 1 % of immune-compromised patients were infected by *Bacillus* through direct skin interaction of infected linen items (Cheng *et al.,* 2017). *B. cereus* outbreaks have been correlated through the utilization of therapeutic devices, such as oral treatment (Kniehl et al., 2003), contaminated alcohol pads (Dolan et al., 2012), reusable ventilator airflow sensors (Kniehl *et al.,* 2003), and as previously mentioned, contaminated hospital linens (Cheng et al., 2017). Numerous factors have led to elevated levels of HAIs caused by *Bacillus* spp. which include traumatic or surgical injuries, intravascular catheters, intravenous therapy (IV), immature immune response, and indwelling devices (Bottone, 2010; Glasset *et al.*, 2018; Sasahara *et al.*, 2011). The current escalation in the number of reported *Bacillus* breakouts, which cause diarrhoea and emetic infectious disease (Table 1-1). *Bacillus* outbreaks related to food have caused severe economic loss and food safety complications (Gao *et al.,* 2018).

Table 1-1: Two toxin-mediated syndromes caused by *Bacillus cereus*, food poisoning, and food-borne infection (Adapted from Granum & Lund, 1997).

Features	Diarrhoeal syndrome	Emetic syndrome
Form of toxin	Two tripartite protein complexes enterotoxins: C*ytK*, *hbl*, n*he*, and *SMase* (Schraft &Griffiths, 2006)	Plasmid encoded emetic toxin (Cereulide) (*ces*)
Source of toxin production	Produced in the small intestines in the host, upon digestion of the toxin-creating *B. cereus* strains within the food.	Small heat-stable depsipeptide formed in the food matrix (Messelhäußer & Ehling-Schulz, 2018)
Infective dose/Number of bacteria in the food	The total recommended for spores is 10^4 to 10^9 cfu, which is lower than the total recommended for vegetative cells. (Logan & Rodrigez-Diaz, 2006)	10^5 to 10^8 cells discovered in the food in question, but cells are not needed for intoxication (Logan & Rodrigez-Diaz, 2006)
Incubation time	Eight to 16 hrs (occasionally >24 hrs) (Tewari & Abdullah, 2015)	30 minutes to six hrs
Length of illnesses	12-24 hrs (infrequently numerous days)	Six-24 hrs
Signs and symptoms	Stomach pain, vomiting(rarely), loose stool, diarrhoea, nausea, and lethality have occurred (Reis *et al.,* 2013)	Nausea, vomiting dysfunction, and vomiting, overall discomfort, and a few fatal incidents due to liver damage (Messelhäußer & Ehling-Schulz, 2018; Tewari & Abdullah, 2015)
Food most commonly involved	Sauces, dairy foodstuffs, meat products, fish, vegetables, and desserts (Messelhäusser *et al.*, 2014)	Pastry, dairy, starch-rich food and fried, cooked rice (Ceuppens *et al.*, 2013)

1.3.2 Extra-intestinal infections

In addition to the ability of *B. cereus* to cause gastrointestinal illnesses, members of the *B. cereus* population have been linked to a variety of life-threatening severe extra-intestinal infectious diseases. (Gao *et al.,* 2018; Messelhäußer & Ehling-Schulz, 2018). Commonly,

diseases of this kind are correlated with immune suppression in the affected patients, but other incidents have been disclosed from immunocompetent individuals. These extra-intestinal or non-gastrointestinal infections frequently occur in premature babies and include conditions ranging from local infections such as severe surgical wounds and infections related to eyes to systemic infections such as fulminant septicemia (Messelhäußer & Ehling-Schulz, 2018).

Extra-intestinal infections brought by *Bacillus cereus* subspecies can be divided into this various category viz systemic and non-systemic diseases. Systemic diseases consist of bacteremia and septicemia. Non-systemic infections are panopthalmitis and endophthalmitis, which consists of eye infections, endocarditis known as heart infections and meningoencephalitis, and brain abscess (Bottone, 2010; Messelhäußer & Ehling-Schulz, 2018). Extra-intestinal *B. cereus* infections have the most significant risk factors, especially for hospitalized patients utilizing intravenous drugs and catheters, having severe wounds (either surgical or injury), and leukaemia (Bottone, 2010; Messelhäußer & Ehling-Schulz, 2018; Mursalin *et al.*, 2020). Additional risk factors include cutaneous infections, endophthalmitis, superficial wounds or skin infections, septic arthritis or fasciitis and myositis, and acute systemic diseases such as pneumonia, meningitis, and endocarditis, and brain absences (Messelhäußer & Ehling-Schulz, 2018).

The actual occurrence of these infections is presently unknown. *Bacillus* isolates from blood and infected wounds are not generally defined, and toxin profiles of these isolates are thereby not systemically evaluated (Messelhäußer & Ehling-Schulz, 2018). The characterization of the toxin profile is of significance as it poses a risk for *Bacillus* outbreaks. These non-gastrointestinal diseases from the *B. cereus* family may be categorized into several main classifications centered on their entry routes. The first route is commonly known as exogenous, which arises from bacterial invasion by infection, open wound, or surgical intervention. The second route is endogenous, of which the origin of the infection remains mostly unknown apart from for patients with a proven record of habitual medicine misuse (Messelhäußer & Ehling-Schulz, 2018).

The non-gastrointestinal outbursts triggered by *B. cereus* were described in National University Hospitals in Singapore during 2010 when construction adjacent to the hospital promoted air and environmental contamination with *Bacillus* species. Of the 146 cases, bacteremia was reported in both immune-compromised patients and patients with indwelling devices (Balm *et al.*, 2012). In 2010, three patients at a tertiary paediatric hospital in Aurora, Colorado, USA, were positive for *Bacillus cereus* blood cultures. It is interesting to note that non-sterile alcohol pillows were identified as a source of infection (Dolan *et al.*, 2012).

1.4 Hospital-acquired infections (HAIs) and spread within the hospital

Contaminated areas in the hospital environment are identified as potential deposits of nosocomial bacterial pathogens (Esteves *et al.*, 2016; Palza *et al.*, 2018; Suleyman *et al.*, 2018). Gastrointestinal illnesses, endophthalmitis, necrotizing meningitis, subarachnoid haemorrhage, and brain abscesses are the most dominant hospital-acquired infections associated with *B. cereus* (Bottone, 2010; Glasset *et al.*, 2018).

Healthcare workers such as doctors, nurses, laboratory workers, and technical professionals, frequently exposed to the hospital environment, contribute to acquiring and spreading HAIs microbes (Mitchell *et al.*, 2015). Contact transmission seems to be the most frequent source of transmission, occurs as a result of direct contact among patients (via saliva, droplets, hands, and other fluids) and workers contaminated during regular patient care (e.g., hands, throat, skin, and nose). Contact transmission may also result in direct implantation of organisms in ordinarily sterile sites via inadequately sterilized instruments (Chinn & Sehulster, 2003; Suleyman *et al.*, 2018). Contamination occurs readily on hospital surfaces with direct hand contact. Hospital equipment such as stethoscopes and bedside tables in the environments can harbour microorganisms. Hands and instruments used by health care workers have been recognized as vectors of transmission of microorganisms (Collins, 2008). Indirect transferal of HAIs in the clinical setting may also occur through the air (Figure 1-1). Airborne transmission can arise when the microorganism originates from the mucous of the nose or throat becomes dispersed into the atmosphere as droplets or dry particles via coughing or sneezing (Chinn & Sehulster, 2003; Suleyman *et al.*, 2018). These particles may travel varying

distances and end up contaminating floors, environmental sources, and non-colonized patients, resulting in acquired infections.

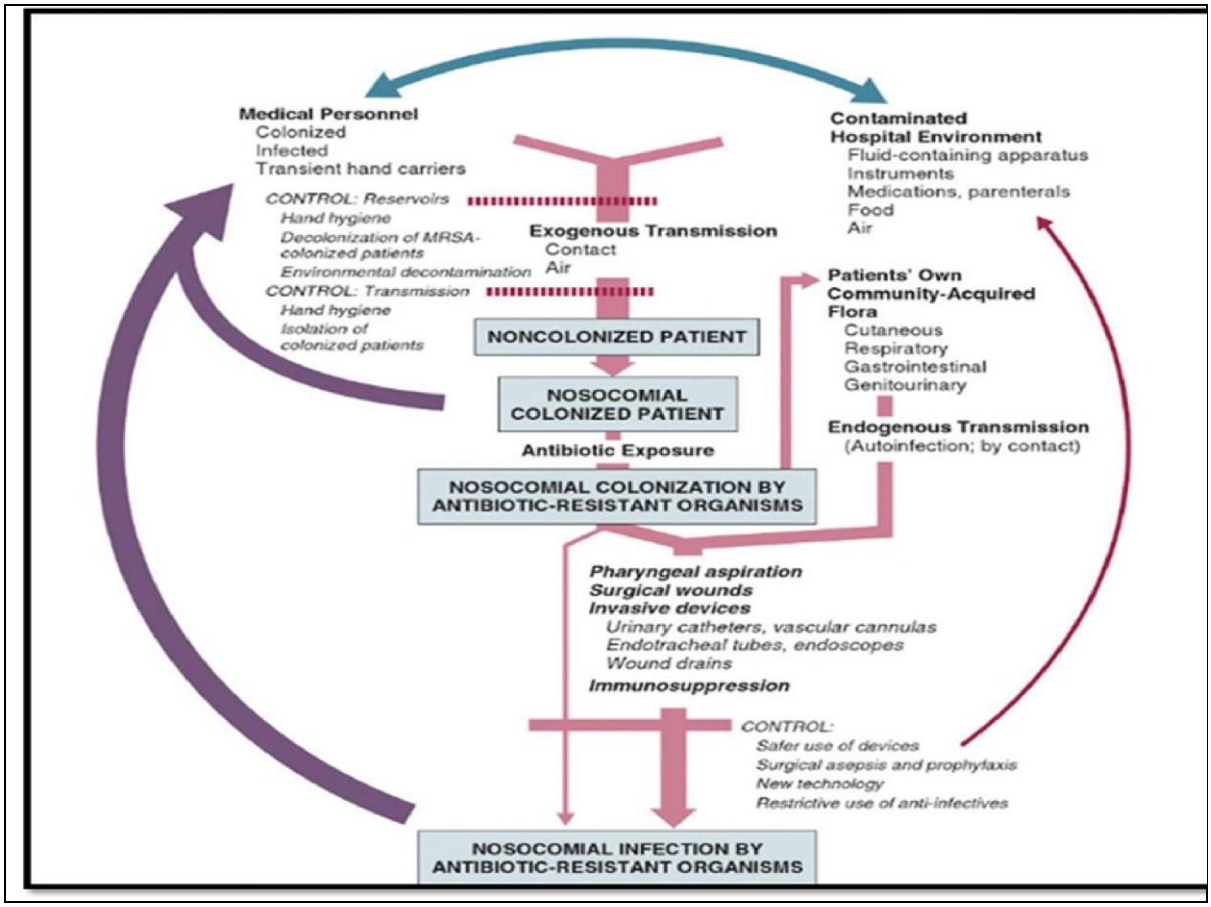

Figure 1-2: The epidemiology of hospital-acquired infections (HAIs) that shows dissemination arises primarily through exogenous transmission, which is contact and airborne contaminated hospital environments enhancing transmission, invasion, and susceptibility to infections (Adapted from Maki, 1978).

1.4.1 Dissemination of *Bacillus* as hospital contaminant

In 2006, two post-operative *Bacillus* meningitis bacteremia reports at Jichi Medical University hospital (Tochigi, Japan) revealed that medical institution linen cleaned in a washing machine harboured *B. cereus* spores even after laundry (Sasahara *et al.*, 2011). The same study reported that 44% of the umbilical swabs obtained from the new-born hospital ward consisted of rare

B. cereus serotypes. Following further investigative processes, similar serotypes were isolated from personnel hands, air samples, and clean cloth diapers from the hospital washing machine. Notably, the diapers were the primary vehicle for spreading *B. cereus* amongst new-born babies (Sasahara *et al.,* 2011).

Various sources are accountable for the contamination of theatre rooms, including drainage of wounds, indoor traffic volume, unfiltered air ventilation systems, operating room gowns, moving of patients between the wards, storage bags, surgical equipment, footwear, and use of inadequately sterilized equipment (Fleischer *et al.,* 2006; Gebremariam & Declaro, 2014; Okon *et al.,* 2012). Systematic review determining the degree of contamination present in operating theatres from published studies from 2000 to 2012 noted *Bacillus* spp. was present in the air of most operating theatres. The air samples showed 60 % of *Bacillus* spp. present in the urology operating theatre, 63 % in the surgical theatre, and 66 % in the orthopaedics theatre (Gebremariam & Declaro, 2014). From other samples, it was reported that *Bacillus* spp. was a diverse organism isolated from a variety of hospital surfaces. Notable in 43 % of surgical operating theatre surfaces, 31 % of the surfaces/articles from the orthopaedic operating theatre, and 77 % of the surfaces sampled from the operating theatre. Neurosurgery (10 %), urology (27 %), and optometry (15 %) operating theatre surfaces and theatre articles revealed the presence of *Bacillus* spp. Stethoscopes have long been a debatable point concerning their attributions to bacteria spread within hospital settings due to their universal use by healthcare professionals (Gupta *et al.,* 2014; Thapa & Sapkota, 2017; Uneke *et al.,* 2010). Gupta *et al.* (2014) conducted a study examining fifty stethoscopes of clinicians and nurses. Fifty-two microorganisms were found from fifty different stethoscopes, with a 2 % incidence for *Bacillus* species. Infections caused by contaminated medical devices may seriously endanger the wellbeing of patients in healthcare facilities, especially those in intensive care units (ICU) (Gupta *et al.,* 2014; Kilic *et al.,* 2011). It was concluded that possible microbial pathogens are harboured on stethoscopes capable of surviving on these exteriors. Therefore, disinfecting procedures are necessary between consecutive patients to minimize the risk of disseminating these pathogens within the healthcare facility (Gupta *et al.,* 2014). *Bacillus* species bloodstream catheter-related diseases were mainly reported in patients suffering from haematological malignancies and immunocompromised patients (Bottone, 2010; Gurler *et al.,* 2012). *Bacillus* species can generate biofilms that have a significant role in attachment to a

catheter (Auger *et al.*, 2009). Biofilms development can result from cell-surface touch as well as cell-to-cell, which contributes toward the development of micro-colonies. Biofilm development can also lead to *B. cereus* persistence due to their protected mode of development on inert surfaces in healthcare settings where reproduction can occur, in addition to the persistence of spores (Bottone, 2010).

1.4.2 Significance of Infection Prevention Control (IPC) practices

In 2012, the National Core Standards for Health Care Establishments in South Africa documented the six qualities of preventing HAIs in hospital settings (National Department of Health (NDoH), 2011). These guidelines postulate that an infection prevention control (IPC) program must be in place to avert HAIs. One of the measures of these standards is the occurrence of a formal system to display IPC to confirm that are suitable measures are engaged to minimize the risk of contamination. Although numerous improvements have been made in IPC programs, the execution of a surveillance system to discover HAIs has lagged; therefore, major measures are needed to prevent outbreaks from occurring (Collins, 2008; Mehta *et al.*, 2014). With many challenges in achieving efficient IPC programs, modern technologies like photocatalytic disinfection and high-intensity narrow-spectrum light to supplement traditional methods need to be implemented. Further research needs to investigate the efficacy and cost effectiveness of IPC programs (Boyce, 2016).

The hospital environment is frequently contaminated with nosocomial potential pathogenic characteristics of great concern (Oumokhtar *et al.*, 2017). There has been growing emphasis on improving the simple cleansing and decontamination of hospital environmental exteriors (Boyce, 2016). The dissemination of pathogenic microorganisms can be prevented if the modes of transmission are disrupted. This can be achieved by implementing effective means of disinfection (Dancer, 2014). A protocol for cleaning and disinfecting the surfaces in the hospital settings must be applied systematically to reduce the population of bacteria present (Lalami *et al.*, 2016).

Experts and professionals in the medical field have provided several recommendations and methods to aid in controlling microbial pathogens. Appropriate application of personal protective equipment (PPE) in hospital settings, correct timing and donning of gloves, and the use of sterile gowns by health care workers when interacting with colonized or infected patients have been viewed as essential risk reduction strategies of transmitting microbial pathogens (Mitchell *et al.,* 2015). A critical tool preventing spread is high-level hand sanitation, which prevents the cross-spread of contagious agents among patients by the medical workers' hands (Collins, 2008; Hongsuwan, 2018). By improving hand hygiene compliance, proper use of alcohol-based hand sanitizers minimizes rates of nosocomial infections by up to 40 % (Kampf *et al.,* 2009). The most known World Health Organization (WHO, 2009) proposed five moments of handwashing to reduce the transmission of pathogenic bacteria (Sax *et al.,* 2007) (see Figure 1-2). These five hand hygiene moments are defined as follows: (a) alcohol-based handshakes must be used before touching the patient; (b) before surgery; (c) after contact with body fluid; (d) after touching the hospitalized individual; as well as (e) after touching the nearby hospitalized individual (WHO, 2009; Salmon *et al.,* 2015). Most commonly used biocidal products, such as antiseptics, disinfectants, are usually used to disinfect environmental surfaces, preservatives of various formulations, and sterilization of medical devices. It also plays a vital role in reducing opportunistic pathogens in hospital settings that lead to the development of HAIs (Maillard, 2005; Olesiak *et al.,* 2012). The *Bacillus* genus is known for its highly resistant endospores, which pose a major concern since most disinfectants are only effective against the vegetative form of bacteria (Gopal *et al.,* 2015; Moeller *et al.,* 2008). Disinfectants containing glutaraldehyde efficiently inactivating against *Bacillus* spores. Glutaraldehyde has a broad spectrum of activity and establishes solid cross-links with amino acid groups in the cell wall and bacterial spore coats (Olesiak *et al.,* 2012). The effectiveness of a cleaning agent often varies based on their concentration and the duration of exposure/action time. Hydrogen peroxide has become a potent, strong antiseptic and is efficient toward spores, mostly at large concentrations as well as at elevated temperatures (Olesiak *et al.,* 2012). Alcohol, e.g., ethanol, isopropanol, and n-propanol, is a common component in several disinfectants for hygienic purposes and scrubbing for disinfecting skin and medical equipment surfaces (Olesiak *et al.,* 2012; Weber *et al.,* 2007). Newer disinfectants introduced or under development include electrolyzed water, polymer guanidine, cold atmospheric plasma pressure, peracetic acid-hydrogen peroxide, and enhanced hydrogen peroxide liquid disinfectants (Boyce, 2016). Hydrogen peroxide hand washing and the routine

washing of clothes have shown reductions in bacteria colonization and overall bacteremia rates in a hospital environment (Gall *et al.*, 2020; Leas *et al.*, 2015).

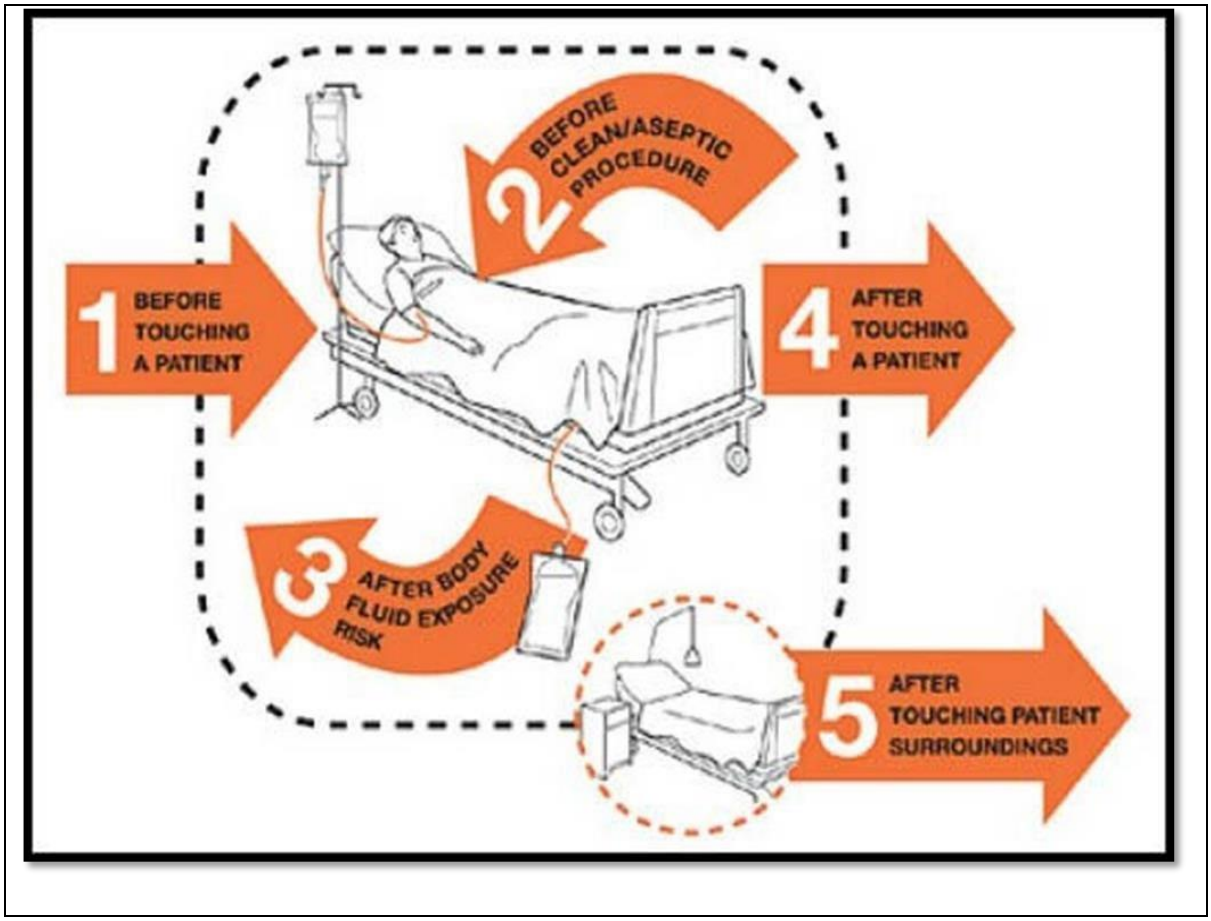

Figure 1-3: The cycle of five moments of hand hygiene that was released by the World Health Organization (WHO, 2009).

1.5 *Bacillus* species main virulence factors and pathogenesis

While *Bacillus* spp. produce various proteins with potential toxigenic activity, the true extent of their virulence factors remains unknown due to the difficulty in isolating and characterizing

these proteins (Bottone, 2010; Celandroni *et al.*, 2016). However, there are eleven commonly identified virulence proteins produced by *Bacillus* viz. *hbl, nhe, bcet, entM, hyll,* and *ces* (see Table 1-2). These are significant proteins that influence the virulence potential of *Bacillus* spp. and can contribute to food poisoning symptoms.

Table 1-2: Toxins produced by genus *Bacillus* species and their components (Adapted from Stenfors Arnesen *et al.,* 2008)

Type of toxins	Syndrome	Name of the gene and proteins	Protein/peptide sizes
Hemolysis BL	Diarrheal	B-*hbl*A, L_1- *hblC* and L_2-*hblD*	35 kDa (B) 36 kDa (L_1) 45 kDa ($L_{2)}$
Cytotoxin K	Diarrheal	*cytK*	34 kDa
Enterotoxin T	Diarrheal	*bceT*	41 kDa
Cereulide	Emetic	*ces*	1.2 kDa
Enterotoxin FM	Diarrheal	*ent FM*	45 kDa
Non-hemolytic enterotoxin	Diarrheal	*nheA, nheB* and *nheC*	45 kDa (A) 39kDa (B) 105kDa (C)
Potential enterotoxin hemolysin II	Diarrheal	*hlyII*	42.6 kDa
Cereolysin	Endophthalmitis	Clo	55 kDa
Phospholipase	Invasive nongastrointestinal	Pc-plc	29.2kDa(PC-LC)
		Pi-plc	34.6kDa (PI-PLC)
Sphingomyelinase	Invasive nongastrointestinal	SMase	34.2 kDa

1.5.1 Haemolysis BL (Hbl)

The components of the enterotoxin factors coded by three major genes, *hblA*, *hblD*, and *hblC*, are known as the unique toxin complex system. Current knowledge of toxic activity *hbl*

includes vascular permeability, necrosis, and hemolysis in the skin (Senesi & Ghelardi, 2010). Hemolysis BL components tandemly arranged genes in the sequence *hblC*, *hblD*, and *hblA* encode components named L1, L2, and B, respectively. (Beecher & Macmillan, 1991; Reis *et al.*, 2013). All these components individually bind to the membrane, but the kinetics are different. Therefore, all three Hbl components must be present for toxin activity to occur (Lindbäck & Granum, 2006; Reis *et al.*, 2013).

There is a proposal that the three well-known Hbl proteins typically attach independently to erythrocytes; subsequently, the membrane bound Hbl component creates a complement membrane attack complex inducing lysis of colloidal osmosis membranes (Jeßberger *et al.*, 2014). In this context, complement lesions, also called membrane attack complexes (MACs), may also be identical (Madegowda *et al.*, 2008). The *hblA* downstream consists primarily of an open reading frame, *hblB*, equivalent to *hblA* with the first 158 estimated amino acids. The *hblB* gene's product has yet to be recognized. The presence of HBL in sheep's blood agar results in abnormal hemolysis breakdown. (Beecher & Wong, 2000b; Ehling-Schulz & Messelhäusser, 2013; Schoeni & Lee Wong, 2005). The spread of *hbl* genes in the *B. cereus* species is quite complex, and the strains display various diarrheal toxin production capabilities. Almost 45 % to 65 % of *B. cereus* strains excrete Hbl (Senesi & Ghelardi, 2010). However, there are differences in the proportion of Hbl-producing strains among clinical strains (81 %) and food / environmental strains (43 %) (Senesi & Ghelardi, 2010; Thaenthanee *et al.*, 2005).

1.5.2 Non-haemolytic enterotoxin (Nhe)

The Nhe enterotoxin comprises three mechanisms (*nheA*, *nheB* and *nheC*) (Didier *et al.*, 2012). It was discovered that the food-borne illness outbreaks that were triggered by *Bacillus cereus* strains were not only caused by the Hbl toxin. After a Norwegian outbreak as a result of an Hbl-negative strain, three other components of the enterotoxin complex, which form the Nhe complex, were also causing illness (Tewari & Abdullah, 2015). The Nhe complex is like the Hbl; for toxicity to be induced, all three components must be present for maximum cytotoxic activity. Within the three-component toxin complex, *NheA* is known as the target antigen. Epithelial cell cytotoxic activity is related to colloid osmotic lysis after pore formation in the

plasma membrane (Fagerlund *et al.,* 2008; Senesi & Ghelardi, 2010). Almost all *B. cereus* strains produce *Nhe* complex. The non-hemolytic (Nhe) toxins are highly toxic to Vero cells (Lindbäck *et al.,* 2010; Sastalla *et al.,* 2013). Under the Hbl complex, proteins are secreted separately in the Vero-cells, and for the maximum toxic stimulation to take place, all the three components are required at a molecular proportion of *(nheA*) =10: (*nheB)* =10: (*nheC*) =1, respectively (Lindback *et al.,* 2004).

1.5.3 Cytotoxin K (CytK)

Cytotoxin K (CytK) was initially believed to be the only toxicity factor causing the hemolytic, necrotic and cytotoxic nature of *B. cereus* strains when first detected in strain NVH391/98 of *B. cereus* (Lund *et al.,* 2000; Ramarao & Sanchis, 2013; Senesi & Ghelardi, 2010). This toxin was implicated in France in 1998 as the cause of a severe incident of food-borne illnesses. It is known as a pore-forming toxin, single-component, and β-barrel having similar toxicity characteristics as *Clostridium perfringens* β-toxin (Ramarao & Sanchis, 2013). About 90 % of the *B. cereus* strains commonly carry this cytotoxin gene. The CytK toxin can produce poorly anion-selective pores, consisting of an impartial mediator (Ramarao & Sanchis, 2013). Potent cytotoxicity toward the human intestinal epithelium (Caco-2) and ability to act as a tissuedestroying protein damages the cohesion of the plasma membrane of many cells, including epithelial cells of the small intestine (Senesi & Ghelardi, 2010). It is also associated with necrotic enteritis and severe diarrhoeal outbreaks. Sequencing of the CytK protein revealed that its toxicity is comparable to the α-hemolysin of β-toxin of *C. perfringens* and *S. aureus* (Fagerlund *et al.,* 2004; Lund *et al.,* 2000; Ramarao & Sanchis, 2013).

