A STANDARDISED APPROACH TO THE TREATMENT AND MANAGEMENT OF SIGNIFICANT *ACINETOBACTER* SPECIES INFECTION AT ACADEMIC COMPLEX HOSPITALS IN KWAZULU-NATAL

By

KHINE SWE SWE-HAN

"A thesis by manuscripts"

Submitted in fulfillment of the academic requirements for the

Degree of Doctor of Philosophy (PhD)

School of Laboratory Medicine and Medical Sciences

College of Health Sciences

University of KwaZulu-Natal

Supervisor: Prof. M Pillay;

Co-supervisors: Prof. K P Mlisana; Dr K Baba

2017

DECLARATION 1: PLAGIARISM

I.....**KHINE SWE SWE-HAN...**.....declare that

(i) The research reported in this dissertation, except where otherwise indicated, is my original work.

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DECLARATION 2: PUBLICATION

The publications (in print, in press and submitted) that constitute this thesis and the contribution I made to each of the manuscripts are presented here.

Adaptations: Changes have been identified as adaptations to published papers on the title of each paper as indicated below.

Publication:

Paper 1

Khine Swe Swe-Han, Manormoney Pillay. Multidrug Resistant *Acinetobacter* species: A significant cause of sepsis in an intensive care unit in a regional hospital, Durban. International Journal of Nursing Didactics. 2015; 5 (8). www.innovativejournal.in. [IJND-ISSN: 2231-5454] http://innovativejournal.in/ijnd/index.php/ijnd

Adaptations: *Acinetobacter baumannii* has been changed to *Acinetobacter* species *(Acinetobacter* spp.) throughout the thesis according to examiner's comments. The reason for this change is due to the difficulty in identification of individual species by their phenotypic traits and that the use of current automated or manual commercial systems will require further confirmatory testing to use *Acinetobacter baumannii.*

References: All references have been revised to conform with the standard format as a requirement of PhD thesis. Page 68-69.

Figure 2: Color has been changed to improve the clarity (suggested by the Examiner). Page 71.

Contributions by first author

Oral presentation at UKZN research conference in 2013.

Authors' contributions

K Swe Swe–Han (first author) contributed to the following; (1) the conceptualisation and design of the study, acquisition of data, analysis and interpretation of data (2) drafting the manuscript, revising it critically for important intellectual content, (3) final approval of the version to be submitted.

Prof. M. Pillay helped to design the study and draft the manuscript. She also provided scientific input and critically reviewed the manuscript.

All authors read and approved the final manuscript.

Paper 2

Khine Swe Swe-Han, Koleka. P Mlisana, Manormoney Pillay. Analysis of clinical and microbiological data on *Acinetobacter* species isolates for the preauthorisation of antibiotics at the patient level for an effective antibiotic stewardship programme.

Published in the Journal of Infection and Public Health (Feb 2017); Reference: JIPH-D-16-00413. http://dx.doi.org/10.1016/j.jiph.2017.01.014

Adaptations: *Acinetobacter baumannii* has been changed to *Acinetobacter* species *(Acinetobacter* spp.) throughout the thesis according to examiner's comments. The reason for this change is due to the difficulty in identification of individual species by their phenotypic traits and that the use of current automated or manual commercial systems will require further confirmatory testing to use *Acinetobacter baumannii.*

References: All references have been revised to conform with the standard format as a requirement of PhD thesis. Page 95-97.

Authors' contributions

K Swe Swe –Han (First author) contributed to the following; (1) the conceptualisation and design of the study, acquisition of data, analysis and interpretation of data with statistician, (2) drafting the manuscript , revising it critically for important intellectual content, communication with the English editor , (3) final approval of the version to be submitted.

Prof. M. Pillay provided scientific input and critically reviewed the manuscript.

Prof M. Pillay and Prof. K. P. Mlisana helped design the study and draft the manuscript.

All authors read and approved the final manuscript.

Paper 3;

Khine Swe Swe-Han, Kamaldeen Baba, Koleka P. Mlisana, Manormoney Pillay. Colistin exhibits diverse and species dependent synergy in combination with different antibiotics against *Acinetobacter* species. Published in the World Journal of Pharmacy and Pharmaceutical Sciences.2017; 6(2) 183-199 (Feb 2017).

Adaptations: ***Acinetobacter baumannii*** has been changed to ***Acinetobacter*** **species** *(Acinetobacter* spp.) throughout the thesis according to examiner's comments. The reason for this change is due to the difficulty in identification of individual species by their phenotypic traits and that the use of current automated or manual commercial systems will require further confirmatory testing to use *Acinetobacter baumannii.*

References: All references have been revised to conform with the standard format as a requirement of PhD thesis, Pages 110-114.

Table 2: The rows of the table 2 have been rearranged. (Comments by Examiners). All data remain the same. Page 117.

Contribution by first author

Oral presentation at international conference Antibiotics -2015;

Poster presentation at FIDSSA-2015.

Author's contributions

K Swe Swe–Han (First author) contributed to the following; (1) the conceptualisation and design of the study, order the reagents for making media with colistin, laboratory synergy test, acquisition of data, analysis and interpretation of data (2) drafting the manuscript and revising it critically for important intellectual content, communication with the English editor (3) final approval of the version to be submitted.

Dr Kamaldeen Baba helped supervise the synergy test method, interpretation of synergy test, design the study and draft the manuscript. He also provided scientific and intellectual input and critically reviewed the manuscript.

Prof. M. Pillay and Prof. K. P Mlisana helped design the study, draft the manuscript and also provided scientific and intellectual input and reviewed the manuscript.

All authors read and approved the final manuscript.

Paper 4

K Swe Swe- Han, Melendhran Pillay, Desmond Schnugh, Koleka P Mlisana, Kamaldeen Baba, Manormoney Pillay. Horizontal transfer of OXA-23-Carbapenemase-producing *Acinetobacter* species in intensive care units at an academic complex hospital in Durban, KwaZulu -Natal, South Africa. (Submitted to South Africa Journal of infectious disease (SAJID). Manuscript Reference number: SAJID - 2016 – 0052). It has been accepted for publication by SAJID in May 2017.

Contribution by first author

Poster presentation at UKZN Research day conference -2016 [2nd winner awarded].

Author's contributions

First author's (K. Swe Swe –Han) contributions:

(1) The conceptualisation and design of the study, ordering of the laboratory reagents for identification, susceptibility testing and molecular work.

(2) As a pathologist (microbiologist), interpretation of laboratory results and regular ward rounds. Based on the collaboration with clinicians and clinical characterisation and laboratory results of patients, the isolates of *Acinetobacter* spp. were selected and stored for the study.

(3) Subculture of the isolates, identification, confirmation of susceptibility by repeating the Vitek 2 and MICs with E- test.

(4) DNA extraction (in-house method) and PCR after training and assistance by the Medical Scientist.

(5) Arranged collaboration and transportation of the stored isolates to the molecular laboratory, NHLS, Johannesburg (JHB) for PFGE typing.

(6) Data acquisition, analysis and interpretation.

(7) Drafted the manuscript, critical revision for intellectual content, communication with the English editor and final approval of the version to be submitted.

Prof. M. Pillay, Prof. K P Mlisana and Dr K. Baba helped design the study and edited the manuscript. Prof. M. Pillay also provided scientific input into the PCR, and critically reviewed the drafts and final revised manuscript.

Melendhran Pillay, Medical Scientist, assisted with the molecular work such as DNA-extraction, PCR and sequencing, contributed the methodology of the in- house methods. He also reviewed the methods section of the manuscript.

At the molecular laboratory in Johannesburg, PFGE typing and the report were performed by Desmond Schnugh, Medical scientist.

All authors read and approved the final manuscript.

Paper 5- manuscript 5

Khine Swe Swe Han, Melendhran Pillay, Koleka P Mlisana, Kamaldeen Ba Ba, Manormoney Pillay. Colistin resistant clinical *Acinetobacter* species may be mediated by the absence of the *Ipx*A gene at an academic complex hospital in Durban, KwaZulu-Natal, South Africa (was submitted to Africa Journal of Laboratory Medicine) (AJLM). Ref. No.AJLM: 597.

Author's contributions

First author's (K. Swe Swe –Han) contributions:

(1) The conceptualisation and design of the study, ordering of the laboratory reagents for identification, susceptibility testing and molecular work.

(2) As a pathologist (microbiologist), interpretation of laboratory results and regular ward rounds. Based on the collaboration with clinicians and clinical characterisation and laboratory results of patients, the isolates of *Acinetobacter* spp. were selected and stored for the study.

(3) Subculture of the isolates, identification, confirmation of susceptibility tests.

(4) DNA extraction (in-house method), primer selection and PCR after training and assistance by the Medical Scientist.

(5) Data acquisition, analysis and interpretation.

(6) Drafted the manuscript, critical revision for intellectual content, communication with the English editor and final approval of the version to be submitted.

Prof. M. Pillay, Prof. K P Mlisana and Dr. K. Baba helped design the study and edited the manuscript. Prof. M. Pillay also provided scientific input into the PCR, and critically reviewed the drafts and final revised manuscript. Melendhran Pillay, Medical Scientist, assisted with the molecular work such as DNA-extraction, PCR and sequencing, contributed the methodology of the in- house methods. He also reviewed the methods section of the manuscript.

All authors read and approved the final manuscript.

Chapter 7

A standardised approach to the treatment and management of significant *Acinetobacter* species infection at academic complex hospitals in KwaZulu-Natal

Khine Swe Swe- Han, K Baba, K P Mlisana, M Pillay.

Author's contributions

K Swe Swe –Han (First author) contributed to:

(1) Definitions and criteria, the conceptualisation and design of the guideline;

(2) Drafting the standard guideline and revising it critically for important intellectual content;

(3) Final approval of the version to be implemented locally (Academic complex hospitals, KZN).

Prof. K P Mlisana, Dr K Baba and Prof. M Pillay helped to design and draft the guideline.

All authors read and approved the final standard guideline.

Name KHINE SWE SWE/ HAN **DATE. 27th March 2017**

Signed … 

DEDICATION

I dedicate this to my family. To my children P. M. Han, A. K. Zaw and K. S.L. Han and my husband K. A. Han - you are my source of strength and pride.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisor Prof. M Pillay for her invaluable guidance and understanding while I completed this work. I would also like to thank my co-supervisors, Prof. K P Mlisana and Dr K Baba, for their support and flexibility.

I am grateful to Mrs. Fikile Nkwanyana, (Biostatistician) for her expertise in statistics.
Thank also to the National Health Laboratory Service (NHLS), Business Manager, Laboratory Manager, medical technologists and Melen Pillay (Medical scientist).
My sincere appreciation to the Medical Education Partnership Initiative for financial assistance, as well as to the National Health Laboratory Service and the University of Kwa-Zulu Natal for facilitating my work.

Many thanks go to Professor Musa Mabandla, Dr Michelle Gordon (credentialing staff meeting team) for encouraging and motivatiing my work.

I am grateful to Prof. M.J. Chimbari, Prof. Mukaratirwa, Prof. Ben Sartorius and Mr. Clement Matasane for their guidance, support for the format of thesis and completion of writing.

Last but not least, thank you to my family for their love and endless support.

TABLE OF CONTENTS

LIST OF ACRONYMS/ABBREVIATIONS

A-A	Aplastic anaemia
AK	Amikacin
A-P	Abruptio placenta
API	Analytical profile index
A-S	Aortic stenosis;
ASWP	Antibiotics Stewardship Programme
A tip	Arterial Tip
BC	Blood culture
BMTU	Bone marrow transplant unit
Carba	Carbapenem
CAZ	Ceftazidime
CDC	Centers of Diseases Control and Prevention
CIP	Ciprofloxacin
CLSI	Clinical Laboratory Standards Institute
CSF	Cerebrospinal fluid
CST	Colistin
CVP	Central venous pressure tip
CXR	Chest X-ray
DC	Discharge
D	Deceased
ECDC	European Centre of Disease Control and Prevention
E-test	Epsilometer test
ETA	Endotracheal aspirate
IALCH	Inkosi Albert Luthuli Central Hospital
ICU	Intensive care unit
IDSA	Infectious Diseases Society of America
IMP	Imipenem
KEH	King Edward Hospital VIII
LW	Labour ward
LZ	Linezolid
LPS	Lipopolysaccharide
MDR	Multidrug- resistant
MEM	Meropenem

MIC	Minimum inhibitory concentration
MVA	Motor vehicle accident
NA	Not available
NHLS	National Health Laboratory Service
PD	Peritoneal dialysis
PDR	Pandrug- resistant
PL	Pleural fluid
R	Resistant
RIF	Rifampicin
RVD	Retroviral disease
S	Sensitive
SHEA	Society for Healthcare Epidemiology of America
SIRS	Systemic inflammatory response syndrome
TB	Tuberculosis
TZP	Piperacillin-tazobactam
UKZN	University of KwaZulu- Natal
VAN	Vancomycin
VAP	Ventilator associated pneumonia
XDR	Extensively drug- resistant

ABSTRACT

Introduction: Carbapenem-resistant *Acinetobacter* species (*Acinetobacter* spp.) are increasingly recognised as important pathogens, whose resistance patterns present a high-risk global challenge. However, there is limited scientific data and a lack of a standardised approach to help the clinician select optimal therapy in local setting. This study aimed to provide a standardised approach for the management of significant *Acinetobacter* spp. infection based on phenotypic and genotypic characterisation of local isolates, as well as clinical characteristics and outcomes of patients at academic complex hospitals in KwaZulu-Natal.

Objectives: The significance of *Acinetobacter* spp. infections and the most effective drug combinations for optimal therapy were determined. *Acinetobacter* spp. isolates were phenotypically and genotypically characterised. This was followed by the development of a standard management guideline for local use, based on the data obtained in the different objectives.

Methods: The research consisted of a retrospective and prospective observational and experimental laboratory component. The laboratory component included synergy testing of colistin, susceptibility to antimicrobial agents in use at local hospitals, polymerase chain reaction and sequencing for analysis of the resistant genes related to carbapenem, colistin and amikacin. Phenotypic, genotypic, and clinical characterisation were utilised to develop a standardised management approach of significant *Acinetobacter* spp. infection.

Results: *Acinetobacter* spp. was identified as a significant cause of sepsis and mortality among patients in a surgical intensive care unit (ICU). Cases of multidrug-resistant (MDR) and extensively drug-resistant (XDR) *Acinetobacter* spp. increased over seven years, together with the emergence of pandrug-resistant (PDR) isolates. The results of synergy testing of colistin combinations with amikacin, carbapemens (imipemen, meropenem), ciprofloxacin, tazocin, linezolid, rifampicin and vancomycin against *Acinetobacter* spp. was highly diverse and species-dependent. Characterisation of *Acinetobacter* spp. isolates showed that oxacillinase β-lactamase (OXA-23)-producing MDR isolates correlated with their antibiogram. Pulsed field gel electrophoresis (PFGE) showed horizontal transfer between seven clusters, each containing two patients each, totalling 14 patients. However, the PFGE typing revealed a diverse collection of MDR *Acinetobacter* spp. clones, and that isolates from not more than two patients were related. This suggests, therefore, that no outbreak had occurred based on the PFGE typing interpretation. Further genetic investigation revealed that the *aph*A6 gene were associated with amikacin resistance and *Ipx*A gene may be associated with colistin resistance in our local setting.

Conclusion: The results highlighted the importance of antibiotic stewardship in the treatment of *Acinetobacter* spp. infection. Individual-specific antibiograms are recommended as the best

approach for treatment in KwaZulu-Natal (KZN) and synergy testing should be performed for individualised direct therapy. The clinical and microbiological indicators of significant infection are crucial when establishing the decision to treat. The study provided a valuable standardised approach, including a flow chart of criteria for sepsis and colonisation; a standardised algorithm for the management; and synergy test at academic complex hospitals, Medical Microbiology laboratory, National Health Laboratory Service (NHLS) in KZN.

Key words: Individual-specific antibiogram approach; standardised algorithm for management; significant *Acinetobacter* species infections; antibiotic stewardship programme; synergy testing; $bla_{\text{OXA-23;}}$ *Ipx*A gene; *aph*A6 gene.

OVERVIEW OF THE THESIS

The current study, with different objectives which address different aspects of the *Acinetobacter* species, was performed on a seven-year collection of clinical and laboratory data of patients infected and colonised with *Acinetobacter* species (*Acinetobacter* spp.) at academic complex hospitals in Durban, KwaZulu-Natal (KZN). Based on the outcomes of this study, a standard treatment management guideline was developed for the local settings.

This thesis is presented in a "thesis by manuscripts" format. The first section of this thesis (Chapter 1) consists of a brief Introduction, literature review, problem statement, research questions/hypothesis/aims/objectives, and general methodology. The research aspects are presented in chapters 2 to 6, a management guideline in chapter 7, and a synthesis of the complete research in chapter 8, describing the link and culminates in a conclusion, with a set of recommendations of future research.

Chapter 2: Multidrug-resistant *Acinetobacter* species: a significant cause of sepsis in an intensive care unit in a regional hospital, Durban

No local literature exists on *Acinetobacter* spp. as a significant source of sepsis, although there has been a worldwide increase in carbapenem-resistant *Acinetobacter* infections. This retrospective observational analytical study investigated the prevalence of significant *Acinetobacter* spp. sepsis through clinical and microbiological data. This study elucidated the significant cause of sepsis with multidrug-resistant *Acinetobacter* species and a high mortality rate (60%) of patients in intensive care units. This manuscript was published in the *International Journal of Nursing Didactics* in 2015.

Chapter 3: Analysis of clinical and microbiological data on *Acinetobacter* species assist the preauthorisation of antibiotics at patient level for an effective antibiotic stewardship programme

This analytical retrospective observational study was performed over seven years from 2008 to 2014. Here, we analysed clinical and microbiological data on *Acinetobacter* spp. isolates in order to produce a flow chart to differentiate significant sepsis from colonisation for pre-authorisation of antibiotics. The data were collected from an electronic system and verified by clinicians and clinical microbiologists during clinical ward rounds. This study elucidated the criteria of difference between significance sepsis *versus* colonisation and local antibiotic resistant patterns. Flow charts, including criteria and relevant definitions, were provided to assist preauthorisation of antibiotics at patient level for an effective antibiotic stewardship programme. This manuscript

was published by the *Journal of Infection and Public Health* (Manuscript Reference number: JIPH-D-16-00413) in Feb 2017. **http://dx.doi.org/10.1016/j.jiph.2017.01.014**

Chapter 4: Colistin exhibits diverse and species-dependent synergy in combination with different antibiotics against *Acinetobacter* species

Although drug-resistant *Acinetobacter* spp. is a serious problem in clinical settings, especially in patients with *Acinetobacter* polymicrobial infections, there was no optimal use of antibiotics in combination therapy at our local academic complex hospitals. Therefore, this analytical observational experimental study was conducted to determine the effectiveness of various antibiotic combinations, using synergy testing. This study was useful and essential to support an effective antibiotic stewardship programme to recommend that an empirical combination regimen is not suitable in this local setting. This manuscript has been published by the *World Journal of Pharmacy and Pharmaceutical Sciences* (Manuscript Reference number: WJPPS/8599/6/2017). 2017; 6(2) 183-199 (published in Feb 2017.)

Chapter 5: Horizontal transfer of OXA-23-carbapenemase-producing *Acinetobacter* species in intensive care units at an academic complex hospital in Durban, KwaZulu-Natal, South Africa

To date, there was no prior genotypic and phenotypic characterisation of MDR *Acinetobacter* species in Inkosi Albert Luthuli Central Hospital (academic complex hospitals) in KwaZulu-Natal. This analytical experimental study determined the association of $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-58}}$ genes with carbapenem-hydrolysing class D β-lactamase (CHDL) production, as well as their relation to the spread of MDR *Acinetobacter* species. This molecular study showed that MDR *Acinetobacter* species carried the $bla_{\text{OXA-23}}$ gene that was responsible for resistance to carbapenems (MIC 8 to >16 mg/L). In addition, the PFGE typing of a diverse collection of MDR *Acinetobacter* spp. clones showed that isolates from not more than two patients were related. Collectively, this data informs the local infection prevention and control programme which in turn plays a major role in supporting the management guideline and antibiotic stewardship programme. This manuscript has been accepted on 24th May 2017 for publication by the *Southern African Journal of Infectious Diseases*. (Manuscript Reference number: Ref.: Ms. No. SAJID - 2016 - 0052R2)

Chapter 6: Colistin resistant clinical *Acinetobacter* species may be mediated by the absence of the *Ipx*A gene report at an academic complex hospital in Durban, KwaZulu-Natal, South Africa

This study investigated the phenotypic and genotypic characteristics of *Acinetobacter* spp. as well as clinical characteristics and outcomes of the patients in an academic complex hospital. This was an analytical, observational experimental study, which highlighted the prevalence of colistin and amikacin resistant *Acinetobacter* species and their associated resistant genes *Ipx*A and *aph*A6 respectively. This finding contributed to the development of the urgently needed management guidelines and ideas for future research, including molecular surveillance.

This manuscript was under review by the *African Journal of Laboratory Medicine.*

(Manuscript Reference number: Ref. No.AJLM: 597).

Chapter 7: Standardised approach for the management of patients with significant *Acinetobacter* species infections at an academic complex hospitals in KwaZulu-Natal, South Africa

Based on the outcomes of this study described in the previous five chapters, we developed a standardised guideline for treatment of *Acinetobacter* spp. infections in academic complex hospitals in Durban, KZN. This was an analytical, observational (combined retrospective and prospective study), experimental study which was submitted to Inkosi Albert Luthuli Central Hospital (IALCH) clinical and medical managers to be implemented via the antibiotic stewardship programme (ASWP) committee in 2017.

Chapter 8: Synthesis of the thesis

This chapter thematically links all the chapters and describes the development, prospects, opportunities and challenges in the utilisation of a standardised approach for the management of patients with significant *Acinetobacter* spp. infections at the academic complex hospitals in KZN, South Africa. The study demonstrates the use of clinical, microbiological, molecular and epidemiological data to develop a standard laboratory and clinical approach to the management of MDR *Acinetobacter* spp. infection. Included in this approach is synergy testing on individual isolate and definitions of the criteria for clinical and microbiologically significant sepsis. Based on this work, steps for developing a standard guideline are recommended, which may be adapted to suit administrative structures and implementation in the KwaZulu-Natal (KZN) areas. For academic complex hospitals, a specific guideline for standard approach of *Acinetobacter* spp. infection needs to be implemented urgently in order to assist the preauthorisation part of the antibiotic stewardship programme (ASWP). The study outcomes revealed that local

Acinetobacter spp. was a significant cause of sepsis, with horizontal transfer of resistant genes, and the results of synergy testing were diversed and species dependent. Therefore, the integration of a standard approach to treatment and prevention is valuable and applicable.

This study showed a potentially novel mechanism of colistin resistance, that may be due to the inactivation of the Lipid A domain as a result of the potential complete loss of the *Ipx*A gene. This is contradictory with other reports and the results require additional molecular techniques to confirm the finding of the absence *Ipx*A gene as a novel resistance mechanism.

Appendix: **Ethical approval**

The series of study was approved by the Ethics Committee of University of KwaZulu- Natal (Ethic approved- BE 283/12).

The consent were approved by the hospitals and Department of Health.

CHAPTER 1: INTRODUCTION, LITERATURE REVIEW AND GENERAL METHODOLOGY

1.1 Introduction

Drug-resistant *Acinetobacter* species (*Acinetobacter* spp.) present a high-risk infection control and preauthorisation challenge for clinicians and microbiologists worldwide (Diekema *et al.*, 2004; Fishbain and Peleg, 2010; Ghafur *et al.*, 2014; Phee *et al.*, 2015). Despite the high prevalence of infection, there is no local standardised approach to help the clinician to select optimal empirical and targeted therapy in hospital settings.

Among *Acinetobacter* species, *Acinetobacter baumannii* is the most relevant common pathogen in the clinical context (Retailliau *et al*., 1979; Peleg *et al*., 2008; Gonzalez-Villoria and Valverde-Garduno, 2016), since it is frequently isolated with nosocomial infections and high mortality rate (Lin *et al*., 2010; Chang *et al*., 2011; Leão *et al*., 2016). Previous reviews have addressed *Acinetobacter* spp. as a successful pathogen, investigating its biological aspects, epidemiology, pathogenicity factors, clinical and pathophysiological overview and global spread (Doughari *et al*., 2011; Mangoni and Zarrilli, 2011; Wong *et al*., 2017). These bacteria have been reported to be associated with bacteraemia, sepsis in intensive care units (ICUs), community acquired meningitis, secondary infections of wounds, and community pneumonia (Doughari *et al.*, 2011; Gonzalez-Villoria and Valverde-Garduno, 2016; Samawi *et al*., 2016).

The community acquired isolates are relatively sensitive to antibiotics and the resistant isolates have been reported almost exclusively in hospitals and intensive care units (Falagas and Karveli, 2007; Perez *et al*., 2007; Sengstock *et al*., 2010). Thus, it is easy to define community-acquired and hospital-acquired *Acinetobacter* spp. isolates according to their antibiotic susceptibility patterns and the clinical admission history. *Acinetobacter* spp. isolated after 48 hours of ICU admission means a hospital-acquired infection. Non-resistant *Acinetobacter* spp. isolated from a patient admitted directly from the emergency room or an outpatient department means community-acquired isolates.

However, it is still difficult to differentiate between a coloniser and significant *Acinetobacter* spp. sepsis isolate at the hospital level (Perez *et al*., 2007; Almasaudi, 2016), which is an important part of the antibiotic stewardship programme. *Acinetobacter* spp. are recognised as common hospital- and community-acquired pathogens and colonisers globally (Lahiri *et al*., 2004; Leung *et al.*, 2006; Sengstock *et al*., 2010; Parandekar and Peerapur, 2012; Almasaudi, 2016).

The early application of effective therapy is the most important step for the survival of patients from *Acinetobacter* infections. Unfortunately, due to the frequency of resistance in *Acinetobacter* infections, initiation of effective therapy is a particular problem. In addition, ineffective

antimicrobial treatment is more common for *Acinetobacter* than most other pathogens, and a dramatic increase in mortality, consequently (Spellberg and Bonomo, 2014; Joly-Guillou, 2005; Wong *et al*., 2017).

The increasing prevalence of carbapenem-resistant isolates, which are also resistant to all commonly used antibiotics, is of particular concern, since colistin is the only preferred agent for the treatment of these MDR organisms (Moffatt *et al*., 2010). However, its resultant renal and neurotoxicity makes it an unattractive alternative (Arıdoğan, 2012). In addition, there have been reports of *Acinetobacter* isolates resistant to colistin (Moffatt *et al*., 2010). Colistin combination therapy has been suggested as the best approach even for colistin-resistant *Acinetobacter* spp. isolates. The synergy effect of combination therapy using colistin with various other agents has been reported (Wareham and Bean, 2006; Pankey and Ashcraft, 2009; Daoud *et al*., 2013; Zafar *et al*., 2015). Unfortunately, unknown factors regarding the effectiveness of synergy with colistin combination therapy still prevail (Ahmed *et al.*, 2014; Zafar *et al*., 2015) and there is a lack of standard approach in the treatment and management of *Acinetobacter* spp. infection. Moreover, the clinical utility of these combinations against PDR *Acinetobacter* spp. remains to be determined (Doughari *et al.*, 2011). The limited options in the treatment is a major concern and research on the use and efficacy of combination therapies (Doughari *et al*., 2011), as well as clinical outcomes, is warranted in local settings (Inkosi Albert Luthuli Central Hospital academic complex of KZN, South Africa).

Drug-resistant *Acinetobacter* spp. presents a serious global infection control challenge. Previous studies identified the resistance-encoding genes that are responsible for MDR *Acinetobacter* spp. (Poirel *et al*., 2010; Antunes *et al.*, 2014; Rolain *et al*., 2016). Whilst epidemiological studies have been conducted to investigate the spread of these bacteria (Almasaudi, 2016), *Acinetobacter* spp. isolates have not yet been characterised at the local academic hospital in Durban, South Africa.

With an exponential rise in infections over the past decade, clinicians and microbiologists face the task of choosing optimal antimicrobial agents for treatment that is essential part of antibiotic stewardship programme (Diekema *et al*., 2004; Fishbain and Peleg, 2010). Antibiotic resistance is a major challenge in healthcare settings, with changes in antibiotic resistant patterns, rising costs and the introduction of new agents making it difficult to choose the best regimens (Van-Belkum *et al*., 2016). Historically, if the optimal usage of antibiotics is not monitored and overuse of the antimicrobial agents occurs, their efficacy will be lost. In response to these challenges, the antibiotic stewardship programme (ASWP) was created, as part of the urgent essential antibiotic policy, in the academic complex hospital, IALCH, KwaZulu-Natal, South Africa. The first task

is to develop a standardised algorithm for the management of patients with significant *Acinetobacter* spp. infection.

Another concern is the significant community acquired and nosocomial infections caused by *Acinetobacter* spp., which has become a serious public health concern worldwide (Doughari *et al*., 2011), including in the local hospital. The aim of the study was to offer a standard guideline for management of infection and perspectives on addressing this global public health problem. *Acinetobacter* spp. infections have risen exponentially over the past decade and many questions remain unanswered. We are unable to find any documented report of the outcomes of *Acinetobacter* spp. infections in local academic hospitals and also unable to find standardised guidelines locally and in other African countries. The general guidelines were initially developed by unit-specific antibiograms and have been revised and expanded annually. However, the standardised management algorithm for *Acinetobacter* spp. infections will constitute the new guideline, based on the outcomes of the studies of this PhD research at the Medical Microbiology Department, National Health Laboratory Service (NHLS), and Inkosi Albert Luthuli Central Hospital (IALCH) academic complex hospital.

This thesis presents a series of studies aimed at determining the epidemiological, clinical, phenotypic and genetic characteristics of the organism. Based on the outcomes of these studies, an algorithm for a standardised approach to the treatment and management of significant *Acinetobacter* spp. infection was developed.

1.2 Literature review

1.2.1 History of Acinetobacter species

Acinetobacter species, of which *Acinetobacter baumannii* is the most common, are non-fermentative aerobic Gram-negative coccobacilli. *Acinetobacter* spp. colonise the skin, mucous membranes and are present in secretions of healthy people (Almasaudi, 2016). *Acinetobacter* species are one of the most common organisms in hospital environments and frequently isolated from the skin and urinary tracts of patients (Kim *et al*., 2014). The organism is found naturally in soil and water and as a human commensal of the skin, throat and secretions (Fournier *et al*., 2006). Although the pathogenicity of *Acinetobacter* was previously believed to be low (Fournier *et al*., 2006), recent data suggests that various innate and acquired resistance factors, as well as the ability to produce biofilm, may contribute to the recent increase in pathogenicity and presence in hospital environments (Kim *et al*., 2014; Almasaudi, 2016; Gonzalez-Villoria and Valverde-Garduno, 2016; Samawi *et al*., 2016).