CytK can exist in two different commonly known forms, which are CytK-1 and CytK2. The CytK-1 was initially identified in strain NVH391/98 of *Bacillus* and highly toxic to Vero cells and CaCo2 human intestinal compared to the CytK-2 form (Ramarao & Sanchis, 2013). The CytK expression is regulated by the phospholipase-C-regulator (PlcR)/pap-R system. Sequence analysis revealed highly conserved palindromic regions within the promoter regions of plcR-regulated genes that act as the exact identification target for PlcR activation (Ceuppens *et al.,* 2011). The amino-terminal sequence CytK contains a putative secretion signal peptide secreted by the Sec-route/pathway. Secretion was indicated not to be affected by crystalline secretion in the *B.thuriengiensis* mutant in *flhA* lacking flagella. This suggests the flagella

transfer mechanism is not related to the excretion of this toxicity (Ghelardi *et al.,* 2002; Senesi & Ghelardi, 2010).

1.5.4 Enterotoxin T (BceT)

The BceT is a protein characterized by being a single component (41 kDa) with enterotoxicity activity, was first reported by Agata *et al.* (1995) (Agata *et al.,* 1995; Burgess & Horwood, 2006). Eventually, BceT was described as a *B. cereus* enterotoxin. The idea was questionable when it stated that the genetically modified BceT was subsequently cloned and found to be silently expressed in *Escherichia coli,* seemingly not posing biological activity (Burgess & Horwood, 2006; Amor *et al.,* 2019).

1.5.5 Potentially enterotoxin hemolysis II (HlyII)

HlyII is known for its hemolytic activity, temperature-labile toxin with a size of 42.6 kDa (Stenfors Arnesen *et al.,* 2008). Commonly found in the *B. cereus* subspecies counting *B. thuriengensis,* it also falls under the members of α-toxin, γ-toxin (from *S. aureus* and *C. perfingens*), leukocidin and β-barrel spore-forming toxin family. HlyII is an oligomeric ß-barrel toxin that forms pores. Toxins in this class include the α-toxin of *Staphylococcus aureus*, the ß-toxin of *Clostridium perfringens*, and the *B. cereus* cytotoxin K (CytK) (Andreeva *et al.,* 2006; Ramarao & Sanchis, 2013).

HlyII was shown to induce phagocytic cell lyses in mouse macrophages, insect hemocytes, dendritic cells, and human monocytes, although not all epithelial cells (Ramarao & Sanchis, 2013). The cells' surface is linked to the oligomeric transmembrane pores where the potential enterotoxin is attached, in a flat lipid bilayer, contributing to cell permeability and lysis (Miles *et al.,* 2002). HlyII induces hemolysis in three major steps. The first phase is known for the temperature-dependent binding of HlyII monomers to the erythrocyte membrane; the second one is the temperature-dependent creation of the trans-membrane pore by multiple hemolysin particles; and lastly, temperature-independent erythrocyte lysis (Baida & Kuzmin, 1996; Ramarao & Sanchis, 2013).

1.5.6 Cereulide (Ces)

Cereulide is characterized as an ionospheric cyclododecadepsipeptide of interchanging amino and hydroxylic acids (D-Oxy-Leu-D-Ala-L-Oxy-Val-L-Val) (Ducrest *et al.,* 2019; Frenzel *et al.,* 2011). Cereulide is formed by the *B. cereus* strains clonal lineage that bears pXO1-like mega-plasmid, called pBCE, which encodes non-ribosomal peptides cereulide synthetase (NRPS) (Ehling-Schulz *et al.,* 2015; Lücking *et al.,* 2015). It is cytotoxic and mitochondrial toxic to humans and other eukaryotic organisms' primary cells and cell lines (Bauer *et al.,* 2018; Decleer *et al.,* 2018). Toxicity to beta-cells fetal and Langerhan's islets suppress oxidation of fatty acids of hepatic mitochondrial that can induce active oxidation (Bauer *et al.,* 2018; Vangoitsenhoven *et al.,* 2015).

Cereulide is mainly found in *B. pumilis, B. licheniformis,* and *B. cereus,* as well as in, *B. pumilis, B. subtilis, and B. mojavensis* (From *et al.,* 2005). Peptides of this form may vary in size of cereulide in particular; the putative emetic toxins are generated easier at 22°C than at 30°C (Ehling-Schulz *et al.,* 2019; From *et al.,* 2005). On the other hand, *B. cereus* demonstrated an optimum production temperature significantly lower, 12 °C to 22 °C, whereas *B. licheniformis* optimally generates an emetic toxin at approximately 28 °C (Logan, 2012; Stenfors Arnesen *et al.,* 2008).

Cereulide is generally referred to as a heat-stable peptide toxin that induces emetic syndrome (Messelhäußer & Ehling-Schulz, 2018). It is substantial and can withstand a wide pH range, enzymatic cleavage, and deactivation through purification or thermal processing during food production or reheating cooked foods. (Frenzel *et al.,* 2011; McDowell *et al.,* 2019; Rajkovic *et al.,* 2008). The *ces* are toxic to mitochondria by acting as a potassium ionosphere and inhibiting human natural killer cells (Paananen *et al.,* 2002). During the initial molecular assays on studies of emetic toxin-producing microbes, the peptide synthetase genes responsible for non-ribosomal production of ces genes were classified (Toh *et al.,* 2004).

1.5.7 Enterotoxin FM (EntFM)

The EntFM is a polypeptide of 45 kDa folded in β-configuration and includes several odd sequence structures, such as 280 residues of amino acid asparagine (Asano *et al.,* 1997; Burgess & Horwood, 2006). EntFM was extracted for the first time in a *B. cereus* FM1 strain, accountable for food-borne poisoning and called enterotoxin FM (EntFM). It was concluded to induce fluid aggregation at a higher dose in mouse and rabbit and genetically modified intestinal loop studies (Boonchai *et al.,* 2008; Tran *et al.,* 2010). The EntFM gene is believed to be situated on the chromosomes and tends to be like the *B. cereus* and *B. thuriengensis* strains, with frequency findings, have shown that EntFM is found in several infection-linked microorganisms (Ngamwongsatit *et al.,* 2008; Tran *et al.,* 2010). Fewer published studies are available about this protein and its contribution to virulence toxicity in *B. cereus* (Boonchai *et al.,* 2008).

1.5.8 Cereolysin O (CLO)

Cereolysin O is primarily known for its hemolytic activity, and temperature-labile can be induced by cholesterol level and is also called haemolysin-I (Ramarao & Sanchis, 2013). Hemolysin-I is known as thuringiolysin-O (TLO) in *B. thuriengensis* and anthrolysin-O (ALO) in *B. anthracis* (Ramarao & Sanchis, 2013). It has been characterized as a cholesteroldependent cytolysin, with the size of 55 kDa, forming pores in the eukaryotic membranes TLO and CLO. It is a part of the CBC family homologous of TLO and anthrolysin (ALO) that regulates the transcriptional activator of the phospholipase-C regulator (Gohar *et al.,* 2002). The CLO triggers the production of lactate dehydrogenase (LDH) in retinal tissue based on the i*n-vitro* method retinal toxicity (Ramarao & Sanchis, 2013). It might impair the necrosis of *B. cereus* endophthalmitis, although ALO can reduce the role of the epithelial boundary by rearing the junctional occluding protein (Beecher *et al.,* 2000; Ramarao & Sanchis, 2013).

1.5.9 Phospholipase

Two main phospholipase enzymes generated by *B. cereus* are known as phosphatidylcholinespecific phospholipase-C (PI-PLC) and phosphatidylcholine-specific

phospholipase-C (PCPLC) (Lindbäck & Granum, 2015). Phosphatidylcholine is smaller in size at 29.2 kDa enzymes. Its active site consists of three zinc atoms, which lead to the enzyme's catalytic and structural characteristics (González-Bulnes *et al.,* 2010). With little or no exclusive substrate present in the system, phospholipase can be attached to a membrane interface (Beecher & Wong, 2000a). Cooperative activity with sphingomyelinase is required for the lysis of erythrocytes of both pigs and humans to suppress sheep erythrocytes, hemolysis BL and to stimulate the hemolysis termination cycle (Beecher *et al.,* 2000; Beecher & Wong, 2000a). Phosphati-dylinosol is a 34.6 kDa enzyme, phosphati-dylinosol and phosphatidylcholine may be essential during respiratory infection consequent in tissue and necrosis haemorrhage (Granum, 1994). Phospholipase-C that usually hydrolyzes the polyglycan-containing membrane anchors and phosphatidylinositol (PI) which are structural essential components that belong to one class of membrane proteins (Celandroni *et al.,* 2014).

1.5.10 Sphingomyelinase (SMase)

Sphingomyelinase is an enzyme with a size of 34.2 kDa, its active site containing two magnesium atoms. It breaks down sphingomyelin to ceramide and phosphorylcholine in the membrane (Beecher & Wong, 2000a).

1.6 *Bacillus* infection therapy

Antibiotic treatment is still the standard treatment tool for *Bacillus* spp. infections (EhlingSchulz *et al.,* 2019). Nevertheless, advent of antibiotic exploitation has generated selective pressure to acquire resistance genes by gene transfer processes in microorganisms, resulting in treatment failures (Davies & Davies, 2010). First-line therapy for *Bacillus* infections involves tetracyclines, ciprofloxacin, vancomycin, clindamycin, and gentamicin. Nonetheless, resistance to β-lactam antibiotics, broad-spectrum cephalosporins, and ticarcillin-clavulanate have been reported for numerous *Bacillus* strains (Bottone, 2010; Tuazon, 2017). Reportedly, the inappropriate antibiotic treatment of *Bacillus* infections resulted in higher mortality rates (Ikeda *et al.,* 2015).

1.6.1 Antibiotic resistance encountered in *Bacillus* species

Antibiotic resistance is a global concern with discovering and synthesizing new antibiotics not discovered rapidly enough (Bebell & Muiru, 2014). Many factors have contributed to this, including expanding challenges in evaluating new drugs, cost increases and time required to develop drugs, the complexity of planning and carrying out final clinical trials, and concerns about drug durability being reduced due to the emergence of resistance. (Altevogt *et al.,* 2014). The incidence of antimicrobial resistance to medically necessary microorganisms in healthcare settings is strongly related to the misuse and prescription of antimicrobial agents, thus expanding the specific antimicrobial pressure in microorganisms (Ayukekbong *et al.,* 2017). The resistance possibly resulted in different resistance pathways by horizontal gene transfer of antimicrobial resistance and virulence genes and enhanced biofilm formation (Schroeder *et al.,* 2017).

Bacillus spp. known as useful for a wide variety of various biotechnology tools, for example, probiotic nutrient enhancements for human and animal feed inoculants, their potential to stimulate the immune response and inhibitory antimicrobial compounds based on the creation of pathogenic microorganisms (Adimpong *et al.,* 2012; Elshaghabee *et al.,* 2017). However, there is increasing public health pressure concerning the potential of microbe cultures used for nutritional supplements or food manufacturing as possible sources for gene transmissions that may induce antibiotic resistance in these microbes (Adimpong *et al.,* 2012; Gevers *et al.,* 2003).

The presence of *Bacillus* spp. highlights this issue. Commercially produced probiotic dietary enhancements for humans and animals are resistant to numerous antibiotic classes such as chloramphenicol, macrolides, tetracycline, lincosamide, and β-lactams (Adimpong *et al.,* 2012; Hoa *et al.,* 2000). Antibiotic resistance in *Bacillus* spp. is induced by either intrinsic properties (natural morphological characteristics) or the advancement of antimicrobial resistance genes via mobile genetic elements (transposable elements from other microbes and plasmids) or mutation of native genes (Adimpong *et al.,* 2012). The inherent or acquired characteristics allow the bacterium to inactivate antibiotics via the deterioration and exportation of antibiotics from the cell (Luthra *et al.,* 2018).

Data on the antimicrobial susceptibility patterns of *Bacillus* species is quite restricted. Resistance to erythromycin, tetracycline, and carbapenem by *B. cereus* have been documented and may jeopardize the selection of empiric alternative treatment options (Adimpong *et al.,* 2012; Turnbull *et al.,* 2004). As wound healing due to *B. cereus* infection, sensitivity to fluoroquinolones has been stable and effective (Bottone, 2010; Ehling-Schulz *et al.,* 2019). The excessive development of β-lactamase enzymes by many microbes, particularly *Bacillus*, is a common trigger of antimicrobial resistance to cephalosporins 3rd generation (Schlegelova *et al.,* 2003). This may result from genetic modification or the creation of a sensitive form in the existence of antibiotics (Davies & Davies, 2010). Some microbes, including the genus *Bacillus*, have been reported to contain β-lactamase production genes (Bush, 2018). Nonetheless, in wildtype *Bacillus* strains, these chromosomal β-lactamases do not always have adequate antibiotic resistance. β-Lactam resistance is due to inactivation of β-lactamases. Extended spectrum β-lactamases does not grant resistance to carbapenem, but the presence of different classes of carbapenem hydrolyzing enzymes do. The hydrolysis of amide bonds that are present in the β-lactam ring is catalyzed by β-lactamase and thus inactivates the antibiotic (Tahmasebi *et al.,* 2014).

High antimicrobial resistance to penicillin, cefepime ampicillin, and oxacillin by *B. cereus* strains is attributed to β-lactamase synthesis (Drawz & Bonomo, 2010). The β-lactamases of Gram-positive microorganisms are mostly extracellular, varying on the growth requirements; some enzymes bind to the cytoplasmic membrane (Tahmasebi *et al.,* 2014). Homologous proteins induce β-lactamases; three types have been identified amongst the various *B. cereus* species. β-lactamase-I is class-A that is known as an extracellular serum penicillinase in the active site. β-lactamase III is a class-A membrane-bound lipoprotein that has a secreted form. β-lactamase II, class-B β-lactamase, promotes the binding of cobalt (II) as well as cobalt (II) ions (Bush, 2018; Drawz & Bonomo, 2010). Erythromycin is one of the most used macrolide antibiotics to treat infections. Erythromycin resistance gene confers resistance by methylation gene regulation of 23S rRNA. Macrolides receptors have been reported in *Bacillus* species, commonly in *B. licherformis*, *B. subtilis* (*ermD* and *ermK*), *B. anthracis* (*ermB*) also *B. claussi* (*erm34*) (Adimpong *et al.,* 2012; Dai *et al.,* 2012). Tetracycline is a broad-spectrum antibiotic utilized to cure infections in humans, animals, and

insects (López *et al.*, 2008). Tetracycline resistance is caused by three mechanisms: bacterial ribosome protection, enzymatic inactivation of the tetracycline molecule, and energy-dependent efflux. (López et al., 2008). Tetracycline resistance genes present in *B. cereus* are accountable for the high level of resistance, which is most likely due to the horizontal transfer of antibiotic resistance genes from other microbes to *B. cereus.* However, the occurrence of such resistant genes in *Bacillus* species is not confirmed (Agersø *et al.*, 2002). There is insufficient data on the prevalence of tetracycline resistance genes to determine the source of these genes. Amplification was detected using plasmid DNA and no amplification when chromosomal DNA was used as a reference. This suggests that tetracycline-resistant characteristics exist more often on the plasmid instead of chromosomal (Rather *et al.*, 2012). Previously it was noted that *B. cereus* cluster plasmid DNA carries the *tetL* gene, another *Bacillus* species-genus carry either *tetK* or *tetL* on the plasmid or chromosomal DNA (Agersø *et al.*, 2002). Most tetracycline-resistant genes frequently originate in Gram-positive microbes. However, although the genes may be generated in the presence of conjugal plasmids, they cannot be transferred independently, restricting their spread within the species. (Agersø *et al.*, 2002; Chopra & Roberts, 2001). There are two primary tetracycline genes, *tetK*, and *tetL*, that encode efflux proteins known for pumping out tetracycline and doxycycline (Grossman, 2016). Among the tetracycline resistance genes, *tetM* has been the most common gene in Gram-positive microbes and is known for encoding ribosomal associated protection protein that protects the bacteria from being susceptible to minocycline, doxycycline, and tetracycline (Chopra & Roberts, 2001; Eliopoulos *et al.*, 2003).No known report on the presence *tetM* in the *Bacillus* genus was found but shown within a laboratory set-up through genetic modification. Presence of a tetracycline antimicrobial resistance gene on a potential mobile element of the sequenced plasmid pBC16 suggested that resistance to antimicrobials such as penicillin, kanamycin, gentamicin, kanamycin, and tetracycline could be attained through plasmid transmission among related Gram-positive organisms such as *B. cereus* (Abdel-Shakour & Roushdy, 2010; Schlegelova *et al.*, 2003).

1.7 Diagnostic challenges of *Bacillus*

The similarity between related species sharing patterns of genetic, biochemical, and morphological characteristics, classifying *Bacillus* species in the laboratory using standard methods is difficult (Celandroni *et al.*, 2019). When using the plating method, a combination

of Gram-stain and colony expression may be used as an appropriate indicator of the presence of *Bacillus* species in the clinical specimen. (Aruwa & Ogunlade, 2016; Carroll *et al.,* 2020). The best approach accessible in the laboratory for identifying species was the use of morphological and physiological protocols, but these methods were demonstrated to be very time-intensive and labour-intensive (Aruwa & Ogunlade, 2016; Franco-Duarte *et al.,* 2019).

In 1876, the first isolated and cultured *B. anthracis* was achieved by Robert Koch. He began by inoculating healthy mice by the tails with splenic tissue from an infected cow using slivers of wood (Compton, 1987). The classical method used for identifying *Bacillus* species requires a unique selective medium, time-consuming, and costly to prepare (Aruwa & Ogunlade, 2016). Numerous media claim to cultivate specific species; however, most of these are not selective. *Bacillus* strains do not form endospores under all cultural conditions (Fritze & Claus, 2003; Fritze & Pukall, 2011). Sporulation is inducible, e.g., by limitation of nutrient factors, including carbon or nitrogen. Further, most *Bacillus* strains form endospores in media supplemented with manganese salts (Fritze & Pukall, 2011; Sinnelä *et al.,* 2019).

The only selective media for aerobic spore-forming bacteria in the mesophilic/neutrophilic range has been established for the *B. cereus* group. Despite the differences in the possible virulence, the separation of the *B. cereus* members remains a challenge. A *Bacillus*-specific medium is needed for the direct isolation of the species under this genus. (Fritze & Pukall, 2011). Polymyxin egg yolk mannitol bromothymol blue agar (PEMBA), mannitol egg yolk polymyxin agar (MYP or MEYP), brilliance agar, and *Bacillus* chromogenic medium (BCM) is the most widely used plating media for *Bacillus* species identification (Tallent *et al.,* 2012). When culturing on MYP, *Bacillus* colonies are pink, on PEMBA colonies appear peacock blue, and on Bacara, a new chromogenic agar, it appears as orange-pink colonies. *B. cereus* colonies on Brilliance and BCM appear turquoise green (Shirmohammadi *et al.,* 2014; Tallent *et al.,* 2012). All three media contain egg yolk, and if the bacteria produce lecithinase, the colony is enveloped by a halo region. Problems encountered with MYP include a lack of clear colonial morphology and lecithinase production. This allows the growing properties to be obscured by the underlying vegetation, which ferments mannitol and creates lecithinase (Tallent *et al.,* 2012; Tewari *et al.,* 2013). The

overlapping precipitation zone and the proclivity for *B. cereus* colonies to coalesce (joining elements to form a multicellular structure to form a clump) impede reliable enumeration (Peng *et al.*, 2001; Tallent *et al.*, 2012). Newer chromogenic media formulations incorporate enzyme substrates to enable the detection of several microbes. The use of this species-specific media can remove the necessity of sub-culturing and biochemical tests, leading to faster identification and reduced costs for reagents required to confirm the organisms (Perry, 2017; Tewari *et al.*, 2013).

The primary aims of modern identification systems are that they reduce identification time, increase throughput of samples and achieve these within a highly automated, reliable and flexible piece of laboratory equipment (Stager and Davis 1992). Automation of conventional tests presented the possibility of using such approaches in industrial and clinical environments, leading to the development of an automated microbial identification system, the VITEK (bioMe´rieux Inc., Durham, NC) (Hilket *et al.,* 2009). The first-generation VITEK BAC (*Bacillus*) card allowed identification of only 17 *Bacillus* species, but its performance was proven reliable in comparison with other phenotypic methods.

1.7.1 Analytical Profile Index (API) tools

Due to similar phenotypic characteristics of *Bacillus* spp. and the need for strictly regulated settings during detection, it is challenging to classify closely related species with traditional techniques (Irenge & Gala, 2012). The API is based on the principle that microorganisms breakdown various substrates during metabolism to release by products and metabolites which can be detected by a change in colour and on addition of specific reagents. Both the API 20E and the API 50 CHB, have been adapted to the identification of this group of organisms.

. The kit includes up to 20 plastic microtubes containing dehydrated substrates for enzymatic activity or sugar consumption/fermentation by inoculated microorganisms. The positives or negatives lead to a numeric value compared to a bacterial database (Dilnessa *et al.*, 2016;

Neppelenbroek *et al.*, 2014). The *Bacillus* spp. API identification system requires both API 50CHB and API 20E (bioMérieux, France) to identify the bacterium and describe genus-level strains (Agamennone, 2018).

The method is based on detecting metabolization of substrates by the microorganisms, revealed by a colour change due to the precipitation or enzymatic conversion. Alternatively, fermentation is detected by a drop in pH. The biochemical panel of the API 20E test measures the metabolism of various carbohydrates and amino acids and detects specific enzymes such as gelatinase and β-galactosidase. The API 50CH test provides a detailed carbohydrate fermentation profile (Agamennone *et al.,* 2019). The use of these identification strips has been shown to offer additional accurate and reproducible results better than conventional techniques such as plating techniques.

1.7.2 Molecular identification with the polymerase chain reaction

Bacillus species have specific identifiable characteristics such as resistance or toxin genes, which requires molecular methods to be detected. With advanced in technology , ribosomal RNA (16S rRNA) gene fragment sequencing is a common substitute to the conventional techniques (Maughan & Van der Auwera, 2011). With the advance in technology, ribosomal RNA (16S rRNA) gene fragment sequencing is a common substitute intended for conventional techniques and has numerous benefits when identifying a microorganism (Srinivasan *et al.,* 2015).

Different PCR identification tests, including real-time PCR approaches, have indeed been established for specified microorganisms. These methods (molecular method) are extremely efficient, provide high-throughput results with simple automation, without any post-PCR detection analyses, and are low-cost resources for rapid detection and identification, particularly in medical or environmental specimens (Forghani *et al.,* 2016; Naravaneni & Jamil, 2005). The most clinically important species in the *Bacillus* genus is *Bacillus Cereus* group encompassing a variety of genetically closely related associated species, which are concise under the designation *Bacillus cereus* sensu-lato. As a result, PCR distinguishes

species within the larger *B. cereus* group as well as *B. cereus* from other bacilli groups and bacterial genera (Kadyan *et al.,* 2013; Němečková *et al.,* 2012).

Cultural distinction and enumeration of putative *B. cereus* is the traditional method for identifying *Bacillus* species in food microbiology laboratories (Celandroni *et al.,* 2019). Due to their associated genetic relationship, members of the *B. cereus* group are difficult to distinguish by traditional plating identification techniques or molecular 16S rDNA sequencing. They are thus assumed to be probable *B. cereus* (Celandroni *et al.,* 2019). Several speciesspecific primer techniques such as phage-typing, mass spectrometry, serotyping, gas chromatography, enzyme-linked immunosorbent assay (ELISA), and plasmid profiles have been used to identify and detect *Bacillus* spp. (Němečková *et al.,* 2012).

1.7.3 Molecular tools for genetic diversity and typing

Microbial typing of the hospital and environmental samples are utilised to identify the source and pathways of diseases, verify, or rule out outbreaks, track cross-transmission of healthassociated infectious diseases, identify drug-resistant strains and examine the efficacy of protection measures. There are various DNA-based typing methods available for identifying or characterizing microorganisms. Molecular typing is recognized as an efficient method of outlining the source of a pathogen and understanding the epidemiology of the pathogens (Ruppitsch, 2016; Van Belkum *et al.,* 2007). Molecular typing tends to discriminate within the same species by using their chromosomal difference (Singh *et al.,* 2006). Typing has shown higher discriminatory power than obtained by conventional phenotypic techniques, such as phage typing, serotyping, pyocin tying, and bio-typing (Ferrari *et al.,* 2017).

Molecular typing is recognized as an efficient method of outlining the source of a pathogen as well as understanding the epidemiology and prevalence of the pathogens (Adzitey *et al.,* 2013; Gao *et al.,* 2018). The most commonly used molecular typing techniques are PCRbased DNA fingerprinting techniques, which include restriction analysis of an amplified region carrying the 16S rRNA spacer region, enterobacterial repetitive intergenic consensus (ERIC)PCR, repetitive extragenic palindromic sequences (REP)-PCR, BOX-A1R-based

repetitive extragenic palindromic (BOX-PCR) (is a fingerprinting analysis based on the BOX gene), random amplification of polymorphic DNA (RAPD), restriction analysis of the amplified 16S rRNA gene (ARDA-16S), and part of the 23S rRNA gene (ARDRA23S+spacer), multi-locus sequence typing (MLST), amplified fragments length polymorphism (AFLP) and pulse-field gel electrophoresis (PFGE).

1.7.3.1 Enterobacterial repetitive intergenic consensus (ERIC-PCR)

ERIC-PCR has been identified as the simplest and most effective method for determining genetic diversity between strains, as it can reasonably justify the correlation of genotypic and phenotypic characteristics. (Gao *et al.,* 2018). It is based on the targeting of repeated DNA sequences with oligo-nucleotide-specific primers, commonly used for microorganisms epidemiologic studies typing and risk assessment of pathogenic bacteria like *Bacillus* strains. Commonly used for microbial epidemiological typing and are widely used for risk assessment of harmful bacteria such as *Bacillus* strains (Gao *et al.,* 2018). ERIC-PCR is an affordable, responsive, and effective molecular typing method that needs no advanced knowledge of the genome (Bakhshi *et al.,* 2018; Ranjbar *et al.,* 2017).

It has been implemented in different types of strains of *B. cereus*. However, few experiments exist on the typical fingerprint analysis of *B. cereus* isolates to associate genetic traits with the biological characteristics of various strains (Gao *et al.,* 2018). Nonetheless, classification relying on 16S rRNA gene sequences is constrained and cannot generate taxon specific markers with different specificities (Poretsky *et al.,* 2014). Gao *et al.* (2018) performed research on the molecular typing of the *B. cereus* using selected primers based on the repetitive element sequences in Chinese pasteurised milk samples. ERIC-PCR analysis revealed high genetic diversity; notably that this technique was appropriate for examining the association amongst genotypic and phenotypic traits. ERIC-PCR is characterized by being quick, simple to implement, and has good discriminatory power. Besides, it has type ability, but low reproducibility compared to the excellent reproducibility of, for example, PFGE (Adzitey *et al.,* 2013).

1.7.3.2 Pulsed-field gel electrophoresis

The agarose gel electrophoresis methodology required to separate large DNA fragments using a charge that determines the gel's three directions is known as a common feature for the pulsed field gel electrophoresis (PFGE) (Adzitey *et al.,* 2013). Restriction enzyme recognizes a specific nucleotide sequence in the DNA, called a restriction site, and cuts the DNA molecule at only that specific sequence. (Adzitey *et al.,* 2013; Shi *et al.*, 2010).These unique patterns will be revealed using pulse filed gel electrophoresis. It is practical for the profiling and epidemiological classification of *B. cereus* strains. It has a high degree of differential control, repeatability, and typology. However, the method is labour intensive and expensive compared to other typing methods (Sharma-Kuinkel *et al.,* 2014). PFGE has proven to be superior to most other methods for biochemical and molecular typing. PFGE provides a high-resolution, macro-restriction analysis at the genome level.

It is widely acknowledged as the gold standard of typing, having been used to investigate many food-borne microbes including other epidemiological research (Adzitey *et al.,* 2013; Alonso *et al.,* 2005). A genotype study investigated HAI's caused by *B. cereus* in a hospital in Tokyo in 2013, causing bacteriemia in 13 patients and, unfortunately, fatal in two patients. PFGE analysis revealed 19 clusters, one-third contained in a single PFGE cluster (Akamatsu *et al.,* 2019).

1.7.3.3 Repetitive extragenic palindromic sequences (REP-PCR*)*

Repetitive extragenic palindromic sequence PCR is based on repeated DNA elements of the bacterium under investigation or present in foodborne pathogens (Adzitey *et al.,* 2013; Figueroa *et al.,* 2015; Wang *et al.,* 2015). Repeated extragenic palindromic DNA elements found throughout the microbial genome are amplified to create profiling of varying sizes unique to each strain (Wang *et al.,* 2015).

It is widely known for being much more cost-effective, less time-consuming, and accessible to minor or significant quantities of isolates. The findings have a working

relationship with the information acquired by PFGE but a lesser discriminating ability (Adzitey *et al.,* 2013). Lee *et al.* (2012) analysed *B. cereus* strains using the Rep-PCR fingerprint technique cultured from Sunsik, a ready-to-eat Korean food made of vegetables, fruits and grains. The typing of *B. cereus* strains revealed a higher degree of genetic variation, with a similarity of less than 31 %. RAP-PCR patterns differed between strains with similar toxic or antibiotic resistance profiles, indicating that the presence of toxin-encoding genes or antimicrobial-resistant determinants was not related to the genetic background (Lee *et al.,* 2012).

1.7.3.4 Random amplified polymorphic DNA (RAPD)

Randomly amplified polymorphic DNA is a technique by which a 10-mer primer is used to randomly amplify sample DNA fractions under less-stringent polymerase chain reaction conditions (Adzitey *et al.,* 2013; Babu *et al.,* 2014). The benefits of RAPD are that it is costeffective, fast, easily accessible, and simple to execute. Nonetheless, the reproducibility is low, moderate discriminatory power, and approximately 80% type-ability (Adzitey *et al.,* 2013; Rezk *et al.,* 2012; Shi *et al.,* 2010). Over the past decades, RAPD-PCR was the established technique for molecular typing of various *Bacillus* species members. It was used to establish the epidemiological relationship between members of *B. cereus* group, *B. lentus,* and *B. anthracis* (Ehling-Schulz & Messelhäusser, 2013).

RAPD may also be an essential testing tool in routine diagnostic laboratories for emetic *B. cereus* illness (Ehling-Schulz *et al.,* 2005). The method showed to be highly discriminatory for the epidemiological examination of infections or outbursts due to *B. cereus* (Ehling-Schulz & Messelhäusser, 2013).

1.7.3.5 Deoxyribonucleic acid sequencing techniques

The sequencing includes methods used to determine a sequence of the DNA bases known as guanine, adenine, thymine, and cytosine. Sequencing is frequently utilised in typing, identification, description, and phylogenetic analysis of unidentified or new bacterial strains

(Adzitey *et al.,* 2013). Notably is its high discriminatory power, 100 % type ability, and robust reproducibility. Disadvantages include extended testing periods (two to three days to complete), expensive, and the equipment often has limited availability compared to equipment utilized by other typing methods (Wassenaar & Newell, 2000).

Zhang *et al.* (2011) conducted a study where *dnaJ*, a housekeeping gene which is known as Hsp40 molecular chaperone protein of *B. cereus* strains. This gene was obtained from the incidents of hospital infections and sequenced using the deoxyribonucleic acid sequence method to separate highly pathogenic incident strains of *B. cereus* from another *Bacillus* spp. According to assays, blood culture dnaJ sequence resemblances ranged from 93 % to 100 % of *B. cereus* strains identified from sequenced cases within hospitals. This method was used to sequence *B. cereus* group strains isolated from outbreaks of hospital infection to distinguish highly pathogenic outbreak strains of *B. cereus* from other sources of *Bacillus* spp. The difference discovered was that six *B. cereus* epidemics showed strains resembling *B. anthracis* at 99 % to 100 % sequencing similarity (Zhang *et al.,* 2011).

1.7.3.6 Multi-locus sequence typing (MLST)

Multi-locus sequence typing, known as a straightforward, compact, and sequence-based procedure for typing microbes, utilises inner fragment sequences of seven housekeeping genes (Ehling-Schulz & Messelhäusser, 2013; Urwin & Maiden, 2003). MLST was established centered on the principle of multi-locus enzyme electrophoresis (MLEE) by allocating alleles to various housekeeping genes by DNA sequencing directly rather than indirectly by electrophoretic mobility (Barker, 2018; Vanhee *et al.,* 2009). For most bacteria, the benefits of MLST include clear, portable, highly accurate, extremely inconsistent, compact, and reliable data entry (Lamon *et al.,* 2015; Urwin & Maiden, 2003). Numerous MLST-based typing systems for *B. cereus* have been produced; effectively used to determine the genetic connection between *B. cereus* strains with diverse background sources such as soil, insects, fruit, and human (Okutani *et al.,* 2019).

Several MLST has been identified for the genotype of *B. cereus*, and a study revealed a clonal population trend for *B. cereus* and associates *B. thuriengensis* and *B. anthracis* (Hoffmaster *et al.,* 2008). The MLST typing method has utilised to assess whether *B. cereus* strains connected with diseases consist of fluctuating degrees of complexity, e.g., severe gastrointestinal versus systemic disease (Hoffmaster *et al.,* 2008). Hoffmaster *et al.* (2008) conducted a study on *B. cereus* strains associated with severe pneumonia and septicemia. Samples were collected from Special Bacteriology Reference Laboratory, Centers for Disease Control and Prevention, with isolates representing strains collected from 19 states in the USA between the year 1954-2004. The analyses of the collective 55 isolates showed diversity and consisted of two distinct clades and 38 sequence types (ST), 27 isolates connected with severe infections. Isolates from these samples are reportedly accommodated in the clade 1/cereus III lineage, linked to severe diseases, whereas clade 1/cereus II has been linked to both acute and emetic illnesses. Out of 38 sequence types, only three ST's were identified repeatedly for epidemiologically distinctive isolates (Hoffmaster *et al.,* 2008).

1.8 Description of public hospital and level of healthcare in South Africa

Public hospital services are usually provided by the government through national healthcare systems, whereas the private hospital offers services for the hospital profit or self-employed practitioners (Basu *et al.,* 2012). Private healthcare in South Africa is usually available to those earning much higher, thus exhibiting great socioeconomics and racial disparities, with 7 % of black Africans having access to private healthcare compared to about 70 % of white South Africans (Urban *et al.,* 2012). Public hospitals provide healthcare services for all citizens, especially disadvantaged or low-income people (Kim & Kim, 2013).

Public health benefits include free care for all citizens, including pharmaceutical care, wheelchairs, grips/crutches, toilet seats, and home care visits. Public hospitals face major medical system challenges, including negative staffing behaviour, long waiting times, and less sterile maintained facilities. Inadequate infection control, as well as a lack of medication, jeopardized the safety and security of both staff and patients (Department of Health, 2017). A survey of 488 Gauteng public hospital patients conducted in 2007 revealed patients' expectations of doctors, nurses, prescription drugs, and adequate medical care. Medical

equipment, bathroom cleanliness, and other facilities were not met (Hasumi & Jacobsen, 2014). South African healthcare system is structured in five layers, namely, primary health care (clinics), district, regional, tertiary (academia), and central (academia) (see Table 1-3).

Table 1-3: Classification of hospitals in the public sector and level of care in South Africa (Department of Health, 2012), and the National insurance policy (Department of Health, 2017).

Level of	No of beds	Description	Service offered	Specialised field hospital
District (Level 1)	Small: 50 to 150 Medium:150 to 300 Large: 300 to 600	• Categorized into the small, medium, and large district. • Support primary care. • Provide healthcare on a 24-hour basis. • Where practical's, provides training for healthcare service providers. • Receive support and outreaches from specialised based at regional hospitals.	• General practisers, clinical nurses practitioners, and primary health care services.	▪ Provide these specialised services: internal medicine, general surgery, family physician, obstetrics and gynaecology and paediatrics health services.
Regional (Level 2)	200 to 800	• Provide service on a 24-hour basis. • Provides training to healthcare providers. • Receives outreach and support from tertiary hospital. • Services are limited to provincial boundaries.	• Provides services in Internal medicine: General surgery: Pediatrics: Obstetrics and gynaecology	• Special services in at least one of the following services: orthopaedics, surgery, psychiatry, anaesthesia, diagnostic radiology, trauma and emergency unit, short-term care and critical care unit.

Tertiary (Level 3)	400 to 800 beds	• Provide specialist level of services. • Provides intensive monitoring services under the joint supervision of experts. • Receives referrals from regional hospitals not limited to the province.	• This level of services includes sophisticated diagnostic and treatment services.	• General experts include diagnostic services such as anaesthesia, general surgery, internal medicine, obstetrics and gynaecology, orthopaedics, pediatric psychiatry: radiology, and pathology.
Central	Maximum of 1200 beds	• Provide tertiary hospital services. • Provide training for health care providers • Accept patients referred from multiple states/provinces. • The medical school needs to be involved as the primary educational platform. • Central and national referral services.	• This level of comprehensive healthcare services includes advanced diagnostic and therapeutic services.	• General specialists in anesthesiology, general surgery, internal medicine, obstetrics, and gynaecology, orthopaedics, paediatrics, psychiatry, radiology, and pathology.

1.9 Justification for the study

The prevalence of bacteria on hospital surfaces has been well-documented, posing a risk to patients. Hospital-acquired infections have raised global concerns in hospital environments. Several infections in hospitals lead to challenging diseases caused by Gram-positive bacteria such as *Bacillus* (Khan *et al.,* 2015; Revelas, 2012).

Although several studies have been published about the bacteria disseminations in hospitals, especially internationally, there is a lack of South African data. This study provides awareness of the recent dissemination of genus *Bacillus* in South African public hospitals and investigates the antibiotic and the virulence profile. The study hopes to provide insight into the current hospital environment looking at different hospital levels (central, district, regional and tertiary) and three distinct wards that face different challenges compromising IPC practices. The study intended to identify resistance and virulence profiles of *Bacillus* strains that survive in the hospital environment. The study would provide health practitioners with some guidelines concerning therapeutic options for patients infected within the hospital environment.

1.10 Aim and objectives

1.10.1 Aim

The study aimed to investigate the prevalence and dissemination, antimicrobial susceptibility profile, antibiotic and virulence genes, and the level of genetic diversity of *Bacillus* species in four public health facilities at different levels of health care in South Africa.

1.10.2 Objectives

The following are the specific research objectives:

- To determine the prevalence of *Bacillus* species in four public hospital environments in the KwaZulu-Natal province, South Africa.
- To assess the *Bacillus* prevalence in three different wards (pediatric, ICU, and general ward).

- To examine common-touch surfaces within the hospital environment, viz. door handle, ward telephone, occupied and unoccupied bed, drip stand, blood pressure monitor, mop, patient file, ventilator, sink, and nurse' table as a risk of contamination with *Bacillus* isolates;
- To determine the antibiotic resistance profile of the *Bacillus* isolates to the following antibiotics: erythromycin, ciprofloxacin, tetracycline, meropenem, amoxicillin, and cefotaxime using minimum inhibitory concentration (MICs) method;
- To determine the occurrence of genes causing virulence (*bceT, cytK, entFM, hblA, hblC, hblD, nheA, nheB, nheC, HIyll,* and *ces*) and antimicrobial resistance genes *(blm, ermB, tetA, tetB, tetK,* and *tetM*) using specific primers through conventional PCR methods and;
- To analyze the genotypic diversity of *Bacillus* species using ERIC-PCR.

1.11 Hypothesis

Alternative hypothesis (H_A): *Bacillus* species are present in the hospital environment within the four public health facilities at different levels of healthcare in KwaZulu-Natal, South Africa.

Null hypothesis (H_o): No notable presence of *Bacillus* spp. in the hospital environment within the four public health facilities at different levels of healthcare in KwaZulu-Natal, South Africa.

1.12 Research Question

What is the prevalence rate of *Bacillus* bacteria in the four public hospitals identified in the eThekwini district, KwaZulu-Natal, South Africa?

1.13 Study Outline

Chapter 1: **Introduction and literature review**

This chapter provides a background to the whole study, including the aims and objectives. It also comprises a brief review of the literature on disseminating bacteria in the hospital environment and the role players contributing to hospital-acquired infections. The review then focuses on the primary study species, the Gram-positive *Bacillus* species, concerning its distribution, virulence, and antimicrobial resistance profile. In addition, the review provides a perception of the different levels of healthcare in South Africa, providing insight into the hospitals that participated in this study.

Chapter 2: **Occurrence, antibiotic resistant, virulence genes, and genetic diversity of *Bacillus* spp. from public hospital environments in KwaZulu-Natal, South Africa.**

This chapter describes the prevalence, phenotypic and genotypic characteristics of *Bacillus* spp. in public hospitals to be conducted using selective media, biochemical testing, and molecular methods. It draws inferences from the results that hospitals harbour bacterial strains that are mostly linked with the diarrheal syndrome and may assist with therapeutic options for hospital-acquired infections. This chapter is prepared for resubmission to an international journal. The presentation of chapter two is in a manuscript format as a requirement by the College of Health Sciences at the University of KwaZulu-Natal. The manuscript was submitted to the *Journal of Microbial Drug Resistance*; Manuscript ID: MDR-2020-0543.

Chapter 3: **Conclusions, limitations, and recommendations**

This chapter examines whether the study was able to address the study's fundamental questions. Thus, it addresses a summary of the conclusions, the contribution of knowledge to the literature on the distribution, virulence, and antimicrobial resistance profile of *Bacillus* species from the hospital environment. It further reports on the limitations encountered and outstanding issues and proposes suggestions for future research.

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CHAPTER 2: OCCURRENCE, ANTIBIOTIC RESISTANCE, VIRULENCE GENES AND GENETIC DIVERSITY OF *BACILLUS* SPP. FROM PUBLIC HOSPITAL ENVIRONMENTS IN KWAZULU-NATAL, SOUTH AFRICA

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Contributions

- Miss Zamile N. Mbhele, as the main author, developed the study protocol, performed laboratory work and data analysis, wrote the manuscript.
- Mrs Christian O. Shobo aided in the execution of laboratory work and assisted in conceptualising the study and review of the manuscript.
- Dr Daniel G. Amoako assisted helped in the, designing the protocol for the study, analyses, and critical reviewing of the manuscript.
- Dr Oliver T. Zishiri, as co-supervisor, aided in the conceptualisation of the study, analysis of data and critical reviewing of the manuscript.
- As the principal investigator, Dr Linda A. Bester conceptualised and funded the study, design and data analysis, and critical reviewing of the manuscript.

ABSTRACT

Aim: Information on the prevalence, phenotypic and genotypic characteristics of *Bacillus* spp. in hospitals in Africa is scarce. This study aimed to determine the dissemination of *Bacillus* species in four public hospitals representing different levels of healthcare within the eThekwini district in KwaZulu-Natal, South Africa.

Methods: The study, conducted over three months during 2017, examined representative samples obtained from three wards (general ward, intensive care unit (ICU), and paediatric unit) from four public hospitals, referred to as 'A' for the central; 'B' for the tertiary; 'C' for the regional and 'D' for the district hospitals. Swabs collected from 11 predetermined hospital surfaces were screened and analysed using selective media, biochemical testing, and molecular viz. PCR. The minimum inhibitory concentrations to evaluate antibiotic susceptibility were used, according to CLSI guidelines, and typing was conducted using enterobacterial repetitive intergenic consensus (ERIC)- PCR.

Results: Overall, 17 % (135/777) isolates were identified, with a prevalence of 24 % (32/135) for the central hospital; 33 % (45/135) for the tertiary hospital; 27 % (36/135) for the regional hospital and 16 % (22/135) for the district hospital. It was further confirmed that the *Bacillus* species belonged to either *B. cereus* (96 % (129/135)) or *B. subtilis* (4 % (6/135)). The prevalence was similar across the wards, averaging 33 % (45/125). The highest prevalence of *Bacillus* isolates was found on the drip stands (12 % (16/135)); sink (12 % (16/135)); ward phone (12 % (15/135)) and nurses' tables (10 % (14/135)). There was a statistically significant difference in *Bacillus* prevalence (p = 0.044) between tertiary and regional hospitals (p = 0.000). There was no statistically significant difference between the wards (p = 0.133). A positive correlation was observed between hospital and wards (r = 0.525, p = 0.000). MICs revealed a high resistance to β-

lactams, fluoroquinolones, and tetracyclines. The most common resistant genes detected were *ermB* 56 % (33/59) (erythromycin resistance) and t*etM* 5 % (5/112). No statistically significant difference was observed between the antibiotics tested ($p \geq 0.05$). Enterotoxin virulence genes showed 77 % (104/135) and 88 % (119/135) for *hblA* and *hblD*, respectively. These genes are associated with diarrhoeal syndrome. However, no *ces* genes (cereulide toxin) for emetic syndrome were detected. The Enterobacterial repetitive intergenic consensus sequences PCR (ERIC-PCR) revealed considerable diversity at the different levels of healthcare, although the clonal dissemination of strains between sites or wards within each specific hospital was revealed.

Conclusion: The study highlighted the prevalence of *Bacillus* spp and, more specifically, *B. cereus* in the public hospital environments studied. The study also revealed that diarrhoeal syndrome is more likely to be the outcome of a *Bacillus* hospital-acquired infection.

Significance and impact of the study: Bacterial strains associated with the diarrhoeal syndrome that hospitals harbour may assist with therapeutic options for *Bacillus* hospital-acquired infections. Also, *Bacillus* may be considered as a target organism when conducting hospital surveillance on hygiene management.

Keywords: hospital-acquired infections, hospital environment, *Bacillus* spp, genetic relatedness, diarrhoeal syndrome.

2.1 Introduction

Contaminated surfaces within hospitals are, potentially, sources of nosocomial microbial pathogens (Palza *et al.,* 2018; Phoon *et al.,* 2018; Suleyman *et al.,* 2018). Contamination on surfaces, frequency of contact and contamination from hands or surfaces, and the ability of bacteria to grow and divide are all factors influencing the transmission of bacteria in hospital settings (Boyce, 2007; Phoon *et al.,* 2018). The primary modes of transmission of these bacteria are through contaminated medical devices such as stethoscopes; respiratory devices; gowns; door handles; bed rails; call buttons; masks; gloves; and the splashing of contaminated water on sterile equipment (Fijan & Turk, 2012; Grass *et al.,* 2011; Inweregbu *et al.,* 2005). These bacteria can be subsequently transferred from the environment to a healthcare worker, janitorial staff, or even a community member. Subsequent contact with a patient's skin may result in an infection, which is referred to as a hospital-acquired infection (Cohen *et al.,* 2012; Pittet *et al.,* 2006).

In South Africa, public hospitals are government institutions that receive support through the national healthcare system. Long waiting times, hurried appointments, outdated services, and inadequate disease control and prevention are all drawbacks of public healthcare (Young, 2016). Most of these are due to social and healthcare deficiencies that are exacerbated by economic difficulties (Rispel, 2016). Nosocomial infections, also known as hospital-acquired infections (HAIs), are infections that appear in hospitalised patients for the first time 48 hours after admission (Khan *et al.,* 2015; Hewlett & Rupp., 2012). These infections were not present upon admission to the hospital (Revelas, 2012). There are two sources of HAIs: the infections that hospitalised patients acquire from the hospital environment and the infections transferred by individuals who enter the hospital or who are working at the hospital (Akbari *et al.,* 2018). According to reported studies, half of all HAIs fall into the former category (Akbari *et al.,* 2018).

This genus has been grouped into two major species groups: *B. cereus* and *B. subtilis,* and consists of both pathogenic and non-pathogenic, harmless species (Kim *et al.,* 2005; Logan, 2011). The *B. subtilis* group is common in soil and comprises *B. amyloliquefaciens; B. licheniformis* ; *B. subtilis; B. atrophaeus*; *B. vallismortis;* and *B. sonorensis* (Logan, 2011). *Bacillus* species are usually considered a contaminant in clinical settings. The overridingly dominant human pathogen in this group is *B. cereus,* which is the common pathogen in food poisoning, causing both local and systemic infections in individuals (Bottone, 2010; Cheng *et al.,* 2017; Ehling-Schulz *et al.,* 2006; Veysseyre *et al.,* 2015). Sporadic infections by *Bacillus* spp. are surgical wounds; panophthalmitis (inflammation of internal and external tissue of the eye) (Rishi *et al.,* 2013); pneumonia; bacteremia (Avashia *et al.,* 2007); meningitis; sepsis; the central nervous system (CNS); and soft tissue infections (Lee *et al.,* 2010) reported. Infections attributed to *B. cereus* were fatal in 10% of the reported cases in hospital settings (Bottone, 2010; Decousser *et al.,* 2013; Evreux *et al.,* 2007; Glasset *et al.,* 2018).

In comparison to the rest of the world, Africa has few studies on hospital *Bacillus* infections. In Morocco, the incidence of nosocomial infections in 2009 was approximately 8 % in provincial hospitals 8 % in regional hospitals, and between 9 and 11 % in a teaching hospital (Lalami *et al.,* 2016). In 2017, the *Bacillus* spp. was reported on the hands (4 %), uniforms (6 %), and throats (14 %) of personnel when studied in different wards in a hospital in Isfahan, Iran (Akbari *et al.,* 2018). According to a multi-centre bloodstream infection surveillance in eight teaching hospitals in Japan, between 2010 and 2014, *Bacillus* spp. peripheral catheter-associated haemorrhagic infections (PCBSI) ranked fourth (7 %) among pathogens, while central nervous system (CNS) infections ranked first (42 %) and *Bacillus* spp. ranked tenth (1 %) among the causative agents of central line-associated haemorrhagic infection (Kurai *et al.,* 2015; Kutsuna *et al.,* 2017).

Bacillus cereus induces two types of gastroenteritis: diarrhoeal and emetic syndromes. (AlAbri *et al.,* 2011; Bottone, 2010; Ehling-Schulz *et al.,* 2006; Messelhäußer & Ehling-Schulz, 2018). Diarrhoeal syndrome is related to numerous enterotoxins such as hemolysin BL (HBL); non-hemolytic enterotoxin (Nhe); cytotoxin K (CytK); enterotoxin FM (EntFM); enterotoxin T

(bceT); and, potentially, enterotoxin hemolysin II (hlyII) (Messelhäußer & Ehling-Schulz, 2018; Schraft & Griffiths, 2006; Yang *et al.,* 2005). Emetic syndrome causative agent is the toxin cereulide, reportedly produced by non-ribosomal peptide synthetase encoded by the *ces* gene cluster (Ehling-Schulz *et al.,* 2005b; Owusu-Kwarteng *et al.,* 2017). Unlike the emetic enterotoxins, cereulide is a thermal and acidic tolerant depsipeptide (Gao *et al.,* 2018; Rajkovic *et al.,* 2008).

Gastroenteritis diarrhoeal syndrome outbreaks were reported in the Royal Hospital in Oman in May 2008, when the most affected patients were adult women and caregivers who presented with diarrhoea (90 %) and mild vomiting (10 %). All affected were treated symptomatically, except for two patients who required intravenous rehydration (Al-Abri *et al.,* 2011). In 2012, a high seasonal outbreak of *Bacillus* bacteremia was linked to contaminated hospital linen at Queen Mary Hospital in Hong Kong was also reported by Cheng *et al*. (2017).

There is a scarcity of data on the prevalence of *Bacillus* spp. in hospitals, as well as their associated virulence genes and antibiotic-resistance profiles. Furthermore, little is known of the sources of this organism in the hospital environment in South Africa, although some studies have revealed faeces as the primary source of these pathogens (Carr & Strauss, 2001). As a result, monitoring the hospital environment is a critical tool in the management of HAIs. This study, therefore, sought to assess the dissemination of *Bacillus* species in public hospitals in KwaZulu Natal.

2.2 Methodology

2.2.1 Study clearance and ethical considerations

The Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal (UKZN) granted ethical clearance, with reference number BE606/16. The research was also registered into the Health Research and Knowledge Management database (HRKM 098/17). Participating hospitals granted gatekeeper approval.

2.2.2 Study sample sites

The research was carried out at four public hospitals in the eThekwini Metropolitan Municipality in KwaZulu-Natal, South Africa, from September to November 2017. The four hospitals (A, B, C, and D) were classified as either central, district, regional or tertiary according to the hospital classification guideline of the South African Department of Health (Department of Health, 2012) and the National Health Insurance Policy (Department of Health, 2017). (Further details about the study sites and their stratification are supplied in Table S1). Each hospital received three random visits, the first at the start of the week, the second in the middle of the week, and the third at the end of the week (for an optimal representation of the contamination).

2.2.3 Sample collection

As shown in Table S1, a total of 777 samples were collected from the four public hospitals. A stratified random sampling method was used to collect the samples. Samples were taken from three wards: a general ward, an intensive care unit (ICU), and a paediatric ward. Eleven surfaces per ward were swabbed (Table S1). Samples were collected by swabbing approximately 5cm of surface at each site using prelabelled nylon flock swabs with transport media (FLOQSwabs™ COPAN Diagnostics Inc, USA). The swabs were transported in cooler boxes with ice packs and were processed within four hours after collection for further analysis. Swabs were placed in 50 mL sterile tubes containing tryptone soya broth (TSB) (Oxoid, Hampshire, England) and stored in the bio-freezer (-80 °C) (BioVac, Cape Town, South Africa) for further analysis.

2.2.4 Phenotypic determination of *Bacillus*

For each sample, 1 mL was inoculated into 9 mL of tryptone soy broth (TSB) (Oxoid, Hampshire England) and incubated at 37 °C for 24 hours. This was subsequently followed by sub-culturing 100 µL onto a *Bacillus* selective medium Brilliance™ *Bacillus* agar medium (Oxoid, Basingstoke Hampshire, England) containing a selective *Bacillus* supplement (SR0230E, Oxoid, Basingstoke Hampshire, England). Plates were incubated (CO_2 SHEL LAB, Sheldon, USA) at 37 °C for 24 hours, and blue-green precipitate colonies were considered as presumptive *Bacillus* isolates. The presumptive *Bacillus* colonies were further streaked onto nutrient agar (Acumedia Lab, Lansing, USA) to obtain pure cultures. Biochemical screening including catalase activities using 3 % H_2O_2;

an oxidase test by strips (Sigma Aldrich, St. Louis, USA); triple sugar iron (TSI) (Oxoid, Basingstoke, UK); and motility tests (Himedia, Edwards and Wing, Mumbai, India). The *Bacillus subtilis* American Type Culture Collection (ATCC) 6051 and *Staphylococcus aureus* ATCC 29213 were used as controls. Presumptive *Bacillus* species were stored in 500 µL of TSB (Oxoid, Hampshire England), supplemented with a 10 % glycerol (VWR Internationals Life Science, Amresco, Parkway) solution in a biofreezer (-80 °C) (BioVac, Model DFU-374CE, AC 220 V. 1 ph 8A/50 Hz, -50 °C to -80 °C) for further analysis using sterile 2 mL cryovial tubes.

2.2.5 Molecular identification of *Bacillus*

Bacillus spp. were confirmed using the polymerase chain reaction (PCR,) targeting the 16S rRNA (for the *B. subtilis* group) and the *motB* gene (for the *B. cereus* group), using previously described primers (Oliwa-Stasiak *et al.,* 2010; Subramanian *et al.,* 2010; Wattiau *et al.,* 2001) (Table S2). Stock cultures were sub-cultured on nutrient agar plates (Acumedia Lab, Neogen®, UK) and incubated (CO_2 SHEL LAB, Sheldon, USA) at 37 °C for 24 hours. Genomic DNA extraction was performed using the conventional boiling method, as previously described by Ribeiro Junior *et al.* (2016). The Nanodrop ND-100 spectrophotometer (Thermo Fischer Scientific, Waltham, USA) was used to determine the concentration and purity of the DNA. The DNA was considered pure when a 260/280 ratio ranging between 1.8-2.0 was measured. DNA was stored at -20 °C for further analyses. Each reaction was performed in a total volume of 15 µL, consisting of 8 µL of DreamTaq Green PCR Master Mix (2X) (Thermo Scientific, Lithuania); 0.5 µL of each primer pair (final concentration of 10 µM of each primer); 2.5 µL of template DNA; and 3.5 µL of nuclease-free water (Ambion, RNA company, USA).

The PCR thermal cycling conditions were similar for both genes and were as follows: 35 cycles of denaturation at 95 °C for 30 secs; annealing at 60 °C for 1 min; extension at 72 °C for 1 min; and a final extension at 72 °C for 7 mins. All the reactions were carried out using the PCR thermal cycler Bio-Rad T100 TM Thermal Cycler (Bio-Rad Laboratories Inc., Singapore) and included a positive control, *B. subtilis* ATCC 6051, and a negative template control, (NTC) containing nuclease-free water (Ambion, RNA company, USA) instead of DNA. The PCR products of 7 µl underwent gel electrophoresis at 75 V for 90 mins on a 1.5 % gel run in Trisborate-EDTA (pH 8.3, 1X) (Thermo Fischer Scientific, Waltham, MA USA) containing 0.5 ug/mL ethidium bromide. A 100 bp DNA ladder (New England, Biolabs, USA) was used as a standard

marker, and the images were captured under ultra-violet light with a Gel-Doc XR TM (Bio-Rad, USA).

2.2.6 Antibiotic susceptibility testing

Susceptibility to antibiotics was evaluated using the micro-dilution inhibitory concentration (MIC) with the cation-adjusted Muller-Hinton broth (Oxoid, Basingstoke, England), according to the performance standards for antimicrobial susceptibility testing of the Clinical and Laboratory Standards Institute (CLSI M02-A12, 2015). Antibiotic resistance was tested against ampicillin and amoxicillin (β-lactams); erythromycin (macrolides); ciprofloxacin (fluoroquinolones); imipenem, and meropenem (carbapenems); tetracycline; and cefotaxime (cephalosporins). The antibiotics were selected, based on their recommendation for the treatment of human *Bacillus* infections, following the study by Schlegelova and colleagues (2003). Due to the scarcity of data on clinical *Bacillus* in the literature, *S. aureus* MIC breakpoints, as described in the CLSI M100S27 (2017), for susceptible, intermediate, and resistant were employed for all antibiotics tested (Ikeda *et al.*, 2015; Kreizinger *et al.*, 2016). *S. aureus* ATCC 29213 and *B. subtilis* ATCC 6051 were used as control strains. Further analyses for multidrug resistance (MDR) were conducted, where MDR was defined as isolates displaying resistance to three or more antibiotics originating from different antibiotic classes (Bassetti *et al.*, 2013). The MIC test for each isolate were done in triplet , to make sure that results were accurate.