Acinetobacter was first identified in 1911 and the different genospecies were distinguished through DNA hybridisation, according to homology groups with more than 70% relatedness. The *Acinetobacter calcoaceticus-baumannii* complex consists of four genospecies: *(Acinetobacter calcoaceticus, Acinetobacter baumannii, Acinetobacter pittii* and *Acinetobacter nosocomialis). Acinetobacter baumannii (A. baumannii)* is the most frequently associated with hospital-acquired infections and the highest mortality rate (Lin *et al.*, 2010; Chang *et al*., 2011; Gonzalez-Villoria and Valverde-Garduno, 2016) and, therefore, the most important in a clinical setting (Retailliau *et al*., 1979; Peleg *et al*., 2008; Gonzalez-Villoria and Valverde-Garduno, 2016).

1.2.1.1 Microbiology and taxonomy

Acinetobacter species belongs to the subclasss γ-Proteobacteria in the family Moraxellaceae, a group of nonhaemolytic Gram-negative coccobacilli, usually diploid or in variable length chains. The genus *Acinetobacter* comprises over fifty highly diverse species of oxidase-negative, catalase-positive, indole-negative, and nitrate-negative bacteria (Howard *et al*., 2012). Identification of individual species by their phenotypic traits is difficult, although it may be facilitated by molecular methods such as 16S rDNA sequencing, DNA-DNA hybridisation (Howard *et al*., 2012), *gyrB* multiplex PCR and *rpoB* gene sequencing (Lee *et al*., 2014). The *A. baumannii* complex, namely *Acinetobacter baumannii, Acinetobacter pittii* (genospecies 3), and *Acinetobacter nosocomialis* (genospecies 13TU), are relevant in nosocomial infections (Jung and Park, 2015). Together with *Acinetobacter calcoaceticus*, which is found mostly in the natural environment, the group is named the *A. calcoaceticus-A. baumannii* complex (ACB complex) (Kim *et al.*, 2014) and are closely related. They are considered important nosocomial pathogens and account for most clinically significant infections (Jung and Park, 2015). Identification of individual species by use of current automated (e.g. Vitek 2) or manual commercial systems, Analytical profile index (API) strips will require further confirmatory testing may be facilitated by molecular methods such as *gyr*B multiplex PCR and *rpoB* gene sequencing (Lee *et al*., 2014).The genus of *Acinetobacter* is complex and historically, there has been confusion about the existence of multiple species, subject to uncertainty in distinguishing the separate species. Community acquired infections caused by *Acinetobacter* spp. has been increasingly reported (Sengstock *et al*., 2010). The organism is robust, particularly in dry environments, and often successful in evading host immunity, factors which enhance its spread and pathogenicity (Kim *et al.*, 2014).

The history of the first isolation of the organism is unknown (Daly *et al*., 1962; Lessel, 1971; Glen *et al.*, 1977) although it is probable that Gram-negative coccobacilli found in 1914 were isolates of *Acinetobacter* (Daly *et al*., 1962; Lessel, 1971; Glen *et al.*, 1977; Howard *et al.*, 2012).

Past literature until quite recently, does not distinguish between *A. baumannii* and *A. calcoaceticus*, and reflected a mixture of the two species (Al-Atrouni *et al.*, 2016). The genus comprises over fifty species (Al-Atrouni *et al.*, 2016), of which *A. baumannii*, *A. calcoaceticus* and *A. lwoffii* are medically relevant (Dijkshoorn and Van-Der-Toorn, 1992) and *A. baumannii* is the most virulent, according to clinical and animal model data (Dijkshoorn and Van-Der-Toorn, 1992). *A. haemolyticus, A. johnsonii, A. junii, A. nosocomialis, A. pittii, A. schindleri* and *A. ursingii* are occasional pathogens (Endo *et al*., 2012; Chiu *et al*., 2015; Salzer *et al.*, 2016) and *A. seifertii*, an emerging pathogen, may sometimes be mistaken for *A. baumannii* (Nemec *et al.*, 2015; Kishii *et al.*, 2016; Yang *et al*., 2016).

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1.2.1.2 Current taxonomy

As a result of molecular and genetic analysis, there have been numerous changes to the naming and classification of these organisms. According to the current classification, which seem to have gained wide acceptance among bacterial taxonomists, this group of bacteria as Gammaproteobacteria is categorised in the order Pseudomonadales and the family Moraxellaceae. Thus, the taxonomical classification is given as; Domain: Bacteria, Phylum: Proteobacteria, Class: Gammaproteobacteria, Order: Pseudomonadales, Family: Moraxellaceae, Genus: *Acinetobacter* (Nemec, 2017; http://apps.szu.cz//anemec/Classification.pdf.). The species *A. baumannii*, *Acinetobacter haemolyticus* and *A. calcoaceticus* are of clinical significance (Rossau *et al*., 1991; Bergogne-Bérézin and Towner, 1996; Jung and Park, 2015).

1.2.2 Increasing impact and prevalence of Acinetobacter spp.

The majority of *Acinetobacter* species are non-pathogenic but nonetheless possess mechanisms for antibiotic resistance, such as carbapenemases and extended-spectrum β-lactamases (ESBLs) (Al-Atrouni *et al.*, 2016) and may thus serve as important environmental reservoirs for resistant clinically relevant isolates. They are found in wet locations such as wetlands, fish farms, wastewater plants and in seawater. In one study in Texas, *A.baumannii-A. calcoaceticus* complex was found as a coloniser in 17% of healthy military staff but was concluded not to be a source of infection since these strains did not match those in infected soldiers (Griffith *et al.*, 2006). Highly pathogenic strains are therefore, seldom present in healthy humans. In the past, *Acinetobacter* spp. were not seen as clinically significant pathogens, but recently there has been a global increase in the prevalence of infection and drug resistant strains of these organisms (Peleg *et al*., 2008; Spellberg and Rex, 2013; Almasaudi, 2016), including both nosocomial and community acquired (Golanbar *et al*., 2011; Almasaudi, 2016). Although drug-sensitive isolates are usually community-acquired and drug-resistant are mostly hospital-acquired (Perez *et al*., 2007), it is not

easy to differentiate between colonisation and sepsis. Hospital surveillance data suggests that *Acinetobacter* has quickly spread throughout the world, with ICUs being most severely affected (Peleg *et al*., 2008; Spellberg and Rex, 2013; Almasaudi, 2016). The United States reported about 45,000 cases of *Acinetobacter* infections annually, while global numbers range from 600,000 to 1,400,000 cases per year (Spellberg and Rex, 2013). According to U.S. National Healthcare Safety Network (NHSN) data, *Acinetobacter* caused 1.8% of all healthcare-associated infections in 2009-2010 (Sievert *et al.,* 2013).

The majority of infection is acquired in healthcare facilities, but it is also reported to cause severe community-acquired pneumonia in alcoholics (Anstey *et al*., 2002). This pathogen was also found to cause infections associated with war-related injuries in the Iraq/Kuwait/Afghanistan regions (Johnson *et al*., 2007; Scott *et al.,* 2007) and in survivors of the Asian tsunami in 2004 (Garzoni *et al*., 2005). Mortality associated with Gram-negative bacteremia was significantly higher in patients with multidrug-resistant *Acinetobacter* spp., compared with other Gram-negative bacilli, which ranged from 19% to 54% (Gaynes and Edwards, 2005; Robenshtok *et al*., 2006; Kim *et al.,* 2016). The changing epidemiology, the increasing incidence and the significant mortality rates has moved *Acinetobacter* spp. into the clinical spotlight (Russo *et al*., 2010; Almasaudi, 2016). It is now recognised that *Acinetobacter* spp. play a significant role in the colonisation, community-acquired infections and nosocomial infections (Parandekar and Peerapur, 2012).

Global surveys show that *Acinetobacter* is a frequent cause of hospital acquired infection (Leung *et al.,* 2006; Sengstock *et al.,* 2010; Wong *et al*., 2017). There are reports of serious infections in immunocompromised hosts and especially in ICUs (Perez *et al*., 2007; Almasaudi, 2016). These include ventilator-associated pneumonia, bacteraemia, urinary tract infections, burn wound infections, endocarditis, secondary meningitis, and septicaemia, mostly involving patients with impaired host defenses, especially in ICUs (Fournier *et al.,* 2006; Patwardhan *et al.,* 2008; Almasaudi, 2016). In one study, *Acinetobacter* spp. accounted for 2% to 10% of Gram-negative infections in Europe (Fournier *et al.,* 2006).

According to the Centers for Diseases Control and Prevention (CDC) and European Centre for Disease Control and Prevention (ECDC), MDR microorganisms are resistant to at least one agent in three or more antimicrobial categories (Magiorakos *et al*., 2012) or MDR *Acinetobacter* spp. are those isolates that showed resistance to carbapenems. Drug resistant isolates have a significant effect on optimal antibiotic use in patients with serious infections (Fishbain and Peleg, 2010; Ghafur *et al*., 2014; Phee *et al*., 2015) and the previous surveillance studies have reported

increased resistance to carbapenems, considered to be the primary treatment against these bacteria (Arıdoğan, 2012; Sievert *et al.*, 2013; Maraki *et al.*, 2016; Zilberberg *et al.*, 2016).

In KZN, the pathogen has emerged as a particularly important organism in late-onset ventilator associated pneumonia, possibly related to an increased invasiveness of ICU procedures (Reddy *et al*., 2015). The study in Morocco reported that the frequency and rates of MDR *Acinetobacter* spp. infections was statistically higher in ICUs ($p < 0.05$), with recommendation of infection prevention and control only (Uwingabiye *et al.*, 2016). ICUs also harbour similar patterns of drug resistant *Acinetobacter* and reviews indicate that novel agents are needed to address resistance (Doughari *et al.*, 2011; Gonzalez-Villoria and Valverde-Garduno, 2016). Despite the high prevalence of infection, there is a lack of a standardised approach for optimal therapy in the African countries and Saudi Arabia (Almasaudi, 2016).

1.2.3 Antibiotic resistance patterns of Acinetobacter spp.

Acinetobacter species (*Acinetobacter* spp.) have acquired resistance to most treatment options, including aminoglycosides (amikacin), quinolones (ciprofloxacin) and broad-spectrum β-lactams (Piperacillin-tazobactam and carbapenems). Most isolates are resistant to cephalosporins and carbapenem resistance has also emerged (Kim *et al.*, 2010; Jung and Park, 2015). The pathogen poses a danger in hospitals due to carbapenem, amikacin and colistin resistance, as seen by outbreak incidents occurring in various countries (Dijkshoorn *et al*., 2007; Almasaudi, 2016).

An emerging challenge both internationally and in South Africa is the prevalence of drug resistant *Acinetobacter*, including multidrug-resistant (MDR), extensively drug-resistant (XDR) and pandrug- resistant (PDR) *Acinetobacter* species that cause serious problems in clinical settings globally (Fishbain and Peleg, 2010; Ghafur *et al.*, 2014; Phee *et al.*, 2015). According to the Centers for Disease Control and Prevention (CDC) and European Centers for Disease Control and Prevention (ECDC), MDR microorganisms are resistant to at least one agent in three or more antimicrobial categories (Magiorakos *et al*., 2012) or MDR *Acinetobacter* spp. are those isolates that showed resistance to carbapenems. XDR *Acinetobacter* spp. demonstrate resistance to all tested agents except tigecycline, rifampicin and polymyxin B, while PDR isolates are resistant to all agents (Falagas and Karageorgopoulos, 2008; Tan *et al*., 2011; Magiorakos *et al*., 2012).

The increasing prevalence of MDR *Acinetobacter* spp. is a serious concern in the management of infections worldwide. This organism has the capacity to acquire putative genetic factors, such as plasmids and pathogenicity islands, which facilitate high-level multidrug and metal resistance (Shakibaie *et al.*, 1998). The global rise in MDR. (Peleg *et al*., 2008; Sahu *et al*., 2012), XDR and

PDR *Acinetobacter* spp. (Falagas and Karageorgopoulos, 2008, Çelik *et al*., 2014) is therefore, a major challenge to current treatment options.

1.2.4 Mechanisms of antibiotic resistance

The mechanisms of antimicrobial resistance in *Acinetobacter* spp. are generally classified into four broad categories: changes in outer membrane proteins (OMPs), efflux pumps, changes in penicillin binding proteins (PBPs) and antimicrobial-inactivating enzymes (Kamolvit *et al*., 2015).

1.2.4.1 Changes in outer membrane proteins (OMPs)

OMPs, or porins, have been implicated in *Acinetobacter* drug resistance but the mechanisms are poorly understood. Membrane changes likely work in concert with β-lactamases to produce β-lactam resistance (Manchanda *et al.,* 2010). Carbapenem resistance has been linked to protein loss through porin channels and it has been suggested that mutations and reduced porin expression may also play a role. The loss of CarO, a 29-kDa protein is linked to imipenem and meropenem resistance; the carO gene disruption by distinct insertion elements leads to loss of the CarO porin in imipenem-resistant *Acinetobacter* species.

Detected clinical outbreaks of carbapenem-resistant *Acinetobacter* species due to porin loss that included reduced expression of a number of OMPs such as 47, 44, and 37 kDa OMPs in *Acinetobacter* species isolates (Bonomo and Szabo, 2006). Heat-modifiable protein HMP-AB, a 33-36-kDa and 43 kDa proteins are also identified OMPs involved in β-lactam resistance (Jain and Danziger, 2004).

1.2.4.2 Efflux pumps

Efflux pumps consist of a pump in the cytoplasmic membrane and an exit portal, linked by a lipoprotein. In the *Acinetobacter* genus, the *adeB* gene, which encodes a resistance-nodulation-cell division (RND), protein type pump, is associated with resistance to aminoglycosides, quinolones, tetracyclines, chloramphenicol, erythromycin, trimethoprim and ethidium bromide. (Nowak *et al.*, 2015).

A wide variety of multidrug efflux pumps are present in multidrug-resistant *Acinetobacter*. The substrate profile of the *Abe*M pump includes fluoroquinolones (Perez *et al.,* 2007). The (RND) familytype pump *Ade*ABC is the most studied so far and it has a substrate profile that includes β-lactams (including carbapenems) and other class of antimicrobials. (Perez *et al.,* 2007). Its

structure consists of *Ade*A forming the inner cytoplasmic membrane protein, *Ade*B creates the linking component and *Ade*C forms the porin (Nowak *et al.*, 2015).

The regulation of *Ade*ABC is controlled by a regulator (*ade*R) and sensor (*Ade*S), where point mutations may lead to overexpression of *Ade*ABC, and therefore higher efflux (Peleg *et al.*, 2008). In combination with OXA, this amplification results in high-level resistance to carbapenems (Perez *et al.*, 2007; Nowak *et al*., 2015).

1.2.4.3 Changes in Penicillin binding proteins (PBPs)

Seven different PBPs (1a, 1c, 2, 3, 4, 4b and 5) have been found in *Acinetobacter* species. Porin deficiency results in decreased uptake of carbapenems, while modification of the PBPs results in decreased affinity. Isolates from Spain have revealed mutations leading to reduced expression of PBP-2 (Perez *et al.*, 2007).

1.2.4.4 Antimicrobial-inactivating enzymes

The most common mechanism is facilitated by β-lactamases. β-lactamases are divided into 4 molecular groups: consisting of Ambler class A, class B (metallo enzymes), Class C (β-lactamases), and class D (oxacillinases) (Jain and Danziger, 2004).

These enzymes, at least partially, hydrolyse carbapenems along with other β-lactams (Jain and Danziger, 2004). Ambler class β-lactamases are mentioned in section; 1.2.5.1.

1.2.5 Mechanisms of resistance to selected antibiotics

The selected appropriate antibiotics that commonly use for *Acinetobacter* spp. are β- lactams (ceftazidime, piperacillin-tazobactam, and carabapenems), aminoglycosides (amikacin) and colistin (CLSI 2014).

1.2.5.1 Resistance to β- lactams

Acinetobacter spp. display resistance to β-lactams through hydrolysis of β-lactams by β-lactamases, (Dijkshoorn *et al*., 2005; Peleg *et al*., 2008; Stoeva *et al*., 2008) changes in penicillin-binding proteins (PBPs), changes in the structure and number of porin proteins leading to decreased permeability, and efflux pumps that decrease the concentration of antibiotic in the cytoplasm (Perez *et al*., 2007). The Ambler scheme divides β-lactamases into four major classes (A to D). The basis of this classification scheme rests upon protein homology (amino acid similarity), and not phenotypic characteristics. In the Ambler classification scheme, β-lactamases of classes A, C, and D are serine β-lactamases. Carbapenemases, oxacillinases (OXA) or metallo-

β-lactamases (MBLs) are of major concern because of their ability for rapid dissemination (Abdalhamid *et al*., 2014).

(i) Class A β-lactamases

Narrow-spectrum β-lactamases, such as TEM β-lactamases (class A) most commonly encountered β-lactamase in Gram-negative bacteria, is able to hydrolyze penicillin and first generation cephalosporins β-lactamase. *Acinetobacter* spp. isolates harboring PER-1, an ESBL, demonstrate high-level resistance to penicillins and extended-spectrum cephalosporins, but fortunately, PER-1 β-lactamase does not confer resistance to carbapenems in *Acinetobacter* spp. PER-1 is very prevalent among *Acinetobacter* spp. isolates. (Perez et al., 2007). Both Extended-spectrum β-lactamases (ESBLs) and Narrow-spectrum β-lactamases (NSBLs) such as TEM-1 and TEM-2, from the Ambler class A group have been identified in *Acinetobacter* (Jain and Danziger, 2004).

(ii) Class B β-lactamases.

A recent phenomenon in β-lactam resistance is the increase in metallo-β-lactamases (MBLs) in *Acinetobacter* spp. (Walsh *et al*., 2005). Metallo-β-lactamases (MBLs) are Ambler class B, or β-lactamases that can hydrolyse carbapenems and all β-lactam antibiotics, excluding aztreonam. Unlike class A and D carbapenemases, class B β-lactamases have a metal ion in the active site, usually zinc, which plays a role in catalysis (Walsh *et al*., 2005(Perez *et al.*, 2007).). Mishra *et al*. (2012) and Altun *et al*. (2013) determined that *Acinetobacter* were the most common MBL-producing isolates and reported a higher incidence of MBL-production than *Pseudomonas aeruginosa* (El-Kazzaz and El-khier, 2015; Potron *et al*., 2015).

The two major metallo-β-lactamases that have been reported in *Acinetobacter* species are "Verona integron-encoded metallo-β-lactamases" (VIM) and ''Imipenem hydrolysing β -lactamase" (IMP). The IMP or VIM family has been described in various areas in the world such as in Japan, Italy, Hong Kong, and Korea. These enzymes are encoded by genetic elements located on chromosomes or on plasmids and are known to hydrolyse all β-lactam antibiotics except aztreonam. Enzymatic degradation by β-lactamases is the most frequent mechanism of β-lactam resistance in *Acinetobacter* species. The highly mobile nature of plasmids poses a risk of transmission among other microorganisms (Urban *et al.*, 2003).

(iii) Class C β-lactamases.

Acinetobacter spp. have a chromosomally encoded class C β-lactamase in the *bla* genes. Class C cephalosporinases hydrolyse penicillins and cephalosporins, both narrow-spectrum and extended-

spectrum, but do not hydrolyse cefepime or carbapenems. Therefore, many *Acinetobacter* spp. isolates are resistant to ceftazidime (Perez *et al.*, 2007).

Metallo-enzymes that hydrolyse carbapenems and other β-lactams except monobactams. These are not inhibited by clavulanic acid. AmpC-type cephalosporinase, or *Acinetobacter*-derived cephalosporinases (ADCs) are produced by *Acinetobacter* species but do not hinder the efficacy of cephalosporins with regular rates of expression (Manchanda *et al.*, 2010). However, over expression of ADC caused by the upstream insertion sequence (IS) element known as IS*Aba1* that codes for class C cephalosporinases, results in resistance. ADCs hydrolyse penicillin and extended spectrum cephalosporins but have no effect on cefepime and carbapenems (Manchanda *et al*., 2010).

(iv) Class D β-lactamases.

Class D β-lactamases, also known as OXA-type enzymes or oxacillinases, are represented by more than 350 genetically diverse enzymes that are widely disseminated in Gram-negative bacteria (Antunes *et al.*, 2014). Although they exhibit weak hydrolysis of carbapenems, the genes encoding OXA associated with insertion sequences that provide strong promoters, leading to overexpression and carbapenem resistance (Turton *et al.*, 2006). Class D OXA β-lactamases are robust penicillinases i.e. oxacillinases. Some, like OXA ESBLs, can hydrolyse extended-spectrum cephalosporins (Aubert *et al*., 2001; Walther-Rasmussen and Hoiby, 2006). The most worrying in this class are the OXA β-lactamases that can inactivate carbapenems, the first of which was discovered in 1985 before carbapenems were introduced. The plasmid-encoded enzyme, OXA-58 has since been discovered. OXA-58 has been described both as chromosomal and as a plasmid-mediated carbapenemase in *A.baumannii* (Brown and Amyes, 2006). β-lactam resistance occurs mainly through carbapenem-hydrolysing class D β-lactamases (CHDLs) (Joseph *et al*., 2010), also known as OXA-type enzymes or oxacillinases (Shakibaie *et al*., 1998; Kim *et al*., 2010). Previously, Class D carbapenemase enzymes are classified into four subgroups according to their amino acid sequence identity: OXA-23 (plasmid-encoded), OXA-24 (chromosomally encoded), OXA-51 (chromosomally encoded) and OXA-58 (plasmid-encoded) (Peleg *et al*., 2008; Kock *et al.*, 2013). Based on their amino acid sequence identity, CHDLs have been subdivided into several subgroups. There are five subclasses of OXA associated with *A. baumannii* reported in literature; the intrinsic chromosomal OXA-51-like, of which there are over 70 variants, and the acquired OXA-23-like, OXA-24 (OXA-40-like), OXA-58-like, and OXA-143-like (Poirel *et al.*, 2010). Acquired OXA are found both chromosomally and on plasmids and can be detected by multiplex PCR (Woodford *et al*., 2006; Higgins *et al.*, 2010). OXA-143-like subgroups are of major clinical importance due to their wide dissemination in bacterial pathogens.

Acquired OXA are found both chromosomally and on plasmids and can be detected by multiplex PCR (Woodford *et al*., 2006; Higgins *et al.*, 2010). In addition, the OXA-235-like, OXA-236-like, and OXA-237-like, first representatives of a novel subclass of CHDLs, were described in *A. baumannii* strains in 2013 that could not be detected by previous PCR methods (Higgins *et al.*, 2013). The majority of these carbapenemases have been identified in various *Acinetobacter* isolates, predominantly in *Acinetobacter baumannii* (Dijkshoorn and Van-Der-Toorn, 1992; Manchanda *et al.*, 2010; Kishii *et al.*, 2016; Yang *et al.*, 2016). These were described in regions such as Scotland, Spain, France, Japan, Singapore, China, Brazil, Cuba, and Kuwait (Manchanda *et al.*, 2010).

Carbapenem-hydrolysing class D enzymes occur globally (Falagas and Karageorgopoulos, 2008; Sahu *et al.*, 2012). Koh *et al*. in 2007, reported that 91% of *Acinetobacter* spp. isolates in Singapore were carbapenemase producers and possessed the $bla_{\text{OXA-23}}$ gene (Koh *et al*., 2007). The high prevalence of OXA-23-like can be linked to plasmids and transposons, although the gene can also be found on a chromosome (Parandekar and Peerapur, 2012). In 2012, Liakopoulos *et al*., reported a 95% prevalence of OXA-23-like in Greece between 2010 and 2011. A 2013 study conducted at Pretoria Academic Hospital (South Africa) revealed a prevalence of OXA-51-like (83%) and OXA-23-like (59%) (Kock *et al*., 2013).

OXA-23 carbapenemase was detected in 1985 before the introduction of carbapenems. Since then, the initially termed ARI-1 (*Acinetobacter* resistant to imipenem), which is a plasmid-encoded enzyme, has been reported in England, Brazil, Polynesia, Singapore, Korea, and China (Manchanda *et al.*, 2010).

Carbapenem-hydrolysing class D β-lactamases (CHDLs) are most problematic clinically, as they produce resistance to the antibiotics of last resort, carbapenems, thus severely limiting therapeutic options.

1.2.5.2 Resistance to aminoglycosides

Resistance is facilitated primarily by aminoglycoside-modifying enzymes (AMEs), including aminoglycoside phosphotransferases, aminoglycoside acetyltransferases, and aminoglycoside nucleotidyltransferases (Perez *et al*., 2007; Jung and Park, 2015).

Genes coding for AME within class 1 integrons are frequently present in multidrug-resistant *Acinetobacter* species isolates.

The AAC (3) enzymes found in Gram-negative bacteria are divided into nine classes. The AAC (3)-I, present in many Gram-negative isolates including *Enterobacteriaceae*, is made up of five enzymes linked to gentamicin, sisomicin, and fortimicin (astromicin) resistance (Ramirez and

Tolmasky, 2010). Resistance to parenteral aminoglycosides, conferred by 16S rRNA methylases, have been reported in Japan, Korea and the United States (Perez *et al.*, 2007; Jung and Park, 2015). The genes are present on transposons in plasmids, making them at risk of horizontal transfer (Doi and Arakawa, 2007; Jung and Park, 2015). The AME that was discovered in Japan plays a role in amikacin resistance (Perez *et al.*, 2007; Jung and Park, 2015).

Other mechanisms of resistance include alterations in the target ribosomal protein, impaired transport of aminoglycosides into the cell, and efflux pump-mediated removal of aminoglycosides (Jung and Park, 2015).

A wide array of AME have been observed in *Acinetobacter* spp. (Perez *et al*., 2007). The predominant AME, an AAC (3) class enzyme occurred in nearly 50% of the isolates resistant to aminoglycosides while some of the AAC (A4)-harbouring *Acinetobacter* spp. remained susceptible to amikacin (Akers *et al*., 2010). The AME, encoded by *aac*A6, which has been reported from Japan, plays a crucial role in amikacin resistance (Doi *et al.*, 2004). Amikacin resistance in *Acinetobacter* spp. is facilitated by APH (3')-VI, encoded by the *aph*A6 gene (Nemec *et al.*, 2004; Aliakbarzade *et al*., 2014). In addition, the *aac*A4 gene, which encodes AAC (6')-Ib, confers resistance to amikacin, netilmicin, and tobramycin (Aliakbarzade *et al*., 2014), while *aad*B is associated with resistance to kanamycin, gentamicin and tobramycin (Aliakbarzade *et al*., 2014).

1.2.5.3 Resistance to polymyxins

The peptides, polymyxin B and polymyxin E (also known as colistin or intravenous colistimethate sodium) have seen increased use as final resort therapy in MDR *Acinetobacter* spp. (Perez *et al*., 2007). Colistin displays bactericidal activity through its interaction with the lipid A components of lipopolysaccharide (LPS), thus disrupting the outer membrane of Gram-negative bacteria (Olaitan *et al.*, 2014). In *Acinetobacter* spp, colistin-resistance is mediated by complete loss of LPS production via mutations within the genes (*lpx*A, *lpx*C and *lpx*D) of the lipid A biosynthesis pathway or by modification of lipid A components of LPS via mutations in the *pmr*A and *pmr*B genes of the two-component regulatory system and *pmr*C that encodes a lipid A phosphoethanolamine transferase (Adams *et al.*, 2009; Park *et al.*, 2015). It was demonstrated in two different isolates of *Acinetobacter* spp. that colistin resistance may occur from a susceptible sample through lipid A biosynthesis mutants. The isolates which do not possess LPS demonstrate high levels of resistance to colistin (Arıdoğan, 2012). *Acinetobacter* isolates that are resistant to colistin have been reported (Adams *et al*., 2009; Moffatt *et al*., 2010). The clinical significance of colistin-heteroresistant isolates following colistin treatment has been highlighted in a case report (Hernan *et al.*, 2009).

Isolates producing OXA-23 and OXA-58 carbapenemases exhibited a high percentage of colistin heteroresistance (Rodriguez *et al.*, 2010). The wide usage of colistin against carbapenem-resistant *Acinetobacter* spp. led to the development of resistance (Pogue *et al.*, 2015).

1.2.6 Antibiotic resistance drives outcomes

Acinetobacter spp. possess extensive drug resistance mechanisms, with a resistance island of 45 genes in its genome (Adams *et al*., 2009; Blackwell *et al*., 2016), with an additional ability to acquire resistance genes from other bacteria (Adams *et al*., 2009; Blackwell *et al*., 2016), and develop resistance to a particular agent during therapy (Cheng *et al*., 2015).

The clinical challenge therefore, lies in providing effective antibiotic therapy and overcoming the pathogen's massive potential for drug resistance (Adams *et al*., 2009; Blackwell *et al*., 2016).

Carbapenem resistance in particular, poses a threat to effective treatment options, with *Acinetobacter* isolates showing an interplay of a number of resistance mechanisms. Among these, oxacillinase (OXA) production and the absence of PBP2 are the most common, while reduced entry of carbapenems caused by downregulation of porin also occurs (Fernandez-Cuenca *et al.*, 2003). Oxacillinases OXA-23-like, OXA-24-like or -40-like, OXA-51-like, OXA-58-like, and OXA-143-like have been linked to most cases of carbapenem resistance worldwide (Higgins *et al.,* 2010; Ben *et al.,* 2011; Principe *et al.,* 2014; Kamolvit *et al.,* 2015; Labarca *et al.*, 2016). OXA-23 is a plasmid- or transposon encoded -lactamase, while OXA-51 is a chromosome-based enzyme and is intrinsic to *Acinetobacter.* OXA-24/40 can be chromosomal or plasmid based, and OXA-58 is plasmid encoded. These class D -lactamases are not very robust carbapenemases, but the presence of an insertion sequence (IS) element, such as IS*Aba*I and IS*Aba*9, increases expression of the carbapenemase significantly, resulting in clinical carbapenem resistance (Higgins *et al.*, 2010; Nigro and Hall, 2015; Warner *et al.,* 2016).

Another element in resistance is the class β-lactamases, or metallo-lactamases (MBLs) (Perez *et al.,* 2007), with the finding that the enzymes IMP, VIM, SIM and NDM are present in *Acinetobacter* (Perez *et al.*, 2007; Dortet *et al.,* 2014). In addition, identification of the composite transposon Tn125 suggests that *Acinetobacter* was the source of bla_{NDM} genes which later spread to *Enterobacteriaceae* (Bonnin *et al.,* 2012; Bonnin *et al.,* 2014; Krahn *et al.,* 2016).

Acinetobacter is disproportionately responsible for an increase in patient mortality, resulting from treatment failure with antimicrobial drugs. Due to the high rates of resistance, early effective treatment is difficult, thus compromising clinical outcomes (Blot *et al.,* 2003; Maragakis and Perl, 2008; Spellberg and Bonomo, 2014; Zilberberg *et al.,* 2016).

XDR strains, namely those demonstrating resistance to all available agents except for those that are more toxic or less effective copered to first-line therapy, is common in *Acinetobacter* species (Infectious Diseases Society of America, 2012). Carbapenem resistance is usually the mark of XDR *Acinetobacter*, which leaves polymixins, tigecycline and sometimes aminoglycosides as treatment options (Chopra *et al*., 2013; Chopra *et al*., 2014; Freire *et al*., 2016). According to National Healthcare Safety Network (NHSN) and Eurofins, over half of *A. baumannii* in ICUs in the United States show carbapenem resistance, which is the highest rate of all pathogens surveyed (Sievert *et al.*, 2013; Zilberberg *et al*., 2016). Data from other countries show even higher rates of resistance and a sharp increase in recent decades, with global figures rising from 4% in 2000 to 60% in 2008 (Kallen *et al.*, 2010) and in some settings the proportion of XDR strains is close to 90% (Sievert *et al.*, 2013; Guo and Xiang. 2016).

Ineffective initial therapy was likely the reason for differences in outcome for XDR, carbapenem resistant strains, and not differences in virulence, since treatment with tigecycline or colistin within 48 hours reduced the mortality rates in these patients from 88% to 38% (Lee *et al.*, 2014). Similar outcomes were observed in ICUs in the U.S., where mortality was doubled in cases of ineffective initial therapy (Zilberberg *et al*., 2016).

1.2.7 Drug susceptibility testing

The standardised method for drug susceptibility testing of *Acinetobacter* spp. includes the disk diffusion test (Kirby-Bauer), the automatic identification and susceptibility system (VITEK 2 system (BioMérieux) and MicroScan Walk-Away® (Becton Dickinson Diagnostic Systems, Sparks, MD). Minimum inhibitory concentrations (MICs) are widely performed using the Epsilometer test ('E-test') (BioMérieux).

1.2.7.1 The Kirby-Bauer disk diffusion test

Agar disk-diffusion test was implemented in 1940 (Heatley, 1944) as per the Clinical Laboratory Standards Institute (CLSI) and is the official method routinely used in many clinical microbiology laboratories for antimicrobial susceptibility testing. The procedure involves the growth of the organism on Mueller-Hinton agar in the presence of various antimicrobial impregnated filter paper disks. The presence or absence of growth around the disks is an indirect measure of the ability of the specific compound to inhibit the organism (Hudzicki, 2009). The antibiogram provides qualitative results by interpreting as susceptible (S), intermediate (I) or resistant (R)

(Reller *et al.*, 2009). [Examples: imipenem (10 µg), ≥ 22 (S), 19-21 (I), ≤18 mm (R); meropenem (10 µg), ≥ 18 (S), 15-17 (I), ≤14 mm (R)] for *Acinetobacter* species (CLSI 2014).

As a useful phenotype of the microbial strain tested, it can guide clinicians in the appropriate selection of initial empiric treatments and antibiotics used for individual patients (Caron, 2012; Balouiri *et al*., 2016). However, since the bacterial growth inhibition does not equate to bacterial death, this method cannot distinguish bactericidal and bacteriostatic effects.

Moreover, the agar disk-diffusion method is not appropriate to determine the minimum inhibitory concentration (MIC), as it is impossible to quantify the amount of the antimicrobial agent diffused into the agar medium. An approximate MIC can be calculated for some microorganisms and antibiotics by comparing the inhibition zones with stored algorithms (Nijs *et al.*, 2009). The disk-diffusion assay provides many advantages over other methods such as simplicity, low cost, the ability to test enormous numbers of microorganisms and antimicrobial agents, and ease of interpretation of results. In addition, several studies have demonstrated the great interest in patients who suffer from bacterial infection of an antibiotherapy based on the antibiogram of the causative agent (Kreger *et al.*, 1980). This fact is due to the good correlation between in *vitro* data and in *vivo* evolution (Caron, 2012; Balouiri *et al*., 2016).

1.2.7.2 The VITEK 2 system (BioMérieux)

This system uses a fluorescence-based technology and is an automated instrument that is capable of rapid, simultaneous identification and antimicrobial sensitivity testing of microorganisms (Funke *et al.*, 1998), in accordance with the guidelines established by the CLSI (CLSI 2014). A transmittance optical system allows interpretation of test reactions using different wavelengths during incubation. Each test reaction is read every 15 mins to measure either turbidity or colour products of substrate metabolism. Both VITEK 2 ID-GNB (an identification system) and VITEK 2 AST-No. 12 (a susceptibility testing system) card systems gave rapid, reliable, and highly reproducible results (Ling *et al.*, 2001). Several advantages of the VITEK 2 system were mentioned; first, it is a closed system that can avoid unwanted cross-contamination or environmental contamination. Second, it has a reliable recheck system if a specimen card is misplaced on the specimen cartridge. Third, the VITEK 2 system is able to handle dozens of specimens automatically at the same time. It is also easy for laboratory staff to prepare and load bacterial specimens. The decreased turnaround and hand-on times greatly improve the efficiencies of routine clinical laboratories. In conclusion, both the VITEK 2 ID-GNB (an identification system) and VITEK 2 AST-No. 12 (a susceptibility testing system) card systems gave rapid, reliable, and highly reproducible results (Ling *et al.*, 2001).

1.2.7.3 The E-test

The E-test is based on diffusion of a preformed antibiotic gradient from a plastic strip. It is technically simple and similar to the disk diffusion method, but it can provide MICs. The versatility and ease of use of the E-test means that it is an attractive alternative to conventional dilution tests. Problems in performing *in vitro* colistin susceptibility testing of *Acinetobacter* spp. have been encountered: the disk diffusion method has been found to be inaccurate and not reproducible for *Acinetobacter* spp. (Gales *et al.,* 2001; Arroyo *et al.,* 2005). Agar dilution and broth microdilution (BMD), currently the recommended susceptibility test methods for this organism, are cumbersome and impractical (Gales *et al.,* 2001; Arroyo *et al.,* 2005). The E-test (bioMerieux), being quick, cost effective and helps in decreasing the laborious work, has been reported to be a simple and accurate alternative method for determining the antimicrobial susceptibilities of various microorganisms. A study suggested that the E-test could be a reliable and suitable alternative to the reference method for the detection of colistin resistance in *Acinetobacter* spp. clinical isolates by clinical laboratories (Arroyo *et al.,* 2005).

1.2.7.4 Conventional dilution tests

These are the earliest methods of antimicrobial susceptibility testing and include the broth macrodilution or tube dilution method.

1.2.7.4.1 Broth dilution method

Broth micro- or macro-dilution is one of the most basic antimicrobial susceptibility testing methods (Balouiri *et al*., 2016). The procedure involves preparing two-fold dilutions of the antimicrobial agent (e.g. 1, 2, 4, 8, 16 and 32 µg/mL) in a liquid growth medium dispensed in tubes containing a minimum volume of 2 mL (macrodilution) or with smaller volumes using 96-well microtitration plate (microdilution) (Washington and Woods, 1995). Then, each tube or well is inoculated with a microbial inoculum prepared in the same medium after dilution of standardised microbial suspension adjusted to 0.5 McFarland scale. After mixing well, the inoculated tubes or the 96-well microtitration plate are incubated (mostly without agitation) under suitable conditions depending upon the test microorganism. The MIC is the lowest concentration of antimicrobial agent that completely inhibits growth of the organism in tubes or microdilution wells as detected by the unaided eye (CLSI 2012) (Washington and Woods, 1995; Jorgensen and Ferraro, 1998).

Unlike microdilution method, the main disadvantages of the macrodilution method are the tediousness, manual undertaking, risk of errors in the preparation of antimicrobial solutions for each test, and the comparatively large amount of reagents and space required (Reller *et al.*, 2009).

Thus, the reproducibility and the economy of reagents and space that occurs due to the miniaturisation of the test are the major advantages of the microdilution method. Nevertheless, the final result is significantly influenced by approach, which must be carefully controlled if reproducible results (intralaboratory and interlaboratory) are to be attained (CLSI 2012). For the determination of MIC endpoint, viewing devices can facilitate reading microdilution tests and recording results with high ability to discern growth in the wells. Moreover, several colorimetric methods based on the use of dye reagents have been developed (Al-Bakri and Afifi, 2007). The Alamar blue dye (resazurin), an effective growth indicator, can also be used for this purpose (Bouhdid *et al.,* 2009; Ouedrhiri *et al.,* 2015).

It is well known that the inoculum size, the type of growth medium, the incubation time and the inoculum preparation method can influence MIC values (CLIS 1998). Therefore, broth dilution has been standardised by CLSI for testing bacteria that grow aerobically (CLSI 2012). The EUCAST broth dilution method is principally similar to that of CLSI with modifications usually concerning some of the test parameters such as inoculum preparation, inoculum size, and the MIC reading method which is visual in CLSI assay and spectrophotometric in EUCAST guidelines (CLSI 1998; Balouiri *et al*., 2016).

The determination of minimum bactericidal concentration (MBC) also known as the minimum lethal concentration (MLC), is the most common estimation of bactericidal activity. The MBC is defined as the lowest concentration of antimicrobial agent needed to kill 99.9% of the final inoculum after incubation for 24 hr under a standardised set of conditions described in document M26-A (CLSI 1998; Balouiri *et al*., 2016), in which the MBC can be determined after broth macrodilution or microdilution by sub-culturing a sample from wells or tubes, yielding a negative microbial growth after incubation on the surface of non-selective agar plates to determine the number of surviving cells (CFU/mL) after 24 h of incubation. The bactericidal endpoint (MBC) has been subjectively defined as the lowest concentration, at which 99.9% of the final inoculum is killed (CLSI 1998; Balouiri *et al*., 2016).

1.2.8 Impact of molecular methods on infection control

Suitable molecular typing methods are essential for epidemiological investigations and infection control studies. The increasing rates of resistance of *Acinetobacter* spp. to the available antimicrobial drugs means that outbreaks should be identified and controlled early. Knowledge is lacking about the diversity within the species and the emergence of epidemic clones (Nemec *et al.,* 2008). Pulsed-field gel electrophoresis (PFGE) is considered the "gold standard" of epidemiological typing among the various genotypic methods for *Acinetobacter* spp. (Smith *et*

al., 1993; Hamouda *et al*., 2010). Among traditional and molecular typing methods, antibiotic resistance typing has been used for the epidemiological investigation of outbreaks caused by *Acinetobacter* species. Multiplex PCR used to identify antibiotic resistance genes encoding the MDR phenotypes in clinical isolates of *Acinetobacter* spp. demonstrated that MDR *Acinetobacter* spp. isolates harboured the same resistance genes (Ghajavand *et al*., 2015). Infection prevention and control measures form a crucial part of the management of MDR *Acinetobacter* spp. (Almasaudi, 2016; Uwingabiye *et al*., 2016; Samawi *et al*., 2016).

1.2.9 Role of synergy testing for combination therapy against Acinetobacter spp.

The high frequency of resistance to standard treatment in *Acinetobacter* spp., as well as the emergence of MDR strains, has changed the options for optimal antibiotic therapy in serious infections (Fishbain and Peleg, 2010; Ghafur *et al*., 2014; Phee *et al*., 2015). Despite toxicity risks, colistin is commonly used in the face of limited options (Peleg *et al*., 2008; Vidaillac *et al*., 2012). Combination therapy with colistin is preferred over monotherapy, due to the drug's toxic effects and the development of colistin resistance (Leu *et al*., 2014).

1.2.9.1 Combination therapy

The emergence of MDR, XDR and PDR *Acinetobacter* spp. is a serious problem globally, and these resistant isolates have a significant effect on the optimal use of antibiotics in patients with serious infections (Fishbain and Peleg, 2010; Ghafur *et al*., 2014; Phee *et al*., 2015). With limited therapeutic options, colistin is an alternative antimicrobial agent, despite concerns about renal toxicity and neurotoxicity, especially in chronic conditions of hospitalised patients who are prone to developing renal failure (Peleg *et al*., 2008; Vidaillac *et al.*, 2012). In addition, patients with *Acinetobacter* spp. bacteremia who are receiving early and appropriate antimicrobial therapy are expected to show favourable clinical outcomes. However, the presence of any underlying pathology and polymicrobial sepsis negatively affect such outcomes (Kim *et al*., 2012).

The emergence of resistance during colistin monotherapy, as well as the potential toxic effects, has led to its increased use in combination with other drugs, instead of merely increasing the dose in monotherapy (Leu *et al*., 2014). Therefore, therapy using various synergistic combinations of antimicrobials (including carbapenems, colistin, rifampicin, and ampicillin-sulbactam) has been suggested as the best approach, and empirical combination of therapies have become common practice (Bonapace *et al*., 2000; Petrosillo *et al*., 2014).

The administration of antibiotic combinations has been proposed for three main reasons: (i) to broaden the spectrum of activity (ii) to minimise the development of antibiotic resistance and (iii) to achieve antibiotic synergy. The latter may be important if an antibiotic with marginal activity is used against the infecting bacterium.

In vitro activity and kill-kinetics of a vancomycin-colistin combination were shown to have a synergistic action against five number of multidrug-resistant *Acinetobacter baumannii* (MDRAB) isolates [defined as resistant to at least three different antimicrobial classes (aminoglycosides/quinolones/β-lactams) but susceptible to colistin] (Falagas *et al.*, 2006), and *A. baumannii* ATCC19606, was used as a drug-susceptible type isolate in United Kingdom. (Gordon *et al*., 2010). Vancomycin, a glycopeptide and an inhibitor of bacterial peptidoglycan synthesis, lacks activity against Gram-negative bacteria due to its large size and hydrophobicity. The cell-permeabilising properties of colistin could however, be exploited to improve the penetration of glycopeptides through the *Acinetobacter* spp. outer membrane, toward their targets in the cell wall (Gordon *et al*., 2010). This mechanism of synergy for colistin and vancomycin against MDR *Acinetobacter* species may be useful for polymicrobial infection with Gram-positive and MDR/XDR *Acinetobacter* spp. mixed infection. However, the clinical utility of these combinations against drug-resistant *Acinetobacter* remains to be determined (Doughari *et al*., 2011). Previous studies reporting on synergy tests of colistin with different combinations of antimicrobials showed contradictory and contrasting outcomes, and further synergy research needs to be conducted to obtain more conclusive results (Falagas *et al.*, 2006; Wareham and Bean 2006; Pankey and Ashcraft. 2009; Vidaillac *et al*., 2012; Petrosillo *et al*., 2014; Temocin *et al*., 2015; Zafar *et al*., 2015).

1.2.9.2 Different methods of synergy testing

In vitro synergy tests are used in XDR and PDR *Acinetobacter* spp. infections to evaluate synergism between available combination agents (Sopirala *et al*., 2010). The desired characteristics of a test are simplicity, accuracy and reproducibility. Although time- kill and checkerboard tests are commonly used, they are time consuming and labour intensive. The E-test is simpler but has not been studied for this purpose previously (White *et al*., 1996). E-test was easier to perform, less time-consuming, less expensive and more accurate (Sopirala *et al.*, 2010). Nevertheless, some literature suggests that accuracy is not pathogen but method dependent (Sopirala *et al.*, 2010). The interesting new rapid synergy testing method, a novel two-dimensional antibiotic gradient technique named Xact™, for meropenem/colistin synergy testing for multidrug-resistant *Acinetobacter baumannii* strains has been recommended in routine microbiology (Van-Belkum *et al.*, 2015). This new test was comparable, shown to be

diagnostically useful, easy to implement and less labour intensive than the classical method (Van Belkum *et al.*, 2015). Therefore, synergy testing should be done using the new method and compared with E-test method in future.

Time–kill and checkerboard titration methods

The time-kill curve, which determines lethal effect, and the checkerboard titration method, which provides bacteriostatic and bactericidal results, are two basic techniques for the quantitative assessment of synergism (Young, 1978; Greenwood, 1979; Moellering, 1979; Hallander, 1982). In a modification of the checkerboard method proposed by Berenbaum (1978), two drugs were mixed in fractions of their MICs for each bacterial isolate and serially diluted (Berenbaum, 1978). MICs and fractional inhibitory concentrations (FICs) were determined after overnight incubation, by examining for turbidity.

1.2.9.2.1 Time-kill test

Time-kill test is the most appropriate method for determining the bactericidal effect. It is a strong tool for obtaining information about the dynamic interaction between the antimicrobial agent and the microbial strain. The time-kill test reveals a time-dependent or a concentration-dependent antimicrobial effect (CLSI 1998). For bacteria, this test has been well standardised and described in M26-A document of CLSI (CLSI 1998). It is performed in broth culture medium using three tubes containing a bacterial suspension of 5×10^5 CFU/mL. The first and the second tubes contain the molecule or the extract tested usually at final concentrations of 0.25×MIC and 1×MIC, and the third one is considered as the growth control. The incubation is done under suitable conditions for varied time intervals (0, 4, 6, 8, 10, 12 and 24 h) (CLSI 1998; Konaté *et al.*, 2012). Then, the percentage of dead cells is calculated relatively to the growth control by determining the number of living cells (CFU/mL) of each tube using the agar plate count method. Generally, the bactericidal effect is obtained with a lethality percentage of 90% for 6 h, which is equivalent to 99.9% of lethality for 24 h (Konaté *et al.*, 2012). In addition, this method can be used to determine synergism or antagonism between drugs (two or more) in combinations (White *et al.*, 1996; CLSI 1998).

1.2.9.2.2 Checkerboard method

The checkerboard, or checkerboard titration, method is the most frequently used evaluation method. The technique derives its name from the square array of tubes, agar plates or microtiter wells used to hold the different antibiotic concentrations against which the offending pathogen is being tested. Twofold dilutions of each antibiotic, both alone and together, are used (Marymont et al., 1983). Any antibiotics which are stored at 2° to 8°C until used. The stock solutions and serial twofold dilutions of each drug at least double the MIC were prepared according to the recommendations method immediately prior to testing (Bajaksouzian et al., 1997).

A total of 50 µL of Mueller-Hinton broth was distributed into each well of the microdilution plates. The first antibiotic of the combination is serially diluted along the ordinate, while the second drug was diluted along the abscissa. An inoculum equal to a 0.5 McFarland turbidity standard was prepared from each isolate in Mueller-Hinton broth (BBL). Each microtiter well was inoculated with 100 µL of a bacterial inoculum of 5 $x10^5$ CFU/ml, and the plates were incubated at 35°C for 48 h under aerobic conditions. The resulting checkerboard contains each combination of two antibiotics, with tubes that contain the highest concentration of each antibiotic at opposite corners. According to the standard guidelines for broth microdilution, the MIC was defined as the lowest concentration of antibiotic that completely inhibited the growth of the organism as detected with the naked eye. Synergy is more likely to be expressed when the ratio of the concentration of each antibiotic to the MIC of that antibiotic was same for all components of the mixture. The fractional inhibitory concentration index (ΣFICs) were calculated as follows: ΣFIC=FIC A + FIC B, where FIC A is the MIC of drug A in the combination/MIC of drug A alone, and FIC B is the MIC of drug B in the combination/MIC of drug B alone. The combination is considered synergistic when the ΣFIC is ≤ 0.5, indifferent when the ΣFIC is ≥ 0.5 to ≤ 2, and antagonistic when the ΣFIC is ≥ 2 (Orhan *et al.*, 2005).

1.2.9.2.3 Synergy test by colistin-incorporated plate and E-test

Synergy testing can also be performed by the E-test method, whereby one drug is incorporated into the agar at a fixed concentration, seeded with the inoculum, after which the second drug is applied on an E-test (Berenbaum, 1978; Gordon *et al*., 2010). The MIC of the second drug is compared in the presence and absence of the first drug. Different drug combinations can be tested in this manner. Hence, the performance of the E-test for synergy testing in comparison to the checkerboard (CB) dilution method, widely used to assess synergy between antibiotics, have been systematically analysed (White *et al*., 1996; Sopirala *et al.*, 2010).

While both the E-test (Plates containing colestimethate) and CB correlated well with time-kill analysis in demonstrating synergy for two-drug combinations, there was better correlation of the

E-test with time-kill results (Sopirala *et al.*, 2010). All the antibiotic combinations that showed synergy in the time-kill analysis in all tested isolates also showed synergy in E-test as documented in the previous study (Sopirala *et al.*, 2010). On the other hand, most of the isolates showed additivity (no synergy) in the CB test. E-test was also easier to perform, less time-consuming, less expensive and more accurate (Sopirala *et al.*, 2010).

1.2.9.2.4 Synergy Testing using the two strips E-test method (fixed ratio method)

This method involves the use of Mueller-Hinton (MH) agar inoculated with bacterial suspensions at an optical density of 0.5 McFarland units. Minimum inhibitory concentration (MICs) of two drugs are determined and also determined on the combination setup on the MH agar plate by placing strips/scales in each gradient's position. Briefly, this involves the addition of E-test strips containing antibiotics to the bacterial lawn in a sequential manner. Plates are incubated with the first E-test strip for 1 h at (20°to 25°C) room temperature, and then removed. This is followed by the addition of the second E-test strip immediately over the imprint of the first E-test strip. After incubation for 18 h at 35°C, MICs are determined by placing strips/scales in each gradient's position.

1.2.10 Clinical impact of Acinetobacter spp.

Community and nosocomial acquired infections caused by *Acinetobacter* spp. have become a serious public health concern in many countries, including South Africa. *Acinetobacter* spp. were reported as the most common organisms isolated from bronchoalveolar lavage specimens in ventilator-associated pneumonia (VAP)-defined patients in a South African pediatric ICU, followed by *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Morrow *et al*., 2009).

The emergence of MDR *Acinetobacter* spp. in South African neonatal and pediatric units has been associated with greater than 50% mortality and significant morbidity (Jeena *et al.*, 2001). These results are similar to a study in Brazil, a developing country with challenges similar to those of South Africa (Abramczyk *et al.*, 2003). In addition, a marked increase in the number of ICU infections due to MDR *Acinetobacter* spp. has been reported in South Africa (Ahmed *et al*., 2012). The first study on the characteristics and outcome of pediatrics intensive care unit (PICU) patients with positive *Acinetobacter* spp. blood culture (distinguishing between colonisation and pathogen) had been published in 2015 (Reddy, 2015).

1.2.11 Antibiotic stewardship programme (ASWPs)

According to a consensus statement from the Infectious Diseases Society of America (IDSA), the Society for Healthcare Epidemiology of America (SHEA), and the Pediatric Infectious Diseases Society (PIDS), antibiotic stewardship is defined as "coordinated interventions designed to improve and measure the appropriate use of [antibiotic] agents by promoting the selection of the optimal [antibiotic] drug regimen including dosing, duration of therapy, and route of administration" (Fishman *et al*.,2012). The benefits of antibiotic stewardship include improved patient outcomes, reduced adverse events improvement in rates of antibiotic susceptibilities to targeted antibiotics, and optimisation of resource utilisation across the continuum of care. IDSA and SHEA strongly suggest that antibiotic stewardship programs (ASPs) are led by infectious disease physicians with additional stewardship training (Barlam *et al.,* 2016).

The 2016 guidelines from the Infectious Diseases Society of America/Society for Healthcare Epidemiology of America (IDSA/SHEA) recommend preauthorisation and prospective review of antibiotics as a measure to improve the efficacy of ASWP (Barlam *et al.,* 2016).

Preauthorisation requires providers to obtain approval before prescribing these antibiotics, while prospective audits allow antibiotic stewards and clinicians to assess and optimise treatment after the antibiotic has been prescribed. The strategies may be used alone or in combination, with the aim of reducing antibiotic misuse and preventing the emergence of resistant strains. Hospitals should use one or both of these methods based on their local resources and expertise (Barlam *et al.,* 2016). Rapid synergy testing supports the improvement of aims of ASWPs and has been published in 2015 (Van-Belkum *et al.,* 2015).

1.2.12 Problem statement

MDR, XDR and PDR *Acinetobacter* spp. isolates are on the rise worldwide (Begum *et al.,* 2013; Ogutlu *et al.,* 2014) and pose a great challenge for physicians and clinical microbiologists regarding Antibiotic Stewardship Programs (ASWPs), both globally and locally. Successful ASWPs are crucial in making sure that available treatment options are preserved (Manchanda *et al.,* 2010; Barlam *et al.,* 2016). The emergence and spread of drug-resistant (MDR, XDR, PDR) *Acinetobacter* spp. are alarming, since the organism is responsible for many healthcare-associated infections (Manchanda *et al*., 2010; Gonzalez-Villoria and Valverde-Garduno, 2016; Samawi *et al*., 2016). Moreover, the resistance patterns of *Acinetobacter* spp. present a high-risk global infection control challenge (Coelho *et al*., 2006; Cheon *et al*., 2016). Despite the prevalence of infection, there is limited scientific data and a lack of a standardised management guideline to assist ASWPs and help the clinician select optimal therapy in local hospital settings.

ASWPs in hospitals aim to optimise antimicrobial prescribing so that individual patient care is improved, antimicrobial resistance is decreased and hospital costs are reduced (MacDougall and Polk, 2005). Guidelines from the Infectious Diseases Society of America/Society for Healthcare Epidemiology of America (IDSA/SHEA) (2016) recommends preauthorisation and prospective review of antibiotics as a measure to improve the efficacy of ASWP (Barlam *et al*., 2016). Preauthorisation means that providers need approval before prescribing antibiotics, while prospective audit allows antibiotic stewards to communicate with clinicians after the antibiotic has been used in order to optimise treatment. These strategies may be used alone or in combination to prevent antibiotic misuse and resistance. Hospitals should use one or both of these methods based on their local resources and expertise (Barlam *et al*., 2016). However, it is difficult to differentiate between isolates that cause sepsis *versus* colonisation when establishing the decision to treat, which is a major part of ASWPs (Swe Swe-Han and Pillay, 2015).

Although *Acinetobacter* was previously, and even now in some units, ignored when isolated from clinical samples, there are now over 1000 references to 'infections and resistant *Acinetobacter*' in the international literature. In addition, the type of infections caused by *Acinetobacter* has changed over the past 30 years (Joly-Guillou, 2005; Mathai *et al*., 2012; Begum *et al*., 2013; Ogutlu *et al.*, 2014). The literature has explored *Acinetobacter* spp. as a successful pathogen; its biological aspects; epidemiology and pathogenicity factors; global spread and surveillance and multilateral system and related outbreak investigation (Naas *et al*., 2005; Higgins *et al.*, 2010; Antunes *et al*., 2014; Kim *et al*., 2015). The literature shows that standard treatment and clinical decisions are difficult in *Acinetobacter* spp. infection due to its ability to develop resistance with unusual resistance patterns, and lack of standard guidelines to determine significant infection (Ogutlu *et al*., 2014; Cheon *et al*., 2016). In order to plan health care policies, a standardised management approach and intervention measures need to be implemented to reduce *Acinetobacter* spp. sepsis in resource-poor settings. It is necessary to have evidence of the prevalence, proportion of sepsis and colonisation groups; drug resistance patterns; effectiveness of synergy of colistin with other drug combinations; and characterisation of *Acinetobacter* spp. isolates in local hospitals to initiate the health care policies (Ntusi *et al*., 2012).

In the KZN province of South Africa, there is a paucity of such data and no guidelines to differentiate the *Acinetobacter* spp. isolates that cause infection *versus* colonisation. Data on the prevalence of resistance patterns of *Acinetobacter* spp. and their significance with regards to sepsis and colonisation is limited in South Africa.

The increasing prevalence of carbapenem-resistant *Acinetobacter* spp. isolates that are also resistant to all commonly available antibiotics is of particular concern. Colistin is the only drug of choice for the treatment of MDR *Acinetobacter* spp. (Moffatt *et al*., 2010). However, its resultant renal toxicity and neurotoxicity makes it an unattractive alternative (Arıdoğan, 2012). In addition, *Acinetobacter* isolates that are resistant to colistin have also been observed (Moffatt *et al*., 2010). Therefore, therapy using combinations of antimicrobials (including carbapenems, colistin, rifampicin, and ampicillin-sulbactam) has been suggested as the best approach. The clinical utility of these combinations against PDR *Acinetobacter* spp. remains to be determined (Doughari *et al.*, 2011). The limited available options are major concern and further work on the use and efficacy of combination therapies, as well as on clinical outcomes, is warranted (Doughari *et al*., 2011). The synergistic effects of colistin in combination with various other agents has been reported, but there is a lack of a standard approach in the treatment and management of infection (Almasaudi, 2016; Gonzalez-Villoria and Valverde-Garduno, 2016).

Drug-resistant *Acinetobacter* spp. presents a serious global infection control challenge (Fishbain and Peleg, 2010; Ghafur *et al.*, 2014; Phee *et al.*, 2015; Almasaudi, 2016). Previous studies have examined its spread and identified genes encoding drug resistance. However, characterisation of *Acinetobacter* spp. clinical isolates in the local academic hospital in Durban (South Africa) has not been previously reported.

With an exponential rise in infections over the past decade, clinicians and microbiologists face the task of choosing optimal antimicrobial agents for treatment regarding ASWPs. Antibiotic resistance is a major challenge to the healthcare system and it is difficult to provide optimal treatment options due to changes in resistance patterns, increased costs and the availability of new agents (Diekema *et al.*, 2004; Fishbain and Peleg, 2010; Ghafur *et al.*, 2014; Phee *et al*., 2015). Historically, it has been shown that if an optimal antibiotics usage guideline is not developed, this can lead to overuse of antimicrobial agents and the loss of their efficacy (Ventola, 2015).
In order to address the above, this study investigated the clinical significance of *Acinetobacter* spp. infections; criteria for colonisation *versus* significant sepsis based on clinical and microbiological data, determined the most effective combinations of drugs and characterised *Acinetobacter* spp. isolates phenotypically and genotypically. A standard approach for the treatment and management of *Acinetobacter* spp. infections for community-centred academic complex hospitals was developed for implementation in the local setting. This was an analytical, observational, experimental study performed at the Microbiology laboratory, NHLS academic complex hospital IALCH, in Durban (South Africa). This research provides a valuable

standardised approach to assist the clinician to select optimal therapy in the form of a standardised algorithm for the management of patients with significant *Acinetobacter* spp. infections, including a synergy test protocol at this unit.