2.2.7 Detection of virulence and antibiotic-resistant genes

The polymerase chain reaction was implemented to detect virulence genes, using primers as previously described (Asano *et al.*, 1997; Ehling-Schulz *et al.*, 2005b; Fagerlund *et al.*, 2004; Hansen & Hendriksen, 2001; Oltuszak-Walczak & Walczak, 2013) (Table: S2). A total of 11 virulence genes that cause either diarrhoeal or emetic syndrome were selected for this study. These included ten entero-toxigenic genes (*hblA; hblC; hblD; nheA; nheB; nheC; cytK*; *BceT*; E*ntFM; hlyII*) and one cereulide synthetase gene (*cesB*). All the reactions were carried out in a T100 TM Thermal Cycler (Bio-Rad, California, USA). Each reaction was performed in a total volume of 25 µL consisting of 12.5 µl of DreamTaq Green PCR master mix (2X) (Thermo Fischer Scientific, Lithuania); 0.5 µL of each primer pair (final concentration of 5 µM each); 8.5 µL of nuclease-free water (Ambion RNA company, Waltham, USA); and 3 µL of template DNA. The following

thermal cycling conditions were used for all the virulence genes: initial denaturation at 94 ℃ for 5 mins; 35 cycles of denaturation at 94 ℃ for 30 secs; annealing at 55.7 ℃ for 30 secs; extension at 72 ℃ for 90 secs; and a final extension at 72 ℃ for 7 mins, and included a positive control DNA of B. *cereus,* with a no-template control (NTC) serving as a negative.

For the determination of resistant genes, *tetA; tetB; tetK* and *tetM;* and *tet39,* which is associated with tetracycline resistance, were tested. In addition, the *ermB* resistance gene for macrolide (erythromycin*)* and metallo beta-lactamase type-2 (*blm)* genes that confer resistance to the different beta-lactams antibiotics, were detected using the selected primers, as previously described (Adelowo & Fagade, 2009; Adimpong *et al.,* 2012; Rather *et al.,* 2012; Tahmasebi *et al.,* 2014) (Table: S2). The PCR reaction mixture (25 µL) consisted of 12.5 µL of DreamTaq Green PCR Master Mix (2X) (Thermo Scientific, Lithuania); 1µl of each forward and reverse primer (final concentration of 10 µM of each); 3 µL of template DNA; and 7.5 µL of the nuclease-free water (Ambion RNA company, Waltham, USA). The optimised thermal cycling conditions for *ermB, tetM* and *tetK* were as follows: initial denaturation at 98 ℃ for 50 secs; followed by 30 cycles of denaturation at 95 ℃ for 50 secs; annealing at 55 ℃ for 60 secs; extension at 72 ℃ for 60 secs; and a final extension at 72 ℃ for 5 mins (Molechan *et al.,* 2019). For the *blm* gene, the thermal cycling conditions were initial denaturation at 94 ℃ for 3 min; followed by 30 cycles of denaturation at 94 ℃ for 45 secs; annealing at 55 ℃ for 45 secs; extension at 72 ℃ for 45 secs; and a final extension at 72 ℃ for 5 mins. For *tet39,* the thermal cycling conditions were as follows: initial denaturation at 94 ℃ for 5 mins; followed by 35 cycles of 94 ℃ for 1 min; annealing at 57 ℃ for 1 min; extension at 72 ℃ for 2 mins; and a final extension at 72 ℃ for 10 mins (Adelowo & Fagade, 2009). For *tetA* and *tetB,* the thermal cycling conditions were as follows: initial denaturation at 95 ℃ for 4 mins; followed by 35 cycles of 95 ℃ for 1 min; annealing at 58 ℃ for 1 min; extension at 72 ℃ for 1 min; and a final extension at 72 ℃ for 7 mins (Rather *et al*., 2012). *Enterococcus faecalis* ATCC 51299, *Staphylococcus aureus* ATCC 29212, and an in-house sequenced control, *Staphylococcus aureus* (SS1, Accession number: RQTH00000000.1), were used as positive controls. A 100 bp DNA ladder (New England Biolabs, Herts, UK) was used as a standard marker. Negative controls consisted of NTC's. All the PCR products were electrophoresed and visualised using the conditions as previously described.

2.2.8 Clonality

Enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) was carried out using primers ERIC-F and ERIC-R (Table: S2), as described by Versalovic *et al.* (1991), to determine the genetic relatedness between the isolates. The DNA extraction was performed using the Quick DNA™ miniprep plus kit (Zymo, Irvine, USA) according to the manufacturer's guidelines. PCR was carried out in 25 µL, consisting of 12.5 µL of DreamTaq Green PCR Master Mix (2X) (ThermoFisher Scientific, Lithuania); 1 µL of 5 µM of each primer; 7.5 µL of nuclease-free water (Ambion RNA company, Waltham, USA); and 3 µL of template DNA. The PCR cycling conditions were as follows: initial denaturation at 94 °C for 3 mins; 35 cycles each of denaturation for 30 secs at 94 °C; annealing at 45°C for 40 secs; extension at 72 °C for 3 mins; and final elongation at 72 °C for 10 mins (Gao *et al.*, 2018). The amplicons were electrophoresed on a 1.5 % agarose gel at 75 V for 3 hours in 1X Tris-acetate EDTA (TAE) buffer (BioConcept Ltd., Switzerland). A quick- load® 100 bp (New England Biolabs, Herts, UK) DNA ladder was used as a standard molecular weight marker to determine the size of the bands. The gels were stained in 0.5 µg/mL ethidium bromide and were visualised using a Gel Doc™ XR Plus imager (Bio-Rad, California, USA). The gel electrophoresis patterns were analysed using Bionumerics software Version 6.6 (Applied Maths NV, Belgium). The similarity of the dendrograms constructed was calculated using Dice coefficient clustering analysis through an unweighted pair group with arithmetic average (UPGMA), using 1 % tolerance and 0.5 % optimisation. Clusters were determined at a 60 % similarity index (Bishi *et al.*, 2008).

2.2.9 Statistical analysis and data interpretation

The data analysis was done using the IBM Statistical Package for the Social Sciences (SPSS) ®, version 25.0 (IBM Corporation, New York, USA). A probability value of ≤ 0.05 was considered statistically significant. To see if there were any significant differences in prevalence between the wards and hospitals, a chi-squared (χ^2) test of homogeneity was used. Any differences observed were further analysed using multiple pairwise comparisons by implementing Post-Hoc testing and applying the Z-test of two proportions with the Bonferroni correction. The chi-square (χ^2) test of independence was conducted to examine the degree of association with, and significant differences between, the different antibiotics, and antibiotic-resistant, and virulence genes.

2.3 Results

2.3.1 Prevalence of *B. cereus* and *B. subtilis* within the hospital facilities

A total of 17 % (135/777) *Bacillus* isolates were obtained from the samples collected from the four public hospitals (Table 2-1). Molecular screening confirmed 4 % (6/135) as *B. subtilis* and 96 % (129/135) as *B. cereus*. The prevalence of *Bacillus* was higher in the tertiary hospital, B, with 33 % (45/135), followed by regional hospital C with 27 % (36/135); and central hospital A had 24 % (32/135) isolates. The fewest isolates were collected from district hospital D, with 16 % (22/135) (Table 2-1).

The prevalence of *Bacillus* species collected from the four different hospitals differed statistically significantly (p = 0.044), indicating that the prevalence varied by the hospital. The Bonferroni test revealed that the significant differences (p =0.000) were between tertiary and regional hospitals. The chi-squared test revealed that there were no statistically significant differences in *Bacillus* prevalence between the three hospital wards (p = 0.133) (ICU, paediatric and general). The prevalence of *Bacillus* isolates collected at different times of the week did not differ statistically (p = 0.766). A positive correlation was observed between wards and hospitals (r = 0.525; p = 0.000), indicating that, as *Bacillus* increased in the hospital wards, so an increase also occurred in the hospitals being studied.

Table 2-1: Distribution of *Bacillus* species isolated from three wards in each of four participating public hospitals at different sites in KwaZulu-Natal, South Africa

Hospital and sites			**Hospital wards**		***P*-value**
Hospitals/Sites	**Hospital prevalence % (n/N)**	**GW*% (n/N)**	**ICU % (n/N)**	**Paediatric % (n/N)**	
Central (A)	24 (32/135)	33 (14/43)	21 (10/47)	18 (8/45)	
Tertiary (B)	33 (45/135)	30 (13/43)	38 (18/47)	31 (14/45)	
Regional (C)	28 (36/135)	26 (11/43)	28 (13/47)	27 (12/45)	
District (D)	16 (22/135)	12 (5/43)	13 (6/47)	24 (11/45)	
Total % (n/N)	17 (135/77)	32 (43/135)	35 (47/135)	33 (45/135)	0.044
Phone	11 (15/135)	26 (11/43)	2 (1/47)	7 (3/45)	
Drip stand	12 (15/135)	12 (5/43)	15 (7/47)	9 (4/45)	
Bp monitor	10 (13/135)	14 (6/43)	9 (4/47)	7 (3/45)	
Patient file	7 (10/135)	9 (4/43)	4 (2/47)	9 (4/45)	
Ventilator	7 (9/135)	2 (1/43)	17 (8/47)	2 (1/45)	
Mop	7 (10/135)	7 (3/43)	9 (4/47)	7 (3/45)	
Sink	12 (16/135)	7 (3/43)	4 (2/47)	22 (10/45)	
Occupied bed	7 (9/135)	2 (1/43)	9 (4/47)	9 (4/45)	
Unoccupied bed	8 (11/135)	2 (1/43)	15 (7/47)	7 (3/45)	
Nurses' table	10 (14/135)	9 (4/43)	11 (5/47)	11 (5/45)	
Door handle	9 (12/135)	9 (4/43)	6 (3/47)	11 (5/45)	

*GW = General ward.

With regards to the wards, although the intensive care unit (ICU) revealed a slightly higher prevalence (35 % (47/135)), followed by the paediatric (33 % (45/135)) and general wards (32 % (43/135)), there was no statistically significant difference in the overall prevalence of *Bacillus* spp. *(p* ≥ 0.05) gathered from three different wards. For the ward sites swabbed, the incidence ranged from 7 % to 15 %. The highest number of isolates observed originated from beds (occupied and unoccupied), with 15 % (20/135); sinks and drip stands, with 12 % (16/135) each; telephones, at 11 % (15/135); the nurses' tables at 10 % (14/135); blood pressure monitors at 10 % (13/135); and patient files and mops, with 7 % (10/135) (Table 2-1).

2.3.2 Testing for Antibiotic Susceptibility

For overall resistance, *Bacillus* showed a high level of resistance towards ampicillin with the prevalence of 100 % (135/135), with MIC values of ≥512 mg/L; amoxicillin exhibited 97 % (131/135); ciprofloxacin, 99 % (134/135); and tetracycline, 83 % (112/135). Resistance against cefotaxime was 51 % (69/135); erythromycin, 44 % (59/135); meropenem, 20 % (27/135); and imipenem, at 19 % (26/135) was also noted (Table 2-2).

Table 2-2: Susceptibility profiles of 135 *Bacillus* species isolates tested against eight antibiotics.

Antibiotics[a]	MIC[b] (mg/L) (N=135)																Isolate % (n/N)			Cut-offs for S, I and R		
	0.0075	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	S %(n/N)	I %(n/N)	R %(n/N)			
ERY	-	1	9	5	26	11	12	11	2	5	5	4	2	2	1	39	39(52)	18(24)	44(59)	S ≤0.5	I (1-4)	R ≥8
CIP	-	-	-	-	1	-	-	-	7	26	39	24	17	9	2	10	1(1)	-	99(34)	S ≤1,	I -1,	R ≥4
TET	-	-	-	-	1	1	1	2	4	17	20	28	6	7	16	32	7(9)	11(4)	83(112)	S ≤4	I-8	R ≥16
MER	-	1	1	14	16	4	9	11	16	35	22	1	1	1	-	3	53(72)	27(36)	20(27)	S ≤4	I-8	R ≥16
AMX	-	1	-	-	1	1	1	2	-	-	1	-	-	-	-	128	3(4)	-	97(131)	S ≤4/2	I-	R ≥8/4
AMP	-	-	-	-	-	-	-		4	6	-	-	-	1	-	124	-	-	100(135)	S ≤0.25	I-	R ≥0.5
IMI	1	13	6	1	6	6	27	17	7	24	6	7	5	-	-	8	63(85)	18(24)	19(26)	S ≤4	I-8	R ≥16
CTX	-	-	-	-	2	4	5	7	5	9	25	8	7	8	1	52	25(34)	24(32)	51(69)	S ≤8	I 16-32	R ≥64
p-value=0.067																						

[a] ERY: Erythromycin; CIP: Ciprofloxacin; TET: Tetracycline; MER: Meropenem; AMX: Amoxicillin; AMP: Ampicillin; IMI: Imipenem; CTX: Cefotaxime. b MIC: minimum inhibitory concentration; S: susceptible; I: intermediate; R: resistant. Highlighted blocks indicate the antibiotics that showed high resistance. Greyscale indicates MIC breakpoints. The circled numbers are those MIC values with the highest number of isolates.

A total of 43 different antibiograms were detected, with a multi-drug resistance rate of 97 % (132/135). The dominant MICs for erythromycin; tetracycline (n = 39); amoxicillin (n = 32); ampicillin (n = 124); and cefotaxime (n = 52) were 512 mg/L. Furthermore, the common MICs for ciprofloxacin, meropenem and imipenem were 16 mg/L (n = 39); 8 mg/L (n = 35); and 1 mg/L (n = 27), respectively (Table 2, circled numbers). Carbapenem, meropenem and imipenem had a susceptibility of 53 % (72/135), 63 % (85/135) and for intermediate, 27 % (36/135) for meropenem and 18 % (24/135) for imipenem. There was no statistically significant difference in resistance between the eight antibiotics tested ($p \geq 0.05$).

2.3.3 Detection of antibiotic-resistant genes

The prevalence of antibiotic-resistant genes was as follows: 56 % (33/59) in *ermB*; 5 % (5/112) in *tetM;* and 4 % (4/112) in *tetK* (Table 2-3). Most (56 % (33/59)) isolates that showed resistance to erythromycin harboured the *ermB* gene. Of the 112 isolates with observed resistance to tetracycline, only 4 % (4/112) of *tetK*, and 5 % (5/112) of *tetM*, genes were detected in isolates showing phenotypic tetracycline resistance. None of the isolates encoded the *tet A, tet B,* and *tet 39* genes. Even though there was a high level of resistance to β-lactams (ampicillin and amoxicillin), none of the isolates investigated encoded the *blm* gene.

Table 2-3: Prevalence of antibiotic-resistant and virulence genes in *Bacillus* spp. collected from the four hospital environments studied (N = 135).

	Resistant genes[a]							**Virulence genes[b]**										
	tetA	***tetB***	***tetK***	***tetM***	***tet39***	***blm***	***ermB***	***hblA***	***hblC***	***hblD***	***nheA***	***nheB***	***nheC***	***cytK***	***entFM***	***bceT***	***Hlyll***	***ces***
No. of isolates	0	0	4	4	0	0	33	104	74	119	86	88	90	104	37	59	50	0
%	0	0	4	5	0	0	56	77	55	88	64	65	67	77	27	44	37	0

[a] Gene confers resistance to the corresponding antibiotics: *tetA, tetB, tetK, M* and *tet39* (tetracycline), *ermB* (erythromycin) and *blm* (β-lactams; ampicillin, amoxicillin). [b] Virulence genes encode the corresponding factors: *hblA, hblC,* and *hblD* (hemolysin BL), *nheA, nheC,* and *nheC* (nonhemolytic), *cyt K* (cytotoxin K), *entFM* (enterotoxin FM), *hlyII (*potentially enterotoxin hemolysin BL), *ces* (cereulide) and *BceT* (enterotoxin T)

2.3.4 Detection of virulence genes

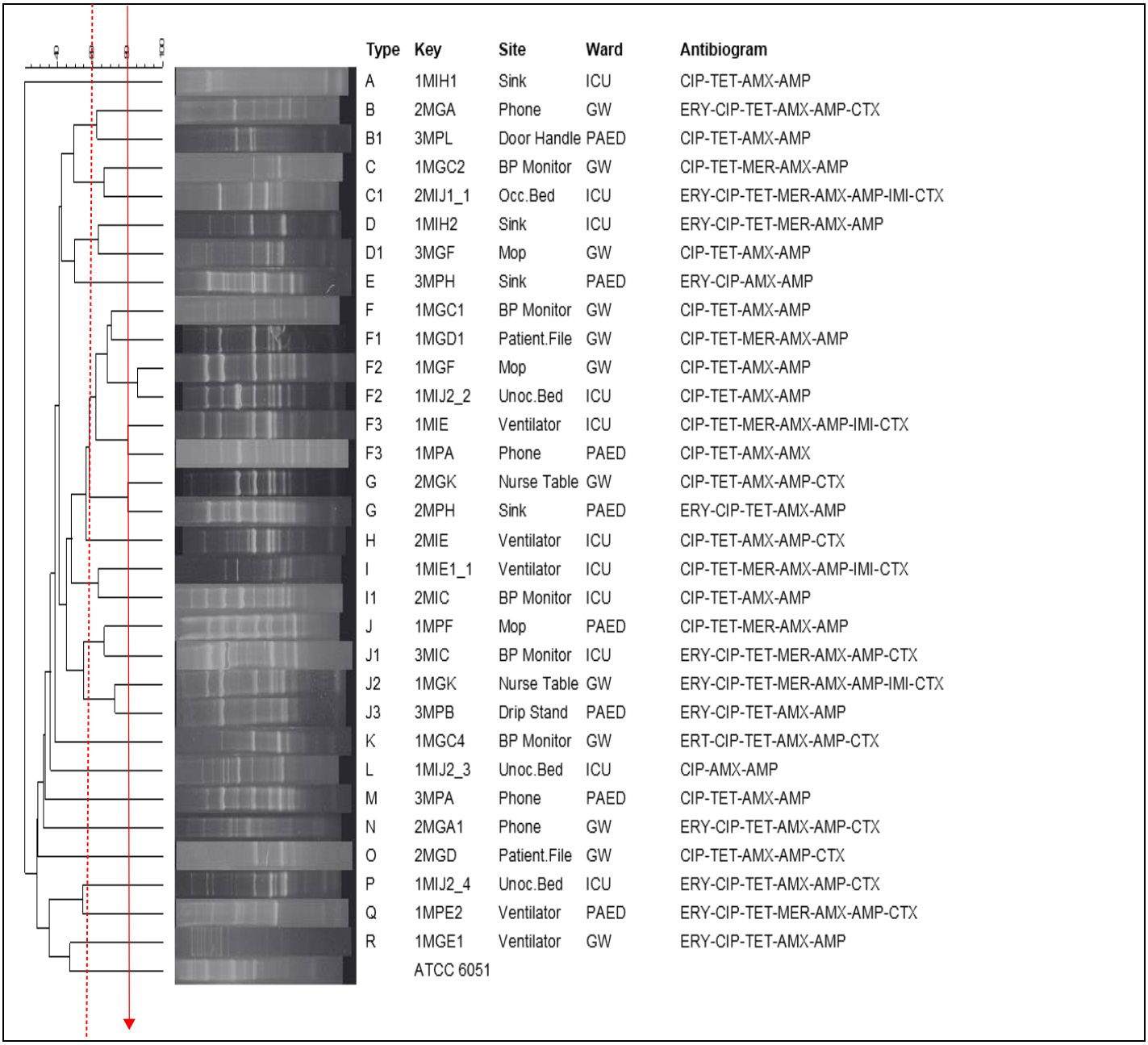
Table 2-3 shows the overall prevalence of virulence genes discovered in *Bacillus* spp. The hemolysin (*hblD*) showed the highest prevalence with 88 % (119/135), and no *ces* gene (cereulide toxin) was detected. The *Bacillus* strains harboured in the hospital environments that were studied were thus more likely to be associated with the diarrhoeal syndrome.

2.3.5 Enterobacterial repetitive intergenic consensus sequences PCR (ERIC-PCR)

The dendrograms obtained by genomic DNA fingerprinting using the ERIC-PCR assisted in identifying the clonal relationships between the isolates from the various hospitals. There was a high degree of heterogeneity/divergence among the isolates from the various hospital environments. Therefore, the isolates were analysed according to the specific hospitals to offer valuable insights into their clonal relationships. The fingerprints generated consisted of distinct bands of different sizes (Figures 2.3 to 2.6). ERIC-clustering revealed a clonal spread of strains between the sites and wards within each specific hospital setting.

Note: In Figures 2-1 to 2-4 (below), the solid red line indicates the major ERIC-type cut-off value, and the dotted red line indicates the ERIC-type cut-off. Abbreviations used are GW: general ward; PAED: paediatric ward; occ. bed: occupied bed; unocc. bed: unoccupied bed; ERY: Erythromycin; CIP: Ciprofloxacin; TET: Tetracycline; MER: Meropenem; IMI: Imipenem; AMP: Ampicillin; AMX: Amoxicillin; CTX: Cefotaxime. *B. subtilis* ATCC 6051 was used as a quality control strain.

Similarity index of *Bacillus*

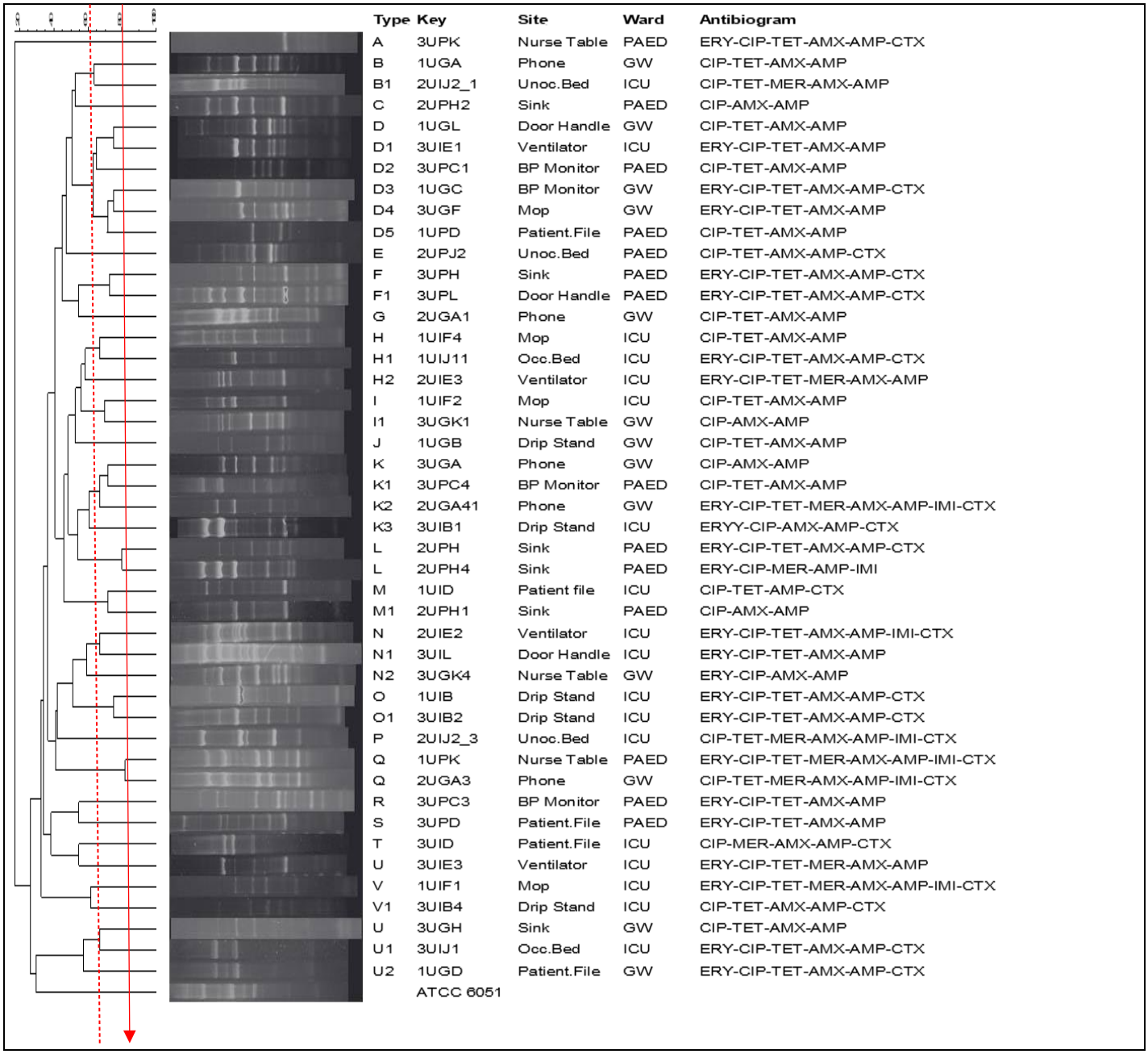


Type	Key	Site	Ward	Antibiogram
A	1MIH1	Sink	ICU	CIP-TET-AMX-AMP
B	2MGA	Phone	GW	ERY-CIP-TET-AMX-AMP-CTX
B1	3MPL	Door Handle	PAED	CIP-TET-AMX-AMP
C	1MGC2	BP Monitor	GW	CIP-TET-MER-AMX-AMP
C1	2MIJ1_1	Occ.Bed	ICU	ERY-CIP-TET-MER-AMX-AMP-IMI-CTX
D	1MIH2	Sink	ICU	ERY-CIP-TET-MER-AMX-AMP
D1	3MGF	Mop	GW	CIP-TET-AMX-AMP
E	3MPH	Sink	PAED	ERY-CIP-AMX-AMP
F	1MGC1	BP Monitor	GW	CIP-TET-AMX-AMP
F1	1MGD1	Patient.File	GW	CIP-TET-MER-AMX-AMP
F2	1MGF	Mop	GW	CIP-TET-AMX-AMP
F2	1MIJ2_2	Unoc.Bed	ICU	CIP-TET-AMX-AMP
F3	1MIE	Ventilator	ICU	CIP-TET-MER-AMX-AMP-IMI-CTX
F3	1MPA	Phone	PAED	CIP-TET-AMX-AMX
G	2MGK	Nurse Table	GW	CIP-TET-AMX-AMP-CTX
G	2MPH	Sink	PAED	ERY-CIP-TET-AMX-AMP
H	2MIE	Ventilator	ICU	CIP-TET-AMX-AMP-CTX
I	1MIE1_1	Ventilator	ICU	CIP-TET-MER-AMX-AMP-IMI-CTX
I1	2MIC	BP Monitor	ICU	CIP-TET-AMX-AMP
J	1MPF	Mop	PAED	CIP-TET-MER-AMX-AMP
J1	3MIC	BP Monitor	ICU	ERY-CIP-TET-MER-AMX-AMP-CTX
J2	1MGK	Nurse Table	GW	ERY-CIP-TET-MER-AMX-AMP-IMI-CTX
J3	3MPB	Drip Stand	PAED	ERY-CIP-TET-AMX-AMP
K	1MGC4	BP Monitor	GW	ERT-CIP-TET-AMX-AMP-CTX
L	1MIJ2_3	Unoc.Bed	ICU	CIP-AMX-AMP
M	3MPA	Phone	PAED	CIP-TET-AMX-AMP
N	2MGA1	Phone	GW	ERY-CIP-TET-AMX-AMP-CTX
O	2MGD	Patient.File	GW	CIP-TET-AMX-AMP-CTX
P	1MIJ2_4	Unoc.Bed	ICU	ERY-CIP-TET-AMX-AMP-CTX
Q	1MPE2	Ventilator	PAED	ERY-CIP-TET-MER-AMX-AMP-CTX
R	1MGE1	Ventilator	GW	ERY-CIP-TET-AMX-AMP
	ATCC 6051			

Figure 2-1: Dendrogram of *Bacillus* isolates collected from central hospital A

Isolates from central hospital A (n = 31) exhibited a high-level genetic diversity, grouped into 18 ERIC-types, designated from A-R. It was observed that 26 % (8/31) of *Bacillus* isolates grouped into two major ERIC-types: F (4) and J (4) (Figure 2-3). Isolates from the same wards did not fall into similar ERIC-types. The major F-cluster showed two isolates with a similarity index of ≥ 90 %, but from different sites and wards. The G-cluster indicated isolates from different wards and sites, but which shared the same ERIC type. Of note, although the strains were in the same cluster, they were collected at different sampling times.