1.2.13 Research questions, hypotheses, aims and objectives

Acinetobacter spp. isolates are recognised as common hospital- and community-acquired pathogens and colonisers (Kim *et al;* 2014; Wong *et al*., 2017). A significant number of *Acinetobacter* spp. nosocomial isolates, including MDR isolates, have been identified in the academic complex hospitals of KZN, South Africa. The synergistic effect of colistin combined with various agents has been reported (Falagas *et al.,* 2006; Wareham and Bean. 2006; Pankey and Ashcraft, 2009; Vidaillac *et al*., 2012; Petrosillo *et al*., 2014; Temocin *et al*., 2015; Zafar *et al.*, 2015; Bae *et al.*, 2016), but there is a lack of a standard approach in the treatment and management of infection.

This thesis describes a standardised approach for the management of patients with significant *Acinetobacter* spp. infections in KZN. The following research questions were addressed:

- What is the prevalence and resistance pattern of *Acinetobacter* spp., both as colonisers and significant pathogen causing significant sepsis, in KZN academic complex hospitals?
- What are the criteria to differentiate between significance for sepsis *versus* colonisation of *Acinetobacter* spp.?
- Which is the most effective drug combinations against these organisms?
- What is the phenotypic and genotypic correlation of resistanct *Acinetobacter* species?
- For the purpose of epidemiology and infection control, what are the genes related to carbapenem, amikacin and colistin resistant *Acinetobacter* spp. in the local academic complex hospital.
- Are there carbapenemase encoded genes ($bla_{\text{OXA-23}}$) spread in the ICUs in the local hospital?
- What are the clinical outcomes of different treatment modalities currently used in the treatment of *Acinetobacter* spp. at academic complex hospitals in KZN?

This thesis presents a series of studies aimed at determining epidemiological and clinical characteristics and outcomes, and includes both genetic and phenotypic characterisation of the organism. Based on the outcomes, an algorithm for a standardised approach to the treatment and management of significant *Acinetobacter* spp. infection was developed and will be implemented at academic complex hospitals, Durban, KZN.

In line with the research questions proposed, the hypotheses, aims and objectives of this study are addressed below.

1.2.14 Hypotheses of the study

1. *Acinetobacter* spp. infections are significantly associated with severe sepsis in patients.
2. Criteria for sepsis and colonisation differ, based on the clinical and microbiological analysis of each patient.
3. The presence of mutations in drug targets or acquired mechanism will correlate with high-level drug resistance.
4. Specific effective combination drug combinations can be used appropriately as standard guidelines.

1.2.15 Aims of the study

1. To determine the significance of *Acinetobacter* spp. infection in patients hospitalised at the IALCH academic complex hospitals, KZN.
2. To characterise *Acinetobacter* spp. isolated from patients.
3. To determine the most effective combinations of drugs against *Acinetobacter* spp.
4. To develop a standardised approach for the treatment and management of significant *Acinetobacter* spp. infection in KZN, in respect of antibiotic stewardship programme.

1.2.15.1 Objectives for each aim

Aim 1

- To determine the prevalence of *Acinetobacter* spp. associated with and without sepsis from 2008 to 2012 retrospectively.
- To determine the prevalence of *Acinetobacter* spp. associated with and without sepsis in 2013 and 2014 prospectively.
- To determine the significance of infections including clinical outcomes.

Aim 2

- To determine drug susceptibility profiles of *Acinetobacter* spp. in patients from 2008 to 2013.
- To determine MICs of colistin, amikacin and meropenem against *Acinetobacter* spp. isolated from patients.
- To perform mutation analysis on genes encoding drug targets in isolates resistant to colistin, aminoglycosides and meropenem, and to determine the spread of the isolates in ICUs and related units (suspected outbreak).

Aim 3

- To determine the drug combination that is most effective against *Acinetobacter* species.

Aim 4

- To determine, prospectively, the clinical outcome of *Acinetobacter*-infected patients after treatment with appropriate antibiotics, such as colistin, meropenem, ciprofloxacin, tazocin (piperacillin-tazobactam), amikacin and combination therapy (colistin and each of the drugs).
- To develop a standardised treatment algorithm for the management of patients with significant *Acinetobacter* species infections.

1.3 General methodology

1.3.1 Study setting

Durban is the largest city in the province of KZN, South Africa. It has a population of almost 10.3 million (https://census2011.adrianfrith.com/place/5), making the combined municipality, one of the biggest African cities on the Indian Ocean coast. The city is served by a regional, tertiary hospital (King Edward VIII Hospital) and a central referral hospital (Inkosi Albert Luthuli Central Hospital). King Edward VIII Hospital is the second largest hospital in the Southern hemisphere, providing regional and tertiary services to the whole of KZN and Eastern Cape provinces. King Edward VIII is a 922 bed hospital. Inkosi Albert Luthuli Central Hospital (IALCH) commissions the outstanding beds and has a total of 892 beds. The hospital accommodates referrals from all KZN regional hospitals, as well as from the Eastern Cape. Both hospitals are under academic complex teaching hospitals for the University of KwaZulu-Natal's Nelson R. Mandela School of Medicine. They are attached to a Nursing College and house the following specialties: orthopedics, ICUs / critical care, pediatrics, cardiology, oncology, hematology, plastic surgery, vascular surgery, burns, and other specialist facilities.

1.3.2 Study design

This thesis used a combination of methods, including analytical, observational (retrospective and prospective) and laboratory experimental studies. The overall study approach includes molecular epidemiology, clinical and experimental components.

The specific study design involved determining the prevalence of *Acinetobacter* spp. infections and their drug susceptibility profiles in KZN academic complex hospitals, by a retrospective and prospective clinical and microbiological data information review by using the criteria of colonisation and significant pathogens for the period of 2008 to 2014. The stored of representative isolates obtained as part of routine standard of care were characterised by MIC determinations and mutation analysis. The MICs of selected drugs (i.e., colistin, amikacin, and carbapenem) were conducted using the Vitek 2 automatic identification and sensitivity method (CLSI 2012) and

confirmed by the E-test. PCR and amplicon sequencing was performed to analyse mutations in the genes encoding drug targets in resistant isolates associated with severe sepsis.

E-test was used to evaluate the effectiveness of the different combination of drugs against the significant sepsis-related *Acinetobacter* species. Clinical outcomes of infected patients after treatment with appropriate drugs (colistin, meropenem, ciprofloxacin, tazocin [piperacillin-tazobactam], amikacin or a combination of colistin with each drug) were documented prospectively and statistically analysed. An algorithm of a standardised approach for the treatment and management of significant *Acinetobacter* spp.-infection was developed.

1.3.3 Study population

All in-patients identified with *Acinetobacter* spp. isolates were included in the study from the academic complex hospitals, following study sites:

- Patients from the ICU at King Edward VIII Hospital in 2008;
- Patients from high-risk areas (ICUs, renal unit, oncology unit, etc.) at IALCH from 2008 to 2014.

1.3.4 Sampling strategy

Significant *Acinetobacter* species sepsis was determined by positive blood culture and isolation from multiple sites repeatedly or multiple sites singly, with clinical significance.

Diagnosis of *Acinetobacter* pneumonia with sepsis was based on endotracheal aspirate results, and clinical manifestations and identification of new infiltrates on chest x-rays. Bacteremia was determined by at least one positive blood culture.

The following data were collected retrospectively from 2008 to 2012 and prospectively for 2013 and 2014: total number of *Acinetobacter* spp. isolated, total number of patients with *Acinetobacter* spp., type of specimens, antibiogram, antibiotic usage in the unit and outcome.

Laboratory data was collected from the completed laboratory worksheets. The selected marker antibiotics against *Acinetobacter* spp. included: aminoglycosides (amikacin), β-lactams (piperacillin-tazobactam), fluoroquinolones (ciprofloxacin), carbapenems (meropenem) and colistin. Colistin drug susceptibility testing against selected *Acinetobacter* spp. was initiated in 2011.

1.3.5 Statistical planning (variables / confounders)

Simple statistical analysis was performed on information captured from the laboratory database and correlated with the clinical data. The prevalence of *Acinetobacter* spp. associated with and

without sepsis was calculated for the period of 2008 to 2014. Only one representative isolate from each specimen per patient, both with or without sepsis, was included in the analysis.

The prevalence rate of significant *Acinetobacter* spp.-infection was calculated using the formula:

$$z = \frac{x}{y} X\ 100$$

x = Total number of patients with significant *Acinetobacter* spp. infection

y = Total number of patients with *Acinetobacter* spp. isolated

z = Percentage

Clinical outcome of treatment with various drugs or drug combinations was recorded. Recorded data was analysed by the investigator and statistician. The data was collected, captured and thereafter analysed using the Statistical Package for Social Sciences (SPSS version 19). The results were summarised using descriptive statistics, such as frequencies and percentages. Pearson chi-square-test or Fisher's exact test was used for the association between underlying conditions and response to antibiotics. Logistic regression tested for factors associated with clinical survival status.

1.3.6 Sample size for laboratory synergy test and molecular characterisation

One hundred and seven isolates were selected based on their antibiograms and the relevant clinical criteria decided upon during clinical wards rounds. Patients' data were collected and recorded prospectively. Sixty isolates of *Acinetobacter* spp. from 107 patients were selected based on the clinical and microbiological significance criteria (sepsis patients who need combination therapy; significant specimens; pure growth or significant growth; ICU patients and chronically ill patients). Sixty isolates were stored at -70°C, prior to molecular characterisation and synergy tests assessment. Synergy test and molecular characterisation were determined based on the antibiogram (antibiotic resistance patterns) and clinical data (clinical units, underlying risk and type of specimens).

Sixty isolates were used for synergy testing, using seven combinations of antimicrobial agents (The isolates were selected based on the patients with significant sepsis and local infections).

Twenty-four (excluding 3 controls) isolates were selected for $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-58}}$ (The isolates were cultured from the patients admitted to ICUs and non-ICUs within the weeks in which significantly increased number of isolates with same antibiogram without MICs were observed).

Twenty-four isolates (excluding 3 controls) for pulsed field gel electrophoresis (PFGE) were selected based on clinical criteria, same antibiogram, same ICUs and specimen collection dates within one week.);

Twenty-four isolates including six colistin resistant isolates for *Ipx*A;

Ten isolates including six amikacin resistant isolates for *aph*A6 and *aac*A4;

Mutation analysis by genomic sequencing was performed on genes associated with resistance as below;

Eighteen carbapenems resistant MDR *Acinetobacter* spp. (OXA-23 band detected isolates) were sequenced for $bla_{\text{OXA-23}}$. Six amikacin resistant isolates were sequenced for *aph*A6.

Six colistin resistance isolates were sequenced for *Ipx*A.

1.3.7 Inclusion / exclusion criteria:

Inclusion criteria*:*

All adult patients (HIV infected and uninfected) with and without sepsis identified with *Acinetobacter* spp. infection and:

- admitted to ICU at KEH in 2008
- admitted to high risk wards (ICUs, renal unit, oncology unit etc.) at IALCH from 2009 to 2014.

Exclusion criteria

- Patients not infected with *Acinetobacter* spp.
- Patients from other non- high risk wards (out patients' clinic, occupational health clinics).

1.3. 8 Ethics consideration of the project

Ethics approval for the study was obtained from Biomedical Research Ethics Committee (BREC) at the University of KwaZulu-Natal. Reference No: BE 283/12 in 18 July 2013, and recertified annually.

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CHAPTER 2: ORIGINAL ARTICLE

Multidrug Resistant *Acinetobacter* species: A significant cause of sepsis in an intensive care unit in a regional hospital, Durban

Published in *International Journal of Nursing Didactics*, 5: 8 August (2015)

ORIGINAL ARTICLE

Title: **Multidrug Resistant *Acinetobacter* species: A significant cause of sepsis in an intensive care unit in a regional hospital, Durban**

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Abstract

Introduction: *Acinetobacter* species *(Acinetobacter* spp.) are common hospital environmental bacteria that have gained importance during the past few decades as important nosocomial pathogens in critically ill patients. This problem has been compounded by the worldwide increase in carbapenem-resistant *Acinetobacter* infections. In South Africa also, multidrug-resistant, including carbapenem resistant *Acinetobacter* spp. causing significant sepsis has recently increased. *Acinetobacter baumannii* remains an important and difficult-to-treat pathogen whose resistance patterns result in significant challenges for the clinician. The study was conducted to determine the prevalence of multidrug-resistant (MDR) *Acinetobacter* spp., and to differentiate between significant infection and colonisation by correlation with clinical data.

Method: All patients identified with *Acinetobacter* spp. isolates after 48 hrs in the intensive care unit (ICU) were included in the study over a year period. Data was recorded prospectively including any underlying chronic disease, type of specimens, antibiogram, antibiotic usage in the unit and outcome during daily ward rounds. Analyses were done retrospectively.

Results: During the study period, there were 187 isolates from different specimens of 86 patients. Significant sepsis was identified in 30/86 (35%), colonisation in 51/86 (59%) and bacteraemia in 5/86 (6%) patients with *Acinetobacter* spp., respectively. Lack of appropriate treatment resulted in the death of 18/86 (21%) patients. *Acinetobacter* spp. was isolated mainly from endotracheal aspirates 67/187 (36%), and the others were from the various types of specimens. Isolates were multidrug-resistant including carbapenem.

Conclusion: MDR- *Acinetobacter* spp. was identified as a significant cause of sepsis and a high mortality rate *(P* <0.001) among the patients in surgical ICU. Our findings highlight the impact of antibiotic stewardship in the treatment of patients in whom *Acinetobacter* spp. is isolated and the urgent need for the development of standardised guidelines for management of patients with *Acinetobacter* spp. sepsis.

Key words*:* Multidrug-resistant; *Acinetobacter* species; colonisation; sepsis; intensive care unit

Introduction

The prevalence of *Acinetobacter* species (*Acinetobacter* spp.) infection in hospitals is increasing worldwide [1] with a concomitant significant increase in mortality associated with bacteraemia (19 to 54%) compared to other bacterial infections [2, 3]. It is now well recognised that in addition to colonisation, *Acinetobacter* spp. play a significant role in community as well as hospital acquired infections [4]. Although it is difficult to differentiate between colonisation and sepsis, community acquired *Acinetobacter* pathogens are relatively sensitive to antibiotics and the resistant isolates are almost exclusively present in hospitals and high risk areas [5].

Acinetobacter spp. had been reported as the cause of serious infectious diseases such as ventilator associated pneumonia, bacteraemia, urinary tract infections, burn wound infections, endocarditis, secondary meningitis, and septicaemia, involving mostly patients with impaired host defences, especially in intensive care units (ICUs) [6, 7]. *Acinetobacter* spp. have emerged as particularly important organisms causing late-onset ventilator associated pneumonia which may have been related to the increasingly invasive diagnostic and therapeutic procedures used in hospital ICUs in recent years [8].

Acinetobacter spp. have acquired resistance to almost all currently available antimicrobial agents, including the aminoglycosides, quinolones, and broad-spectrum β-lactams. The spectrum of antibiotic resistance of these organisms, together with their survival capabilities, makes them a threat in hospital environments, as documented by recurring outbreaks both in highly developed countries and elsewhere [9]. Most strains are resistant to cephalosporins, while resistance to carbapenems is being reported increasingly [9].

There has been a worldwide increase in infections caused by multidrug-resistant *Acinetobacter baumanni* (MDRAB) [1]. In South Africa also, an increase in carbapenem resistant *Acinetobacter* spp. has been recently reported [10, 11].

The challenges of treating multidrug-resistant bacteria continue to be at the forefront of the clinician's practice in caring for hospitalised patients. *Acinetobacter baumannii* has proven to be an increasingly important and challenging species in health care–associated infections. The drug-resistant nature of the pathogen, its unusual and unpredictable susceptibility patterns and poor clinical understanding of significant sepsis, make empirical and therapeutic decisions even more difficult [12].

During our routine standard of care, we have observed that a significant proportion of nosocomial isolates include MDR *Acinetobacter* spp. in the ICU at a regional hospital in Durban. The clinical significance of this has yet to be elucidated. In this retrospective study, we determined the proportion of MDR *Acinetobacter* spp. in an adult surgical ICU, differentiated significant infection from colonisation and clinical outcomes of treatment. Outcomes of both significant infection and colonisation were recorded. Our findings highlight the impact of antibiotic stewardship in the treatment of patients in whom *Acinetobacter* spp. are isolated in order to develop guidelines for treatment and management of *Acinetobacter* spp. infection.

Methods

Study setting:

The regional academic hospital accommodates 950 beds and includes multi-discipline speciality wards. There is one ICU (13 bed ward) for the management of mainly surgical adult patients.

Study design and patient population:

In this analytical, descriptive cross-sectional study, all patients identified with *Acinetobacter* spp. after 48 hrs in ICU was included over a year study period.

Ethical consideration:

The study was approved by the Biomedical Research Ethics Committee, University of KwaZulu-Natal (Ref: BE 283/12).

Data collection:

The data collection included the total number of *Acinetobacter* spp. isolated, total number of patients with *Acinetobacter* spp., specimen type, antibiogram, antibiotic usage in the unit and clinical outcomes of the patients from whom *Acinetobacter* spp. was isolated. The data was prospectively recorded during routine daily ward rounds during the one year study period. The clinical and laboratory data were analysed retrospectively.

Case definitions:

Diagnosis of *Acinetobacter* pneumonia was based on the results of endotracheal aspirates together with clinical manifestations and identification of new infiltrates on CXR.

Acinetobacter bacteremia was diagnosed on at least one positive blood culture. Significant *Acinetobacter* spp. sepsis was based on positive blood culture and repeated isolation from multiple sites.

Colonisation was defined as *Acinetobacter* spp. isolated from a single specimen of a clinically stable patient on whom a sepsis screen was performed.

Data analysis:

Frequency distributions were calculated for the number of *Acinetobacter* spp. isolated from the specimen types, antibiograms and stratification of patients with clinical symptoms or colonisation. Chi-squared test was used to calculate statistical significance, which was set at $P \leq 0.05$.

Results

During the study period, isolates of *Acinetobacter* spp. were cultured from 187 different specimens of 86 patients. The most predominant specimen type was endotracheal aspirates 67/187(36%) followed by blood 24/187 (13%), CVP tips, peritoneal fluid, arterial line tip, pus and catheter urine. Abdominal drains, tissue, pleural fluid and others were less commonly sampled 3/187 (2% and less) (Figure 1).

Colonisation was observed in the majority 51/86 (59%) of the 86 patients. Significant clinical sepsis was observed in 30/86 (35%) of patients, whilst 5/86 (6%) were diagnosed with bacteremia (Figure 2).

The majority of isolates were multidrug-resistant, including resistance to carbapenems (Figure 3). Amikacin sensitive *Acinetobacter* spp. was isolated from 39/86 (45%) of the 86 patients. The other patients were infected with isolates sensitive to meropenem 10/86 [12%], ciprofloxacin 9/86 [10%], TZP (piperacillin and tazobactam) 4/86 [5%], and ceftazidime 4/86 [5%].

The majority of patients; 68/86 (79%) recovered and were discharged in a stable condition, whilst 18/86 (21%) died. *Acinetobacter* was significantly associated with sepsis in 30 patients. Of these, 18/30 (60%) died and 12/30 (40%) recovered ($P < 0.001$) (Table 1).

Discussion

Acinetobacter spp. are aerobic Gram-negative coccobacilli that are commonly found in hospital environments and easily colonise skin and mucous membranes. In the past, *Acinetobacter* spp. were considered to be of little clinical significance, but the appearance of drug resistant *Acinetobacter* infections have increased worldwide frequently [13].

This study showed that *Acinetobacter* spp. were more commonly colonisers, especially from endotracheal aspirates of patients in ICU. Although it is difficult to differentiate between colonisation and sepsis with *Acinetobacter* spp., the former increases the risk of the latter. Therefore, appropriate infection control and good oral hygiene practices are of paramount importance during the collection of ETA and management of patients.

Although the proportion of colonisation was higher, clinical sepsis was identified in a large proportion of patients (35%) with multiple sites being culture positive. The majority of isolates in this study was MDR, including resistance to carbapenem. *Acinetobacter* spp. were regarded as colonisers in general and therefore, not directly targeted for therapy in surgical ICUs and other clinical units. The patients in ICU during that study period were treated for hospital acquired infections with tazocin (piperacillin-tazobactam) and followed by carbapenem empirically according to the local antimicrobial therapy protocols. Despite an exponential rise in *Acinetobacter baumannii* infections over the past decade, the treatment regimen remains controversial and many questions remain unanswered on the issue of appropriate therapy [14].

Difference between case patients and control subjects in most previous studies did not show statistical significance; however, higher mortality was observed consistently among the case patients [12]. Although, the data in the local hospital did not reach statistical significance in previously, it is evident during daily ward rounds for the management of the patients that *Acinetobacter baumannii* is also important as other common pathogens during study period**.** To implement the optimal usage of antimicrobial agents using the local antibiogram is the challenge for clinicians. The prescription of colistin combination therapy (both empirical and directed) for the *Acinetobacter* spp. has not yet been used often but hopefully, future studies would demonstrate the synergy effect of combination therapy.

The in-hospital mortality attributable to *Acinetobacter* spp. sepsis reported in other studies ranged from 8% to 23%, while in the intensive care unit, it was found to be 10% to 43% [12]. Until now, clinical outcomes of patients with *Acinetobacter* spp. infections were not documented in our local

setting. Our study documented for the first time significant mortality rates (60%) associated with patients diagnosed with sepsis compared to those who were colonised (P<0.001) (Table 1).

The twelve (40%) patients who recovered from sepsis were treated with tazocin, which is used to treat the common known pathogens empirically. Tazocin is chosen for both empirical and direct therapy of common pathogens as second line therapy in the current treatment guideline in the study hospital.

Community and nosocomial infections caused by *Acinetobacter* spp. have become a serious public health concern in many countries [15, 16]. In this study at a regional hospital in KwaZulu-Natal (KZN) province in South Africa, we have shown that MDR *Acinetobacter* spp. contributes significantly to nosocomial isolates causing sepsis. This problem is compounded by the lack of information on clinical significance and a recommended policy guideline for *Acinetobacter* spp. infection.

In conclusion, MDR *Acinetobacter* spp. is a significant cause of sepsis in surgical ICUs. This highlights the impact of antibiotic stewardship in the treatment of patients in whom *Acinetobacter* spp. is isolated and the urgent need for the development of standardised guidelines for the management of patients with *Acinetobacter* spp. sepsis.

There is a lack of surveillance studies on antibiotic-resistance patterns and their associated genes of *Acinetobacter* spp. in local clinical settings. Further research should include the determination of genetic relatedness of circulating *Acinetobacter* spp. to study transmission dynamics. In addition, the comparisons of phenotypic and molecular antibiotic resistance patterns should be studied. This would serve to identify the possible sources of these strains and **to** introduce intervention strategies to interrupt the transmission chains.

ACKNOWLEDGEMENT

M. Pillay (supervisor): For overall editing of English in manuscript.

Clinicians (consultants and registrars in ICU, KEH): For clinical data and clinical ward rounds.

HOD, Laboratory Manager, Laboratory technologists: For laboratory identification of organisms.

Medical Education Partnership Initiative (MEPI), UKZN organisation: For the funding received.

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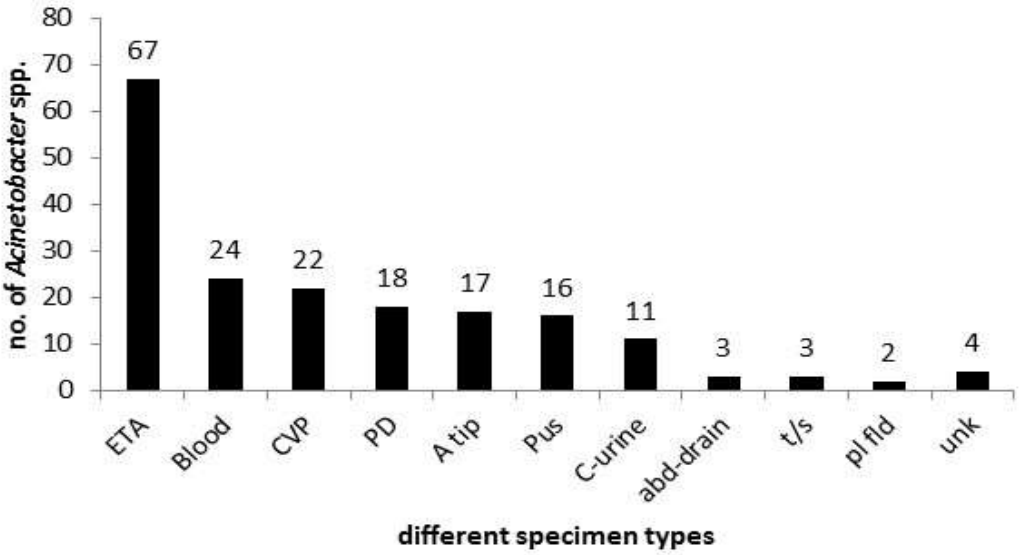

Figure 1. The number of *Acinetobacter* species isolated from different specimen types in 86 patients from the surgical ICU between January to December 2008. The commonest specimen was ETA.

Key: ETA (Endotracheal aspirate); CVP (Central venous pressure tip); PD (Peritoneal fluid); A tip (arterial line tip); C-urine (catheterised urine); abd drain (specimen from abdominal drain); t/s (Tissue); pl fluid (pleural fluid); unk (unknown specimen)

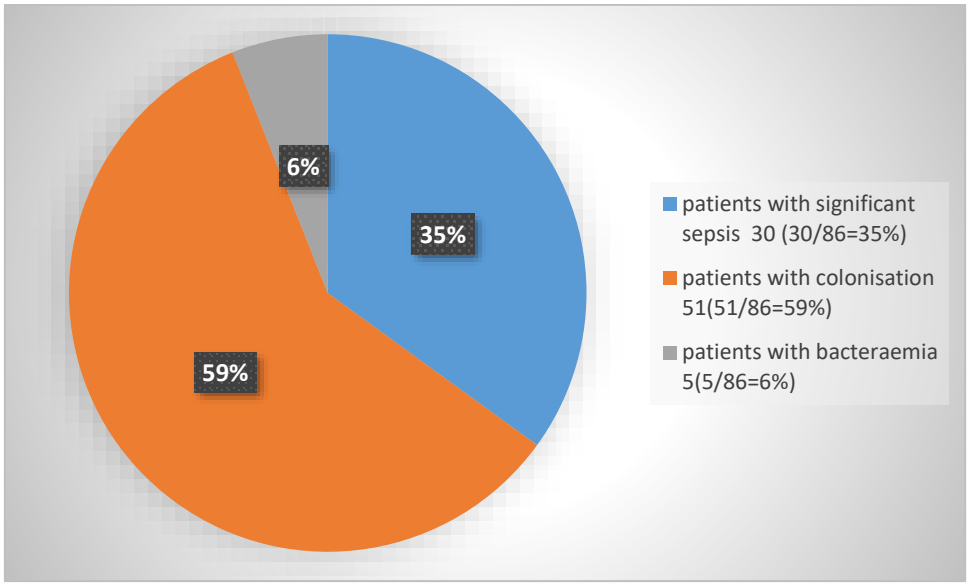

Figure 2. Stratification of patients with significant clinical symptoms or colonisation. Patients with bacteremia comprised 6% and significant clinical sepsis, 35%. The majority of patients (59%) were colonised.

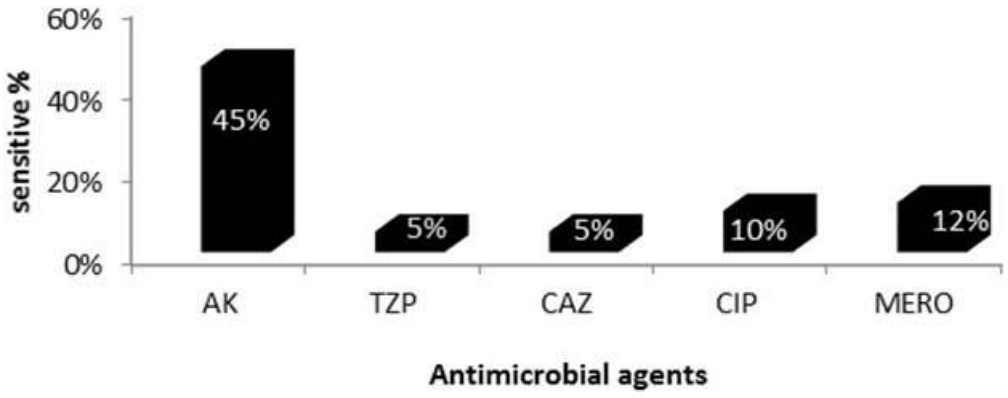

Figure 3. Antibiogram of *Acinetobacter* species isolated from sepsis patients (total= 86) surgical-ICU in 2008

AK (Amikacin); TZP (Tazocin: piperacillin + tazobactam); CAZ (Ceftazidime); CIP (Ciprofloxacin); MERO (Meroenem)

Table 1: Clinical outcomes of patients following treatment in surgical ICU during study period

	Number of patients			
Outcome	**Sepsis**	**colonised**	**Total**	***P***
Recovered	**12**	**56**	**68**	
Deceased	**18**	**0**	**18**	**<0.001**
Total	**30**	**56**	**86**	

CHAPTER 3: ORIGINAL ARTICLE

Analysis of clinical and microbiological data on *Acinetobacter* species assist the preauthorisation of antibiotics at patient level for an effective antibiotic stewardship programme

Journal of Infection and Public Health; **Reference: JIPH-D-16-00413**
http://dx.doi.org/10.1016/j.jiph.2017.01.014 (published)

ORIGINAL ARTICLE

Title: Analysis of clinical and microbiological data on *Acinetobacter* species assist the preauthorisation of antibiotics at the patient level for an effective antibiotic stewardship programme

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Abstract

Background: Drug-resistant *Acinetobacter* species (*Acinetobacter* spp.) poses serious treatment challenges and is on the rise worldwide. The Infectious Diseases Society of America/Society for Healthcare Epidemiology of America recommends preauthorisation of antibiotics to ensure successful antibiotic stewardship programs (ASWPs). This study investigates and analyses the microbiological and clinical characteristics of *Acinetobacter* spp. with differentiating criteria for sepsis *versus* colonisation, in order to support preauthorisation and assist ASWPs at the patient level.

Methods: A retrospective observational study was performed from 2008 to 2014. The clinical and microbiological characteristics of *Acinetobacter* spp. were correlated to assess pathogenic status and antibiotic resistance patterns. A flow chart was produced to differentiate between sepsis and colonisation amongst patient groups.

Results: *Acinetobacter* spp. were cultured in 2656 cases, with a prevalence of 0.9% to 2.4% during seven years study periods. There was a statistically significant difference between the sepsis and colonisation groups (*P*: 0.02). Sepsis accounted for 37% to 51% of *Acinetobacter* spp. isolates and colonisation for 49% to 63% ($P < 0.01$). Multidrug-resistant (MDR), extensively drug-resistant (XDR) and pandrug-resistant (PDR) *Acinetobacter* spp. were detected in 53% to 60%, 1% to 19% and 1% respectively, of cultures in the sepsis group, and 75%, 8-23% and 1% respectively, in the colonised group. There was a high percentage of polymicrobial infection in the sepsis group and pure growth was not always significant for sepsis.

Conclusion: Cases of MDR and XDR *Acinetobacter* spp. increased over the seven year study, while PDR strains emerged. For a successful ASWP, both clinical and microbiological information should be interpreted when establishing preauthorisation/decision to treat.

Key words: MDR-XDR-PDR *Acinetobacter* species; prevalence; sepsis, colonised group; preauthorisation; antibiotic stewardship programme

Introduction

Antibiotic stewardship programs (ASWPs) in hospitals seek to optimise antimicrobial prescribing in order to improve individual patient care, slow the spread of antimicrobial resistance and reduce hospital costs [1]. The 2016 guidelines from the Infectious Diseases Society of America/Society for Healthcare Epidemiology of America (IDSA/SHEA) recommend preauthorisation and prospective review of antibiotics as a measure to improve the efficacy of ASWPs [2].

Preauthorisation requires providers to obtain approval before prescribing these antibiotics, while prospective audit allows antibiotic stewards and clinicians to assess and optimise treatment after the antibiotic has been prescribed. The strategies may be used alone or in combination, with the aim of reducing antibiotic misuse and preventing the emergence of resistant strains. Hospitals should use one or both of these methods based on their local resources and expertise [2].

Multi-, extensive- and pandrug- resistant (MDR, XDR, PDR) *Acinetobacter* spp. [3-6] isolates are on the rise worldwide [7, 8] and present infection control and treatment challenges for clinicians and clinical microbiologists. ASWP have become a crucial tool in preserving the efficacy of antimicrobial agents [1, 6].

Acinetobacter spp. are ubiquitous in the natural environment [9] and are occasionally found as skin and throat commensal, and in the secretions of healthy people [10]. Although *Acinetobacter* was previously ignored when isolated from clinical samples, there are now over 1000 references to "infections and resistant *Acinetobacter*" in the international scientific literature [11]. In addition, the types of infection caused by *Acinetobacter* has changed over the past 30 years [7, 11-13].

The emergence and spread of *Acinetobacter* spp. resistant to most of the available antimicrobial agents poses problems for future management, since the pathogen plays a role in nosocomial infections [6]. *Acinetobacter* isolates from the community are usually sensitive to antibiotics, while drug- resistant isolates are mainly found in hospitals and high risk areas. Therefore, it is fairly easy to differentiate between community and hospital acquired isolates based on hospitalisation history and antibiogram. However, it is more difficult to differentiate innocuous colonisers from strains that cause sepsis when establishing decision to treat [14].

Evidence regarding *Acinetobacter* spp. prevalence and the proportion of sepsis *versus* colonisation, as well as drug resistance patterns, is essential when planning policies and interventions to reduce ICU-associated *Acinetobacter* spp. sepsis in resource-poor settings [15]. In the KwaZulu-Natal (KZN) province of South Africa, there is a paucity of such data and no guidelines to differentiate the *Acinetobacter* spp. that cause infection *versus* colonisation. This study was conducted to guide the decision to treat based on the analysis of microbiological and clinical aspects of *Acinetobacter* spp. isolates, in order to assist ASWP at patient level.

We investigated the prevalence and proportion of *Acinetobacter* spp. infections that caused sepsis and colonisation using both clinical and microbiological criteria, including demographic data of patients cultured with *Acinetobacter* spp.; accuracy of correlation between clinical diagnosis and microbiological significance; pure and mixed growth of *Acinetobacter* spp. in the sepsis group and colonised group, and drug resistance patterns of *Acinetobacter* species. These aspects were studied retrospectively from 2008 to 2014 in patients at Inkosi Albert Luthuli Central Hospital (IALCH), a specialised referral facility in Durban, KZN.

Methods

An analytic, retrospective observational study was performed on clinical and laboratory patient data from January 2008 to December 2014, at the IALCH academic complex hospital, Microbiology department, National Health Laboratory Service (NHLS) in Durban, South Africa. This research was approved by the Biomedical Research Ethics Committee of University of KwaZulu-Natal (Ethics approval: BE283/12).

The antibiograms were studied to identify MDR, XDR and PDR- *Acinetobacter* species. The clinical and laboratory data from patient groups of sepsis and colonisation were correlated, including the identification of pure and mixed growth and an assessment of pathogenic status. A flow chart was produced to differentiate the two groups according to the abovementioned criteria (Figure 1).

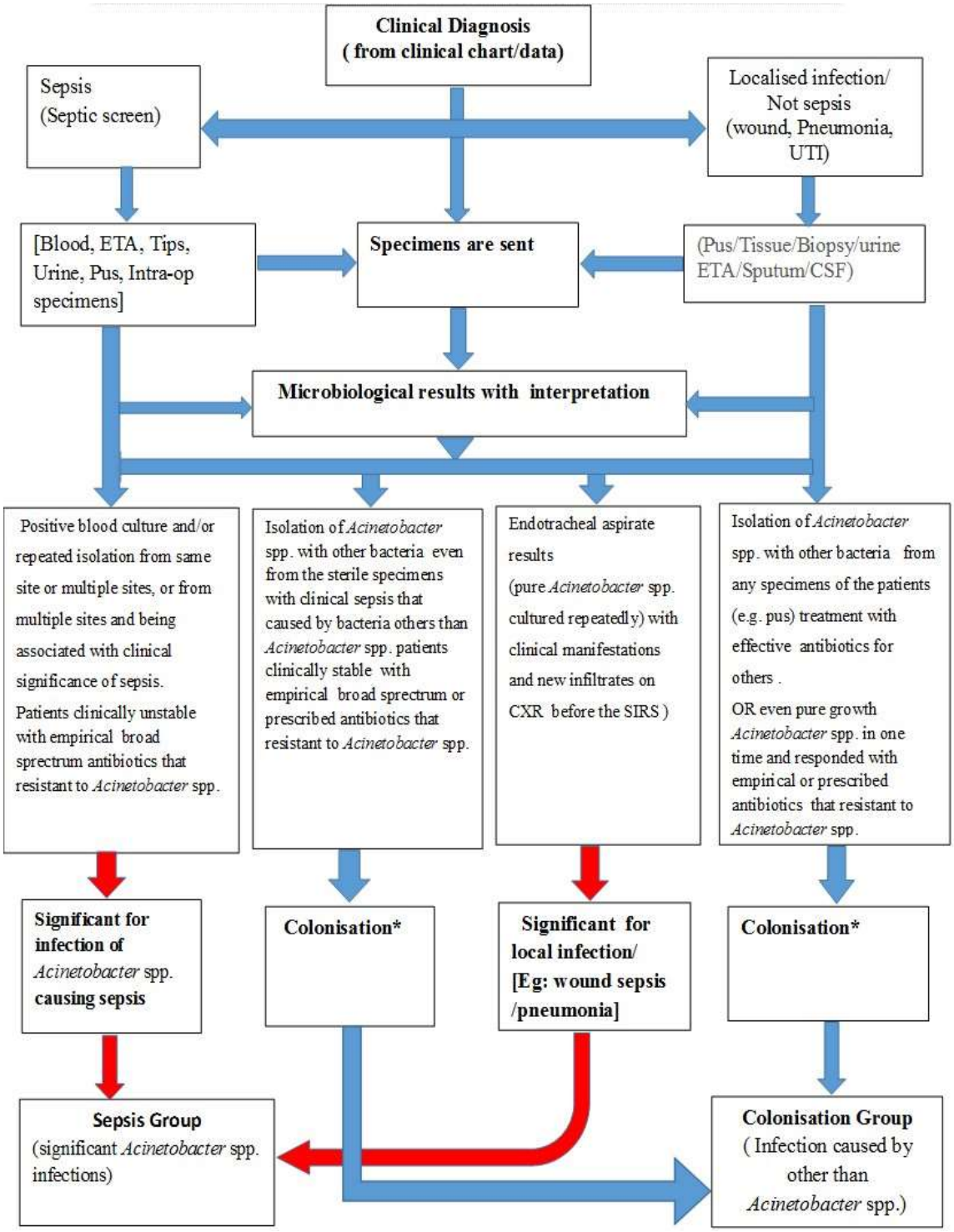

Figure 1. Flow chart of clinical and microbiological criteria of sepsis and colonisation

There was no infectious diseases specialist at IALCH nor a hospital antibiotic policy for *Acinetobacter* spp. in place during the study period. Colistin drug susceptibility testing was initiated in 2011.

Definitions

Clinical diagnosis:

Microbiology specimens were sent to the laboratory marked by the clinician with an indication of either clinical sepsis or localised infection.

Sepsis is based on the indicators of systemic inflammatory response syndrome (SIRS) [16, 17].

Clinically localised infection is based on clinical examination.

Pure growth:

The isolation of only *Acinetobacter* spp. on culture media was regarded as pure growth, isolated with no other bacteria.

Mixed growth:

The isolation of *Acinetobacter* spp. mixed with other bacteria on culture media was regarded as mixed growth.

Microbiological significance:

Acinetobacter spp. isolated from any biological site combined with a compatible clinical picture warranting antibiotic treatment.

Criteria for microbiological significance:

Pure growth *Acinetobacter* spp. cultured from a blood culture bottle, sterile specimen, or repeated specimens from the same or multiple sites in patients not responsive to empirical treatment and broad-spectrum antibiotics;

Or

Mixed growth *Acinetobacter* spp. cultured from repeated specimens from the same site in patients with clinical symptoms.

Microbiological criteria for colonisation:

Acinetobacter spp. isolated from the blood culture from the first specimen sent and with repeat specimens showing growth of other organisms or no growth;

Or

Acinetobacter spp. isolated from endotracheal aspirate (ETA) from the first specimen sent and with repeat specimens showing growth of other organisms or *Acinetobacter* spp. no growth;

Or

Mixed growth *Acinetobacter* spp. cultured in any one site of a septic screen; and patient responded to empirical or current broad spectrum antibiotics that resistant to *Acinetobacter* species.

Clinical significance:

Acinetobacter spp. cultured from specimens from a clinically relevant site.

Sepsis group:

Microbiologically significant *Acinetobacter* spp. infection together with clinically significant *Acinetobacter* spp. infection.

Criteria for inclusion in the sepsis group:

Acinetobacter spp. from sterile specimens in clinically unstable patients;

or

Acinetobacter spp. mixed with other bacteria from non-sterile specimens in a patient with clinically significant sepsis who did not respond to empirical or current broad spectrum antibiotics that resistant to *Acinetobacter* species.

Criteria for inclusion in the colonised group:

Acinetobacter spp. mixed with other bacteria from non-sterile specimens in a clinically stable patient; or

Acinetobacter spp. from sterile specimens in a clinically stable patient.

MDR, XDR, PDR [3-6]

Criteria for defining MDR, XDR and PDR in *Acinetobacter* species [5]:

MDR: Non-susceptible to ≥ 1 agent in ≥ 3 antimicrobial categories.

XDR: Non-susceptible to ≥ 1 agent in all but ≤ 2 categories.

PDR: Non-susceptible to all antimicrobial agents listed.

The isolates were divided into three groups based on their resistance pattern to six different classes of antimicrobials i.e. aminoglycosides (amikacin), β-lactams with inhibitors (piperacillin–tazobactam), cephalosporin (ceftazidime), fluoroquinolones (ciprofloxacin), carbapenem (imipenem, meropenem) and colistin.

Group 1: Resistant to all six groups of antimicrobials; defined as PDR.

Group 2: Resistant to five groups of antimicrobials except colistin; defined as XDR.

Group 3: Resistant to carbapenem or any three groups of antimicrobials; defined as MDR.

Data extraction

The data included the total number of specimens received in each year, number of patients with *Acinetobacter* spp., type of specimen, hospital ward, clinical history, demographic data and antibiogram. To determine significant infections, *Acinetobacter* spp. were cultured from aerobic blood culture and other relevant specimens of sepsis patients; pure growth of isolate from sterile site or non-sterile sites; and repeated isolated *Acinetobacter* spp. from multiple sites with clinically sepsis.

Data analysis

Acinetobacter spp. growth combined with relevant clinical signs and symptoms was considered to be one episode of infection. *Acinetobacter* spp. isolated within 48 hours of ICU admission was a hospital-acquired infection. Non-resistant *Acinetobacter* spp. isolated from a patient admitted directly from the emergency room or an outpatient department was community-acquired.

Only one representative infection per patient within a seven day period was considered in the analysis. Prevalence was the total number of patients with *Acinetobacter* spp. out of the total number of patients' specimens sent each year.

The sepsis and colonised groups were determined according to clinical and microbiological data analysis. The proportion of sepsis to colonised groups was calculated. The accuracy of the interpretation of microbiology results on the correlation of clinical history was analysed from 2011 to 2014.

Statistical analysis

The data were analysed using the Statistical Package for Social Sciences (SPSS version 19) and summarised with descriptive statistics. The relationship between underlying conditions and treatment response was analysed using the Pearson chi-square test (*P* value was calculated for large sample size) and Fisher's exact test (a statistical significance test, if sample size was <1000). Factors associated with patient survival were tested using logistic regression.

Results

Analysis of the microbiological and clinical aspects of *Acinetobacter* spp. isolates

The prevalence of *Acinetobacter* spp. ranged from 0.9% to 2.4% during the study period (2008-2014). The prevalence of *Acinetobacter* spp. in colonised group and sepsis group ranged from 0.5% to 1.5% and 0.4% to 1.1% respectively during the study period (2008-2014) [Figure 2].

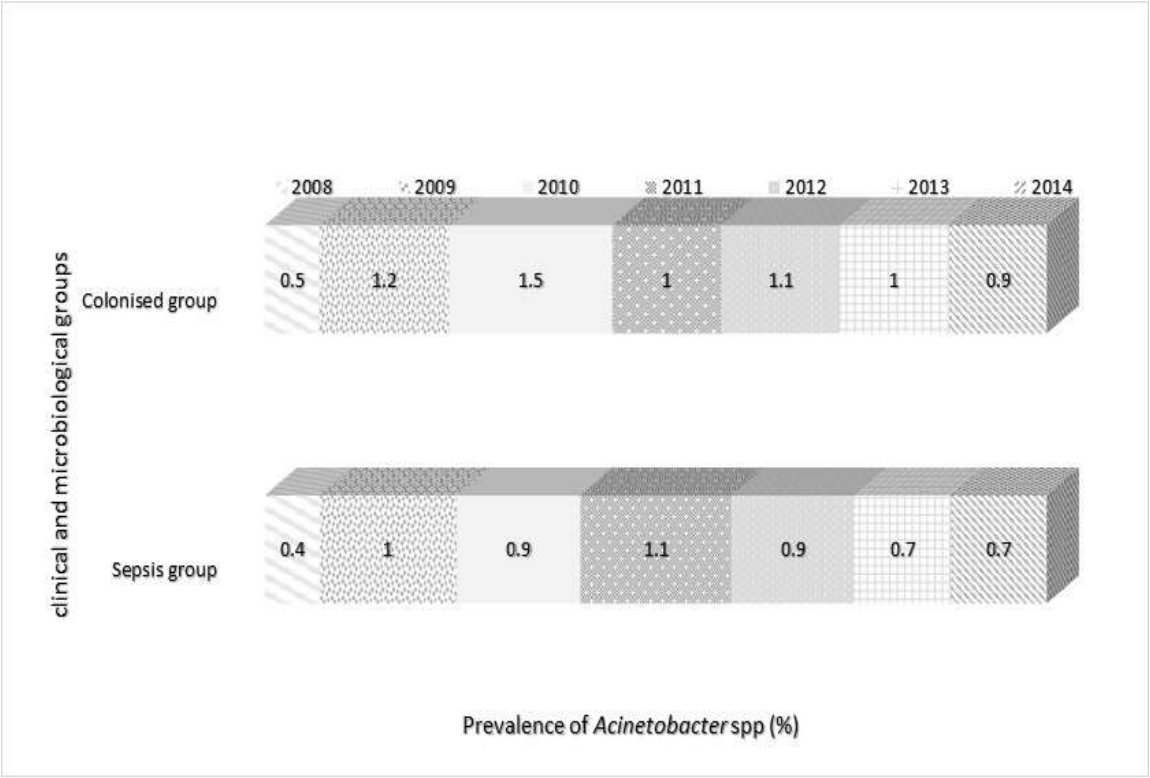

Figure 2. Prevalence of *Acinetobacter* spp. at IALCH from 2008 to 2014

Key: Data is presented as % of patients.

The number of specimens received were 17511, 17266, 18073, 20557, 23200, 24561, 24253 in 2008, 2009, 2010, 2011, 2012, 2013, 2014 respectively.

The numbers of patients per year with *Acinetobacter* spp. at IALCH ranged from 155 to 453 within the seven-year study period, being statistically significant for the increase from 2008 to 2012 and the decrease from 2013 to 2014 (*P:* 0.001). The proportion of significant sepsis ranged from 37% to 51%, and of colonisation from 49% to 63% [Figure 2].

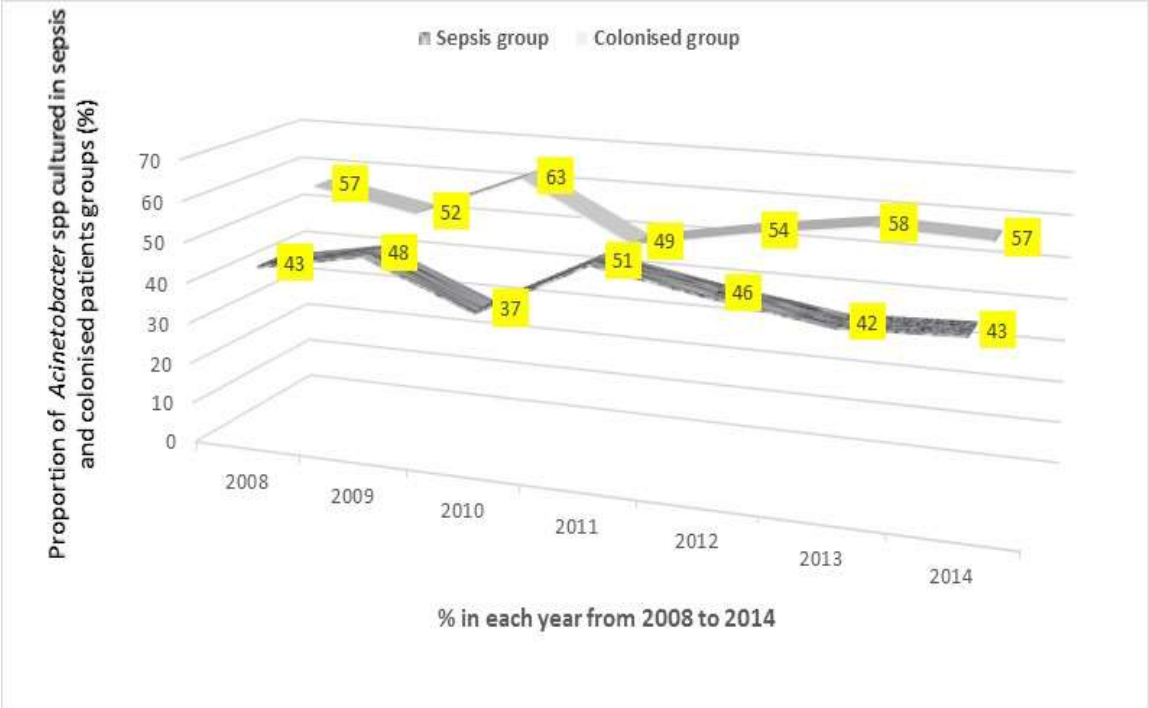

Figure 3. **Proportion [%] of *Acinetobacter* spp. sepsis and colonisation at IALCH from 2008 to 2014.**

Key: Data is presented as % of patients.

The proportion of of *Acinetobacter* spp. sepsis and colonisation were ranged from 37% to 51% and 49% to 63% during study period (2008-2014).

Demographic characteristics

The presence of *Acinetobacter* spp. was statistically insignificant for gender in the sepsis and colonised groups (P >0.05) [Figure.4]. There was a greater risk of infection for patients less than one year old in the sepsis group, and for patients aged 13 to 60 years old patients in the highly colonised group. The proportion of sepsis and colonisation cases was not significantly different in ICU and non-ICU units in the years 2010, 2011, 2013 and 2014, but more sepsis occurred in ICU units in the years 2008 and 2009.

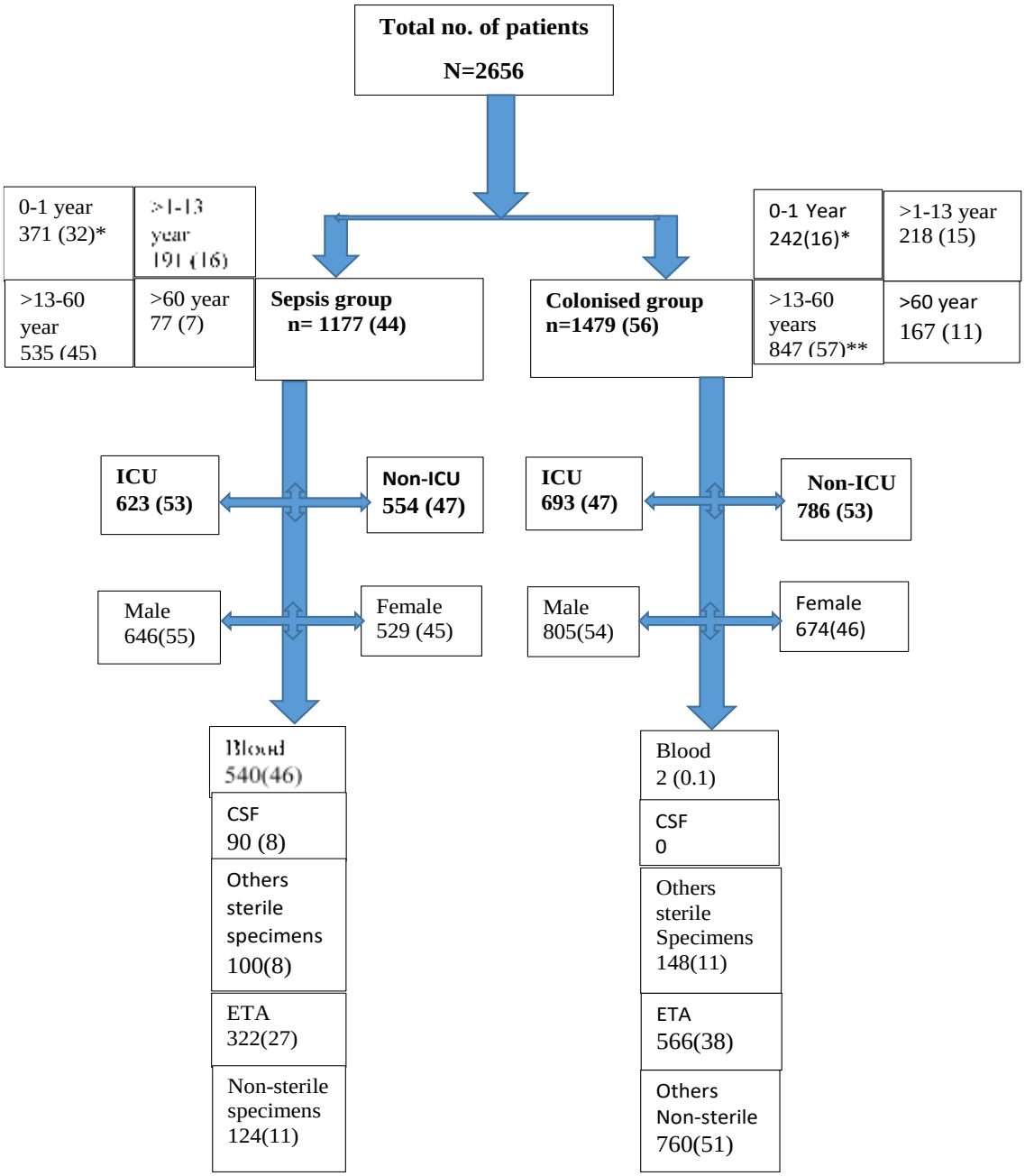

Figure 4. **Flow chart.** Demographic data of patients cultured with *Acinetobacter* spp. from 2008 – 2014.

Key: Data is presented as n (%) of patients.

* a greater risk of infection in patients less than one year old in the sepsis group;

** highly colonised group in 13 to 60 year old;

ICU: Intensive care unit, ETA: Endotracheal aspirate, CSF: Cerebrospinal Fluid.

Sites and source of infection

Respiratory tract specimens from endotracheal aspirate (ETA) were common sites for *Acinetobacter* spp. in both the sepsis and colonised groups. *Acinetobacter* spp. were isolated most commonly from blood (46%) followed by ETA (27% and 38% in the sepsis and colonised groups respectively). Cerebrospinal fluid (CSF) was the only specimen from which *Acinetobacter* spp. were isolated from patients in the sepsis group who had neurological-related infections (8%) [Figure.4].

Clinical history of sepsis and local infection *versus* microbiological significance of infection and colonisation

A significant number of clinically diagnosed sepsis cases 397/684 (58%) was caused by *Acinetobacter* spp. infection whereas the remaining cases 287/684 (42%) were merely colonised with *Acinetobacter* species. Among the clinically localised infections, 337/829 (41%) were caused by *Acinetobacter* spp. and 492/829 (59%) were colonised with *Acinetobacter* spp. [Figure. 5].

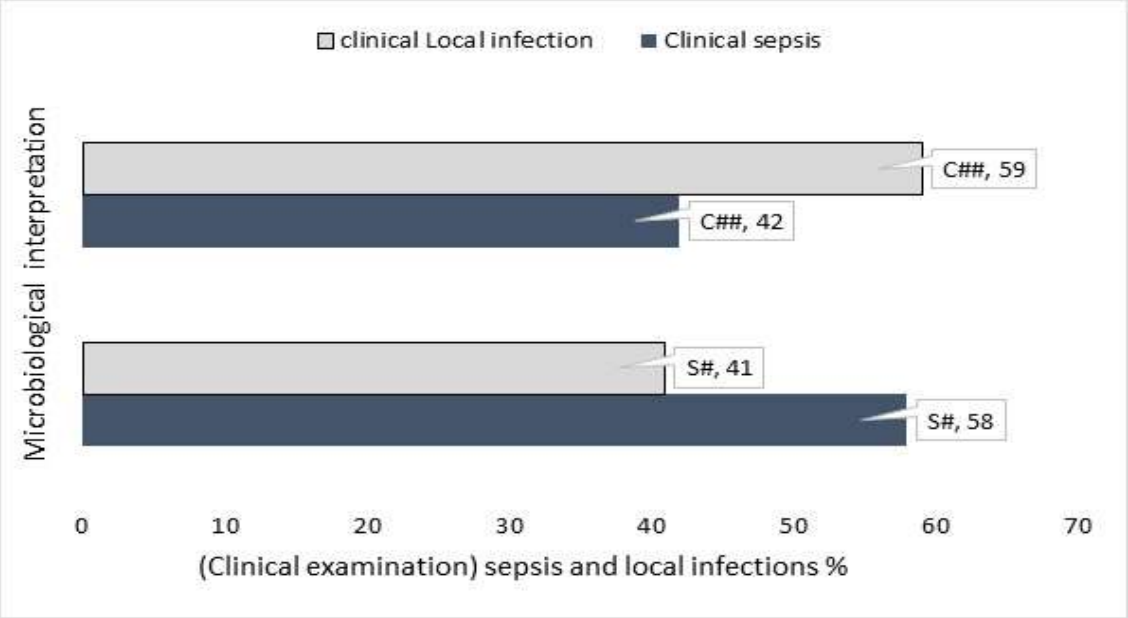

Figure 5. Clinical History *versus* Microbiological Results of *Acinetobacter* spp. during a four year period (2011-2014 (n: 1513).

Key: Data is presented as % of patients.

397 (58) # were microbiological and clinically significant sepsis;

337(41) # were microbiological and clinically significant local infections;

the appropriate antibiotics needed to be prescribed for the *Acinetobacter* spp.;
287(42) ## were microbiological colonisation in clinically significant sepsis group;
492(59) ## were microbiological colonisation in clinically significant local infections;
a prescription was not needed for the *Acinetobacter* species;
S, Sepsis group; C, Colonised group.

Accuracy of the interpretation of microbiology results in correlation with clinical history

According to the correlation between the clinical and microbiological results, both infection with *Acinetobacter* spp. and colonisation percentages in the sepsis and colonisation groups were statistically significant [<0.05 (*P*: 0.02)] [Figure.5]. Therefore, it is important to correlate the clinical and microbiological analysis when interpreting sepsis or colonisation caused by *Acinetobacter* spp. isolates in individual infection.

Pure and mixed growth of *Acinetobacter* spp. in sepsis and colonised groups of patients

Pure growth of *Acinetobacter* spp. in the sepsis group was statistically significant in 2013 [*P*<0.05 (0.001)], but not in 2012 and 2014 [*P* >0.05 (*P* 0.835 in 2012; *P* 0.267 in 2014)]. The specimens from patients with clinical sepsis showed pure growth in 72 (36%), 117 (59%) and 97 (51%) of specimens in 2012, 2013 and 2014 respectively. Pure growth of *Acinetobacter* spp. was also obtained from specimens with local infection; namely 55 (22%), 55 (23%), and 94 (43%) in 2012, 1013 and 2014 respectively. Similarly, mixed cultured *Acinetobacter* spp. and other bacteria were found from specimens of both clinical sepsis and colonised groups; namely 130 (64%), 80 (48%), 95 (49%) in the sepsis group and 190 (78%), 181 (77%) and 125 (57%) in the colonised group for 2012, 2013 and 2014 respectively [Figure.6].