Similarity index of *Bacillus*



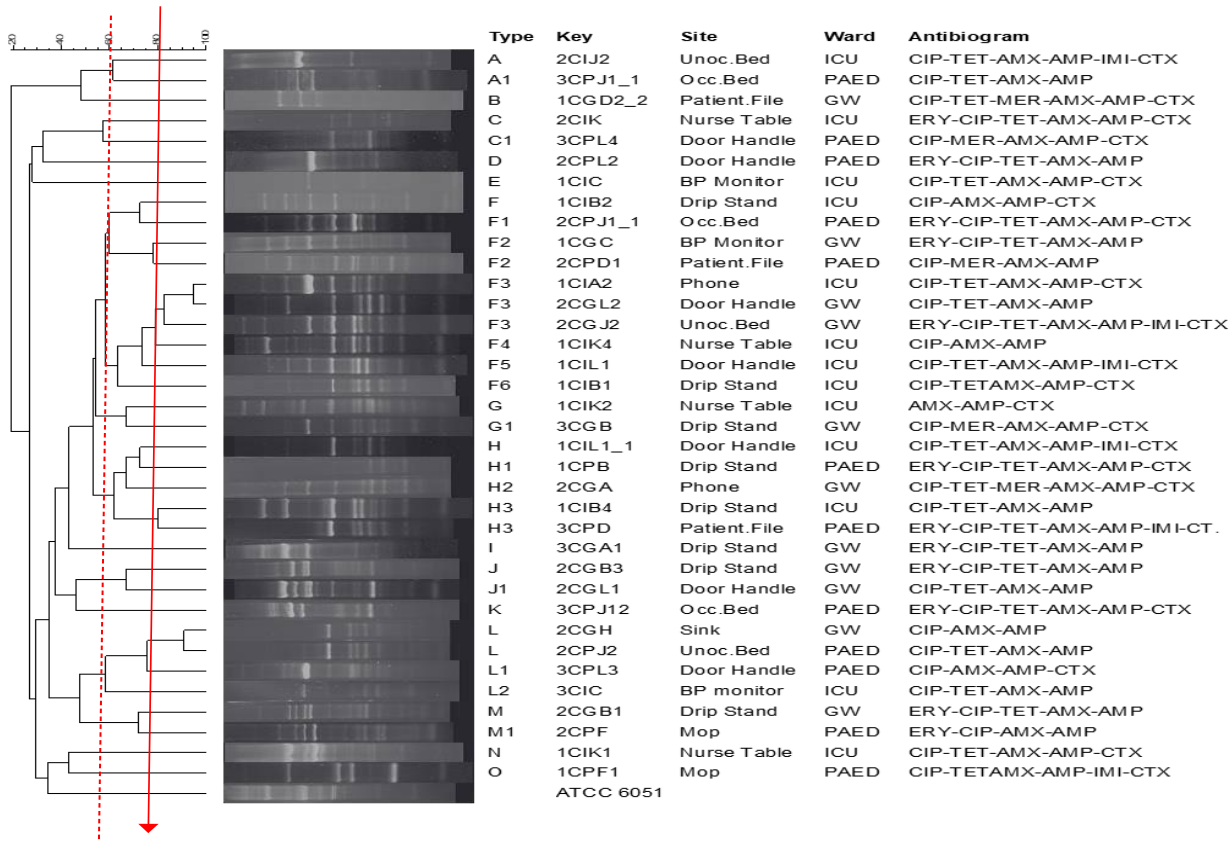
Type	Key	Site	Ward	Antibiogram
A	3UPK	Nurse Table	PAED	ERY-CIP-TET-AMX-AMP-CTX
B	1UGA	Phone	GW	CIP-TET-AMX-AMP
B1	2UIJ2_1	Unoc.Bed	ICU	CIP-TET-MER-AMX-AMP
C	2UPH2	Sink	PAED	CIP-AMX-AMP
D	1UGL	Door Handle	GW	CIP-TET-AMX-AMP
D1	3UIE1	Ventilator	ICU	ERY-CIP-TET-AMX-AMP
D2	3UPC1	BP Monitor	PAED	CIP-TET-AMX-AMP
D3	1UGC	BP Monitor	GW	ERY-CIP-TET-AMX-AMP-CTX
D4	3UGF	Mop	GW	ERY-CIP-TET-AMX-AMP
D5	1UPD	Patient.File	PAED	CIP-TET-AMX-AMP
E	2UPJ2	Unoc.Bed	PAED	CIP-TET-AMX-AMP-CTX
F	3UPH	Sink	PAED	ERY-CIP-TET-AMX-AMP-CTX
F1	3UPL	Door Handle	PAED	ERY-CIP-TET-AMX-AMP-CTX
G	2UGA1	Phone	GW	CIP-TET-AMX-AMP
H	1UIF4	Mop	ICU	CIP-TET-AMX-AMP
H1	1UIJ11	Occ.Bed	ICU	ERY-CIP-TET-AMX-AMP-CTX
H2	2UIE3	Ventilator	ICU	ERY-CIP-TET-MER-AMX-AMP
I	1UIF2	Mop	ICU	CIP-TET-AMX-AMP
I1	3UGK1	Nurse Table	GW	CIP-AMX-AMP
J	1UGB	Drip Stand	GW	CIP-TET-AMX-AMP
K	3UGA	Phone	GW	CIP-AMX-AMP
K1	3UPC4	BP Monitor	PAED	CIP-TET-AMX-AMP
K2	2UGA41	Phone	GW	ERY-CIP-TET-MER-AMX-AMP-IMI-CTX
K3	3UIB1	Drip Stand	ICU	ERYY-CIP-AMX-AMP-CTX
L	2UPH	Sink	PAED	ERY-CIP-TET-AMX-AMP-CTX
L	2UPH4	Sink	PAED	ERY-CIP-MER-AMP-IMI
M	1UID	Patient file	ICU	CIP-TET-AMP-CTX
M1	2UPH1	Sink	PAED	CIP-AMX-AMP
N	2UIE2	Ventilator	ICU	ERY-CIP-TET-AMX-AMP-IMI-CTX
N1	3UIL	Door Handle	ICU	ERY-CIP-TET-AMX-AMP
N2	3UGK4	Nurse Table	GW	ERY-CIP-AMX-AMP
O	1UIB	Drip Stand	ICU	ERY-CIP-TET-AMX-AMP-CTX
O1	3UIB2	Drip Stand	ICU	ERY-CIP-TET-AMX-AMP-CTX
P	2UIJ2_3	Unoc.Bed	ICU	CIP-TET-MER-AMX-AMP-IMI-CTX
Q	1UPK	Nurse Table	PAED	ERY-CIP-TET-MER-AMX-AMP-IMI-CTX
Q	2UGA3	Phone	GW	CIP-TET-MER-AMX-AMP-IMI-CTX
R	3UPC3	BP Monitor	PAED	ERY-CIP-TET-AMX-AMP
S	3UPD	Patient.File	PAED	ERY-CIP-TET-AMX-AMP
T	3UID	Patient.File	ICU	CIP-MER-AMX-AMP-CTX
U	3UIE3	Ventilator	ICU	ERY-CIP-TET-MER-AMX-AMP
V	1UIF1	Mop	ICU	ERY-CIP-TET-MER-AMX-AMP-IMI-CTX
V1	3UIB4	Drip Stand	ICU	CIP-TET-AMX-AMP-CTX
U	3UGH	Sink	GW	CIP-TET-AMX-AMP
U1	3UIJ1	Occ.Bed	ICU	ERY-CIP-TET-AMX-AMP-CTX
U2	1UGD	Patient.File	GW	ERY-CIP-TET-AMX-AMP-CTX
	ATCC 6051			

Figure 2-2: Dendrogram of *Bacillus* isolates collected from tertiary hospital B

The ERIC profiling of isolates from tertiary hospital -B (n = 45) exhibited fingerprints with high genetic diversity. Twenty-three ERIC-types were observed (A-U) (Figure 2-4). A total of 42 % (19/45) of *Bacillus* isolates are grouped into five major ERIC-types: D (6), H (3), K (4), N (3), and U (3). The largest clone was the D-cluster with six isolates (from different sites) from the paediatric, ICU, and general wards. Strains in ERIC-types U1 (occupied bed) and U2 (patient file) were in the same cluster as the same antibiogram but were collected at different sites and from different wards. The O-cluster showed isolates that were collected from the same sites (drip stand) and same ward (ICU) at different sampling times (an interval

of six weeks), with similar antibiograms. Similarly, Cluster-D had isolated from different wards (ICU and general ward), and different sites (occupied bed and patient file), had the same antibiogram, although they were collected at different times.

Similarity index of *Bacillus*

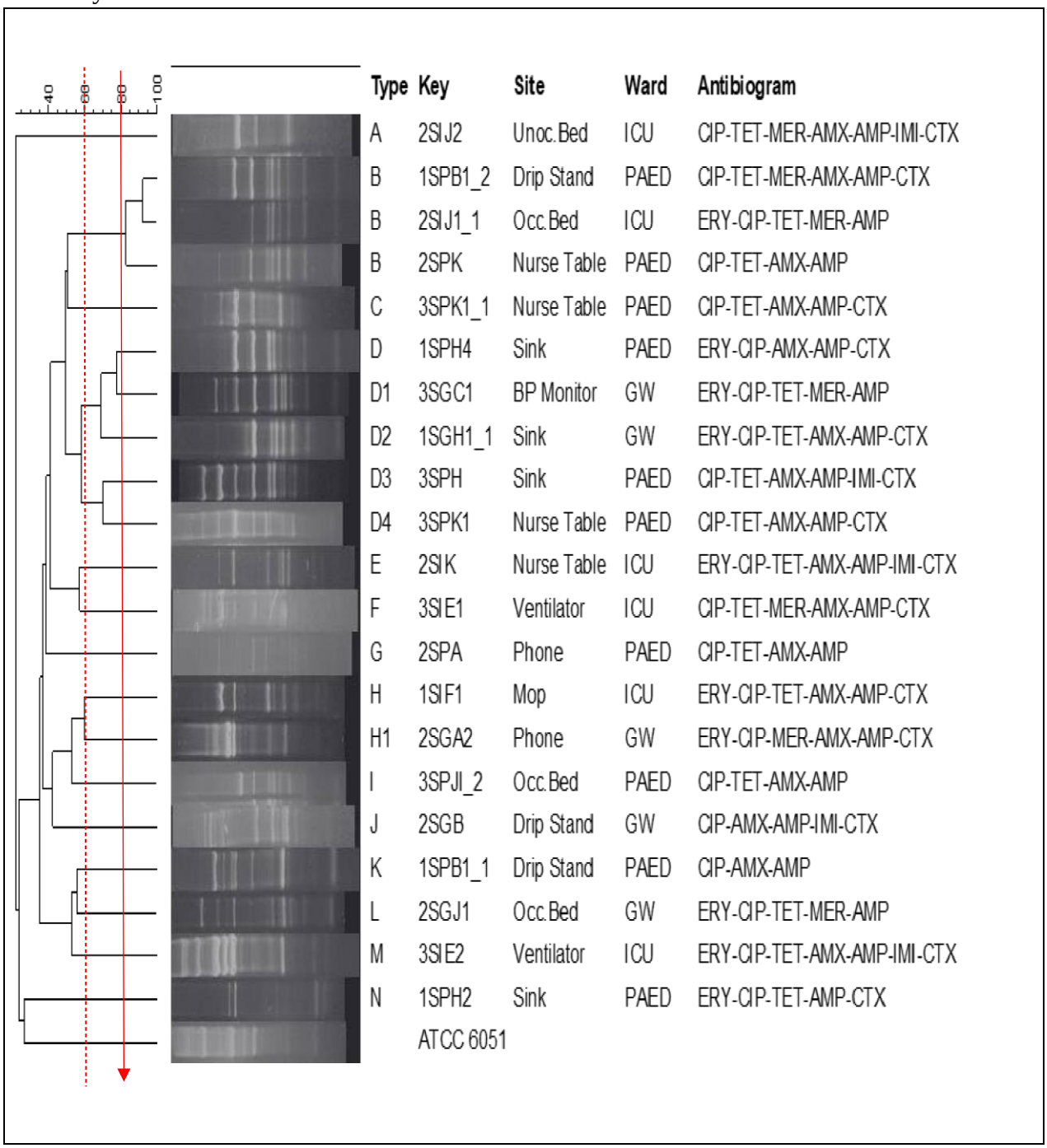


Type	Key	Site	Ward	Antibiogram
A	2CIJ2	Unoc.Bed	ICU	CIP-TET-AMX-AMP-IMI-CTX
A1	3CPJ1_1	Occ.Bed	PAED	CIP-TET-AMX-AMP
B	1CGD2_2	Patient.File	GW	CIP-TET-MER-AMX-AMP-CTX
C	2CIK	Nurse Table	ICU	ERY-CIP-TET-AMX-AMP-CTX
C1	3CPL4	Door Handle	PAED	CIP-MER-AMX-AMP-CTX
D	2CPL2	Door Handle	PAED	ERY-CIP-TET-AMX-AMP
E	1CIC	BP Monitor	ICU	CIP-TET-AMX-AMP-CTX
F	1CIB2	Drip Stand	ICU	CIP-AMX-AMP-CTX
F1	2CPJ1_1	Occ.Bed	PAED	ERY-CIP-TET-AMX-AMP-CTX
F2	1CGC	BP Monitor	GW	ERY-CIP-TET-AMX-AMP
F2	2CPD1	Patient.File	PAED	CIP-MER-AMX-AMP
F3	1CIA2	Phone	ICU	CIP-TET-AMX-AMP-CTX
F3	2CGL2	Door Handle	GW	CIP-TET-AMX-AMP
F3	2CGJ2	Unoc.Bed	GW	ERY-CIP-TET-AMX-AMP-IMI-CTX
F4	1CIK4	Nurse Table	ICU	CIP-AMX-AMP
F5	1CIL1	Door Handle	ICU	CIP-TET-AMX-AMP-IMI-CTX
F6	1CIB1	Drip Stand	ICU	CIP-TETAMX-AMP-CTX
G	1CIK2	Nurse Table	ICU	AMX-AMP-CTX
G1	3CGB	Drip Stand	GW	CIP-MER-AMX-AMP-CTX
H	1CIL1_1	Door Handle	ICU	CIP-TET-AMX-AMP-IMI-CTX
H1	1CPB	Drip Stand	PAED	ERY-CIP-TET-AMX-AMP-CTX
H2	2CGA	Phone	GW	CIP-TET-MER-AMX-AMP-CTX
H3	1CIB4	Drip Stand	ICU	CIP-TET-AMX-AMP
H3	3CPD	Patient.File	PAED	ERY-CIP-TET-AMX-AMP-IMI-CT.
I	3CGA1	Drip Stand	GW	ERY-CIP-TET-AMX-AMP
J	2CGB3	Drip Stand	GW	ERY-CIP-TET-AMX-AMP
J1	2CGL1	Door Handle	GW	CIP-TET-AMX-AMP
K	3CPJ12	Occ.Bed	PAED	ERY-CIP-TET-AMX-AMP-CTX
L	2CGH	Sink	GW	CIP-AMX-AMP
L	2CPJ2	Unoc.Bed	PAED	CIP-TET-AMX-AMP
L1	3CPL3	Door Handle	PAED	CIP-AMX-AMP-CTX
L2	3CIC	BP monitor	ICU	CIP-TET-AMX-AMP
M	2CGB1	Drip Stand	GW	ERY-CIP-TET-AMX-AMP
M1	2CPF	Mop	PAED	ERY-CIP-AMX-AMP
N	1CIK1	Nurse Table	ICU	CIP-TET-AMX-AMP-CTX
O	1CPF1	Mop	PAED	CIP-TETAMX-AMP-IMI-CTX
	ATCC 6051			

Figure 2-3: Dendrogram of *Bacillus* isolates collected from regional hospital C

The analysis of isolates collected from regional hospital C (n = 36) revealed a clonality similarity index of 60 % and showed 14 ERIC-types (A-O) (Figure 2-5). A total of three major clusters were observed, with a total of 39 % (14/36), which grouped into ERIC-types: F (7), H (4), and L (3). The F-cluster, which was the primary cluster, had seven subtypes obtained from different wards and sites.

Similarity index of *Bacillus*



Type	Key	Site	Ward	Antibiogram
A	2SIJ2	Unoc.Bed	ICU	CIP-TET-MER-AMX-AMP-IMI-CTX
B	1SPB1_2	Drip Stand	PAED	CIP-TET-MER-AMX-AMP-CTX
B	2SIJ1_1	Occ.Bed	ICU	ERY-CIP-TET-MER-AMP
B	2SPK	Nurse Table	PAED	CIP-TET-AMX-AMP
C	3SPK1_1	Nurse Table	PAED	CIP-TET-AMX-AMP-CTX
D	1SPH4	Sink	PAED	ERY-CIP-AMX-AMP-CTX
D1	3SGC1	BP Monitor	GW	ERY-CIP-TET-MER-AMP
D2	1SGH1_1	Sink	GW	ERY-CIP-TET-AMX-AMP-CTX
D3	3SPH	Sink	PAED	CIP-TET-AMX-AMP-IMI-CTX
D4	3SPK1	Nurse Table	PAED	CIP-TET-AMX-AMP-CTX
E	2SIK	Nurse Table	ICU	ERY-CIP-TET-AMX-AMP-IMI-CTX
F	3SIE1	Ventilator	ICU	CIP-TET-MER-AMX-AMP-CTX
G	2SPA	Phone	PAED	CIP-TET-AMX-AMP
H	1SIF1	Mop	ICU	ERY-CIP-TET-AMX-AMP-CTX
H1	2SGA2	Phone	GW	ERY-CIP-MER-AMX-AMP-CTX
I	3SPJI_2	Occ.Bed	PAED	CIP-TET-AMX-AMP
J	2SGB	Drip Stand	GW	CIP-AMX-AMP-IMI-CTX
K	1SPB1_1	Drip Stand	PAED	CIP-AMX-AMP
L	2SGJ1	Occ.Bed	GW	ERY-CIP-TET-MER-AMP
M	3SIE2	Ventilator	ICU	ERY-CIP-TET-AMX-AMP-IMI-CTX
N	1SPH2	Sink	PAED	ERY-CIP-TET-AMP-CTX
	ATCC 6051			

Figure 2-4: Dendrogram of *Bacillus* isolates collected from regional hospital D

District hospital D's (n = 21) isolates had 14 ERIC-types, denoted by A-N (Figure 2-6). A single major cluster was detected with a total of 24 % (5/21), which grouped as cluster D (5). The Bcluster indicated isolates from different wards with a similarity index of ≥ 90. There was a high genetic diversity in the *Bacillus cereus* within this hospital, as the isolates had different ERIC types.

2.4 Discussion

The environment in the healthcare facility plays a vital role in the transmission of pathogens associated with nosocomial infections. These pathogens can be transferred from person to person or through touching inanimate objects such as phones, drip stands, and stethoscopes (Nurain *et al.,* 2015; Schmidt *et al.,* 2014; Umar *et al.,* 2015). Understanding the prevalence, genetic content, and interrelationships of bacteria in hospital settings could provide insight into their transmission and the risk of nosocomial infections. *Bacillus cereus* has been linked to infections such as food poisoning, eye infections, fulminant sepsis, and fatal central nervous system infections. (Bottone, 2010; Glasset *et al.,* 2018; Sasahara *et al.,* 2010; Turabelidze *et al.,* 2013). However, research on the spread of *Bacillus* spp. in African hospitals is limited. The weaknesses of this study demonstrate the importance of monitoring *Bacillus* in a hospital environment.

In the current study, the genus *Bacillus*. found in different sampling points in three different wards in four public hospital environments classified according to the central, tertiary, regional and regional classifications. An overall prevalence rate of 17 % (135/777) for *Bacillus* spp. was detected in the four hospitals. This finding was not consistent with the higher rate of 50 % (147/296) reported in a Sudanese hospital survey (Nurain *et al.*, 2015). However, the prevalence of *B. cereus* in this study was higher than the 10 % prevalence rate, with a prevalence of 7 % from workers' hands and 16 % from the hospital setting, which was obtained in the St. Azzhria University Hospital in Isafan, Malaysia (Jalalpoor *et al.,* 2010). Various prevalence for *Bacillus* spp. were observed across the four public hospitals studied, with the tertiary and central hospitals showing the lowest prevalence. The central and tertiary hospitals are both referral hospitals with a constant exchange of patients from other health facilities, such as clinics. This constant movement may increase community exposure and, therefore the prevalence of the *Bacillus* bacteria, when inefficient infection prevention and control measures are practised. From an epidemiological perspective, healthcare facilities are connected through shared patients (Donker *et al.,* 2012).

The prevalence of *Bacillus* spp. had no statistical significance ($p \geq 0.05$). Although ICUs had a higher prevalence than the other wards, there was no difference between them. This result differs from a study by Kunwar *et al.* (2019), which investigated eight hospitals in the Kathmandu district of Nepal (South Asia) and reported a prevalence of 56 % for ICUs, and 44 % in standard general wards, of *Bacillus* spp. Infectious surfaces are known to spread disease, and ICU nurses who care for critically ill patients are aware of the dangers of pathogen cross-contamination. (Falk *et al.,* 2000; Russotto *et al.,* 2015; Seki *et al.,* 2013). Moreover, the proximity of ICU beds to the equipment necessary for focussed observation and care, with many hand-touch sites, adds to the risk, requiring sophisticated and specific cleaning procedures (Russotto *et al.,* 2017; Russotto *et al.,* 2015). Out of the eleven different sites where *Bacillus* spp. was sampled, unoccupied beds; drip stands; sinks; ward phones; and nurses' tables were the most contaminated, which is inconsistent with other studies (Akbari *et al.,* 2018). However, this outcome was not inconsistent with the study that was conducted to investigate the prevalence of *B. cereus* in the hospital environment at a tertiary care hospital in Isfahan, Iran, where a prevalence of 57 % of *Bacillus* was found on high-contact surfaces (doorknobs; beds; windows and nurses tables), with 29 % on low contact surfaces (floors and ceilings) in different hospital wards (Jalalpoor, 2011). Identifying high-risk sites and the most common contaminants can play an essential role in promoting infection control practices and innovations (Russotto *et al.,* 2015).

Bacillus cereus is the most notable of the enteropathogenic *Bacillus* species. It is the cause of several gastrointestinal disorders including local and systemic infections that may have fatal consequences (Ehling-Schulz *et al.,* 2019; Rather *et al.,* 2012; Schoeni & Leew Wong, 2005). *Bacillus* species are usually susceptible to broad-spectrum antibiotics like tetracycline, ciprofloxacin, and erythromycin, which are utilised therapeutically for gastroenteritis disease triggered by this bacterium (Fiedler *et al.,* 2019; Tuazon, 2017). However, the development of resistance to these antibiotics, as well as cloxacillin and streptomycin, has been reported (Fiedler et al., 2019). *Bacillus* spp. are genetically resistant to β-lactams, excluding carbapenems (Citron & Appleman, 2006; Fiedler *et al.,* 2019; Tahmasebi *et al.,* 2014). They may also develop resistance to antibiotics namely erythromycin, tetracycline, ciprofloxacin, streptomycin, and cloxacillin, which are widely used to treat infectious diseases (Citron & Appleman, 2006). However, the degree to which

the *B. cereus* group's stress-transmitting antibiotic-resistant genes can operate in the food chain has yet to be determined (Fiedler *et al.,* 2019).

The high resistance to tetracyclines and fluoroquinolones (ciprofloxacin) shown in this study is not in agreement with other reports, as *Bacillus* spp. is usually susceptible to these classes of antibiotics (Fiedler *et al.,* 2019; Kim *et al.,* 2015); but the bacterium has already been shown to resist tetracycline in the United States and Europe (Luna *et al.,* 2007). Glasset *et al.* (2018) reported the susceptibility of *Bacillus* species to tetracyclines and fluoroquinolones when the epidemiology of *Bacillus* was studied in a French Voluntary Hospital. However, this was not observed in this study as most isolates were resistant to tetracycline and ciprofloxacin (fluoroquinolone). Resistance to tetracycline is generally conferred by the acquisition of resistant genes associated with mobile genetic elements (Partridge *et al.,* 2018; Rather *et al.,* 2012).

Among the 83 % (112/135) of isolates that were resistant to tetracycline, only 4 % (4/112) and 5 % (5/112) of the isolates contained *tetK* (active efflux) and *tetM* (ribosomal protection), respectively; and none of the other (*tetA, tetB,* and *tet39)* resistant genes were detected. Resistance may be due to horizontal transfer of antibiotic-resistant genes from other bacterial species through other mechanisms, such as mutation, which is the most important element in the current antimicrobial resistance (AMR) epidemic (Sun *et al.*, 2019; Von Wintersdorff *et al.,* 2016). Currently, there is a lack of information reported in the literature on the mechanisms regarding tetracycline resistance in *Bacillus* to confirm this assertion.

Erythromycin revealed a moderate resistance 44 % (56/135), which was contrary to a study by Gao *et al.*, 2018 of a different *B. cereus* isolated from pasteurised milk in China, where 81% of the isolates were susceptible to erythromycin (Gao *et al.*, 2018). The *ermB*, which mediates erythromycin resistance, was detected in 56 % (33/59) of the resistant isolates. The remaining isolates with phenotypic resistance to erythromycin did not contain the *ermB* gene, indicating the occurrence of other resistance mechanisms, like *ermD, ermE,* and *ermJ*, which were not investigated in this study but have been reported in another *Bacillus* spp.

(Adimpong *et al.*, 2012; Bozdogan *et al.*, 2004). This proves that the prevalence of macrolides, lincosamides, and streptogramin B (so-called MLSB) phenotypes and genotypes vary according to country, patterns of infections, and drug use (Khodabandeh *et al.*, 2019; Misic *et al.*, 2017).

Most *B. cereus* isolates produce beta-lactamases (Yim *et al.*, 2015), and are resistant to penicillin and cephalosporins. This explains the resistance rate of ampicillin and amoxicillin in our study, which is comparable to the outcomes from other studies (Fiedler *et al.*, 2019; Kim *et al.*, 2015; Lee *et al.*, 2012). The resistance of all isolates to beta-lactams was observed, and these findings are consistent with others reported (Gao *et al.*, 2018; Ikeda *et al.*, 2015; Kim *et al.*, 2015). The abundant production of beta-lactamases by *Bacillus* spp. may have triggered resistance to betalactams (Bottone, 2010; Lee *et al.*, 2012; Tahmasebi *et al.*, 2014). It has been found that the wild genomes of many microorganisms, including the *Bacillus* spp., have genes for the production of βlactamase. However, this chromosome β-lactamase does not provide active antibiotic resistance in wild-type bacilli in general; and the genes are completely silent (Chen *et al.*, 2003; OwusuKwarteng *et al.*, 2017; Park *et al.*, 2009). The *blm* gene that confers resistance to β-lactams was not found. Further investigation is needed to understand the mechanism of beta-lactam resistance in *Bacillus* spp.

Antibiotic resistance can be triggered by the overuse of antibiotics in medicine, agriculture, and aquaculture, and is attributable to several mechanisms (Beceiro *et al.*, 2013; Economou & Gousia, 2015). These mechanisms include the horizontal gene transfer (HGT) of virulence and resistant genes, frequently facilitated by biofilm formation in *Bacillus* spp. (Schroeder *et al.*, 2017; Sharma *et al.*, 2019). The carbapenems are considered the last line of antibiotics used for diseases caused by multidrug-resistant bacteria, due to their stability against beta-lactamases, penicillinases, and cephalosporins, as well as their broad spectrum of action (Meletis, 2016; Nicolau, 2007). The lack of information or available published studies on the determination of antibiotic susceptibility profiles in *Bacillus* spp. collected from distinct geographical areas indicates that more studies are necessary for comparison. Furthermore, to allow the proper management of *Bacillus* species infections, clinically relevant antibiotic breakpoint values for this genus will be needed.

Several virulence factors were recognised to enhance the pathogenic potential of *Bacillus* spp. Most of the *Bacillus* isolates were positive for genes responsible for developing diarrhoeal syndrome, with none positive for genes for emesis syndrome (*ces* gene). Interestingly, the same result has been reported in Japan by Horii *et al.* (2011), where a profile of virulence genes in *Bacillus* spp. was studied, showing that the hemolysin BL (*hblACD*) and nonhemolytic *nhe* (*ABC*) and cytotoxin K (*cytK*) genes were dominant. None of the isolates carried the emetic toxin (*ces*) gene (Horii *et al.,* 2011). This indicated that the diarrhoeal *Bacillus* strains are most widely distributed and dominant in hospital environmental settings. However, the prevalence is higher than that described by (Gao *et al.,* 2018; Glasset *et al.,* 2018), where 54 % was reported for the *hbl* gene and 34 % for the *nhe* gene. The production of toxin genes such as *hbl, nhe,* and *CytK* by *Bacillus* spp. are familiar agents of human gastrointestinal diseases (Ehling-Schulz *et al.,* 2006; Glasset *et al.,* 2018; Ramarao & Sanchis, 2013). These results support the previous report that the presence of two or more enterotoxins in *Bacillus* spp. can produce illnesses such as diarrhoea in humans (Glasset *et al.,* 2018). Furthermore, this indicates that the *Bacillus* from these hospital environments would probably not trigger emetic syndrome associated with the cereulide gene. Most isolates 77% (104/135) harboured the *cytK* gene, although *cytK* was reported dominant in isolates recovered from the environment or soil.