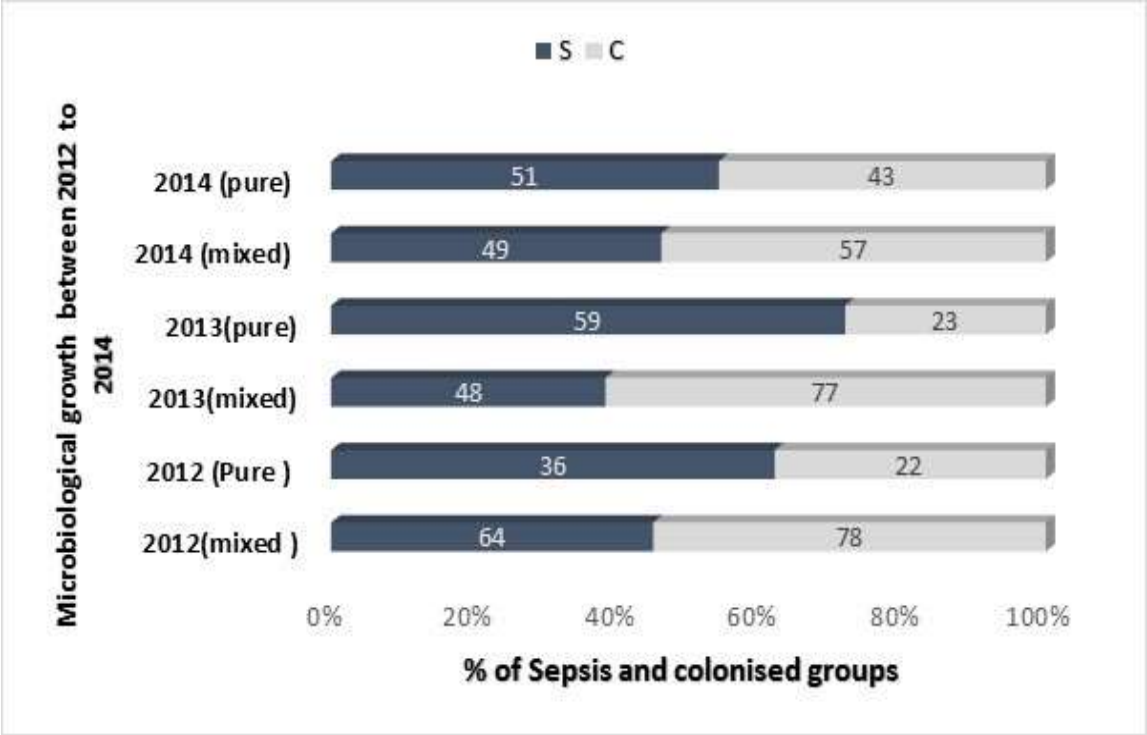

Figure 6. Pure and Mixed Growth of *Acinetobacter* spp. in sepsis and colonised group (2012-2014).

Key: Data is presented as (%). C: colonised patients group; S: sepsis patients group with *Acinetobacter* spp. significant infection; pure growth: *Acinetobacter* spp. cultured only; Mixed growth: cultured *Acinetobacter* spp. cultured along with other bacteria

Antibiotic resistance patterns

During the study period, the drug resistance patterns of *Acinetobacter* spp. in the sepsis group for MDR, XDR and PDR was 53 to 60%, 1% to 19%; 1% respectively, and 22% to 75%, 8% to 23%; 1% respectively in the colonised group. Amikacin sensitivity was high (59% to 90%) and 99% of *Acinetobacter* spp. remained sensitive to colistin throughout the seven years [Figure. 7].

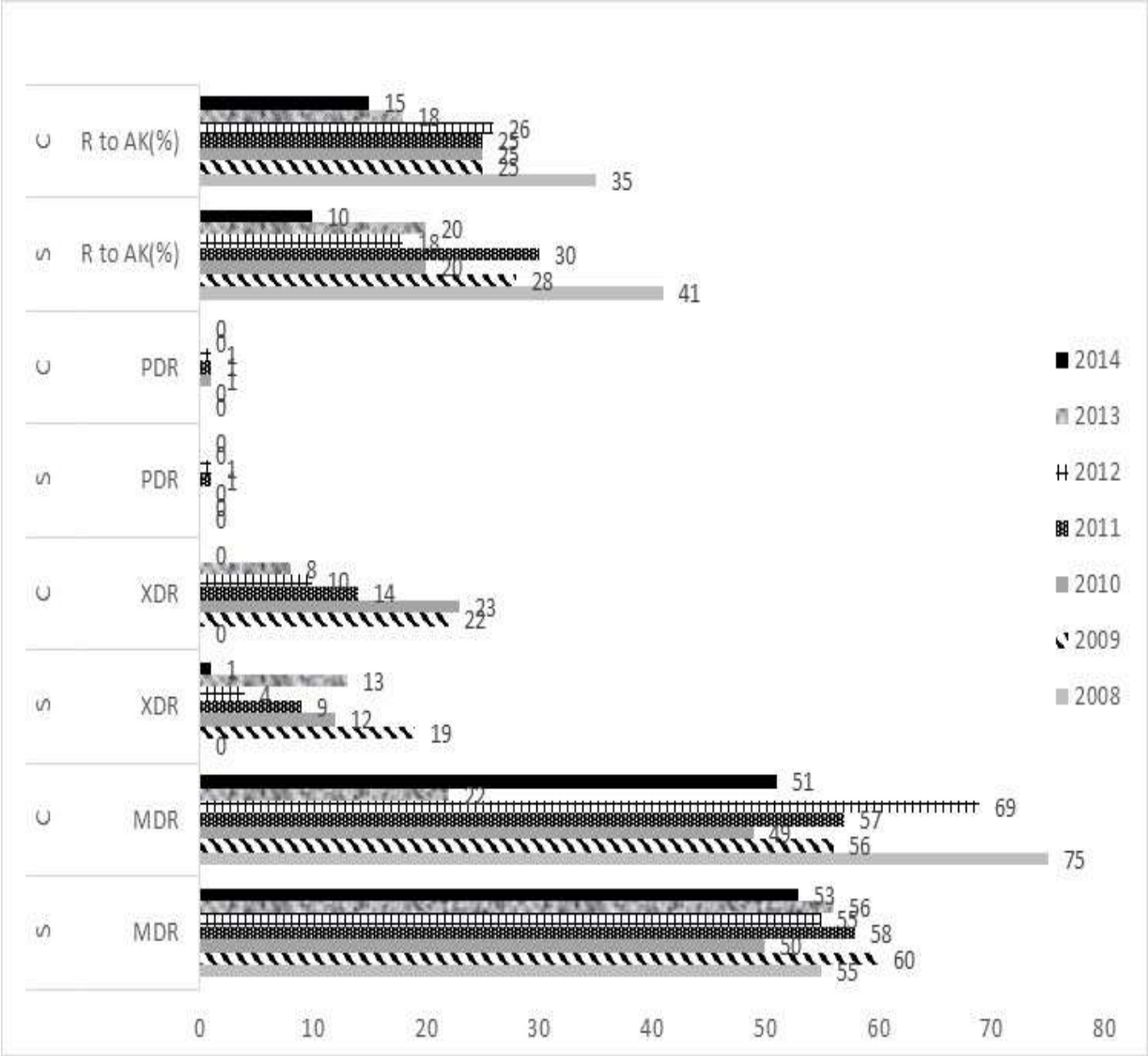

Figure 7. Antibiotic resistant pattern % with MDR, XDR, PDR, and resistant to colistin and amikacin from 2008-2014.

Key: Data is presented as (%).

MDR, multi-drug resistant; XDR, extensively-drug resistant; PDR, pandrug-resistant, CST, colistin; AK, amikacin; S, Sepsis group; C, Colonised group

The percentage of community acquired *Acinetobacter* spp. strains sensitive to all appropriate antibiotics was 10% to 70% in the colonised group and 7% to 36% in the sepsis group [Figure 8].

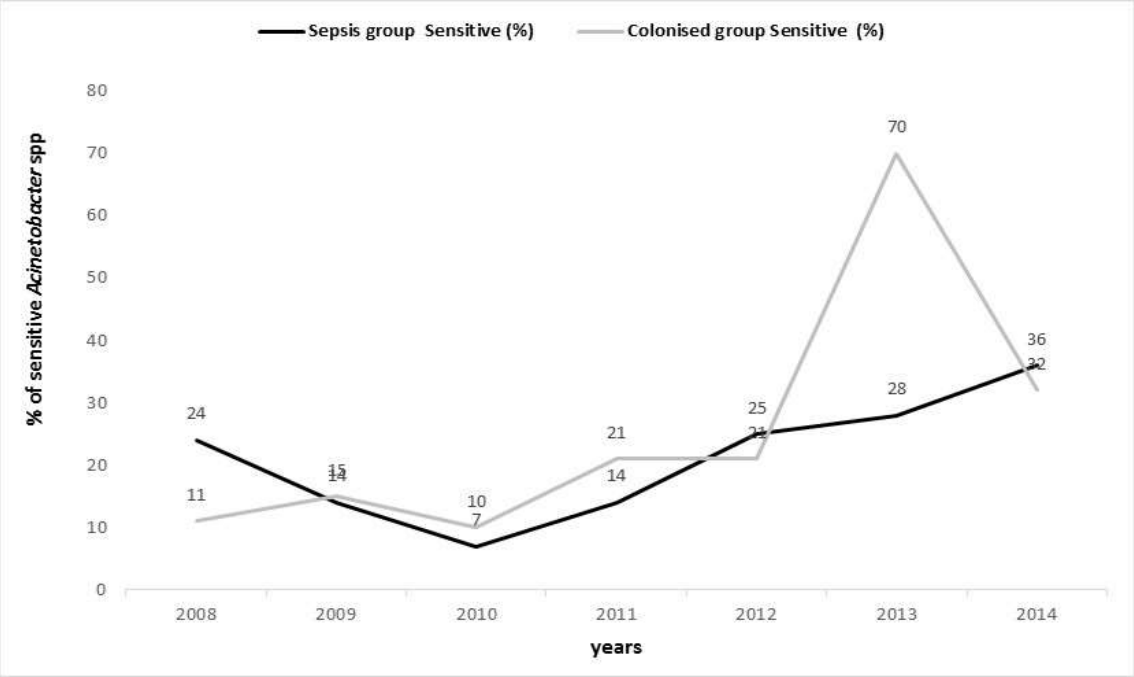

Figure 8. The Percentage of community acquired sensitive *Acinetobacter* spp.* during study period (from 2008 to 2014)

Key: Data is presented as n (%).

* Community acquired *Acinetobacter* spp. are sensitive to all appropriate antibiotics (CAZ, TZP, AK, CIP, IMP, MEM, CST).

CAZ, Ceftazidime; TZP, Piperacillin-tazobactam; AK, Amikacin;

CIP, Ciprofloxacin; IMP, Imipenem; MEM, Meropenem; CST, Colistin.

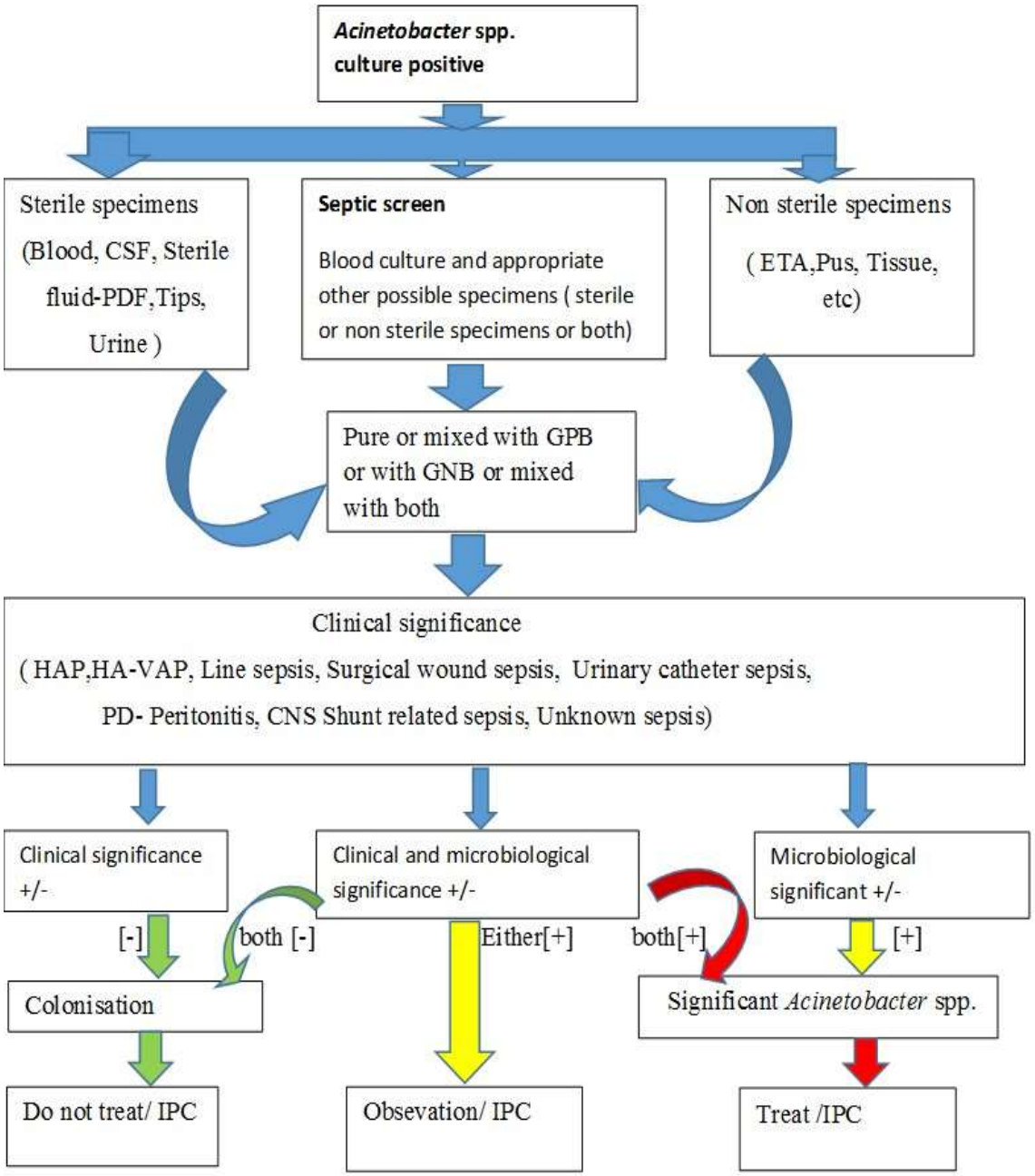

Figure 9. Preauthorisation decision assist the ASWP

Key: ICU: Intensive care unit; ETA: Endotracheal aspirate; CSF: Cerebrospinal fluid; PDF: Peritoneal dialysis fluid; GPB: Gram positive bacteria; GNB: Gram- negative bacteria; HAP: Hospital acquired pneumonia; HA-VAP: Hospital acquired ventilator associated pneumonia; IPC: Infection prevention control.

Discussion

Management of MDR, XDR and PDR *Acinetobacter* spp. infections currently poses serious clinical and epidemiological challenges [6]. The problem of antimicrobial resistance is both an international concern and a local issue that occurs in individual hospitals and communities [18, 19]. Thus, it will take a widespread effort at the individual and institutional level to impact on antimicrobial usage. The clinical and microbiological indicators of significant infections are crucial in establishing preauthorisation and prospective review of antibiotics in order to assist the ASWP at the patient level.

In this study, *Acinetobacter* spp. were found to be frequent cause of significant sepsis in both ICU and non-ICU wards. There was a high risk of infection in non-ICU units, with the hospital receiving patients, specifically those with chronic illnesses, across KZN. The results show a recorded prevalence of *Acinetobacter* spp. at 0.9% in 2008. At that time it was not recognised as a pathogen in this local hospital. The increased prevalence in 2009 (2.2%) and 2010 (2.4%) led to the recognition of *Acinetobacter* spp. as a potential pathogen and the implementation of strict infection prevention control for patients with positive *Acinetobacter* spp. isolated. In 2014, the prevalence was reduced to 1.6% due to reinforced infection prevention practices. . This rate is lower than that reported in global surveillance reports (19.2%: highest in Asia; 3.7%: lowest reported in North America) [7, 11-13, 15].

Although *Acinetobacter* spp. was cultured proportionally more in the colonised group *versus* the sepsis group, the organism was persistently isolated as a potential pathogen in the sepsis group (*P*: 0.001) during every year of this local study, in ICUs and non-ICUs. Previous studies reported that *Acinetobacter* infection rates vary among countries [7, 12, 13].

This study results showed the highest percentage of *Acinetobacter* spp. in blood culture specimens (41% to 50%), followed by ETA (17% to 48%) and CSF (1% to 18%) from 2011 to 2014 in the sepsis group. ETA specimens constituted the largest proportion of samples in both the sepsis and colonised groups. This result was similar to other studies [7, 13] that found most isolates from the respiratory tract [7]. The respiratory tract was the most common site affected, especially in the neonatal sepsis group of patients, and similar results were reported by Reddy *et al* in 2015 [20]. Patients with ETA specimens from both the sepsis and colonised groups experienced a significantly higher rate ($P < 0.5$) of *Acinetobacter* spp. lung infection than those with other sites of infection. Known risk factors for *Acinetobacter* spp. colonisation and infection include prolonged hospital or ICU stay, previous admission to another unit, immunosuppression, debilitation, and the previous use of third-generation cephalosporins [5, 21].

This study also revealed that the percentage of polymicrobial infections was high in the sepsis group (48% to 64%). Literature suggests that most ICU patients infected with these organisms

also have the highest rate of polymicrobial bacteremia (25.2%), which is associated with *Pseudomona aeruginosa*, coagulase-negative *Staphylococci* and *Escherichia coli* bacteria, representing 13.7% to 15.5% of total bacteremic episodes [7]. The high polymicrobial bacteremic rate of *Acinetobacter* spp. may reflect the potential polymicrobial sources of infection [22, 23].

Pure growth of *Acinetobacter* spp. in the sepsis group was statistically significant (P <0.05) in 2013, but not in 2012 and 2014 (P >0.05). These results show that although mixed growth of *Acinetobacter* spp. may result in colonisation, it also may be a pathogen among the mixed bacteria in sepsis group. Similarly, pure growth of *Acinetobacter* spp. was not always interpreted as a significant pathogen for sepsis, indicating the importance of checking other factors, such as the microbiological and clinical indicators, before deciding on the course of treatment. Correlation of the clinical and microbiological data showed a statistically significant difference between the pathogen and colonisation (P <0.05), indicating that interpretation of both clinical and microbiology data is essential before prescribing treatment for *Acinetobacter* spp. infections or sepsis in order to reduce over and under medicating (Figure 9).

The high prevalence of MDR *Acinetobacter* spp. and the rates of resistance to polymyxins were of concern. Colistin has generally been considered as the last bastion against such infections, within the context of a lack of new antimicrobial agents against developing PDR [12]. In IALCH, tigecycline is not used routinely to treat *Acinetobacter* spp. infections or sepsis, and consequently, drug susceptibility testing of this antibiotic was not performed until 2012.

Kim *et al*., reported that PDR was responsible for more than 60% of *Acinetobacter* spp. isolates causing hospital-acquired pneumonia in Asian countries [24, 25].

This local study showed that a large proportion of sepsis group patients had infections with MDR, XDR and PDR *Acinetobacter* species. Although similar resistance patterns of MDR, XDR and PDR were found in both sepsis and colonised groups over the seven years, amikacin sensitivity was high but the majority of *Acinetobacter* spp. isolates (99%) were sensitive to colistin. In the local hospitals, amikacin inhaler/nebulisation is commonly prescribed for pneumonia cases.

Imipenem, meropenem, and piperacillin/tazobactam were the most potent antibiotics, although resistance for these drugs emerged, highlighting the need to use broad-spectrum antibiotics with caution. Hospitals, being the main site for the development of antimicrobial-resistant organisms, are responsible for the stewardship of the available antimicrobial agents. This local study indicated that *Acinetobacter* spp. isolates acquired by colonised patients from the community were sensitive to the appropriate antibiotics. The Percentage of community acquired sensitive *Acinetobacter* spp were reange from 10% to 70% in the colonised groups and 7% to 36% in the sepsis group of patients during study period (2008-2014).

Acinetobacter spp. are part of the natural flora of the human skin and environmental areas and is linked to the high temperature and humidity in Durban, KZN. Moreover, significant community and nosocomial infections caused by *Acinetobacter* spp. have become a serious public health concern in many countries, including South Africa. The emergence of MDR *Acinetobacter* spp. in South African neonatal and pediatric units has been associated with greater than 50% mortality and significant morbidity [26] and a marked increase in the number of ICU infections due to MDR *Acinetobacter* spp. has been reported [27]. These results are similar to a study in Brazil, a developing country with some challenges common to those of South Africa [28].

The first study on the characteristics and outcome of pediatrics intensive care unit (PICU) patients with positive *Acinetobacter* spp. culture, which distinguished between sepsis and colonisation based only on clinical factors, was published in 2015 [20]. The current study is the first in South Africa to describe the characteristics of different age groups of ICU and non-ICU patients with positive *Acinetobacter* spp. culture, and attempt to distinguish between colonisation and infection through analysis of microbiological and clinical indications.

This study revealed a high prevalence of *Acinetobacter* spp. infections or sepsis, specifically MDR *Acinetobacter* spp., in both ICU and non-ICU settings. The particular concern was XDR and PDR *Acinetobacter* spp. which emerged in both the significant sepsis and colonised patient groups. Recognising vulnerable patient groups who are at a higher risk of morbidity and making appropriate antibiotic choices is critical in managing patients with *Acinetobacter* spp. infection. To the best of our knowledge, no such data has been reported from Durban, KZN, South Africa.

This study analyses several relevant factors that could be associated with *Acinetobacter* infections using ICU and non-ICU data over seven years. It differentiates *Acinetobacter* infections and colonisations according to the source of infection, types of specimens, quantity of organisms and microbiology results, and identifies factors, that may reduce adverse outcomes. The results will aid clinicians in using early and appropriate antibiotic regimens, particularly in patients at risk of more virulent MDR infection, as well as in those with late onset ventilator associated pneumonia (VAP) who are at the highest risk for mortality.

Clinical and microbiological indicators of sepsis patients should be analysed by collaboration between clinical microbiologists, clinicians, and infectious disease specialists. This information is crucial when establishing decision to treat in order to assist the preauthorisation and prospective review of antibiotics as part of ASWP at the patient level (Figures 1 and 9).

Since this was a retrospective analysis based on laboratory and clinical data of a large sample of patients, it was not possible to obtain a more detailed analysis of antibiotic usage and clinical outcomes. Further prospective studies should be done to confirm the findings, specifically the

effect of an intervention, using combination *versus* monotherapy as well as outcomes in the context of multidrug- resistance. The increasing emergence of drug-resistant *Acinetobacter* spp. means that continuous surveillance is needed to determine the prevalence and epidemiology of resistant *Acinetobacter* species.

Authors' contributions

First author's (K. Swe Swe –Han) contributions:

(1) The conceptualisation and design of the study;

(2) As a pathologist (microbiologist), interpretation of laboratory results and regular ward rounds. Based on the collaboration with clinicians and clinical characterisation and laboratory results of patients, acquisition of data, analysis and interpretation of data with statistician, for the study. Drafted the manuscript, critical revision for intellectual content, communication with the English editor and final approval of the version to be submitted.

Prof. M. Pillay and Prof. K. Mlisana helped design the study and edited the manuscript.

Prof. M. Pillay also provided scientific input and critically reviewed the drafts and final revised manuscript.

All authors read and approved the final manuscript.

Acknowledgements

The authors would like to express their gratitude to Fikile Nkwanyana for the assistance with the statistical analysis;

Funding information: The study was funded by Medical Education Partnership Initiative (MEPI), UKZN.

Compliance with ethical guidelines

Ethical standard: This study was approved by the research ethics committee at the University of Kwazulu Natal Biomedical Research Ethics Committee Reference No: REF: BE 283/12.

Informed consent:

The requirement of obtaining written informed consent from each patient was waived by the research ethics committee, because this study was retrospective and based on the clinical and laboratory data. The consent and permission were provided by hospitals.

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CHAPTER 4: ORIGINAL ARTICLE

Colistin exhibits diverse and species dependent synergy in combination with different antibiotics against *Acinetobacter* species

World Journal of Pharmacy and Pharmaceutical Sciences; Vol 6, Issue 2, 2017.Feb; 183-199 (published)

ORIGINAL ARTICLE

Title: Colistin exhibits diverse and species dependent synergy in combination with different antibiotics against *Acinetobacter* species

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Abstract

Background: Drug resistant- *Acinetobacter* species (*Acinetobacter* spp.) is a serious problem in clinical settings worldwide and has a significant effect on the optimal use of antibiotics, especially in patients with polymicrobial infections. This study was conducted to determine the effectiveness of various antibiotics with colistin combinations against multidrug-resistant *Acinetobacter* spp. using synergy testing.

Methods: *Acinetobacter* spp. were analysed for antibiotic susceptibility and synergistic efficacy of colistin in combination with other seven antimicrobial agents. These included carbapenems, amikacin, piperacillin-tazobactam, ciprofloxacin, vancomycin, linezolid and rifampicin. In *vitro* synergy tests were performed using a colistin-incorporated plate with the Epsilometer test (E-test) strip method.

Results: Of the sixty isolates tested, 90% were susceptible to colistin and amikacin, 25% to carbapenems and ciprofloxacin, and 20% to piperacillin-tazobactam. The combination of colistin and rifampicin showed synergistic effects against 28% of tested isolates, while colistin combinations with carbapenems, piperacillin-tazobactam, ciprofloxacin and vancomycin each showed synergistic effects in range 2-3% of tested isolates. Seventeen isolates (28%) showed antagonistic effects against colistin in combination with rifampicin.

Conclusion: Synergy testing of colistin combinations yielded highly diverse and species dependent results. Our findings suggest that such combinations should not be used for empirical treatment of *Acinetobacter* spp. infections in Durban, synergy testing should rather be performed for individualised direct therapy. Optimal treatment and the role of combination therapy should be addressed in future research.

Key words: Extensively drug resistance (XDR); combination therapy; synergy test; individualised direct therapy

Introduction

The emergence of multidrug-resistant (MDR), extensively-drug resistant (XDR) and pandrug-resistant (PDR) *Acinetobacter* species (*Acinetobacter* spp.) is a serious problem in clinical settings worldwide, including South Africa.[1,2,3] According to the Centers for Diseases Control and Prevention (CDC) and European Centre for Disease Control and Prevention (ECDC), MDR microorganisms are resistant to at least one agent in three or more antimicrobial categories or MDR *Acinetobacter* spp. are those isolates that showed resistance to carbapenems.[4] In this study, MDR *Acinetobacter* spp. are those isolates that showed resistance to carbapenems or any three groups of antimicrobials. *Acinetobacter* spp. resistant to five groups of antimicrobials excluding colistin are defined as XDR isolates, while those resistant to all six groups of antimicrobials are defined as PDR isolates.[4]

Drug resistant isolates have a significant effect on optimal antibiotic use in patients with serious infections[1,2,3] and recent surveillance studies have reported increased resistance to carbapenems, considered to be the primary treatment against these bacteria.[5] With limited therapeutic options for MDR *Acinetobacter* spp. infection, colistin has been accepted as an alternative agent and is often prescribed in spite of toxicity concerns.[6,7] However, the emergence of resistance during single therapy, as well as the potential toxicity, have led to the use of colistin in combination with other drugs instead of merely increasing the dose in monotherapy.[8] Increasing resistance rates seen in *Acinetobacter* spp. isolates have resulted in the administration of combination therapies, often prescribed empirically, as an alternative choice.[9, 10]

The rationale for using colistin combination treatment is its synergistic effect against resistant isolates, prevention of further resistance and reduced risk of dose dependent side effects.[11] It is also used to treat polymicrobial infections and severe infections with high mortality rates.[12]

In our local hospital, the common challenge is to choose the optimal combination therapy for polymicrobial infections, such as MDR, XDR *Acinetobacter* spp. with other Gram- positive and negative bacteria. Among the various combinations, meropenem showed the best synergy results and no antagonistic effect.[13,14] However, imipenem achieved a superior results to meropenem in another study.[15] Additional research showed that colistin/meropenem combination has a better synergistic effect in colistin-susceptible Gram- negative bacteria than colistin/imipenem.[15] Other various antibiotic combinations have a better synergistic effect with different tests in various studies.[12,16] There are several reports on the synergistic effects of colistin,[17,18] all with contradictory results.[19,20]

Rifampicin in combination with colistin has shown effective synergy in multiple studies involving colistin-resistant isolates, although this combination has not yet been implemented clinically.[16, 21]

A conflicting report showed no synergy with colistin in combination with imipenem, rifampicin

or azithromycin.[17] In addition, antagonism was detected in a colistin/sulbactam combination.[12] Because these drug combination studies are relatively new and demonstrate contrasting outcomes, further research is needed for conclusive results.[7] To determine their efficacy, in this study, we examined the synergy effect of colistin and other drug combinations commonly used in local hospitals, including colistin/carbapenem (imipenem or meropenem), colistin/rifampicin, colistin/piperacillin–tazobactam, colistin/aminoglycoside, colistin/ciprofloxacin, colistin/vancomycin and colistin/linezolid, each against sixty *Acinetobacter* spp. isolates from patients in the high risk units of Inkosi Albert Luthuli Central Hospital (IALCH), Durban, KZN, South Africa. Our investigations may be used to plan future guidelines for an effective standard management policy in the high-risk wards of IALCH and potentially in other hospitals worldwide.

Methods

The study was performed from January 2014 to January 2015 at the Medical Microbiology laboratory, National Health Laboratory Service, Inkosi Albert Luthuli Central Hospital Academic complex, Durban, KwaZulu-Natal, South Africa. The research was approved (Ref: BE283/12) by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal.

Sixty isolates of *Acinetobacter* spp. from 107 patients were selected based on the clinical and microbiological significance criteria.[22] All sixty isolates were subjected to antimicrobial sensitivity testing by the Kirby-Bauer disc diffusion method and the Vitek 2 (bioMérieux, France) automated method. The antimicrobials tested were ceftazidime (30 μg), ciprofloxacin (5 μg), amikacin (30 μg), piperacillin-tazobactam (100/10 μg), imipenem (10 μg) and meropenem (10 μg).

Based on their resistance patterns to different classes of antimicrobials, i.e. aminoglycosides (amikacin), β-lactams with inhibitors (piperacillin–tazobactam), cephalosporin (ceftazidime), fluoroquinolones (ciprofloxacin), carbapenems (imipenem, meropenem), and colistin, the isolates were divided into three groups. Group 1 isolates were resistant to all six groups of antimicrobials, defined as pandrug-resistant (PDR). Group 2 isolates were resistant to five groups of antimicrobials, except colistin, defined as extensively drug resistant (XDR). Group 3 isolates were resistant to carbapenem or any three groups of antimicrobials, defined as multidrug- resistant (MDR) based on the locally used different classes of antimicrobial agents.

Detection of Minimum inhibitory concentration (MIC) by drug susceptibility testing

Susceptibility results were obtained using the Vitek 2 (bioMérieux France) bacterial identification as per the manufacturer's instructions. The identification was repeated for confirmation before performing the synergy test. The Epsilometer test (E-test) (bioMérieux France) was used to test the (MIC) for each of the 60 representative isolates exposed to colistin in combination with different antimicrobials. MIC results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) breakpoint criteria for *Acinetobacter* spp.[23] Since there are no such criteria for vancomycin, linezolid and rifampicin, the susceptibility breakpoints for these antibiotics were based on the MIC interpretive standards of CLSI for Gram-positive bacteria.[24] A control isolates (ATCC 19606) was included.

Synergy testing

The synergy test was performed using the E-test method[24, 25], on colistin with seven different combinations such as amikacin, piperacillin-tazobactam, ciprofloxacin, carbapenems (imipenem, meropenem). Gram- positive appropriate drugs (vancomycin, linezolid) were also included against the 60 clinical *Acinetobacter* spp. isolates to test for synergistic effect. Synergy testing of colistin with vancomycin and linezolid are essential for both the polymicrobial infection and for synergy effect against MDR and XDR *Acinetobacter* spp. sepsis.

We excluded ceftazidime for colistin combination synergy testing due to it not being commonly used.

Media preparation

Two sets of Iso-Sensitest agar (CMO471 OXOID LTD, ENGLAND) plates were prepared, namely, drug-free media without colistin and plates with a fixed concentration of colistin at 0.5 µg/mL.[24]

Synergy testing by an E-test method.

Synergy testing was performed using the E-test® method.[24, 25] Plates were inoculated with a bacterial suspension of optical density equivalent to the 0.5 McFarland standard. Thereafter, E-test strips (bioMérieux) were applied to the plate containing colistin and the colistin-free plate that were prepared in-house at the NHLS laboratory. Plates were incubated aerobically at 37°C for 24 hrs. The MIC values were recorded for each drug in the presence and absence of colistin. The synergy tests were performed twice for each isolate against seven combination of agents. The procedure was repeated twice or more to ensure reproducible results. The average of 2 to 3 MICs

for each isolate was calculated. The MICs values of single agents were correlated with those of results from the Vitek 2 automated method.

Interpretation I [26]

Definitions,

MICA = MIC of drug A alone

MICB = MIC of drug B alone

MICAB = MIC of drug A in the presence of drug B

Synergy

Synergy defined as MIC of combination is ≥ 2 dilutions l**ess** than MIC of the most active drug alone.

E.g. MICA = 8, MICB = 16 (i.e. A= most active);

MICAB = 2. MIC of A is reduced from 8 to 2 in combination with B i.e. by 2 dilutions

Antagonism

Antagonism defined as MIC of combination is ≥ 2 dilutions higher than MIC of the most active drug alone.

E.g. MICA = 4, MICB = 16 (i.e. A= most active);

MICAB = 16. MIC of A is increased from 4 to 16 in combination with B i.e. by 2 dilutions

Indifference/Additive

MIC of combination is within +/- 1 dilution compared to the most active drug alone.

E.g. MICA= 1, MICB = 2 (i.e. A= most active); MIC of A or B in combination = 1

Combination of A with B shows no change in MIC of A, the most active drug (Indifference)

Data analysis

Excel data analysis was performed using the functions of Sort, Filter, Pivot Table and Formulas.

Results

Among the sixty *Acinetobacter* spp. isolates obtained from patient specimens during the study period (Table 1), the susceptibility rate was highest against colistin and amikacin, followed by ceftazidime, carbapenems (imipenem and meropenem), ciprofloxacin, piperacillin/tazobactam

and rifampicin (Figure 1). No isolates were susceptible to vancomycin and linezolid, which are appropriate antibiotics for Gram- positive bacteria (Figure 1).

The MIC values were estimated for the 60 isolates, which were representative of differing levels of drug resistance. *Acinetobacter* spp. showed a high degree of sensitivity to amikacin and colistin at 90% (Figure 1).

Forty-five isolates (75%) were found to be MDR, six isolates (10%) were XDR and six isolates (10%) were resistant to colistin but sensitive to other agents. There were no PDR isolates in our sample (Figure 1). Our interpretation of the synergy effects in the isolates by using the interpretation I criteria of synergistic, additive/ineffective (indifferent) and antagonistic is presented in Table 2 and Table 3, along with data on the percentages of interactions. Minimum inhibitory concentration (MIC) values showed synergistic effects in twenty-three different isolates (Table 2). The combination of colistin and rifampicin showed a synergistic effect in 17 (28%) of the 60 bacterial isolates tested. Colistin with piperacillin-tazobactam and colistin with ciprofloxacin showed synergy in 2 (3%) isolates, while colistin with carbapenem and colistin with vancomycin showed synergy in 1 (2%) isolates. None of the E-tested isolates showed synergistic effects for colistin with amikacin and colistin with linezolid. Combination tests revealed an antagonism effect and also an indifference/additive effect in the majority of isolates tested for colistin with seven combinations (Table 3). The study demonstrated no synergy effects in a number of isolates, although the MIC values of the combined drugs was lower than the MIC values of each individual drug.

Discussion

Acinetobacter spp., being one of the most important causes of nosocomial infection, poses a global public health problem[3] and a serious threat to hospitalised patients.[3] Drug-resistant *Acinetobacter* spp. infections are increasingly becoming a challenge to health care [5, 9], since isolates have limited treatment options due to their resistance to a wide range of agents.

In this study the proportion of carbapenem resistant *Acinetobacter* spp., classified as MDR, was 75% out of sixty isolates and the proportion of XDR was 10%. Although 10% of isolates was resistant to colistin, these isolates were sensitive to other agents. No PDR isolates were found in tested isolates during study time (Figure1).

Acinetobacter is a leading cause of nosocomial infections[1, 20]; which are severe and life-threatening. In addition, the organism is difficult to manage because antibiotic resistance often emerges during treatment [9] and results in severe adverse outcomes.

However, antimicrobial susceptibility testing of *Acinetobacter* spp. demonstrates equally high susceptibility rates for polymyxin B (95% to 99%) and colistin (98% to 100%) in other studies.[27, 28] This is comparable to our own data in this study, where maximum sensitivity to appropriate commonly used antimicrobial agents, such as amikacin and colistin was 90% and some isolates were found to be sensitive to several other drugs (Figure 1). Therefore, this study suggests that direct therapy, rather than empirical therapy, is still a good approach for *Acinetobacter* spp. infections.

Although the isolates in this study had a low degree of sensitivity to imipenem and ciprofloxacin at 25% and to penicillins with inhibitors (piperacillin-tazobactam) at 20 %, we still use these drugs as an appropriate choice for direct therapy. Therefore, monotherapy may be preferred if the bacterial isolates are susceptible to one of the tested agents[18] except colistin and amikacin that can be used as nebuliser monotherapy. However, due to its poor diffusion into lung epithelial lining fluid, the use of colistin as a single agent may have limited effects in *Acinetobacter* spp. pneumonia.[29]

This study showed that the *Acinetobacter* spp. isolates were mostly grown from blood culture followed by endotracheal aspirate (Table 1). Hence, the synergy test of colistin with vancomycin and linezolid combination were performed against MDR, XDR- *Acinetobacter* species from polymicrobial pneumonia cases. The synergy results revealed no synergy effect with a colistin-linezolid combination, and a species dependent synergy effect with a vancomycin-colistin combination. The pneumonia cases with polymicrobial culture, benefit from combination therapy, especially the combination of linezolid-colistin cannot be used as this study showed an antagonistic effect (Table 3).

Literature has reported that the intensive use of antimicrobials inevitably leads to the appearance of isolates resistant to these drugs, with increasing resistance for carbapenems which are still the main treatment option.[5] No new antibiotics have been available the treatment of XDR Gram-negative pathogens including *Acinetobacter* spp. for at least a decade.[30] For MDR *Acinetobacter* spp. infection, the practise is to prescribe either colistin, amikacin with carbapenem, or amikacin with piperacillin-tazobactam.[6] Amikacin appears to retain activity against many *A. baumannii* isolates. As with all antimicrobial agents and multidrug-resistant pathogens, resistance is increasing, and susceptibility testing is required to determine activity. Aminoglycosides are not often used as single agents for treatment, and the toxicity profiles often hinder their use for longer treatment courses. Historically, aminoglycosides have been used mostly in combination therapy, and monotherapy appears to be inferior to other agents and there are concerns regarding the development of resistance, also enhanced by the pressure of increased amikacin use.[31, 32] However, the efficacy of inhaled antibiotics, including aminoglycosides, outside the cystic

fibrosis population is of increasing interest.[1, 33,34] Therefore, the usage of amikacin with guided right dose, frequency, duration and susceptibility is crucial.

This current study supports this approach according to susceptibility results in the local setting, since the pathogen demonstrated sensitivity to colistin with amikacin in 90% of isolates, and lower sensitivity to cabapenems in 25% of the isolates. Carbapenems are still considered the primary treatment if the bacteria is sensitive.

Previous studies suggested that colistin should be combined with another antibiotic for adequate pharmacological effect. Those in vitro studies combined carbapenem and a polymyxin for carbapenem-resistant *Acinetobacter* spp. isolates[17, 18, 35], an approach that results in higher levels of synergy.

Pongpech *et al*., (2010) examined 30 MDR *Acinetobacter* spp. isolates all resistant to imipenem and meropenem and found 100% synergistic activity between imipenem and colistin, which may be related to the ability of colistin to weaken the cell wall or membrane. [5, 31] It is likely that this combination would play a major role in the treatment of *Acinetobacter* spp. infection. According to Pankey and Ashcraft (2009), meropenem and polymyxin B provided *in vitro* synergy against genetically unique meropenem-resistant *Acinetobacter* species.[18] In our setting, another option is to combine colistin with other drugs (such as amikacin, carbapenems, ciprofloxacin, piperacillin-tazobatam, vancomycin, linezolid and rifampicin) in order to achieve lower dose-related toxicity and also for polymicrobial infection with MDR-XDR-*Acinetobacter* spp. sepsis. However, the effectiveness of synergy results was diverse in this study. The estimated MIC for 60 representative isolates of differing levels of drug resistance suggests that drug-resistant *Acinetobacter* spp. is an increasingly challenging pathogen.

In another study, no synergy was detected with the combinations of colistin-carbapenems or rifampicin, while decreases in carbapenem and rifampicin MIC values were detected [17], which were consistent with the current study. Most prior research did not detect an antagonism effect with other combinations. However, one recent study reported antagonism in a sulbactam-colistin combination in 6.66% of isolates.[12] The current study found an antagonism effect with colistin combinations in some isolates and an indifference/additive effect in most isolates. Colistin can be used against multidrug-resistant and colistin-sensitive *Acinetobacter* infections, although the synergistic effect is specific for individual isolates and efficacy of treatment varies among species.[36]

The ***in vitro*** effects of antibiotics vary depending on the test methodology and at the moment there is no standardised method for *in vitro* synergy testing of resistant isolates (Sopirala *et al*., 2010).[37] The limitation of the current is that only one method was used to test synergy.

However, the colistin-incorporated plate and E-test strip method has been used in previous research to demonstrate synergy[38,39,40] and may be more reliable that the sequential E-test strip method.[13,38] When using combination therapy, synergy testing with the direct individualised isolates and employing pre-existing antibiotics is a plausible alternative approach for the treatment of infections due to multidrug-resistant isolates (Tangden, 2014).[41] The available literature on combination therapy for Gram-negative sepsis is diverse and contradictory.

One recommendation based on retrospective analysis is to use combinations of agents, including a carbapenem if the MIC for carbapenem is <4 mg/L (Tangden, 2014).[41]However, this study showed a synergy effect of combination of colistin and carbapenems, regardless of the MICs. This study indicated that synergy was apparent when isolates were susceptible or resistant to the combination drugs. Surprisingly, synergy was not affected by the MIC value of each drug against the specific isolate if either of the isolates had high or low MIC values.

In conclusion, the need for effective, first-line treatment options necessitates synergistic combinations of drugs that are a suitable alternative to amikacin, carbapenems, or colistin. Additionally, these combinations may be used for mixed polymicrobial infections. The results of synergy testing of colistin in combination therapy against *Acinetobacter* spp. are highly diverse. However, among these combinations, the synergistic effect of colistin with rifampicin was most promising. The potential of rifampicin to act synergistically with colistin against resistant isolates may prove advantageous when selecting antimicrobial therapy in settings with high rates of drug resistant *Acinetobacter* spp. pneumonia especially in those patients initiated on anti-tuberculosis (TB) treatment. Because of the TB endemic in South Africa, patients who had been on anti-TB medication with PDR-*Acinetobacter* sepsis cases, the synergy effect on colistin and rifampicin should be tested.

Previous *in vitro* studies suggest that if combination therapy is the treatment of choice, proven combinations may be used, since synergistic activity may depend on bacterial isolates and susceptibility testing methods. The study also discovered the antagonistic effects of colistin and rifampicin combination. Therefore, it is important to note that combinations should be evaluated using synergy tests as a guide to treatment. This study suggest that colistin in combination with another agent should not be prescribed as empirical therapy of standard of care. Rather, synergy testing must routinely be performed for *Acinetobacter* spp. isolated from each patient for individualised therapy and hence, a standard operational procedure (SOP) for synergy testing should be implemented.

Nevertheless, some literature suggests that accuracy is not pathogen but method dependent (Sopirala *et al.*, 2010).[37] The interesting new rapid synergy testing method, a novel two-dimensional antibiotic gradient technique named Xact™, for meropenem/colistin synergy testing for multidrug-resistant *Acinetobacter baumannii* strains has been recommended in routine microbiology (Van-Belkum *et al.*, 2015).[42] This new test was comparable, shown to be diagnostically useful, easy to implement and less labour intensive than the classical method (Van Belkum *et al.*, 2015).[42] Therefore, synergy testing should be done using the new method and compared with E-test method in future. Further research is needed in the form of comprehensive studies with clinical evidence. Synergy mechanisms need to be explored in order to facilitate understanding of our results and predict the effects of other antibiotic combinations.

Funding

The studies was funded by Medical Education Partnership Initiative (MEPI- 5R24TW008863), National Health Laboratory Service and University of KwaZulu-Natal.

Acknowledgement

Medical Education Partnership Initiative (MEPI- 5R24TW008863), University of Kwa-Zulu Natal. Medical Technologists, Laboratory Manager, Medical Microbiology Department, National Health Laboratory Service.

Conflicts of interest

All authors declare no conflicts of interest.

Ethics approval

The study was conducted at the Department of Medical Microbiology, University of KwaZulu-Natal (UKZN)/National Health Laboratory Service (NHLS). Ethics approval was obtained from the Biomedical Research Ethics Committee, UKZN (Reference no. BE 283/12).

Author's contributions

K Swe Swe–Han (First author) contributed to the following; (1) the conceptualisation and design of the study, project management, laboratory work, order the reagents for making media with colistin, laboratory synergy test, (2) acquisition of data, analysis and interpretation of data; (3) drafting the manuscript and revising it critically for important intellectual content, communication with the English editor (4) final approval of the version to be submitted.

Dr Kamaldeen Baba helped supervise the synergy test method, interpretation of synergy test, design the study and draft the manuscript. He also provided scientific and intellectual input and critically reviewed the manuscript.

Prof. M. Pillay and Prof. K. P Mlisana helped design the study, draft the manuscript and also provided scientific and intellectual input and reviewed the manuscript.

Prof. M Pillay also provided scientific input and critically reviewed the manuscript.
All authors read and approved the final manuscript.

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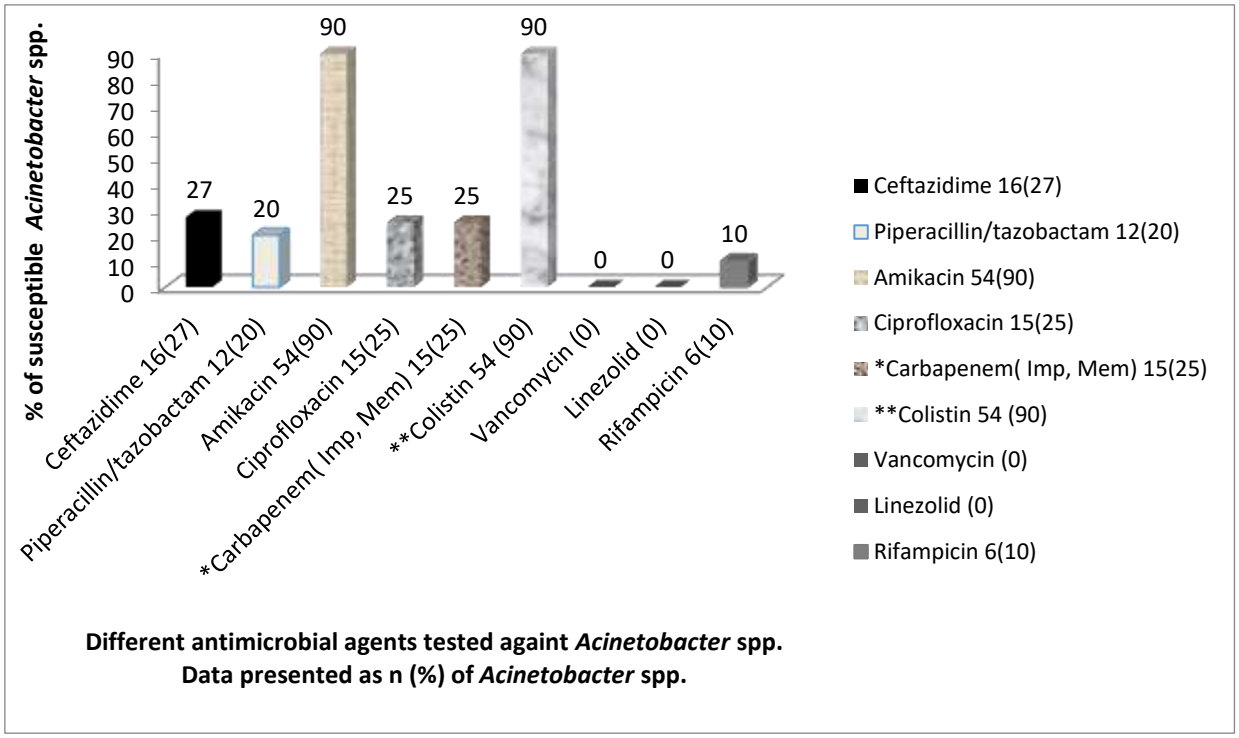

Figure 1. Antibiotic susceptibility of *Acinetobacter* spp. isolates.

Ceftazidime 16(27), Piperacillin/tazobactam 12(20)
Amikacin 54(90), Ciprofloxacin 15(25)
*Carbapenem (Imp, Mem) 15(25), **Colistin 54 (90)
Vancomycin (0) Linezolid (0)
Rifampicin 6(10)

Data presented as n (%) of *Acinetobacter* spp. isolates

* Carbapenem resistant *Acinetobacter* spp. isolates, MDR (multidrug- resistance), were 45 out of 60 isolates; 45 (75%)

** Colistin resistant *Acinetobacter* spp. isolates were 6 out of 60 isolates; 6 (10%).

Table 1: Distribution of 60 *Acinetobacter* spp. clinical isolates according to wards and specimen type

	Specimen type								
Wards	Blood culture	CSF	Tracheal aspirate	Pus	PDF	Tip	Tissue	Urine	Total
ICU	10	2	8	3	3	1	-	2	29
Burns/Plastic unit	1	-	-	1	-	-	1	-	3
Neurosurgery	-	3	2	-	-	1	-	-	6
PDU	-	-	-	-	2	-	-	-	2
BMT	2	-	-	-	-	-	-	-	2
HCU	3	1	1	-	-	-	1	-	6
Vascular unit	1	-	-	1	2	-	-	-	4
Labour unit	6	-	-	1	-	1	-	-	8
Total	23	6	11	6	7	3	2	2	60

Keys: Data presented as (n) of *Acinetobacter* spp. clinical isolates.
ICUs: Intensive care surgery; Intensive care medical unit; Intensive care trauma; Intensive care neonatal and pediatric unit;
PDU: Peritoneal dialysis unit;
BMT: Bone marrow transplant unit;
HCU: High care unit;
PDF: Peritoneal dialysis fluid;
CSF: Cerebrospinal fluid

591 **Table 2: Minimum inhibitory concentration (MIC) values for colistin, other**
592 **combined drugs and colistin combined with each drug for**
593 ***Acinetobacter* spp. isolates in which a synergistic effect[a] was demonstrated**

isolates no.	MIC (µg/mL)			Interpretation I Analysis
	CST	**RIF**	**CST+RIF**	**less 2 dilution/ actual dilution**
2	12	4	1	1
6	6	1	0.18	0.25
7	0.125	3	0.023	0.31
15	0.38	3	0.012	0.095
18	0.125	3	0.008	0.031
26	0.125	2	0.023	0.031
37	0.25	2	0.008	0.062
42	0.125	4	0.002	0.031
45	0.125	2	0.032	0.031
46	8	3	0.5	0.75
47	3	4	0.5	0.75
49	0.25	2	0.003	0.062
52	0.125	6	0.008	0.031
56	0.125	3	0.03	0.031
57	0.125	8	0.008	0.031
58	0.094	1.5	0.023	0.023
59	0.125	32	0.006	0.031
	CST	**Carb(IMP/MEM)**	**CST+Carb**	
6	6	0.94	0.094	0.23
	CST	**TZP**	**CST+TZP**	
1	32	1	0.023	0.25
34	0.38	0.094	0.016	0.023
	CST	**CIP**	**CST+CIP**	
2	12	0.5	0.125	0.125
49	0.25	0.47	0.016	0.062
	CST	**VAN**	**CST+VAN**	
20	0.25	256	0.02	0.062

594 **Key: [a],** Synergistic effect means; MIC of combination is ≥ 2 dilutions lower than MIC of
595 the most active drug alone.

CST, Colistin; RIF, Rifampicin; TZP, piperacillin +tazobactam; Cip, Ciprofloxacin; Carb, Carbapenem (IMP, imipenem and MER, meropenem); VAN, vancomycin;

Table 3: Synergy test results for colistin–combined with other antibiotics against *Acinetobacter* spp. isolates.

	No. of isolates (%)		
Combination	**Synergistic[a] effect**	**Additive/Indifferent[b] effect**	**Antagonistic[c] effect**
CST+ RIF	17 (28)	26 (43)	17 (28)
CST+ TZP	2 (3)	10 (17)	48 (80)
CST+CIP	2 (3)	11 (19)	47 (78)
CST+ Carb*	1 (2)	17 (28)	48 (80)
CST+ VAN	1 (2)	6 (10)	53 (88)
CST+ LZ	0	0	60 (100)
CST+ AK	0	14 (23)	46 (77)

Key: Data presented as n (%) of bacterial isolates

[a] Synergistic effect means MIC of combination is ≥ 2 dilutions lower than MIC of the most active drug alone.

[b] Additive/Indifferent effect means MIC of combination is within +/- 1 dilution compared to the most active drug alone.

[c] Antagonistic effect means MIC of combination is ≥ 2 dilutions higher than MIC of the most active drug alone.

CST, colistin; RIF, Rifampicin; TZP, piperacillin +tazobactam; CIP, ciprofloxacin; Carb, Carbapenem (IMP, Imipenem and MEM, meropenem); VAN, vancomycin; LZ, Linezolid; AK, Amikacin.

CHAPTER 5: ORIGINAL ARTICLE

Horizontal transfer of OXA-23-carbapenemase-producing *Acinetobacter* species in intensive care units at an academic complex hospital, Durban, KwaZulu-Natal, South Africa

South African Journal of Infectious Diseases: (Manuscript Reference number: SAJID - 2016 – 0052) accepted to publish.

ORIGINAL ARTICLE

Title:

Horizontal transfer of OXA-23-carbapenemase-producing *Acinetobacter* species in intensive care units at an academic complex hospital, Durban, KwaZulu-Natal, South Africa

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Abstract

Introduction: Carbapenemase production in multidrug-resistant *Acinetobacter* species is an important mechanism of carbapenem resistance. This study investigated the presence of the carbapenem-hydrolysing class D β–lactamase- encoding genes, *bla*$_{\text{OXA-23}}$ and *bla*$_{\text{OXA-58,}}$ and their association with the spread of MDR *Acinetobacter* species in intensive care units at an academic hospital.

Method: Forty-four MDR *Acinetobacter* species from sixty stored isolates were confirmed using VITEK®2. The minimum inhibitory concentrations (MICs) of imipenem and meropenem were determined using VITEK®2 and Epsilometer tests. The *bla*$_{\text{OXA-23}}$ and *bla*$_{\text{OXA-58}}$ genes were detected by polymerase chain reaction (PCR) in twenty-four selected isolates. The *bla*$_{\text{OXA-23}}$ amplicons were sequenced and compared to the GenBank database. Genotypic relatedness of isolates was determined by pulsed field gel electrophoresis (PFGE). Clinical and laboratory data were analysed.

Results: Among the twenty-four isolates, eighteen were carbapenem resistant and six were carbapenem sensitive. The *bla*$_{\text{OXA-23}}$ gene, but not *bla*$_{\text{OXA-58}}$, was detected in the eighteen resistant strains. The *bla*$_{\text{OXA-23}}$ amplicons showed 100% identity with the GenBank database of *bla*$_{\text{OXA-23}}$. The MICs of carbapenems against *Acinetobacter* species carrying the *bla*$_{\text{OXA-23}}$ gene were 8 to >16 µg/mL. Genetic relatedness was evident among isolates of seven pairs from fourteen patients. Of these patients, twelve were in the same ICU and two were adjacent to another ICU during the same hospitalisation period.

Conclusion: The selected MDR *Acinetobacter* species carried the *bla*$_{\text{OXA-23}}$ gene responsible for resistance to carbapenems (MICs 8 to >16 mg/L), while molecular and clinical data analysis suggested horizontal transmission in ICUs. In addition, the PFGE typing of a diverse collection of MDR *Acinetobacter* species clones showed that isolates were related from no more than two patients, suggesting that no outbreak had occurred. Continuous molecular surveillance for resistance genes is recommended.

Key words: *bla*$_{\text{OXA-23}}$ genes; carbapenem-hydrolysing class D β -lactamases (CHDLs); horizontal transmission; molecular surveillance

Background

Multidrug-resistant (MDR) *Acinetobacter* species (*Acinetobacter* spp.), are both community acquired and nosocomial opportunistic infection and have been responsible for outbreaks around the globe, especially in intensive care units ICUs settings.[1-5] The past twenty years has seen an increase in the prevalence of the pathogen [6-8] with MDR outbreaks reported in the United States.[9-11] A significant reservoir is the large number of chronically ill patients [5] from whom colonisation of recently hospitalised patients may take place.[6, 7]

There has been an increase of *Acinetobacter* spp. resistance to cephalosporins and carbapenems over the years,[12-14] leaving clinicians with limited therapeutic options.[12-14] Resistance to carbapenems, which display high efficacy and low toxicity, is of global concern.[12-14] Surveillance reports from China determined that carbapenem resistance in *Acinetobacter* spp. doubled from 30% in 2006 to 63% in 2013.[12-14] South African studies revealed *Acinetobacter* spp. resistance towards carbapenems and cephalosporins,[15,16] specifically imipenem (86%), meropenem (86 %) cefepime (90%) and ceftazidime (89%).[16]

The major mechanism of resistance to β-lactams in *Acinetobacter* spp. is carbapenem-hydrolysing class D β-lactamases (CHDLs), also known as OXA-type enzymes or oxacillinases.[16] Previous studies classified the class D carbapenemases into four subgroups: associated with OXA-23, OXA-58 as plasmid-encoded and OXA-24, OXA-40, OXA-51 as chromosomally encoded.[17,18] There are five subclasses of OXA associated with *Acinetobacter* spp.*;* the intrinsic chromosomal OXA-51-like, of which there are over 70 variants, and the acquired OXA-23-like, OXA-24 (OXA-40-like), OXA-58-like, and OXA-143-like.[19] In addition, the OXA-235, OXA-236, and OXA-237, the first representatives of a novel subclass of CHDLs, were described in *A. baumannii* strains in 2013.[20] Enzymes belonging to the OXA-23, OXA-24/ 40, OXA-48, OXA-51, OXA-58, and OXA-143 subgroups are of major clinical importance due to their wide dissemination in bacterial pathogens.[21-23] The genes encoding these enzymes are widespread [20] and have been found on both chromosome and plasmids, allowing for spread across *Acinetobacter* species.[20, 24] The prevalence of OXA-23 can be attributed to the acquisition of genetic elements, such as plasmids and transposons, as the OXA-23 gene is located on a plasmid or chromosome.[16] In a 2012 study, Liakopoulos *et al.* reported the prevalence of OXA-23 in Greece from 2010 to 2011 to be 95%,[25] while Koh *et al.* reported that 91% of *Acinetobacter* spp. isolates from Singapore produced carbapenemase and carried the $bla_{\text{OXA-23}}$ gene.[26] Previous studies conducted at Pretoria Academic Hospital revealed the high prevalence of OXA-51 at (83%, 99%) and of OXA-23 at (59%, 77%) in 2013 and 2015 respectively.[16, 18] Isolates of carbapenem resistant *Acinetobacter* spp. are often extensively drug-resistant (XDR), since they are susceptible to one or two agents only.[27] Additionally, the recent rise not only in XDR but also pandrug-resistant

(PDR) *Acinetobacter* spp.[22] is of global concern. Due to the problem of resistance and antimicrobial availability, the Infectious Diseases Society of America determined that *Acinetobacter* spp. is a particularly concerning pathogen.[28] Moreover, the Centers for Diseases Control and Prevention has highlighted the importance of MDR *Acinetobacter* spp. transmission in nosocomial and community acquired infections.[5] In our setting, the latter half of 2008 discovered the emergence of *Acinetobacter* spp. clinical strains with resistance to multiple classes of antimicrobials, including carbapenems, piperacillin-tazobactam, ceftazidime and fluoroquinolones. Hence, treatment options were restricted to salvage agents like colistin and amikacin.

In this study, we investigated a representative sample of *Acinetobacter* spp. from Inkosi Albert Luthuli Hospital (IALCH) from 2013 to 2014. We determined the presence of two carbapenem resistance genes using PCR, sequencing and correlated the MIC of the carbapenems with the genes. We also investigated the clinical data of patients, including ward of admission, site of specimen, prescribed antibiotics and outcome. Nosocomial spread of the strains was investigated through pulsed field gel electrophoresis (PFGE).

Methods

Study setting

The study was conducted at the Department of Medical Microbiology, University of KwaZulu-Natal (UKZN)/National Health Laboratory Service (NHLS). Ethics approval was obtained from the Biomedical Research Ethics Committee, UKZN (Reference No BE 283/12).

The stored *Acinetobacter* spp. (n=60) isolates had previously been isolated from the patients hospitalised at IALCH from January 2013 to January 2014. Of the sixty, forty-four MDR *Acinetobacter* spp. were confirmed by using VITEK 2 (BioMérieux, France). The MICs of imipenem and meropenem were determined using the VITEK 2 and Epsilometer tests (E-test) (BioMérieux, France).