Molecular typing of bacteria is the standard method of determining the source and thereby understanding the epidemiology, of the bacteria (Gao *et al.,* 2018). It separates strains below the species or subspecies level and may be used to identify the cause of an outbreak, allowing the study of microbial population dynamics, and may be useful in the epidemiological surveillance of microbial illnesses (Ruppitsch, 2016). The ERIC-PCR is a simple and effective method for studying genetic diversity among strains and assessing the relationship between phenotypic and genetic characteristics of bacterial species (Gao *et al.,* 2018).

A study on the typing of *B. cereus* strains in a hospital of Zhejiang province in China, using showed that the *Bacillus* isolates are genetically diverse, belong to a complicated

complex, not a simple clone or single clade, and exhibit distinct genetic diversity (Castiaux *et al.,* 2014; EhlingShulz *et al.,* 2005a; Yang *et al.,* 2017). In this study, ERIC-PCR DNA fingerprints isolated from different hospitals differed, indicating that these isolates were not epidemiologically related. However, further analysis revealed a complex spread of this *Bacillus* spp. strains between the sites and/or wards within each specific hospital setting. The tertiary hospital showed isolates from different wards belonging to the same cluster (cluster-D; Figure 2-4). Within this group, *Bacillus* strains with similar resistance profiles from different hospital wards have been grouped, suggesting possible transmission of such strains from different locations such as doorknobs, blood pressure monitors, and patient files. In hospital wards, healthcare workers usually touch potentially contaminated surfaces. Healthcare workers may also use contaminated equipment, which plays a primary role in spreading these strains when proper hygienic standards are not applied (Collins, 2008; Fijan & Turk, 2012). In the regional hospital (Cluster-F; Figure 2-5), an exchange of strains between wards (ICU and general ward) and from different sites, was observed. The frequency of contact in hospital wards or special units may also contribute to the spread of the virus. Medical records, for example, are modified by doctors, nurses, and other medical personnel as they are recorded and transferred to the next shift. (Chen *et al.,* 2014). Patient files are sent to health posts, medical records, or beds for examination, surgery, or treatment rooms; and all are therefore vulnerable to bacterial contamination. One file's movement can lead to the spread of these strains between nurses' tables, occupied beds, phones, and door handles. It has been reported that the contamination from a file is greater in special wards (medical, surgical, and paediatric intensive care units) than in general wards (Chen *et al.,* 2014; Teng *et al.,* 2009). This point emphasizes the importance of handwashing before and after handling patient files, as effective hand washing is the most effective way to prevent pathogen transmission from vector to vector and vector to host. (Chen *et al.,* 2014; Panhotra *et al.,* 2005; Sax *et al.,* 2009; Tacconelli, 2011; WHO, 2009). The ERIC results also showed that isolates collected at different periods cluster together through bacterial persistence and tolerance. This confirms *Bacillus* spp. ubiquitous nature, which allows it to colonize easily, and its spores' ability to withstand dry heat, environmental stress and certain chemical disinfectants for an extended period of time (Tagoe & Desbordes, 2012; Ulrich *et al.,* 2018). This suggests that, despite stringent cleaning procedures, *Bacillus* spp. can persist in the hospital environment and continue to be a source of infection for patients (Doll *et al* ., 2018; Glasset *et al.,* 2018).

2.5 Conclusion

In summary, the overall findings highlight the prevalence, phenotypic, and molecular characterisation of *Bacillus* spp. in the hospital environment, as well as its spread within the same hospital, but in different wards. The high rates of resistance that were observed in this study may warn of the potential challenges in the treatment of patients infected by these strains. The *Bacillus* strains harboured in the studied hospital environments, and more likely causing diarrhoeal syndrome may assist with therapeutic options for this bacterium's hospital-acquired infections. The ERIC-PCR typing analysis showed high genetic diversity in *Bacillus* species from different hospital environments. However, there were *Bacillus* spp. clones present in the wards of the same hospital. Our findings highlight the need to screen for *Bacillus* within the hospital environment. The authors would like to suggest that *Bacillus* maybe suited as an indicator organism for hospital hygiene surveillance. Intervention programs to constantly develop improved cleaning methods to decrease the environmental transmission of pathogenic microorganisms in the hospital environment are imperative.

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Supplementary materials

Table S1: Description of the samples collected from hospital, wards and sampling sites (Department of Health, 2012) and the National Health Insurance Policy (Department of Health, 2017).

Hospitals classification	Hospital wards	Sites
A • Central with tertiary care level-3 facility and known as a referral hospital. Large as consisted of approximately 800 beds • Provides training for healthcare providers and conduct research. • Central quaternary care hospital. It provides comprehensive healthcare services that include sophisticated diagnostics and treatment services • Subspecialty services provided include advanced trauma care organ transplants with complex technical equipment and diagnostic support services.	• General ward • Intensive Care Unit • Paediatrics •	• BP monitor • Door handle • Drip stand • Mop • Nurses table • Occupied bed • Unoccupied bed Patient file Phone Sink Ventilation
B • Central with services rendered through the level-3 facility and providing regional and tertiary services to the whole of KZN and Eastern Cape. • Medium-sized hospital with approximately 900 beds. • Also, a teaching hospital. • Provide highly specialised staff and healthcare facilities		

Continue Table S1: Description of the samples collected from hospital, wards and sampling sites **(**Department of Health, 2012) and the National Health Insurance Policy (Department of Health, 2017).

Hospitals classification	Hospital wards	Sites
C • Regional and formerly classified as district and classified as a level-2 hospital. • It operates 24 hours with trauma and emergency services. • Approximately 500 beds. • Receive support from tertiary hospitals • Limited to province boundaries. • Provide services that can be provided by generalists in general surgery, clinical radiology, pathology and health services for aliens.		
D • District hospital, a level-1 facility that has 24hours trauma services. • Receives referral from several districts' hospitals. • Consist of 500 beds. • Provides services such as in-patients and emergency health services. • Services are provided by general practitioners, including surgery under anesthesia.		

Table S2: Primers used in this study for identification, virulence, resistance genes and ERIC PCR for *Bacillus.*

Primer	Gene target	Sequence 5'-3'	Target fragment length (bp)	References
BCFomp1 F BCRomp1 R	Mot-B	ATCGCCTCGTTGGATGACGA CTCATATCCTACCGCAGCTA	575	(Oliwa-Stasiak *et al.*, 2010)
BSub5F BSub3R	16S rRNA	AAGTCGAGCGGACAGATGG CCAGTTTCCAATGACCCTCCCCC	595	(Subramanian *et al.*, 2010)
HblA-F HblA-R	Hemolysin BL	GTGCAGATGTTGATGCCGAT ATGCCACTGCGTGGACATAT	320	(Hansen & Hendriksen, 2001)
HblC-F HblC-R	Hemolysin BL	AATGGTCATCGGAACTCTAT CTCGCTGTTCTGCTGTTAAT	750	(Hansen & Hendriksen, 2001)
HblD-F HblD-R	Hemolysin BL	AATCAAGAGCTGTCACGAAT CACCAATTGACCATGCTAAT	430	(Hansen & Hendriksen, 2001)
NheA-F NheA-R	Non-hemolytic enterotoxin (NHE)	TACGCTAAGGAGGGGCA GTTTTTATTGCTTCATCGGCT	500	(Hansen & Hendriksen, 2001)
NheB-F NheB-R	Non-hemolytic enterotoxin (NHE)	CTATCAGCACTTATGGCAG ACTCCTAGCGGTGTTCC	770	(Hansen & Hendriksen, 2001)
NheC-F NheC-R	Non-hemolytic enterotoxin (NHE)	CGGTAGTGATTGCTGGG CAGCATTCGTACTTGCCAA	583	(Hansen & Hendriksen, 2001)
BceT-F BceT-R	Enterotoxin	CGTATCGGTCGTTCACTCGG GTTGATTTTCCGTAGCCTGGG	661	(Hansen & Hendriksen, 2001)
CytK-F CytK-R	Cytotoxic protein (CytK)	AAAATGTTTAGCATTATCCGC TGTACCAGTTGTATTAATAACGGC AATC	238	(Oltuszak-Walczak & Walczak, 2013)
Ces-F Ces-R	Cereulide	GGTGACACATTATCATATAAG GGGTAAGCGAACCTGTCTGTAAC AA	1271	Ehling-Schulz *et al.*, (2005b)
Hly II-F Hly II-R	Potential enterotoxin Haemolysin II	GATTCTAAAGGAACTGTAG GGTTATCAAGAGTAACTTG	867	Fagerlund *et al.* (2004)
EntFM-F EntFM-R	Enterotoxin (EntFM)	ATGAAAAAAGTAATTTGCAGG TTAGTATGCTTTTGTGTAACC	1269	(Asano *et al.*, 1997)

Continued Table S2: Primers used in this study for identification, virulence, resistance genes and ERIC PCR for *Bacillus.*

Primer	Gene target	Sequence 5'- 3'	Target fragment length (bp)	References
ERIC -F **ERIC-R**	ERIC-PCR	ATGTAAGCTCCTGGGGATTCA CAAGTAAGTGACTGGGGTGAGCG	200 up	Versalovic *et al.* (1991)
erm B-1 ***erm B-2***	Erythromycin B	CTATCTGATTGTTGAAGAAGG ATT GTTTACTCTTGGTTTAGGATG AAA	142	Adimpong *et al.* (2012)
tet K -1 ***tet K -2***	Tetracycline K	TTAGGTGAAGGGTTAGGTCC GCAAACTCATTCCAGAAGCA	718	(Aarestrup *et al.*, 2000)
tet M-1 ***tet M-2***	Tetracycline M	GTTAAATAGTGTTCTTGGAG CTAAGATATGGCTCTAACAA	657	Aarestrup *et al.* (2000)
tet A-F ***tet A-R***	Tetracycline A	GGCGGTCTTCTTCATCATGC CGGCAGGCAGAGCAAGTAGA	502	Rather *et al.* (2012)
tet B-F ***tet B-R***	Tetracycline B	CATTAATAGGCGCATCGCTG TGAAGGTCATCGATAGCAGG	930	Rather *et al.* (2012)
tet 39-F ***tet 39-R***	Tetracycline 39	CTCCTTCTCTATTGTGGCTA CACTAATACCTCTGGACATCA	701	(Adelowo & Fagade, 2009)
blm-F ***blm-R***	Beta-lactamase	CGCGGAAGATTAAATGGTTAT AATGGATGCTGGCGTAATCAA CAGATTCAG	2900	Tahmasebi *et al.* (2014)

CHAPTER 3: **GENERAL CONCLUSIONS AND FUTURE WORK**

3.1 Summary on the incidence of *Bacillus* species in the environment of the public hospitals studied

This study describes the dissemination, antimicrobial resistance pattern, virulence and resistance genes, and genetic relatedness of 135 *Bacillus* spp. isolates from the public hospital environment of four public hospitals in KwaZulu-Natal, South Africa.

3.2 Conclusion and significant findings

The study showed a moderate prevalence of the *Bacillus* spp. identified from the four different hospital environments, and a high level of phenotypic resistance to antibiotics compared to genotypic resistance. Most genes causing gastrointestinal illness were observed, and major genetic diversity was detected.

The following conclusions were drawn concerning the study:

- A total of 17 % (135/777) *Bacillus* spp. was identified from the environment of the four different hospitals with detection in each hospital as follows: Central with 24 % (32/135), tertiary with 33 % (45/135), regional 27 % (36/135) and district with 16 % (22/135).
- There were statistically significant differences in the prevalence of *Bacillus* species collected from the four different hospitals ($p = 0.044$). The significant differences were between the tertiary and regional hospitals ($p = 0.000$). There was no statically significant difference in the prevalence of *Bacillus* isolates collected at different time points during the week ($p = 0.766$).
- High *Bacillus* bacterial prevalence was present in the tertiary hospital, which yielded the highest, 13 % (45/135), detection. Less bacterial prevalence was found in the district hospital, which consisted of 16 % (22/135) isolates. The tertiary hospital serves as a teaching hospital; therefore, a diverse group of people can facilitate the spread of *Bacillus* pathogens between healthcare workers, patients, and students attending their clinical training.
- The overall incidence of *Bacillus* spp. among three wards, viz. ICU, general ward, and paediatric of the four public hospitals averaged 33 % (45/135). ICU

might be slightly more at risk, although this could not be statistically shown. The chi-squared test showed that there were no statistical differences ($p = 0.133$) in the prevalence of *Bacillus* amongst the three hospital wards (ICU, paediatric and general ward). Intensive care units, where repeated use of invasive procedures and multiple therapies, puts patients at increased risk, increasing HAI prevalence.

- A positive correlation was observed between wards and hospitals ($r = 0.525$; $p = 0.000$); these positive correlations indicated that as *Bacillus* increases in the hospital wards, a correlative increase also occurred in the studied hospitals.

- Of the eleven hospital sites, it was revealed that unoccupied beds (8% (11/135), drip stands (12% (16/135)), sinks (12% (16/135), ward phones (12% (16/135), and nurses' tables (10% (14/135) indicated the highest prevalence compared to other sites. Ward telephones can be the source of HAIs as the contamination and transmission occur due to ear and hand contact of the healthcare workers. The same contamination occurs when operating sinks in the different wards.

- The isolates indicated complete resistance to 100 % (135/135) ampicillin, 99 % (134/135) to ciprofloxacin, 97 % (131/135) resistant to amoxicillin, 82 % (112/135) resistant to tetracycline, and 51 % (69/135) cefotaxime, 44 % (59/135) to erythromycin, 20 % (27/135) to meropenem, and 19 % (26/135) to imipenem. Multi-drug resistance which is antimicrobial resistance shown by the species to three or more antimicrobial classes was found in 98 % (132/135) of the *Bacillus* spp. collected. A total of 43 different MDR antibiograms were detected with CIP-TET-AMXAMP as the most common antibiogram (refer to Appendix 7 for the values of the MIC's of the isolates). Therefore, antibiotic susceptibility profiles showed the presence of highly resistant *Bacillus* spp.
- Resistance to a large number of antibiotics is a severe threat to public health, especially from a therapeutic point of view, which can disrupt experimental therapy in the event of an outbreak. Therefore, it is essential to evaluate *Bacillus*'s resistance against multiple antibiotics to improve infectious disease management. The irresponsible and indifferent use of antimicrobials in animals has been linked to environmentally resistant bacteria's growth and spread.

Subsequent transmission to humans via the food chain has the potential to have serious public health consequences.

- In a hospital setting, the presence of a *Bacillus* species may serve as a risk factor for the occurrence of hospital-acquired infections (HAI's) as well as for the treatment of medical care recipients. This issue is more pronounced in developing countries, as there are constraints such as limited financial resources. Most public hospitals are overcrowded, which contributes to bacteria variability, the inability to adequately clean the environment, and, most importantly, beds are not released long enough to implement disinfection practices. Lack of knowledge of aseptic practices by janitors and healthcare providers, non-observance of safe practices by healthcare workers are factors that can increase the prevalence of bacteria in a hospital.

- The frequency of the resistance genes investigated was as follows: *ermB*, *tetM*, and *tetK* were 56 % (5/112), 5 % (5/112), and 4 % (4/112), respectively, but *tet A, tetB, tet39,* and *blm* were not detected in any of the *Bacillus* spp. This may be due to the presence of mutated pseudogenes in susceptible strains, or resistance may depend on other unknown (or unexplored) genes and mechanisms.

- Virulence genes causing diarrheal syndrome were the most dominant with *hblA* 77 % (104/135) and *hblD* 88 % (119/135), but there was no emetic syndrome *ces* gene detected, as shown in (Appendix 7). The variability of virulence genes can be traced back to different geographic locations and multiple strain sources.

- The ERIC-PCR revealed highly diverse *Bacillus* spp. isolates with major clusters consisting of the isolates from different wards and sites within the same hospital indicating cross-transmission of the bacteria within the hospital. Clonality reveals that the hospital contaminant environment is the link between the transfer of infectious microorganisms acquired in the hospital. A high level of genetic diversity was also detected in isolates collected in the same wards but different hospitals, as shown in Appendices 4 to 7. This also indicates cross-contamination between the different environmental surfaces alluding to inefficient disinfection protocols.

- There was a moderate prevalence of *Bacillus* spp. 17 % (135/777) present in the environment of the four public health facilities at various levels of healthcare services in South Africa. Therefore, the research question was answered. Affected surfaces are frequently more likely to transmit HAI, whereas the latter refers to areas with minimal hand contact and thus less likely to be a route of contamination. In the absence of sufficient environmental remediation, the risk of infection increases if a patient is immediately placed in an environment where another patient was previously infected with the same pathogen, highlighting the environment as a transmission route for HAIs. There was a significant difference in the prevalence of *Bacillus* spp. in the four public hospitals' environment; the null hypothesis was rejected as the results observed were significant.

3.3 Limitations

This study consisted of the following limitations:

- Antibiotic breakpoint scores for *Bacillus* species are identified at the category of the genus due to the lack of existing data on the antimicrobial resistance profile of *Bacillus* genus. Breakpoint values for *Bacillus* spp. are required. to be revealed at the species level rather than just at the genus level.

- Samples have been preserved (frozen) for extended periods during processing and analyses, which may have undermined the degree to which *Bacillus* has been retrieved.

3.4 Future study and recommendations

The following recommendations were assembled based on the results of this study:

- Infection control and prevention should also include molecular epidemiological investigations of the *Bacillus* species in the hospital environment.

- There is still a gap in the epidemiological cut-off values of *Bacillus* species.
- There is a need for standardized antibiotics breakpoint of *Bacillus* spp. to be established at the level of the species, not only at the level of the genus.

- Further molecular research into the mechanism of tetracycline resistance in *Bacillus* spp. is needed.

- Additional research into the current sequence of resistance genes found in the *Bacillus* genome, as well as the likelihood of horizontal gene transfer between strains and species that results in antimicrobial resistance in *Bacillus* species, is required. Therefore, more studies focussing on virulence and resistance genes investigating if these genes are carried on the plasmid or chromosome. Ore gene expression research is needed to lead to a deeper understanding of the relationship between resistance and virulence genes, investigating if the presence of virulence genes leads to the bacteria being resistant to antimicrobials.

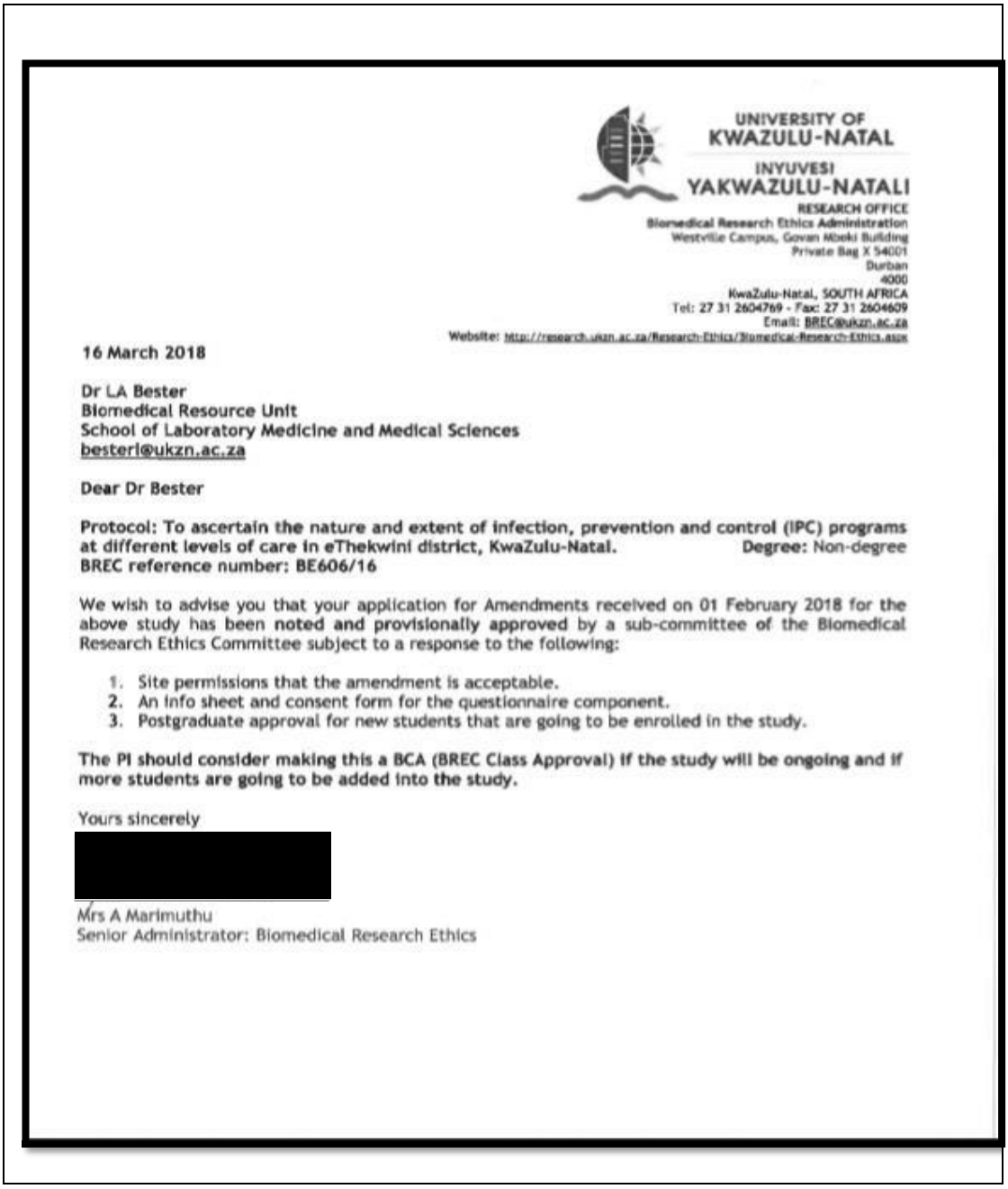
- The differences obtained in the *Bacillus* ERICPCR fingerprints may represent phenotypic and genotypic differences, which can be further studied by sequence analysis, such as the whole genome sequence, to check whether they are different strains as shown at the time of typing.

- This study highlights the need for an intervention program to develop cleaning methods to reduce the environmental transmission of pathogenic microorganisms in the hospital environment.

- Biofilm formation can reduce the effects of ineffective cleaning practices even on dry surfaces, as bacteria can remain in the environment longer and, therefore, more resistant to widely used microbes. Therefore, there is a need to determine the biopotential of an organism.

APPENDICES

Appendix 1: Biomedical Research Ethics Committee (BREC) of the University of Kwa-Zulu-Natal approval letter reference number: BE606/16.



UNIVERSITY OF KWAZULU-NATAL
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RESEARCH OFFICE
Biomedical Research Ethics Administration
Westville Campus, Govan Mbeki Building
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KwaZulu-Natal, SOUTH AFRICA
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Email: BREC@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

16 March 2018

Dr LA Bester
Biomedical Resource Unit
School of Laboratory Medicine and Medical Sciences
besterl@ukzn.ac.za

Dear Dr Bester

Protocol: To ascertain the nature and extent of infection, prevention and control (IPC) programs at different levels of care in eThekwini district, KwaZulu-Natal. **Degree:** Non-degree
BREC reference number: BE606/16

We wish to advise you that your application for Amendments received on 01 February 2018 for the above study has been **noted and provisionally approved** by a sub-committee of the Biomedical Research Ethics Committee subject to a response to the following:

1. Site permissions that the amendment is acceptable.
2. An info sheet and consent form for the questionnaire component.
3. Postgraduate approval for new students that are going to be enrolled in the study.

The PI should consider making this a BCA (BREC Class Approval) if the study will be ongoing and if more students are going to be added into the study.

Yours sincerely

[redacted]

Mrs A Marimuthu
Senior Administrator: Biomedical Research Ethics

Appendix 2: TRREE certificates for completion of research ethics course that included introduction research ethics and evaluation, informed consent, good clinical practice, HIV vaccine trials, adolescent involvement in HIV prevention trials.

TRREE

Zertifikat
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Promouvoir les plus hauts standards éthiques dans la protection des participants à la recherche biomédicale
Promoting the highest ethical standards in the protection of biomedical research participants

Clinical Trials Centre
The University of Hong Kong

Certificat de formation - Training Certificate

Ce document atteste que - this document certifies that

zamile mbhele

a complété avec succès - has successfully completed

Introduction to Research Ethics

du programme de formation TRREE en évaluation éthique de la recherche
of the TRREE training programme in research ethics evaluation

[redacted]

January 24, 2018

Professeur Dominique Sprumont
Coordinateur TRREE Coordinator

FMH Continuing Education Program (5 Credits)
Programme de Formation continue (5 Crédits)

Fédération Pharmaceutique Helvétique FPH Programmes de formation continue Continuing Education Programme Programmes de formation continue

Ce programme est soutenu par - This program is supported by :

TRREE

Zertifikat
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Certificate

Promouvoir les plus hauts standards éthiques dans la protection des participants à la recherche biomédicale
Promoting the highest ethical standards in the protection of biomedical research participants

Clinical Trials Centre
The University of Hong Kong

Certificat de formation - Training Certificate

Ce document atteste que - this document certifies that

zamile mbhele

a complété avec succès - has successfully completed

Research Ethics Evaluation

du programme de formation TRREE en évaluation éthique de la recherche
of the TRREE training programme in research ethics evaluation

[redacted]

January 28, 2018

Professeur Dominique Sprumont
Coordinateur TRREE Coordinator

FMH Continuing Education Program (5 Credits)
Programme de Formation continue (5 Crédits)

Fédération Pharmaceutique Helvétique FPH Programmes de formation continue Continuing Education Programme Programmes de formation continue

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Appendix 3: Submission confirmation from the *Journal of Microbial Drug Resistance* (Manuscript ID: MDR-2020-0543).

Microbial Drug Resistance
Mechanisms, Epidemiology, and Disease

Microbial Drug Resistance: http://mc.manuscriptcentral.com/mdr

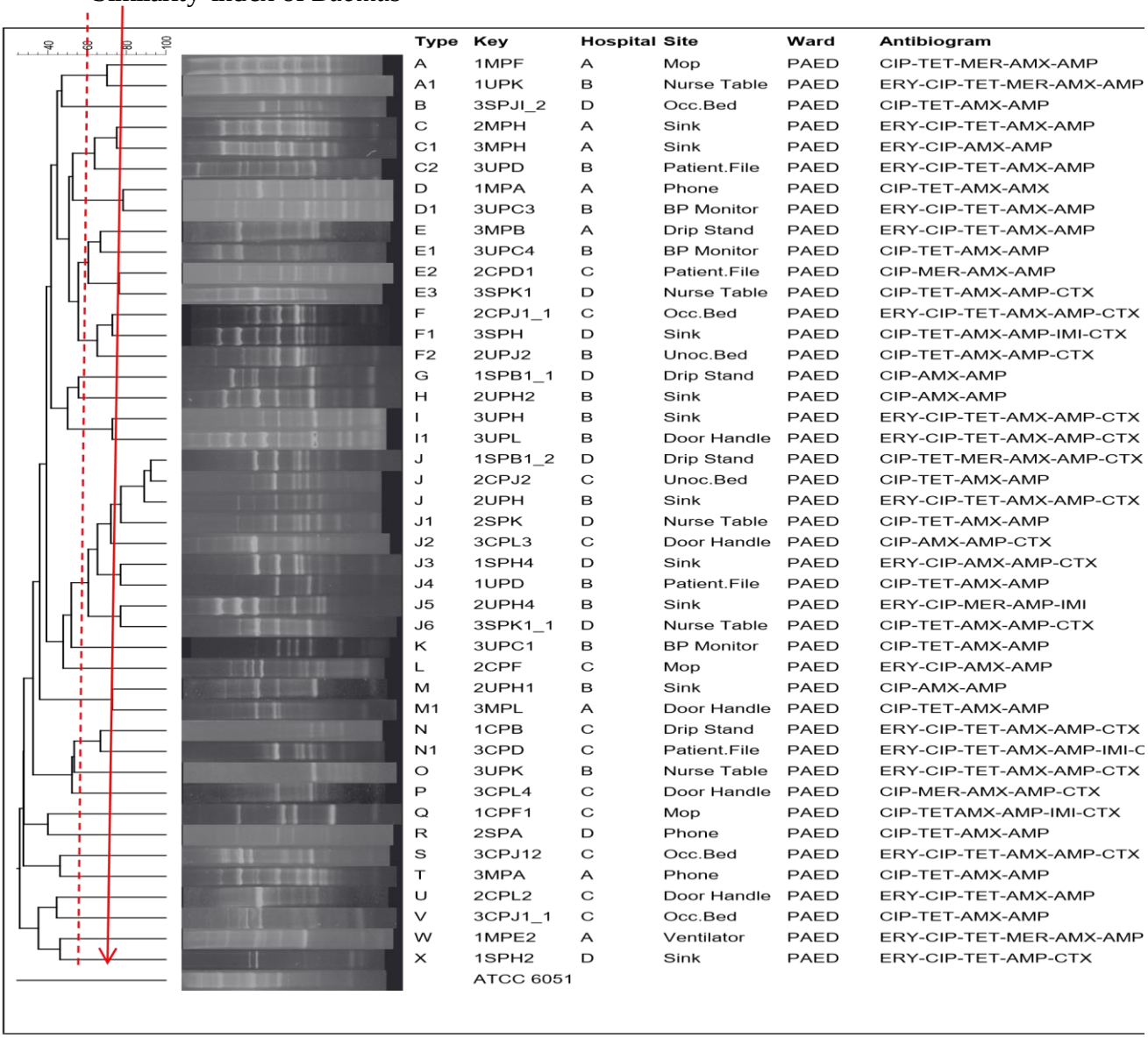
Contamination, antibiotic resistance, virulence factors and genetic diversity of Bacillus spp. from public hospital environments in South Africa

Journal:	*Microbial Drug Resistance*
Manuscript ID	MDR-2020-0543
Manuscript Type:	Epidemiology
Date Submitted by the Author:	14-Nov-2020
Complete List of Authors:	Mbhele, Zamile N. ; University of KwaZulu-Natal Nelson R Mandela School of Medicine Shobo, Christiana; University of KwaZulu-Natal College of Health Sciences, Biomedical Resource Unit Amoako, Daniel; University of KwaZulu-Natal College of Health Sciences, Antimicrobial Research Unit; University of KwaZulu-Natal Zishiri, Oliver T. ; University of KwaZulu-Natal Faculty of Science and Agriculture Bester , Linda ; University of KwaZulu-Natal College of Health Sciences, Biomedical Resource Unit
Keyword:	Antibiotics, Microbial Drug Resistance, Epidemiology, Virulence, Genotyping
Manuscript Keywords (Search Terms):	Bacterial contamination, hospital environment, Bacillus spp, antibiotic resistance, genetic relatedness, South Africa
Abstract:	This study aimed to assess the molecular dissemination of Bacillus species in public hospitals in South Africa. The study conducted over three months during 2017 involved representative samples obtained from three wards (general, Intensive care unit (ICU) and paediatric unit) from four public hospitals denoted as A (Central), B (Tertiary), C (Regional), and D (District). Swabs collected from 11 distinct hospital surfaces were screened using selective media, biochemical testing, and molecular methods. Overall, 135/777 (17%) isolates were identified with a prevalence of (32/135; 24%) for central, (45/135; 33%) for tertiary, (36/135; 27%) for regional, and (22/135; 16%) for district hospital. Bacillus species were further grouped to belong to either cereus or subtilis. It was confirmed (6/135;4%) B. subtilis and (129/135; 96%) B. cereus. Prevalence was similar across the wards, averaging (45/135; 33.3%). The highest prevalence of Bacillus isolates was found on the drip stands (11.8%), sink (11.8%), ward phone (11.5%) and nurses' tables (10.3%). Minimum inhibitory concentration analyses revealed high resistance to β-lactams, fluoroquinolones, and tetracyclines. The most common resistance genes detected were ermB (56%) and tetM (5%). Enterotoxin virulence genes hblA (77%) and hblD (88%) were most detected; however, no ces genes (cereulide toxin) were found. The ERIC-PCR revealed considerable diversity among the different levels of health care, although the clonal-spread of strains between the

Appendix 4: Figure of dendrogram of *Bacillus* isolates collected from the Pediatric ward of four public hospitals KwaZulu-Natal South Africa.

The solid red line indicated the major ERIC-type cut-off and the red dotted line indicated the ERIC-type cutoff. The dotted line indicated the ERIC-type cut-off while the solid line indicates major ERIC-type cut-off. Abbreviations: PAED: Pediatric; OCC.bed: Occupied bed; Unoc. Bed: Unoccupied bed; ERY: erythromycin; CIP: Ciprofloxacin; TET: Tetracycline; MER: Meropenem; IMI: Imipenem; AMP: Ampicillin; AMX: Amoxicillin; CTX: Cefotaxime. *B. subtillis* ATCC 6051 was used as quality control strain (Similarity Index of *Bacillus).*

Similarity index of *Bacillus*

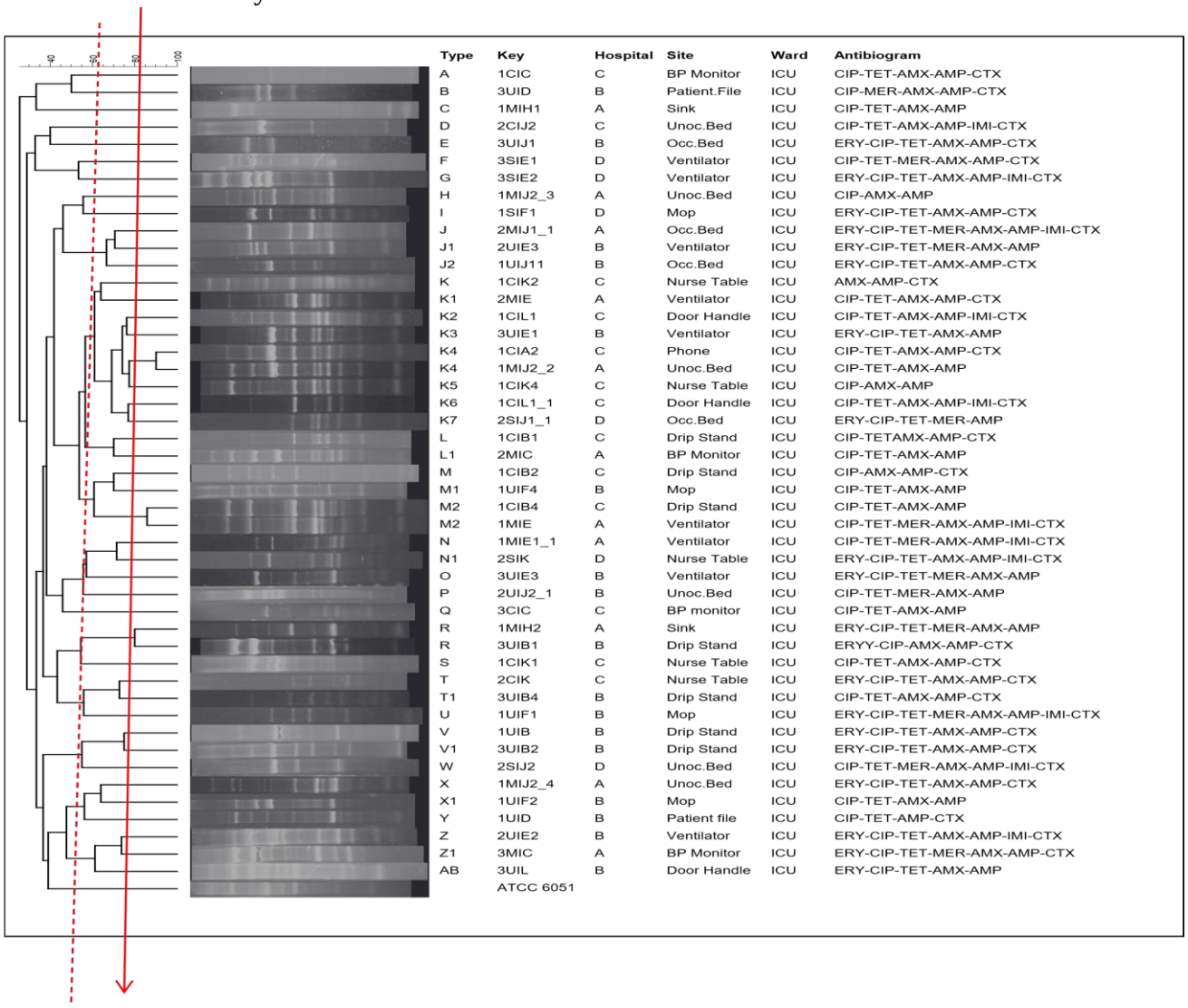


Type	Key	Hospital	Site	Ward	Antibiogram
A	1MPF	A	Mop	PAED	CIP-TET-MER-AMX-AMP
A1	1UPK	B	Nurse Table	PAED	ERY-CIP-TET-MER-AMX-AMP
B	3SPJI_2	D	Occ.Bed	PAED	CIP-TET-AMX-AMP
C	2MPH	A	Sink	PAED	ERY-CIP-TET-AMX-AMP
C1	3MPH	A	Sink	PAED	ERY-CIP-AMX-AMP
C2	3UPD	B	Patient.File	PAED	ERY-CIP-TET-AMX-AMP
D	1MPA	A	Phone	PAED	CIP-TET-AMX-AMX
D1	3UPC3	B	BP Monitor	PAED	ERY-CIP-TET-AMX-AMP
E	3MPB	A	Drip Stand	PAED	ERY-CIP-TET-AMX-AMP
E1	3UPC4	B	BP Monitor	PAED	CIP-TET-AMX-AMP
E2	2CPD1	C	Patient.File	PAED	CIP-MER-AMX-AMP
E3	3SPK1	D	Nurse Table	PAED	CIP-TET-AMX-AMP-CTX
F	2CPJ1_1	C	Occ.Bed	PAED	ERY-CIP-TET-AMX-AMP-CTX
F1	3SPH	D	Sink	PAED	CIP-TET-AMX-AMP-IMI-CTX
F2	2UPJ2	B	Unoc.Bed	PAED	CIP-TET-AMX-AMP-CTX
G	1SPB1_1	D	Drip Stand	PAED	CIP-AMX-AMP
H	2UPH2	B	Sink	PAED	CIP-AMX-AMP
I	3UPH	B	Sink	PAED	ERY-CIP-TET-AMX-AMP-CTX
I1	3UPL	B	Door Handle	PAED	ERY-CIP-TET-AMX-AMP-CTX
J	1SPB1_2	D	Drip Stand	PAED	CIP-TET-MER-AMX-AMP-CTX
J	2CPJ2	C	Unoc.Bed	PAED	CIP-TET-AMX-AMP
J	2UPH	B	Sink	PAED	ERY-CIP-TET-AMX-AMP-CTX
J1	2SPK	D	Nurse Table	PAED	CIP-TET-AMX-AMP
J2	3CPL3	C	Door Handle	PAED	CIP-AMX-AMP-CTX
J3	1SPH4	D	Sink	PAED	ERY-CIP-AMX-AMP-CTX
J4	1UPD	B	Patient.File	PAED	CIP-TET-AMX-AMP
J5	2UPH4	B	Sink	PAED	ERY-CIP-MER-AMP-IMI
J6	3SPK1_1	D	Nurse Table	PAED	CIP-TET-AMX-AMP-CTX
K	3UPC1	B	BP Monitor	PAED	CIP-TET-AMX-AMP
L	2CPF	C	Mop	PAED	ERY-CIP-AMX-AMP
M	2UPH1	B	Sink	PAED	CIP-AMX-AMP
M1	3MPL	A	Door Handle	PAED	CIP-TET-AMX-AMP
N	1CPB	C	Drip Stand	PAED	ERY-CIP-TET-AMX-AMP-CTX
N1	3CPD	C	Patient.File	PAED	ERY-CIP-TET-AMX-AMP-IMI-C
O	3UPK	B	Nurse Table	PAED	ERY-CIP-TET-AMX-AMP-CTX
P	3CPL4	C	Door Handle	PAED	CIP-MER-AMX-AMP-CTX
Q	1CPF1	C	Mop	PAED	CIP-TETAMX-AMP-IMI-CTX
R	2SPA	D	Phone	PAED	CIP-TET-AMX-AMP
S	3CPJ12	C	Occ.Bed	PAED	ERY-CIP-TET-AMX-AMP-CTX
T	3MPA	A	Phone	PAED	CIP-TET-AMX-AMP
U	2CPL2	C	Door Handle	PAED	ERY-CIP-TET-AMX-AMP
V	3CPJ1_1	C	Occ.Bed	PAED	CIP-TET-AMX-AMP
W	1MPE2	A	Ventilator	PAED	ERY-CIP-TET-MER-AMX-AMP
X	1SPH2	D	Sink	PAED	ERY-CIP-TET-AMP-CTX
	ATCC 6051				

Appendix 5: Figure of dendrogram of *Bacillus* isolates collected from the Intensive Care Unit (ICU) of four public hospitals KwaZulu-Natal South Africa.

The solid red line indicated the major ERIC-type cut-off and the red dotted line indicated the ERIC-type cutoff. The dotted line indicated the ERIC-type cut-off while the solid line indicates major ERIC-type cut-off. Abbreviations: OCC.bed: Occupied bed; Unoc. Bed: Unoccupied bed; ERY: erythromycin; CIP: Ciprofloxacin; TET: Tetracycline; MER: Meropenem; IMI: Imipenem; AMP: Ampicillin; AMX: Amoxicillin; CTX: Cefotaxime. *B. subtillis* ATCC 6051 was used as quality control strain.

Similarity index of *Bacillus*

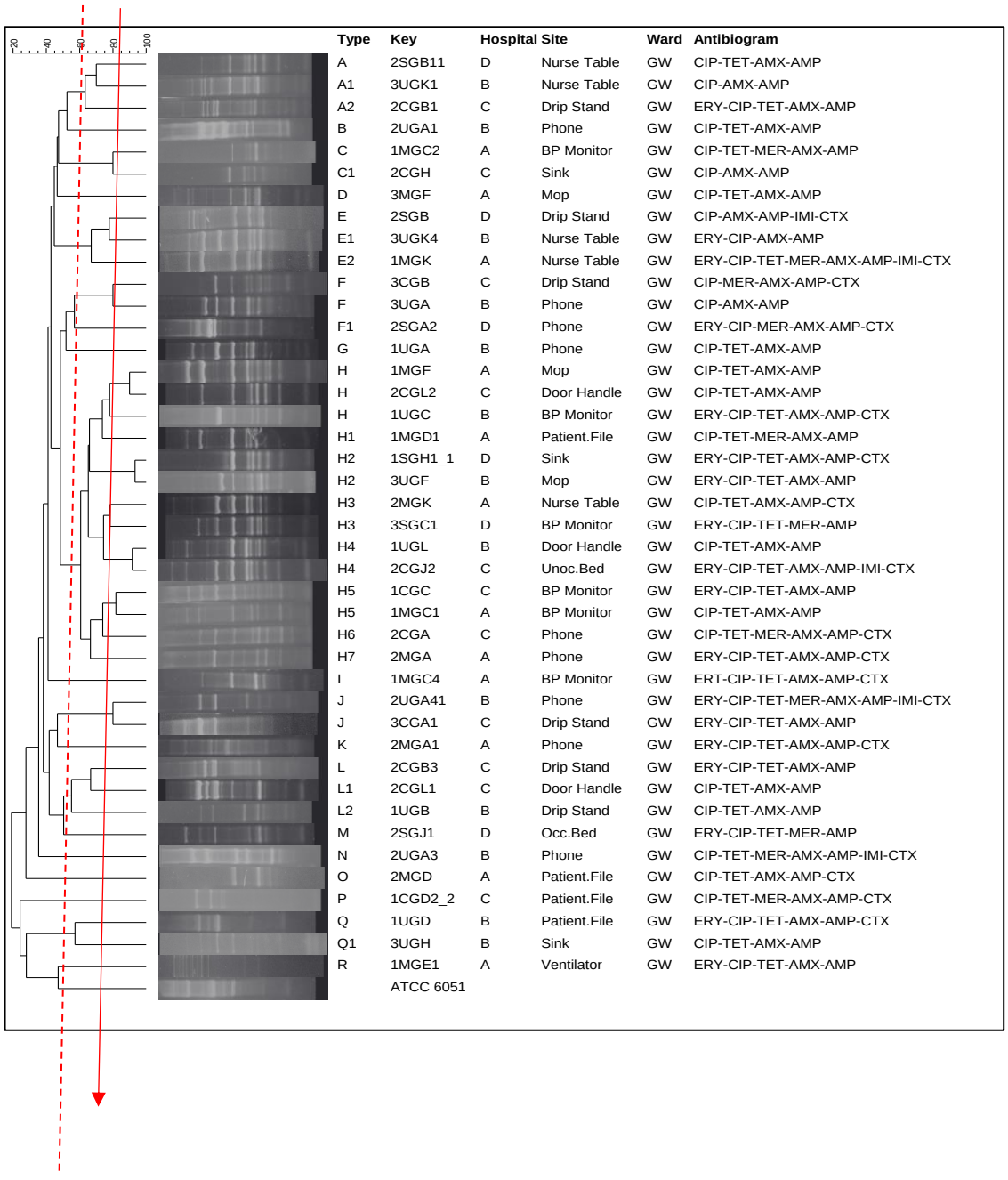


Type	Key	Hospital	Site	Ward	Antibiogram
A	1CIC	C	BP Monitor	ICU	CIP-TET-AMX-AMP-CTX
B	3UID	B	Patient.File	ICU	CIP-MER-AMX-AMP-CTX
C	1MIH1	A	Sink	ICU	CIP-TET-AMX-AMP
D	2CIJ2	C	Unoc.Bed	ICU	CIP-TET-AMX-AMP-IMI-CTX
E	3UIJ1	B	Occ.Bed	ICU	ERY-CIP-TET-AMX-AMP-CTX
F	3SIE1	D	Ventilator	ICU	CIP-TET-MER-AMX-AMP-CTX
G	3SIE2	D	Ventilator	ICU	ERY-CIP-TET-AMX-AMP-IMI-CTX
H	1MIJ2_3	A	Unoc.Bed	ICU	CIP-AMX-AMP
I	1SIF1	D	Mop	ICU	ERY-CIP-TET-AMX-AMP-CTX
J	2MIJ1_1	A	Occ.Bed	ICU	ERY-CIP-TET-MER-AMX-AMP-IMI-CTX
J1	2UIE3	B	Ventilator	ICU	ERY-CIP-TET-MER-AMX-AMP
J2	1UIJ11	B	Occ.Bed	ICU	ERY-CIP-TET-AMX-AMP-CTX
K	1CIK2	C	Nurse Table	ICU	AMX-AMP-CTX
K1	2MIE	A	Ventilator	ICU	CIP-TET-AMX-AMP-CTX
K2	1CIL1	C	Door Handle	ICU	CIP-TET-AMX-AMP-IMI-CTX
K3	3UIE1	B	Ventilator	ICU	ERY-CIP-TET-AMX-AMP
K4	1CIA2	C	Phone	ICU	CIP-TET-AMX-AMP-CTX
K4	1MIJ2_2	A	Unoc.Bed	ICU	CIP-TET-AMX-AMP
K5	1CIK4	C	Nurse Table	ICU	CIP-AMX-AMP
K6	1CIL1_1	C	Door Handle	ICU	CIP-TET-AMX-AMP-IMI-CTX
K7	2SIJ1_1	D	Occ.Bed	ICU	ERY-CIP-TET-MER-AMP
L	1CIB1	C	Drip Stand	ICU	CIP-TETAMX-AMP-CTX
L1	2MIC	A	BP Monitor	ICU	CIP-TET-AMX-AMP
M	1CIB2	C	Drip Stand	ICU	CIP-AMX-AMP-CTX
M1	1UIF4	B	Mop	ICU	CIP-TET-AMX-AMP
M2	1CIB4	C	Drip Stand	ICU	CIP-TET-AMX-AMP
M2	1MIE	A	Ventilator	ICU	CIP-TET-MER-AMX-AMP-IMI-CTX
N	1MIE1_1	A	Ventilator	ICU	CIP-TET-MER-AMX-AMP-IMI-CTX
N1	2SIK	D	Nurse Table	ICU	ERY-CIP-TET-AMX-AMP-IMI-CTX
O	3UIE3	B	Ventilator	ICU	ERY-CIP-TET-MER-AMX-AMP
P	2UIJ2_1	B	Unoc.Bed	ICU	CIP-TET-MER-AMX-AMP
Q	3CIC	C	BP monitor	ICU	CIP-TET-AMX-AMP
R	1MIH2	A	Sink	ICU	ERY-CIP-TET-MER-AMX-AMP
R	3UIB1	B	Drip Stand	ICU	ERYY-CIP-AMX-AMP-CTX
S	1CIK1	C	Nurse Table	ICU	CIP-TET-AMX-AMP-CTX
T	2CIK	C	Nurse Table	ICU	ERY-CIP-TET-AMX-AMP-CTX
T1	3UIB4	B	Drip Stand	ICU	CIP-TET-AMX-AMP-CTX
U	1UIF1	B	Mop	ICU	ERY-CIP-TET-MER-AMX-AMP-IMI-CTX
V	1UIB	B	Drip Stand	ICU	ERY-CIP-TET-AMX-AMP-CTX
V1	3UIB2	B	Drip Stand	ICU	ERY-CIP-TET-AMX-AMP-CTX
W	2SIJ2	D	Unoc.Bed	ICU	CIP-TET-MER-AMX-AMP-IMI-CTX
X	1MIJ2_4	A	Unoc.Bed	ICU	ERY-CIP-TET-AMX-AMP-CTX
X1	1UIF2	B	Mop	ICU	CIP-TET-AMX-AMP
Y	1UID	B	Patient file	ICU	CIP-TET-AMP-CTX
Z	2UIE2	B	Ventilator	ICU	ERY-CIP-TET-AMX-AMP-IMI-CTX
Z1	3MIC	A	BP Monitor	ICU	ERY-CIP-TET-MER-AMX-AMP-CTX
AB	3UIL	B	Door Handle	ICU	ERY-CIP-TET-AMX-AMP
	ATCC 6051				

Appendix 6: Figure of dendrogram of *Bacillus* isolates collected from the general ward of four public hospitals in KwaZulu-Natal, South Africa.

The solid red line indicated the major ERIC-type cut-off and the red dotted line indicated the ERIC-type cut-off. The dotted line indicated the ERIC-type cut-off while the solid line indicates major ERIC-type cut-off. Abbreviations: GW: general ward; OCC.bed: Occupied bed; Unoc. Bed: Unoccupied bed; ERY: erythromycin; CIP: Ciprofloxacin; TET: Tetracycline; MER: Meropenem; IMI: Imipenem; AMP: Ampicillin; AMX: Amoxicillin; CTX: Cefotaxime. *B. subtillis* ATCC 6051 was used as quality control strain.

Similarity of *Bacillus*



Type	Key	Hospital	Site	Ward	Antibiogram
A	2SGB11	D	Nurse Table	GW	CIP-TET-AMX-AMP
A1	3UGK1	B	Nurse Table	GW	CIP-AMX-AMP
A2	2CGB1	C	Drip Stand	GW	ERY-CIP-TET-AMX-AMP
B	2UGA1	B	Phone	GW	CIP-TET-AMX-AMP
C	1MGC2	A	BP Monitor	GW	CIP-TET-MER-AMX-AMP
C1	2CGH	C	Sink	GW	CIP-AMX-AMP
D	3MGF	A	Mop	GW	CIP-TET-AMX-AMP
E	2SGB	D	Drip Stand	GW	CIP-AMX-AMP-IMI-CTX
E1	3UGK4	B	Nurse Table	GW	ERY-CIP-AMX-AMP
E2	1MGK	A	Nurse Table	GW	ERY-CIP-TET-MER-AMX-AMP-IMI-CTX
F	3CGB	C	Drip Stand	GW	CIP-MER-AMX-AMP-CTX
F	3UGA	B	Phone	GW	CIP-AMX-AMP
F1	2SGA2	D	Phone	GW	ERY-CIP-MER-AMX-AMP-CTX
G	1UGA	B	Phone	GW	CIP-TET-AMX-AMP
H	1MGF	A	Mop	GW	CIP-TET-AMX-AMP
H	2CGL2	C	Door Handle	GW	CIP-TET-AMX-AMP
H	1UGC	B	BP Monitor	GW	ERY-CIP-TET-AMX-AMP-CTX
H1	1MGD1	A	Patient.File	GW	CIP-TET-MER-AMX-AMP
H2	1SGH1_1	D	Sink	GW	ERY-CIP-TET-AMX-AMP-CTX
H2	3UGF	B	Mop	GW	ERY-CIP-TET-AMX-AMP
H3	2MGK	A	Nurse Table	GW	CIP-TET-AMX-AMP-CTX
H3	3SGC1	D	BP Monitor	GW	ERY-CIP-TET-MER-AMP
H4	1UGL	B	Door Handle	GW	CIP-TET-AMX-AMP
H4	2CGJ2	C	Unoc.Bed	GW	ERY-CIP-TET-AMX-AMP-IMI-CTX
H5	1CGC	C	BP Monitor	GW	ERY-CIP-TET-AMX-AMP
H5	1MGC1	A	BP Monitor	GW	CIP-TET-AMX-AMP
H6	2CGA	C	Phone	GW	CIP-TET-MER-AMX-AMP-CTX
H7	2MGA	A	Phone	GW	ERY-CIP-TET-AMX-AMP-CTX
I	1MGC4	A	BP Monitor	GW	ERT-CIP-TET-AMX-AMP-CTX
J	2UGA41	B	Phone	GW	ERY-CIP-TET-MER-AMX-AMP-IMI-CTX
J	3CGA1	C	Drip Stand	GW	ERY-CIP-TET-AMX-AMP
K	2MGA1	A	Phone	GW	ERY-CIP-TET-AMX-AMP-CTX
L	2CGB3	C	Drip Stand	GW	ERY-CIP-TET-AMX-AMP
L1	2CGL1	C	Door Handle	GW	CIP-TET-AMX-AMP
L2	1UGB	B	Drip Stand	GW	CIP-TET-AMX-AMP
M	2SGJ1	D	Occ.Bed	GW	ERY-CIP-TET-MER-AMP
N	2UGA3	B	Phone	GW	CIP-TET-MER-AMX-AMP-IMI-CTX
O	2MGD	A	Patient.File	GW	CIP-TET-AMX-AMP-CTX
P	1CGD2_2	C	Patient.File	GW	CIP-TET-MER-AMX-AMP-CTX
Q	1UGD	B	Patient.File	GW	ERY-CIP-TET-AMX-AMP-CTX
Q1	3UGH	B	Sink	GW	CIP-TET-AMX-AMP
R	1MGE1	A	Ventilator	GW	ERY-CIP-TET-AMX-AMP
	ATCC 6051				

Appendix 7: Supplementary material of hospital data, Minimum Inhibitory Concentration (MIC) values, Molecular identification, virulence and resistance genes for 135 *Bacillus* isolates

ID	Clinical data			Species[d]		MIC (mg/l) [e]								Resistance genes[f]					Virulence genes[g]										
	Class[a]	Ward[b]	Site[c]	*BC*	*BS*	ERY	CIP	TET	MER	AMX	AMP	IMI	CTX	*ermB*	*tetK*	*tetM*	*tet39*	*blm*	*hblA*	*hblC*	*hblD*	*nheA*	*nheB*	*nheC*	*CytK*	*entFM*	*BceT*	*HIyll*	*ces*
1MGC2	CENT	GW	Bp	+	_	0.06	8	16	16	>512	>512	8	16	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+	_
1MGC4	CENT	GW	Bp	+	_	>512	32	512	4	>512	>512	8	>512	+	_	_	_	_	+	+	+	+	+	+	+	_	+	_	_
1MIH1	CENT	ICU	Sink	+	_	0,25	8	16	1	512	>512	1	8	_	_	_	_	_	+	_	+	+	+	+	_	+	+	+	_
1MIH2	CENT	ICU	Sink	+	_	8	4	16	>512	>512	>512	8	16	_	_	_	_	_	+	+	+	_	+	+	_	+	_	_	_
1MPE2	CENT	Paed	Vent	+	_	512	>512	256	16	2	>512	2	>512	+	_	_	_	_	_	_	+	+	+	+	_	_	+	_	_
1MGE1	CENT	GW	Vent	_	+	1	256	128	0,25	>512	>512	0,03	4	_	_	+	_	_	+	+	+	_	+	+	+	_	_	+	_

a. Central; TERT: Tertiary; REG: Regional; DIST: District, b. GW: General Ward; Paed: Paediatric; ICU: Intensive Care Unit, c. Bp. Blood pressure monitor; Vent. Ventilator; Tab: Nurse's table; Door: Door handle; Drip: Drip stand; Ph Phone; File: Patient File, d. Bc: *Bacillus cereus*; BS: *Bacillus subtillis*, e. ERY: erythromycin; CIP: ciprofloxacin; TET: tetracycline; MER: Meropenem; AMX: amoxicillin; AMP: ampicillin; IMI: imipenem and CTX: Cefotaxime., f. *tet K*, *tet M* and *tet 39* (Tetracycline), *ermB* (Erythromycin) and *blm* (β-lactams; ampicillin, amoxicillin), g. *hbl* (hemolysin BL), *nhe* (non-hemolytic), *cytK* (Cytotoxin K), *entFM* (enterotoxin FM), *HlyII* (potential enterotoxin hemolysin BL), *bceT* (enterotoxin T) and *ces* (cereulide).

Continued Appendix 7: Supplementary material of hospital data, minimum inhibitory concentration (Mic) values , molecular identification, virulence and resistance genes for 135 *Bacillus* isolates

ID	Clinical data			Species[a]		MIC (mg/L)[e]								Resistance genes[f]					Virulence genes[g]										
	Class[a]	Ward[b]	Site[c]	BC	BS	ERY	CIP	TETE	MER	AMX	AMP	IMI	CTX	*ermB*	*tetK*	*tetM*	*tet39*	*blm*	*hblA*	*hblC*	*hblD*	*nheA*	*nheB*	*nheC*	*cytK*	*entFM*	*BceT*	*HIyll*	*ces*
1MGK	CENT	GW	Tab	+	_	32	8	32	32	>512	>512	32	>512	_	_	_	_	_	+	+	+	+	+	_	+	_	+	+	_
1MGF	CENT	GW	Mop	+	_	0,5	16	32	0,125	>512	>512	0,03	0,5	+	_	_	_	_	_	+	+	_	+	+	+	_	+	+	_
1MGD	CENT	GW	File	+	_	1	64	64	16	>512	>512	8	16	_	_	_	_	_	+	+	+	+	+	+	+	_	+	+	_
1MPF	CENT	Paed	Mop	+	_	2	32	16	8	>512	>512	64	>512	_	_	_	_	_	+	+	+	+	+	+	+	_	+	+	_
2MIJ11	CENT	ICU	Bed	+	_	512	>512	512	16	>512	>512	>512	>512	_	_	_	_	_	+	_	+	_	_	_	+	_	_	_	_
2MGA1	CENT	GW	Ph	+	_	>512	32	512	4	>512	>512	0,25	128	+	_	_	_	_	+	_	_	_	_	_	+	_	_	_	_
2MIC	CENT	ICU	Bp	+	_	0,25	16	32	0,25	>512	>512	1	16	_	_	_	_	_	_	+	+	_	+	+	+	_	+	_	_
2MGD	CENT	GW	File	+	_	0,06	32	32	8	>512	>512	1	64	_	_	_	_	_	+	+	+	+	+	+	+	_	_	+	_
2MGA	CENT	GW	Ph	+	_	>512	64	512	0,25	>512	>512	1	16	_	_	_	_	_	+	+	+	+	+	_	+	_	_	_	_
2MIE	CENT	ICU	Vent	+	_	1	64	256	0,5	>512	>512	8	>512	+	_	_	_	_	+	+	+	+	+	+	+	+	+	_	_
2MGK	CENT	GW	Tab	+	_	2	4	>512	8	>512	>512	4	>512	_	_	_	_	_	+	+	+	+	+	+	+	_	_	+	_
2MPH	CENT	Paed	Sink	+	_	>512	32	>512	2	>512	>512	1	32	_	_	_	_	_	+	+	+	+	+	+	+	_	+	+	_

Continued Appendix 7: Supplementary material of hospital data, Minimum Inhibitory Concentration (MIC) values, molecular identification, virulence and resistance genes for 135 *Bacillus* isolates

ID	Clinical data			Species[d]		MIC (mg/L)[e]								Resistance genes[f]					Virulence genes[g]											
	Class[a]	Ward[b]	Site[c]	BC	BS	ERY	CIP	TET	MER	AMX	AMP	IMI	CTX	*ermB*	*tetK*	*tetM*	*tet39*	*blm*	*hblA*	*hblC*	*hblD*	*nheA*	*nheB*	*nheC*	*cytK*	*entFM*	*bceT*	*HIyll*	*ces*	
3MPL	CENT	Paed	Door	+	_	0,25	32	32	0,125	>512	>512	0,06	1	-	-	-	-	-	+	+	+	_	+	+	_	_	+	_	_	
3MPB	CENT	Paed	Door	+	_	128	128	256	0,25	>512	>512	0,03	2	-	-	-	-	-	+	-	+	+	+	+	+	-	-	-	-	
3MPH	CENT	Paed	Sink	+	_	>512	8	8	0.125	>512	>512	0.03	0,5	-	-	-	-	-	+	+	+	+	+	+	+	-	+	-	-	
3MIC	CENT	ICU	Bp	+	_	16	16	16	16	>512	>512	64	>512	_	_	_	_	_	+	_	+	_	+	+	_	_	_	_	_	
3MGA	CENT	GW	Ph	+	_	1	32	4	8	>512	>512	0,008	1	_	_	_	_	_	_	_	_	+	+	+	+	+	+	_	_	
3MPA	CENT	Paed	Ph	+	_	0,5	32	8	0,25	>512	>512	1	8	_	_	_	_	_	+	_	+	+	+	+	+	_	+	_	_	
3MGF	CENT	GW	Mop	+	_	0,06	32	32	0,5	>512	>512	0,03	0,25	_	_	_	_	_	_	_	_	_	_	+	_	+	_	_	_	
3MGL	CENT	GW	Door	+	_	0.125	16	16	0.5	>512	>512	1	16	-	-	-	-	-	+	-	+	+	+	+	+	-	-	-	-	
1UIJ11	TERT	ICU	Bed	+	_	>512	32	512	8	>512	512	1	128	_	_	_	_	_	+	_	+	_	_	_	+	_	_	_	_	
1UIF1	TERT	ICU	Mop	+	_	64	16	256	16	>512	>512	>512	>512	_	_	_	_	_	+	+	_	_	+	+	+	_	_	+	_	
1UIF2	TERT	ICU	Mop	+	_	0,06	16	16	8	>512	>51	2	32	_	_	+	_	_	+	+	+	+	+	+	+	_	+	+	_	
1UIF4	TERT	ICU	Mop	+	_	0,06	64	32	2	>512	>512	0,5	0,25	_	_	_	_	_	+	+	+	+	+	+	+	+	+	+	_	

Continued Appendix 7:Supplementary material of hospital data, minimum inhibitory concentration (MIC) values, molecular identification, virulence and resistance genes of 135 *Bacillus* isolates

ID	Clinical data			Species[d]		MIC (mg/L)[e]								Resistance genes[f]					Virulence genes[g]									
	Class[a]	Ward[b]	Site[c]	BC	BS	ERY	CIP	TETE	MER	AMX	AMP	IMI	CTX	*ermB*	*tetK*	*tetM*	*tet39*	*blm*	*hblA*	*hblC*	*hblD*	*nheA*	*nheB*	*nheC*	*cytK*	*entFM*	*bceT*	*HIyll*	*ces*
1UIB	TERT	ICU	Drip	+	_	>512	64	512	0,25	>512	16	1	128	_	_	_	_	_	+	+	+	+	+	+	+	+	_	+	_
1UGD	TERT	GW	File	+	_	>512	128	16	0,125	>512	>512	1	8	_	_	_	_	_	+	+	+	+	+	+	+	_	+	_	_
1UID	TERT	ICU	File	+	_	0,25	16	16	2	1	>512	8	>512	_	_	_	_	_	+	_	+	+	_	+	+	_	_	_	_
1UGB	TERT	GW	Drip	+	_	0,06	16	16	0,125	>512	>512	0,03	1	_	_	_	_	_	+	+	+	+	_	+	+	_	_	_	_
1UPK	TERT	Paed	Tab	+	_	16	16	16	16	>512	>512	64	>512	_	_	_	_	_	+	+	+	+	+	_	+	_	+	+	_
1UGL	TERT	GW	Door	+	_	0,5	8	8	0,125	>512	>512	2	16	_	_	_	_	_	+	_	+	+	+	+	+	_	_	_	_
1UPD	TERT	Paed	File	+	_	0,25	16	16	0,25	>512	>512	0,03	0,5	_	_	_	_	_	+	_	+	+	+	+	+	_	_	_	_
1UGA	TERT	GW	Ph	+	_	0,25	64	512	0,25	>512	>512	2	16	_	_	_	_	_	+	_	+	+	+	+	+	_	_	_	_
1UGC	TERT	GW	Bp	+	_	>512	64	512	8	>512	>512	1	>512	+	_	_	_	_	+	+	+	_	+	+	+	_	+	_	_
2UPH1	TERT	Paed	Sink	+	_	0,5	8	1	8	>512	512	0,25	8	_	_	_	_	_	+	+	+	+	+	+	+	+	+	_	_
2UPH2	TERT	Paed	Sink	+	_	0,5	8	0,5	2	>512	512	2	8	_	_	_	_	_	+	_	+	+	+	_	+	+	+	_	_

2UPH4	TERT	Paed	Sink	+	_	8	4	8	128	0,25	>512	>512	16	_	_	_	_	_	+	_	+	+	+	_	+	+	+	_	_

Continued Appendix 7: Supplementary material of hospital data, minimum inhibitory concentration (MIC) values, molecular identification, virulence and resistance genes for 135 *Bacillus* isolates

ID	Clinical data			Species[d]		MIC (mg/L)[e]								Resistance genes[f]					Virulence genes[g]										
	Class[a]	Ward[b]	Site[c]	BC	BS	ERY	CIP	TET	MER	AMX	AMP	IMI	CTX	*ermB*	*tetK*	*tetM*	*Tet39*	*blm*	*HblA*	*hblC*	*hblD*	*nheA*	*nheB*	*nheC*	*CytK*	*EntFM*	*BceT*	*HIyII*	*Ces*
2UIJ211	TERT	ICU	Bed	+	_	0,06	32	512	16	>512	>512	8	16	_	_	_	_	_	+	_	+	_	_	_	_	_	_	_	_
2UIJ23	TERT	ICU	Bed	+	_	2	32	256	>512	16	>512	>512	16	_	_	_	_	_	+	_	+	_	_	_	_	_	_	_	_
2UIE2	TERT	ICU	Vent	+	_	512	4	512	8	>512	>512	>512	>512	_	_	_	_	_	+	_	+	_	_	_	_	_	_	_	_
2UIE3	TERT	ICU	Vent	+	_	>512	4	0,25	2	>512	8	1	>512	_	_	_	_	_	_	_	+	_	_	_	_	_	_	_	_
2UGA1	TERT	GW	Ph	+	_	2	16	16	8	>512	>512	8	16	+	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
2UGA3	TERT	GW	Ph	+	_	0,25	16	128	16	>512	>512	>512	>512	_	_	_	_	_	+	_	+	_	_	+	_	_	_	_	_
2UGA4	TERT	GW	Ph	+	_	8	8	128	16	>512	>512	>512	>512	_	_	_	_	_	+	_	+	_	_	+	+	_	_	_	_
2UPJ (UNOC)	TERT	Paed	Bed	+	_	0,25	8	64	0,5	>512	>512	1	16	+	_	_	_	_	_	+	+	+	+	+	+	_	_	+	_
2UPH	TERT	Paed	Sink	+	_	>512	16	>512	4	>512	8	2	>512	_	_	_	_	_	+	+	+	+	+	_	+	_	+	+	_
3UPC1	TERT	Paed	Bp	+	_	0,25	16	256	8	>512	>512	16	4	_	_	_	_	_	+	+	+	+	+	+	+	_	_	+	_

3UPC3	TERT	Paed	Bp	+	_	16	16	32	0,125	>512	>512	0,03	0,125	_	_	_	_	_	+	+	+	+	+	+	+	+	+	_	_
3UPC4	TERT	Paed	Bp	+	_	0,06	4	32	8	>512	>512	4	16	_	_	_	_	_	+	+	+	+	+	+	+	+	+	+	_

Continued Appendix 7

: Supplementary material of hospital data, minimum inhibitory concentration (MIC) values, molecular identification, virulence and resistance genes for 135 *Bacillus* isolates

ID	Hospital data			Species[d]		MIC (mg/L)[e]								Resistance genes[f]					Virulence genes[g]									
	Class[a]	Ward[b]	Site[c]	BC	BS	ERY	CIP	TET	MER	AMX	AMP	IMI	CTX	*ermB*	*tetK*	*tetM*	*tet39*	*blm*	*hblA*	*hblC*	*hblD*	*nheA*	*nheB*	*nheC*	*CytK*	*EntFM*	*BceT*	*HIyll*	*ces*
3UIB4	TERT	ICU	Drip	+	_	0,25	16	64	2	>512	>512	1	256	+	_	_	_	_	+	+	+	_	_	_	+	_	_	+	_
3UIB1	TERT	ICU	Drip	_	+	>512	32	4	8	>512	>512	8	128	+	+	_	_	_	+	+	+	_	_	_	_	_	_	_	_
3UIB2	TERT	ICU	Drip	+	_	>512	>512	>512	8	>512	8	2	128	_	+	_	_	_	+	+	+	_	_	_	_	_	_	_	_
3UGK1	TERT	GW	Tab	+	_	1	8	8	0,25	>512	>512	1	32	_	_	_	_	_	_	_	+	+	_	_	+	_	_	_	_
3UGK4	TERT	GW	Tab	+	_	256	16	2	8	>512	>512	8	16	+	_	_	_	_	+	_	+	_	+	_	+	_	_	+	_
3UIE1	TERT	ICU	Vent	+	_	>512	512	512	2	>512	>512	0,06	16	+	_	_	_	_	+	+	+	+	+	+	+	_	+	_	_
3UIE3	TERT	ICU	Vent	+	_	32	8	32	16	>512	>512	0,25	32	_	_	_	_	_	+	_	+	_	_	_	+	_	_	_	_
3UPD	TERT	Paed	File	+	_	>512	64	64	0,25	>512	>512	1	8	_	_	_	_	_	+	+	+	_	+	+	+	+	_	+	_
3UGA	TERT	GW	Ph	+	_	2	16	8	1	>512	>512	0,5	2	_	_	_	_	_	+	+	+	+	+	+	+	+	_	+	_
3UIJ1	TERT	ICU	Bed	+	_	>512	64	512	4	>512	>512	16	>512	_	_	_	_	_	+	+	+	+	+	+	+	_	_	+	_

Continued Appendix 7: Supplementary material of hospital data, minimum inhibitory concentration (MIC) value, molecular identification, virulence and resistance genes for 135 *Bacillus* isolates

ID	Clinical data			Species [d]		MIC (mg/L)[e]							Resistance genes[f]					Virulence genes[g]										
	Class[a]	Ward[b]	Site[c]	BC	BS	ERY	CIP	TET	MER	AMX	AMP	IMI	CTX	*ermB*	*tetK*	*tetM*	*tet39*	*blm*	*hblA*	*hblC*	*hblD*	*nheA*	*nheB*	*nheC*	*CytK*	*EntFM*	*BcetT*	*HIyll*	*Cess*
3UIL	TERT	ICU	Door	+	_	>512	64	>512	0,25	>512	16	0,03	2	_	_	_	_	_	+	+	+	+	+	+	+	_	+	_	_
3UPH	TERT	Paed	Sink	+	_	8	8	256	1	>512	>512	1	>512	_	_	_	_	_	+	_	+	_	_	+	+	_	_	_	_
3UPK	TERT	Paed	Tab	+	_	32	32	32	0,125	>512	>512	1	>512	_	_	_	_	_	+	_	+	+	+	_	+	_	+	_	_
3UID	TERT	ICU	File	+	_	0,25	8	8	16	>512	512	1	512	_	_	_	_	_	_	+	_	+	+	+	+	+	_	+	_
3UPL	TERT	Paed	Door	+	_	>512	128	>512	0,25	>512	>512	1	>512	+	_	_	_	_	+	_	+	+	_	+	+	_	+	_	_
1CIK1	REG	ICU	Tab	+	_	0,125	8	16	4	>512	>512	2	>512	_	_	_	_	_	+	+	+	+	+	_	+	_	+	_	_
1CIK2	REG	ICU	Tab	+	_	0,25	0,25	8	2	>512	>512	4	>512	_	_	_	_	_	+	_	+	+	+	+	+	+	+	_	_
1CIK4	REG	ICU	Tab	+	_	0,25	8	8	2	>512	>512	0,5	16	_	_	_	_	_	+	_	+	+	+	+	+	+	+	_	_
1CIB1	REG	ICU	Drip	+	_	0,125	16	32	4	>512	>512	8	>512	_	_	_	_	_	+	_	+	+	+	+	+	+	+	_	_

Continued Appendix 7: Supplementary material of hospital data, minimum inhibitory concentration (MIC) value, molecular identification, virulence and resistance genes for 135 *Bacillus* isolates

ID	Clinical data			Species[d]		MIC (mg/L)[e]								Resistance genes[f]					Virulence genes[g]									
	Class[a]	Ward[b]	Site[c]			ERY	CIP	TET	MER	AMX	AMP	IMI	CTX	*ermB*	*tetK*	*tetM*	*tet39*	*blm*	*hblA*	*hblC*	*hblD*	*nheA*	*nheB*	*nheC*	*CytK*	*EntFM*	*BceT*	*HIyII*	*Ces*
1CIA2	REG	ICU	Ph	+	_	0,06	64	32	0,25	>512	>512	1	128	_	_	_	_	_	+	_	+	+	+	+	+	_	+	_	_
1CIL1	REG	ICU	Door	+	_	2	16	32	8	>512	>512	32	>512	_	_	_	_	_	+	_	_	+	+	+	+	+	_	+	_
1CPF1	REG	Paed	Mop	+	_	2	16	32	8	>512	>512	32	>512	_	_	+	_	_	_	_	_	_	_	_	_	_	_	_	_
1CPB	REG	Paed	Drip	+	_	>512	128	256	1	>512	>512	1	>512	_	_	_	_	_	+	+	+	+	+	+	+	_	+	+	_
1CIC	REG	ICU	Bp	+	_	0,25	8	32	4	>512	>512	8	512	_	_	_	_	_	+	+	+	+	+	+	+	_	_	+	_
1CIL	REG	ICU	Door	+	_	2	16	32	8	>512	>512	32	>512	_	_	_	_	_	+	+	+	+	+	+	+	_	+	_	_
1CGC	REG	GW	Bp	+	_	32	32	256	0,125	>512	8	0,03	0,5	_	_	_	_	_	+	+	+	+	+	+	+	_	_	+	_
1CIB2	REG	ICU	Drip	+	_	0,25	8	8	4	>512	>512	8	>512	_	_	_	_	_	+	+	+	_	+	+	+	+	+	+	_
1CIB4	REG	ICU	Drip	+	_	0,25	8	16	8	>512	>512	2	32	_	_	_	_	_	+	+	+	_	+	+	+	_	+	_	_
1CGD22	REG	GW	File	+	_	0,5	8	512	16	>512	>512	0,25	>512	_	_	_	_	_	+	+	+	+	_	_	+	_	_	_	_

Continued Appendix 7: Supplementary material of hospital data, minimum inhibitory concentration (MIC) value, molecular identification, virulence and resistance genes for 135 *Bacillus* isolates

2CGB1	REG	GW	Drip	+	_	>512	512	128	8	>512	>512	2	2	_	_	_	_	_	_	_	+	_	_	_	_	_	+	_	_
2CGB3	REG	GW	Drip	+	_	>512	128	256	0,25	>512	>512	0,06	2	+	_	_	_	_	_	_	+	+	_	_	+	_	_	_	_
2CPL2	REG	Paed	Door	_	+	>512	>512	>512	1	>512	>512	0,25	1	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
2CPF	REG	Paed	Mop	+	_	>512	8	8	4	>512	>512	2	4	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
2CPJ1	REG	Paed	Bed	+	_	>512	64	256	16	>512	>512	8	>512	_	_	_	_	_	+	_	+	_	_	+	+	+	_	_	_

ID	Clinical data			Species[d]		MIC(mg/L)[e]								Resistance genes[f]					Virulence genes[g]										
	Class[a]	Ward[b]	Site[c]	BC	BS	ERY	CIP	TET	MER	AMX	AMP	IMI	CTX	*ermB*	*tetK*	*tetM*	*tet39*	*blm*	*hblA*	*hblC*	*hblD*	*nheA*	*nheB*	*nheC*	*cytK*	*entFM*	*bceT*	*HIyll*	*ces*
2CIK	REG	ICU	Tab	+	_	>512	512	>512	1	>512	>512	0,03	>512	_	_	_	_	_	+	_	+	+	_	+	+	+	_	_	_
2CGL1	REG	GW	Door	+	_	1	8	32	0,125	>512	>512	0,06	1	_	_	_	_	_	+	_	+	+	+	+	+	+	+	_	_
2CGL2	REG	GW	Door	+	_	0,03	8	128	0,125	>512	>512	0,03	0,125	_	_	_	_	_	+	_	+	+	_	+	_	+	_	_	_
2CPD1	REG	Paed	File	+	_	0,125	32	8	16	>512	>512	2	8	_	_	_	_	_	+	_	+	+	_	+	+	+	_	_	_
2CGH	REG	GW	Sink	+	_	1	8	8	0,125	>512	>512	0,06	2	_	_	_	_	_	+	+	+	+	+	+	+	+	+	+	_
2CPJ2	REG	Paed	Bed	+	_	4	32	32	0,125	>512	>512	0,06	4	_	_	_	_	_	+	+	+	+	+	+	+	+	+	_	_

Continued Appendix 7: Supplementary material of hospital data, minimum inhibitory concentration (MIC) value, molecular identification, virulence

2CGJ2	REG	GW	Bed	+	_	>512	512	>512	8	>512	>512	16	>512	+	_	_	_	_	+	+	+	+	+	_	+	+	+	_	_
2CIJ2	REG	ICU	Bed	+	_	0,125	128	128	4	>512	>512	64	>512	_	_	_	_	_	+	_	+	+	+	+	+	_	_	_	_
2CGA	REG	GW	Ph	+	_	0,25	16	32	16	>512	>512	64	>512	_	_	_	_	_	+	+	+	+	+	+	+	+	_	+	_
3CPL3	REG	Paed	Door	+	_	0,25	16	4	1	>512	>512	2	32	_	_	_	_	_	+	+	+	+	+	+	_	+	+	+	_
3CPL4	REG	Paed	Door	+	_	0,25	16	8	16	>512	>512	8	>512	_	_	_	_	_	+	_	+	_	+	+	+	_	+	+	_
3CGA1	REG	GW	Ph	+	_	128	128	256	16	4	16	0,25	64	_	_	_	_	_	_	_	+	_	_	_	_	_	_	_	_

Continued Appendix 7: Supplementary material of hospital data, minimum inhibitory concentration (MIC) value, molecular identification, virulence and resistance genes for 135 *Bacillus* isolates

ID	Clinical data			Species[d]		MIC (mg/L)[f]								Resistance genes[g]					Virulence genes[h]									
	Class[a]	Ward[b]	Site[c]	BC	BS	ERY	CIP	TET	MER	AMX	AMP	IMI	CTX	*ermB*	*tetK*	*tetM*	*tet39*	*blm*	*hblA*	*hblC*	*hblD*	*nheA*	*nheB*	*nheC*	*cytK*	*entFM*	*bceT*	*HIyll*	*ces*
3CPJ11	REG	Paed	Bed	_	+	0,25	32	32	8	>512	>512	4	16	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
3CPJ12	REG	Paed	Bed	_	+	16	16	32	8	>512	>512	8	128	_	_	_	_	_	+	_	+	_	_	_	_	_	+	_	_
3CIC1	REG	ICU	Bp	+	_	0,5	16	16	8	>512	>512	4	16	_	_	_	_	_	+	+	+	+	+	+	+	_	_	_	_
3CGB	REG	GW	Drip	+	_	>512	128	256	4	>512	>512	2	64	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
3CPD	REG	Paed	File	+	_	0,25	16	16	8	>512	>512	32	512	_	_	_	_	_	+	_	+	+	_	+	+	_	_	+	_
1SPH2	DIST	Paed	Sink	+	_	64	512	512	2	1	128	1	64	_	_	_	_	_	_	_	+	_	_	_	+	_	_	_	_
1SPH4	DIST	Paed	Sink	+	_	0,25	16	2	2	>512	>512	8	>512	+	_	_	_	_	+	+	+	+	+	+	+	+	_	+	_
1SGH11	DIST	GW	Sink	+	_	>512	64	512	8	>512	>512	8	64	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
1SPB11	DIST	Paed	Drip	+	_	0,5	64	4	8	>512	>512	0,5	32	+	_	_	_	_	_	_	_	_	_	_	+	_	_	_	_
1SPB12	DIST	Paed	Drip	+	_	2	64	256	64	>512	>512	4	64	+	_	+	_	_	_	_	_	_	_	_	_	_	_	_	_
1SIF11	DIST	ICU	Mop	+	_	>512	32	512	8	>512	512	1	128	+	_	_	_	_	+	+	_	_	_	_	_	_	_	_	_

2SIJ1	DIST	ICU	Bed	+	_	>512	8	512	0,25	>512	16	0,03	64	_	_	_	_	_	+	+	+	+	+	+	+	+	_	_	_

Continued Appendix 7: Supplementary material of hospital data, minimum inhibitory concentration (MIC) value, molecular identification, virulence and resistance genes for 135 *Bacillus* isolates

ID	Clinical data			Species[d]		MIC (mg/L)[e]								Resistance genes[f]					Virulence genes[g]										
	Class[a]	Ward[b]	Site[c]	BC	BS	ERY	CIP	TET	MER	AMX	AMP	IMI	CTX	*ermB*	*tetK*	*tetM*	*tet39*	*blm*	*hblA*	*hblC*	*hblD*	*nheA*	*nheB*	*nheC*	*cytK*	*entFM*	*bceT*	*HIyll*	*ces*
2SIK	DIST	ICU	Tab	+	_	>512	64	>512	4	>512	>512	32	>512	_	_	_	_	_	+	+	+	+	+	+	+	_	+	+	_
2SGB	DIST	GW	Drip	+	_	1	256	8	1	>512	>512	16	>512	_	_	_	_	_	+	+	+	+	+	+	+	_	+	+	_
2SIJ2	DIST	ICU	Bed	+	_	1	16	32	16	>512	>512	16	>512	_	_	_	_	_	+	+	+	+	+	+	+	_	+	+	_
2SPK	DIST	Paed	Tab	+	_	0,5	16	32	0,03	>512	>512	0,5	16	_	_	_	_	_	+	+	+	+	+	+	+	_	_	+	_
2SPJ2	DIST	Paed	Bed	+	_	>512	64	256	4	>512	512	2	8	+	_	_	_	_	+	+	+	+	+	+	+	_	_	+	_
2SGA2	DIST	GW	Ph	+	_	8	4	8	16	>512	>512	4	>512	_	_	_	_	_	_	_	+	+	+	+	+	+	+	_	_
2SPA	DIST	Paed	Ph	+	_	1	16	32	0,25	>512	>512	1	16	_	_	_	_	_	+	+	+	+	+	_	+	_	_	_	_
2SGJ1	DIST	GW	Bed	+	_	>512	32	64	16	4	16	8	16	+	_	_	_	_	+	+	+	+	+	+	+	_	_	_	_
3SPJ12	DIST	Paed	Bed	+	_	0,5	16	128	4	>512	512	2	8	+	_	_	_	_	_	+	+	_	_	_	_	_	_	+	_
3SGC1	DIST	GW	Bp	+	_	>512	32	512	8	>512	>512	4	>512	+	_	_	_	_	+	_	+	_	_	_	+	_	_	_	_

3SPK11	DIST	Paed	Tab	+	_	4	16	256	8	>512	>512	1	4	+	_	+	_	_	_	+	+	+	_	_	_	_	_	_	_
3SIE2	DIST	ICU	Vent	+	_	>512	32	512	1	>512	>512	16	>512	+	_	_	_	_	_	+	+	_	_	_	+	_	_	+	_

Continued Appendix 7: Supplementary material of hospital data, minimum inhibitory concentration (MIC) value, molecular identification, virulence and resistance genes for 135 *Bacillus* isolates

ID	Hospital data			Species[c]		MIC (mg/L)[e]								Resistance genes[f]					Virulence genes[g]										
	Class[a]	Ward[e]	Site[c]	BC	BS	ERY	CIP	TET	MER	AMX	AMP	IMI	CTX	*ermB*	*tetK*	*tetM*	*tet39*	*blm*	*hblA*	*hblC*	*hblD*	*nheA*	*nheB*	*nheC*	*cytK*	*entFM*	*bceT*	*HIyII*	*ces*
3SIE1	DIST	ICU	Vent	_	+	2	32	512	16	>512	>512	8	>512	+	_	_	_	_	_	_	+	_	_	_	_	_	_	_	_
3SPH	DIST	Paed	Sink	+	_	2	16	32	4	>512	>512	32	>512	_	_	_	_	_	+	+	+	+	_	+	+	+	+	+	_
3SPK1	DIST	Paed	Tab	+	_	0,5	16	16	8	>512	>512	8	>512	+	_	_	_	_	+	+	+	+	+	+	+	_	+	_	_
1MIJ22	CENT	ICU	Bed	+	_	0,25	16	16	4	>512	>512	0,5	32	_	_	_	_	_	_	_	+	_	+	+	_	_	_	_	_
1MIJ23	CENT	ICU	Bed	+	_	0,25	8	8	8	>512	>512	8	16	_	+	_	_	_	_	+	+	+	+	+	+	_	+	+	_
1MIJ24	CENT	ICU	Bed	+	_	16	32	32	8	>512	>512	8	>512	+	_	_	_	_	_	+	+	_	+	+	+	+	+	+	_
1MGC1	CENT	GW	Bp	+	_	1	16	64	8	>512	>512	8	16	_	_	_	_	_	+	+	+	+	_	+	+	+	+	+	_
1MPA	CENT	Paed	Ph	+	_	0,25	8	16	0,125	>512	16	0,125	2	_	_	_	_	_	_	+	+	+	+	+	+	_	+	_	_
1MIE	CENT	ICU	Vent	+	_	0,25	16	32	>512	>512	>512	>512	>512	_	_	_	_	_	+	+	+	+	+	_	+	_	_	+	_