Using the antibiogram, four XDR and fourteen MDR isolates with the same sensitivity patterns were selected for molecular investigation. PCR, sequencing and PFGE typing were used to investigate carbapenem-hydrolysing class D β –lactamase production, the presence of the $bla_{\text{OXA-23}}$ *and* $bla_{\text{OXA-58}}$ genes, as well as the association between drug resistance and presence of the genes.

The *A. baumannii* ATCC 19606 strain was used as quality control for antimicrobial susceptibility tests and molecular methods. The $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-58}}$ carrying *Acinetobacter* spp. isolates

obtained from the National Institute for Communicable Diseases (NICD) served as controls for PCR, sequencing and PFGE.

Drug susceptibility tests and MIC

The stored isolates were confirmed as MDR *Acinetobacter* spp. before PCR. Identification and antibiotic susceptibility testing (AST) of MDR *Acinetobacter* spp., including MICs, was performed using the VITEK® 2 GN Card automatic method (BioMérieux, France) and the Epsilometer test (E-test®) (BioMérieux, France) as per the guidelines from the Clinical and Laboratory Standards Institute (CLSI), 2013.[29]

Carbapenem-resistant *Acinetobacter* spp. was resistant to both imipenem and meropenem with MICs of 8 µg/mL, whereas carbapenem-susceptible *Acinetobacter* spp. possessed a MIC of <1 µg/mL and carbapenem-intermediate *Acinetobacter* spp. a MIC of 1-2 (<4) µg/mL.[29]

Detection of *bla*$_{\text{OXA-23}}$ and *bla*$_{\text{OXA-58}}$

Eighteen MDR *Acinetobacter* spp. belonging to the same antibiogram groups were selected for amplification by PCR to detect the carbapenem resistance genes *bla*$_{\text{OXA-23}}$ and *bla*$_{\text{OXA-58}}$. Resistance mediating genes were assessed for the presence of polymorphisms with the Big Dye Terminator v3.1 Cycle Sequencing kit (Life Technologies, ThermoFisher Scientific, South Africa)

DNA extraction of *Acinetobacter* spp. isolates [30, 31]

Genomic DNA, from each of twenty-seven isolates, comprising eighteen clinical MDR strains, three controls and six sensitive clinical isolates, was extracted from an overnight culture using a loopful of colonies suspended in 500 µL of 1x TE buffer (Tris EDTA) (Capital Laboratory Supplies, Durban, South Africa). Cell lysis and protein digestion were performed using a combination of 10% Sodium Dodecyl Sulfate and 10 mg/mL proteinase K (Capital Laboratory Supplies, Durban, South Africa). Proteins were precipitated with 10% Cetyltrimethyl ammonium bromide (CTAB)/4% NaCl and polysaccharides were extracted by the addition of 24:1 chloroform-isoamyl alcohol (Capital Laboratory Supplies, Durban, South Africa). The DNA was precipitated with isopropanol, washed with 70% cold ethanol (Capital Laboratory Supplies, Durban, South Africa) and dissolved in an appropriate volume of 1xTE buffer. The DNA was electrophoresed in a 1% agarose gel (Capital Laboratory Supplies, Durban, South Africa) to determine quality and quantity. The extracted genomic DNA was stored at -20°C (Defy Ltd, Multimode, SA) until further analysis.

Amplication of the *bla*$_{OXA-23}$ and *bla*$_{OXA-58}$ genes

The *bla*$_{OXA-23}$ and *bla*$_{OXA-58}$ gene regions were amplified using primers (Roche Diagnostics, Randburg, South Africa) specific to the up and downstream regions of the gene sequences (Table 1). The PCR master mix consisted of 5 µL template DNA, 10x PCR buffer, 0.2 µM of each primer, 2.5 mM dNTPs, 1 U Taq DNA Polymerase (Thermofisher Scientific, United States), 1.5 µM MgCl and nuclease free water, with a total volume of 25 µL. The initial denaturation steps were performed for 5 min at 95°C, followed by 40 cycles of 20 s at 95°C, 1 min at 55°C and 30 s at 72°C. This was followed by a final extension step of 5min at 72°C. Both positive and negative controls were used during the PCR amplification process. PCR products were electrophoresed on a 1.5% agarose gel (Capital Laboratory Supplies, Durban, South Africa) with markers of known molecular weights (ThermoFisher Scientific, Waltham, Massachusetts) and visualised under UV light.

Table 1: Primer sequences and the corresponding annealing temperatures

Primers	Sequence (5' to 3')	Annealing temperature (°C)	Reference
bla$_{OXA-23}$ Forward	TCTGGTTGTACGGTTCAGC	53	[32]
bla$_{OXA-23}$ Reverse	AGTCTTTCCAAAAATTTTG	53	[32]
bla$_{OXA-58}$ Forward	ATGAAATTATTAAAAATATTGAGTTTAG	55	[32]
bla$_{OXA-58}$ Reverse	TTATAAATAATGAAAAACACCCAAC	55	[32]

Purification of PCR products

The PCR product were purified using 2 U Shrimp Alkaline Phosphatase (SAP) (AEC-Amersham, Little Chalfont, United Kingdom) and 10 U Exonuclease (Amersham, Little Chalfont, United Kingdom) as per manufacturer's instructions. After the addition of 2 U of SAP and 10 U of Exonuclease I, the PCR tubes were briefly incubated at 32°C for 30 min and then at 80°C for 15 min in a water bath to deactivate the enzymes.

DNA sequencing of *bla*$_{OXA-23}$ amplicons

Gene sequencing was performed with the ABI Big Dye Terminator v 3.1 Cycle Sequencing kit (ThermoFisher Scientific, Waltham, Massachusetts). The sequenced products were separated by capillary array electrophoresis using the ABI 3500 Genetic Analyser (ThermoFisher Scientific, Waltham, Massachusetts).

The National Center for Biotechnology Information (NCBI) database was accessed and the sample sequences were aligned and compared to a reference sequence using the Basic Local Alignment Search Tool (BLAST) (https://www.ncbi.nlm.nih.gov/).

Genotypic relatedness of clinically selective *Acinetobacter* spp. determined by PFGE

Macro-restriction analysis of multidrug-resistant *Acinetobacter* spp. obtained from IALCH was performed using an adaptation of the method by Seifert *et al*., [33] and Sader *et al*.,[34] Pure isolates from blood agar plates were grown in brain heart infusion (BHI) broth for 15-18 hr at 37°C in a shaking incubator (Vacutec, South Africa) for plug preparation. A volume of broth corresponding to 1 optical density (OD) at 600 nm was used to make a 1 % PFGE agarose plug for each isolate. DNA was extracted within the agarose blocks by cell lysis over 18hr. Plug slices for each isolate were digested with 20U *Apal* restriction endonuclease (New England Biolabs, USA) for 4hr at 37ºC. The restricted DNA was electrophoresed n a 1 % Pulsed–Field Electrophoresis Gel (BioRad) in 0.5X Tris-Borate-EDTA buffer (Merck) at 6V/cm with a 5-25 sec switch time for 24 hours using the Chef-DR 3 system (BioRad).

Cluster analysis was performed according to criteria outlined in Van Belkum *et al.*[35] and a type was defined as PFGE banding patterns differing by 4 or less bands. The results were interpreted according to the Tenover criteria.[36]

Clinical and laboratory data collection

The clinical characteristics, namely type of specimen, ward of admission, prescribed antibiotics and patient outcome, were documented from the patient records. In addition, the laboratory data were recorded from the laboratory computer system. Both the clinical and laboratory data were verified during wards rounds and analysed.

Statistical analysis of clinical characteristics and patient outcomes

Data was captured and analysed using the Statistical Package for Social Sciences (IBM® SPSS Statistics version 19). Results were presented using descriptive statistics such as frequency and percentage. The association between underlying patient condition and response to antibiotic

agents was determined using the Pearson chi-square test or Fisher's exact test. Logistic regression was used to test for factors associated with patient mortality.

Results

Drug susceptibility tests and MICs

During the study period, forty-four of the sixty stored *Acinetobacter* spp. isolates were confirmed MDR by the MICs. The selected isolates were resistant to carbapenems, with MIC values for imipenem and meropenem at 8 to >16 µg/mL as determined by the Vitek (BioMérieux, France) automatic system and confirmed with E-test (BioMérieux, France). The control isolate (ATCC 19606) showed sensitivity to imipenem and intermediate sensitivity to meropenem, with MICs of 0.25 and 1 µg/mL respectively. The $bla_{\text{OXA-23}}$ positive control isolate was resistant to imipenem and meropenem with a MIC of >16 µg/mL for both. The $bla_{\text{OXA-58}}$ positive control strain was resistant to imipenem and meropenem with a MIC of 8 and 4 µg/mL respectively (Table 2).

Table 2: MICs of imipenem and meropenem for *Acinetobacter* spp. isolates from the central Hospital in the academic Complex and the occurrence of the corresponding carbapenemase gene

Number of isolates (n=24)	**IMP**	**MEM**	***bla*$_{\text{OXA-23}}$ + or -**	***bla*$_{\text{OXA-58}}$ + or -**
17	>16	>16	+	-
1	8	>16	+	-
6	<0.25	<0.25	-	-
*$bla_{\text{OXA-23}}$	>16	>16	+	-
**$bla_{\text{OXA-58}}$	8	4	-	+
ATCC 19606	<0.25	1	-	-

Key: +, detected; -, not detected; IMP, Imipenem; MEM, Meropenem;
*, positive control for $bla_{\text{OXA-23}}$ known *Acinetobacter* spp.; **, positive control for $bla_{\text{OXA-58}}$ known *Acinetobacter* spp.

The clinical characteristics and patient outcomes were analysed in those patients whose isolates showed the presence of the *bla*$_{OXA-23}$ gene (n=18)

Among the eighteen isolates, four (22%) were XDR isolates which possessed the same antibiogram and fourteen (78%) were resistant to all agents except amikacin and colistin (Table 3).

Table 3: Clinical characteristics and outcomes of the patients with *Acinetobacter* spp. isolates with *bla*$_{OXA-23}$ gene

	Clinical outcomes (n=18)	
	Deceased, n (%) 7 (39%)	**Discharge n (%)** 11(61%)
Antibiotics	Resistance n (%)	Resistance n (%)
Ceftazidime	7 (100%)	11(100%)
Ciprofloxacin	7(100%)	11(100%)
Piperacillin-tazobactam	7(100%)	11100%)
Imipenem	7(100%)	11(100%)
Meropenem	7(100%)	11(100%)
Amikacin	3(17%)	1(6%)
Colistin	0	0
	p>0.05 (0.288)	
ICUs	5(28%)!	9(50%)!
Non-ICUs	2 (11%)	2(11%)
	p <0.05 (0.001)!	
	p >0.05 (0.515)	
Sterile specimens	5(28%)	6(33%)
Non-sterile specimens	2(11%)	5(28%)
	p >0.05 (0.417)	
Monotherapy	3(17%)	5(28%)
Combination therapy	4(22%)	6(33%)
	p >0.05 (0.648)	
***Horizontal transfer (n=14; 7 pairs)**		
****Both in one pair**	4(22%)	6(33%)
*****One patient in one pair**	2(11%)	2(11%)
	p >0.05 (0.643)	

Key: *, PFGE typing showed horizontal transfer that strains were related from no more than two patients and thus no outbreak occurred during the study period.

**, among the 7 pairs of patients, 2 pairs were deceased and 3 pairs were discharged;

***, one patient in each pair was deceased and other one patient was discharged.

! P <0.05: the outcomes in ICU was statistically significance 28% in ICU deceased whereas 50% survived.

Sterile specimen (BC, blood culture; CSF, cerebrospinal fluid);

Non-sterile specimen (ETA, endotracheal aspirate; pus; CT, catheter tip);

ICUs (N-ICU, neonatal intensive care unit; ICUT, intensive care unit trauma);

Non-ICUs (LW, labour ward; neurosurgical ward; high care unit, surgical unit, vascular unit)

Amplification result of the *bla*$_{\text{OXA-23}}$ and *bla*$_{\text{OXA-58}}$ genes by PCR (n=24)

The *bla*$_{\text{OXA-58}}$ gene was not detected in any of the isolates tested. The *bla*$_{\text{OXA-23}}$ gene was detected in 18 clinical isolates, but not in 6 carbapenem sensitive *Acinetobacter* spp. isolates (Figure 1, A-D). The majority (17 out of 18) of *Acinetobacter* spp. isolates carrying the carbapenemase gene exhibited high MICs (>16 µg/mL) to carbapenems (Table 2).

Sequencing finding of *bla*$_{\text{OXA-23}}$ amplicon

Sequencing of the *bla*$_{\text{OXA-23}}$-like amplicon of 18 *Acinetobacter spp*. isolates revealed 100 % identity with that from the GenBank database.

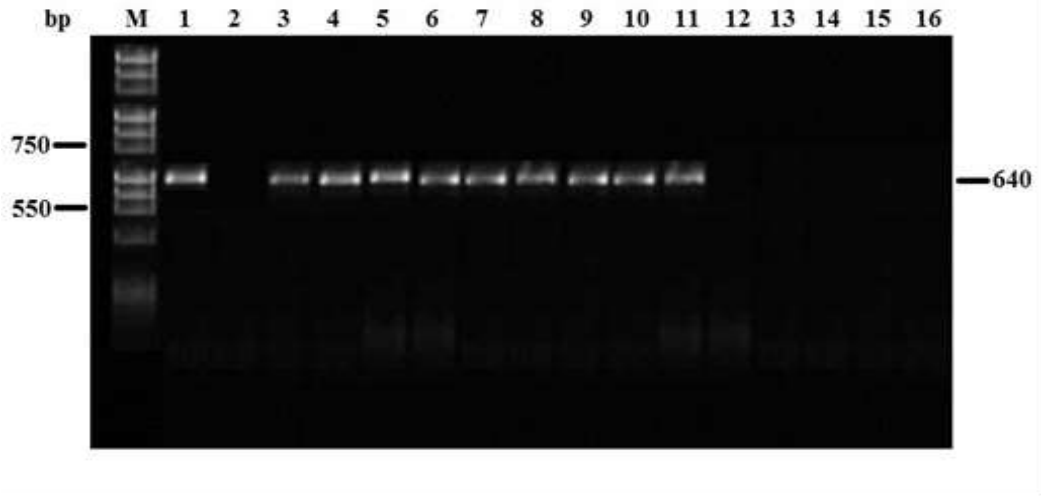

Figure 1 A, B, C and D: Detection of the Oxacillinase genes **(***bla*$_{\text{OXA-23}}$ and *bla*$_{\text{OXA-58}}$ genes) in *Acinetobacter* spp. in a 1.5% agarose gel following amplification by PCR.

Phenotypic resistant *Acinetobacter* spp. (MDR *Acinetobacter* spp., 18 isolates)

Key: **Figure 1A:** *bla*$_{OXA-23}$ amplication product of 640 bp

M, molecular weight marker (mw); lane 1, positive control (PC); lane 2, negative control (NC); lanes 3 - 11, phenotypic resistant *Acinetobacter* spp. (9 isolates);
lanes 12-15, (four isolates were phenotypic sensitive *Acinetobacter* spp.)
lane 16, ATCC 19606 isolate was included.

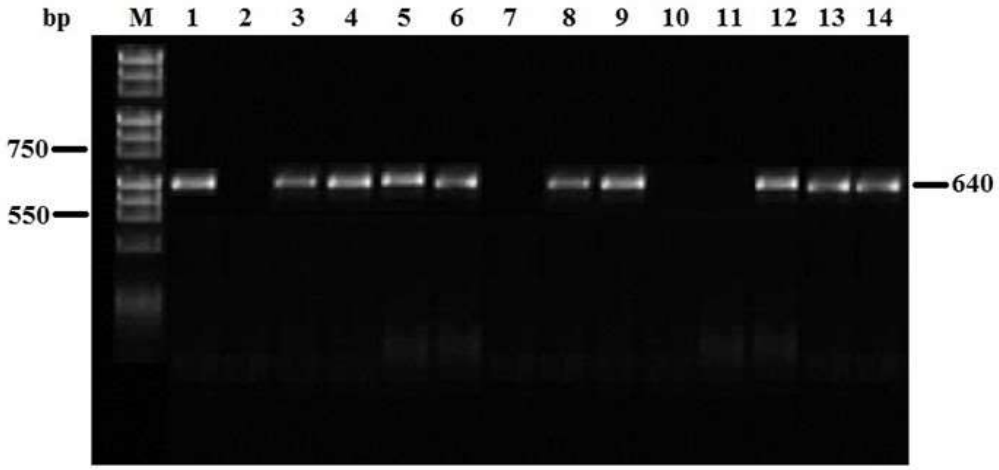

Figure 1B: ***bla***$_{OXA-23}$ **amplication product of 640 bp.**

Key: M, molecular weight marker (mw); lane 1, positive control (PC); lane 2, negative control (NC); lanes 3-6, 8, 9 and 12-14, phenotypic resistant *Acinetobacter* spp. (9 isolates); lanes, 10, 11 (two isolates phenotypic sensitive *Acinetobacter* spp.); lane 7, ATCC19606.

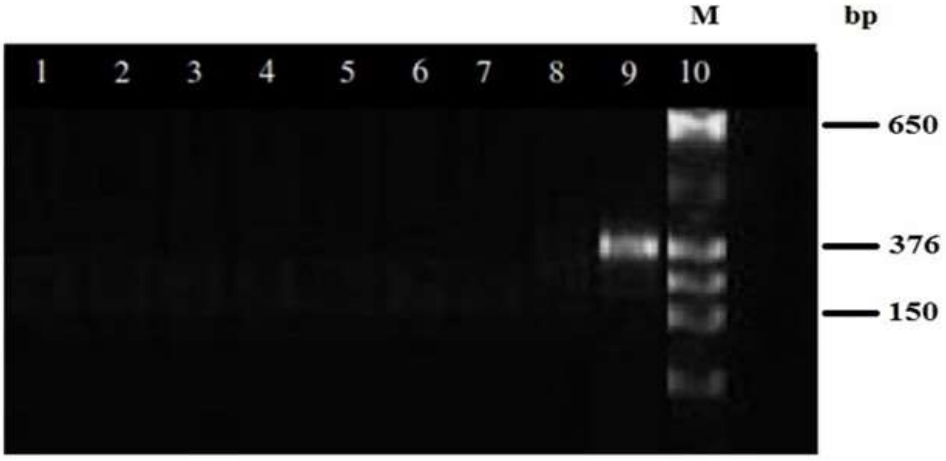

Figure 1C: ***bla***$_{OXA-58}$ **amplication product of 376 bp**

Key: Lanes 1- 7, phenotypic MDR *Acinetobacter* spp., lane 8, ATCC19606,
Lane 9, positive control; lane 10, molecular weight markers.

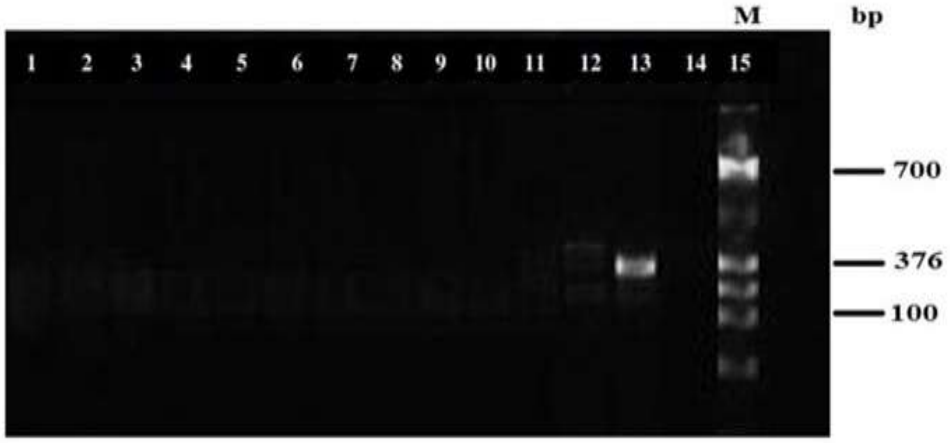

Figure ID: ***bla*$_{OXA-58}$ amplication product of 376 bp.**

Key: Lanes 1- 11, phenotypic MDR *Acinetobacter* spp. (*bla*$_{OXA-58}$ gene was not detected) lanes 13, positive controls; lane14, negative control;
Lane 15, molecular weight marker (376 bp)
ATCC 19606 isolate was included.

Correlation between PCR results and MICs of MDR *Acinetobacter* spp. (n=18)

Table 2 shows the MICs of the eighteen MDR *Acinetobacter* spp. isolates, correlated with the presence of the CHDL genes. The *bla*$_{OXA-23}$ gene was detected in all the isolates which were phenotypically resistant to carbapenems, with MICs of 8 to >16µg/mL for imipenem and meropenem. This gene was not detected in the four carbapenem sensitive strains tested (Figure 1A).

Genotypic relatedness of clinically selective *Acinetobacter* spp. determined by PFGE (n=24)

Twenty-four out of forty-four MDR *Acinetobacter* spp. isolates were selected according to clinical characteristics for genotyping by PFGE. [37]All isolates except for four in lanes 3, 5, 19 and 25 for the strains 12, 9, 16 and 14 were successfully typed (Figure 2). The MICs of those four isolates were different although they possessed the same antibiogram. Therefore the PFGE typing was not repeated for those four isolates. The control isolates (ATCC 19606), lane 28 (OXA-23) and lane 29 (OXA-58) showed a fingerprinting pattern different to the rest of the isolates, indicating that the technique was suitably discriminatory for the investigation of their spread (Table 4).

Two clusters consisting of two isolates each (30 and 19 in lanes 13 and 15) and (24 and 25 in lanes 9 and 10) were indistinguishable and identified as horizontal transfer between each pair (Table 4; Figure 2). According to the Tenover criteria[36], isolates that demonstrate no band differences are regarded as indistinguishable, and 2-3 band differences are closely related, and are therefore, most likely related to each other and interpreted as horizontally transmitted. Isolates with 4-6 band differences are possibly related. The interpretation of PFGE typing results according to the Tenover criteria are shown in Table 4. Based on this interpretation, MDR *Acinetobacter* spp. were most likely horizontally transferred among seven pairs of patients: six pairs in ICUs and one pair in the vascular unit (VU).

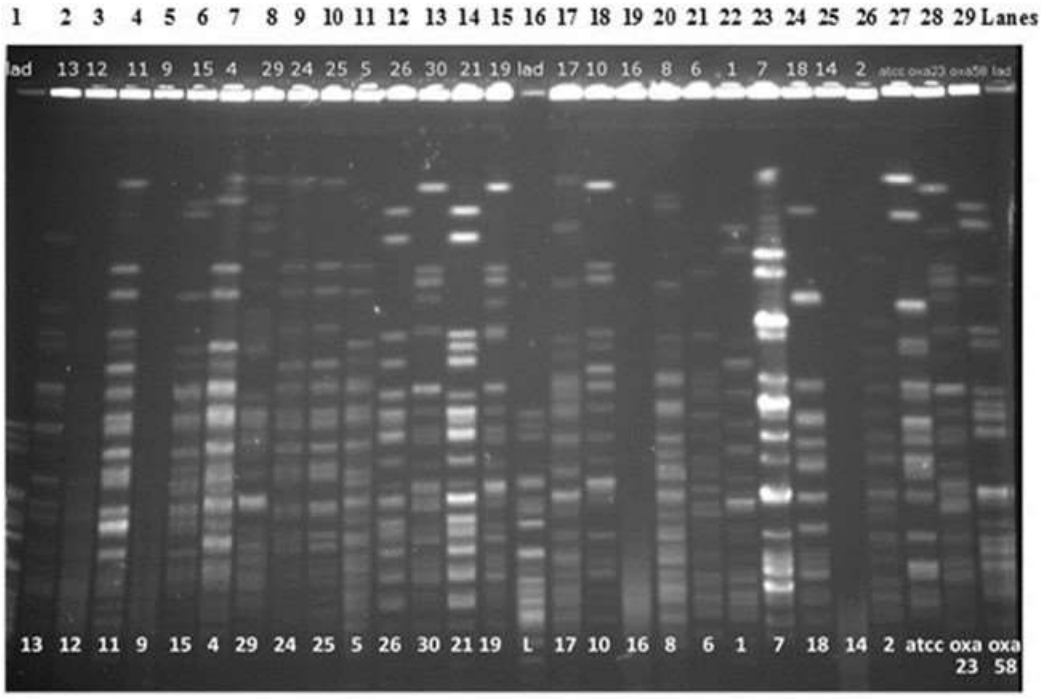

Figure 2: Pulsed field gel electrophoresis results of the OXA-23 producing *Acinetobacter* spp.

Key: Lane 27: ATCC 19606; Lane 28: OXA-23, lane 29: OXA-58 strains controls; lanes 2-15 and lanes 17-26, *Acinetobacter* spp. (n=24 isolates); lane 16, *Salmonella Braenderup* (H9812) used for the DNA ladder.

Table 4: PFGE typing interpretation (n=24)

No. of pairs (patients)	Typical no. of fragment differences (pulsotypes)	Admission Date	Specimens	Wards	Epidemiologic interpretation /Comments *
1	0	Within a week	BC-CSF,	ICU2A-ICU2 B	Indistinguishable horizontal transfer
1	0	Same date	CSF-ETA	ICU2A	Indistinguishable horizontal transfer
1	1	Within a week	BC- BC	ICU2B- NICU	Closely related horizontal transfer
2	2	Same date	BC- CVPtip CSF-Pus	ICU2A x 2**	Closely related horizontal transfer
1	2	Same date	ETA-Pus	ICUT	Closely related horizontal transfer
1	3	Within a week	ETA-ETA	VU	Closely related horizontal transfer
4	5	Within a week	CVPtip-CSF CVPtip-BC CVPtip-Pus CVPtip-ETA	ICU2A x 4**	Possible related
4	6	Within a week	BC-Pus BC-ETA	ICU2A-ICUT ICU2A-VU x 2** ICU2B-NICU	Possible related
7	≥7	>one week	ETA-ETA ETA-CSF ETA-Pus CSF-Pus	ICU2A- NICU ICU2A-VU x 4** VU-VU, VU-ICUT	Unrelated

Key: **PFGE typing interpretation according to Tenover criteria[36]**

* One pair (2 patients) (horizontal transfer between 2 patients/one pair);

** pairs; 2 pairs of patients in ICU2A and horizontal transfer in each pair, possible related in each pair x 4 pairs in ICU2A, possible related in each pair x 2pairs in ICU2A and VU, unrelated in each pair x 4 pairs; VU, vascular unit; BC, Blood culture;

CSF, Cerebrospinal fluid, ETA, Endotracheal aspirate; CVP tip, Central venous pressure line tip.

Correlation between clinical characteristics and laboratory results (n= 44)

Of the forty-four stored MDR *Acinetobacter* spp. isolates, thirty-eight (86%) and forty-four (100%) were sensitive to amikacin and colistin respectively (Table 5). The MICs of both imipenem and meropenem against OXA-23 carrying *Acinetobacter* spp. were high (8 to >16µg/mL) (Table 2). The clinical characteristics and outcomes of patients with *Acinetobacter* spp. producing $bla_{\text{OXA-23}}$ gene are shown in Table 3. Fourteen (78%) of the eighteen strains showed identical antibiograms sensitive to amikacin and colistin. Four (22%) showed the same phenotypic antibiogram and were only sensitive to colistin, therefore defined as XDR. The fourteen (78%) colstin and amikacin sensitive isolates were obtained from patients in ICUs and the remaining four (22%) from non-ICUs.

Eleven (61%) of the eighteen OXA-23 carrying MDR *Acinetobacter* spp. cultured were from sterile sites, namely blood cultures and CSF, while seven (39%) were from non-sterile sites such as ETA, pus, catheterised urine and catheter tip.

Eight (45%) patients were treated with monotherapy and ten (55%) with combination therapy. Eleven patients (61%) were discharged. The seven (39%) patients who demised were mostly admitted at ICUs among the patients with OXA-23-carrying *Acinetobacter* spp. (Table 3). Of the seven pairs of patients from whom the horizontally transferred strains were cultured from ICUs (six pairs) and the vascular unit (one pair), both in two pairs demised and three pairs were discharged. Of the remaining two pairs of patients, one of pair demised and one of other pair was discharged (Table 3).

The results clearly indicate there are several pulsotypes of *Acinetobacter* spp. within wards studied at IALCH (Table 4). There were four distinct pulsotypes identified suggestive of horizontal transfer of organisms among the ICUs: (NICU, ICU2A and ICU2B), (ICUT and ICU2A and within ICU2A itself) and between the ICU2A and the non- ICU (Vascular unit) ward. There were several isolates which were unrelated to the four main pulsotypes. No conclusion could be made regarding how they were acquired in the patients. However, the PFGE typing showed diversity in these collection of MDR *Acinetobacter* spp. clones, where isolates were related from no more than two patients. Therefore it is likely that no outbreak had occurred.

Table 5: MICs of appropriate antibiotics (n=44)

MICs µl/mL									
No. of isolates (n=44)	TZP	AK	CAZ	CIP	IMP	MEM	CST	Specimen	Ward
21	>128	16	64	>4	>16	>16	<0.5	BC	ICU 2A ,2B
3*	>128	>64	64	>4	>16	>16	<0.5	ETA	ICU2A
3	>128	8	64	>4	>16	>16	<0.5	BC	ICU2B
2	>128	8	16	>4	>16	>16	<0.5	TIP	ICU2B
1	>128	4	8	>4	>16	>16	<0.5	BC	ICU2B
1	>128	8	8	2	>16	>16	<0.5	ETA	ICU2A
1	>128	16	64	>4	8	>16	<0.5	ETA	ICU2A
2	>128	<2	64	>4	>16	>16	<0.5	PDF	ICUT
1	>128	8	8	>4	>16	>16	<0.5	PUS	ICUT
1	>128	16	64	>4	8	>16	1	URINE	ICUT
1*	>128	64	64	>4	8	>16	1	PUS	Non-ICU
2	>128	8	32	>4	>16	>16	<0.5	CSF	Non-ICU
2*	>128	64	64	>4	>16	>16	<0.5	BC	Non-ICU
1	>128	16	2	>4	8	>16	<0.5	PF	Non-ICU
1	>128	16	8	<0.25	>16	>16	<0.5	ETA	Non-ICU
1	>128	4	64	>4	>16	>16	<0.5	FLUID	Non-ICU

Key: TZP, piperacillin-tazobactam; AK, amikacin; CAZ, ceftazidime; CIP, ciprofloxacin; IMP, imipenem; MEM, meropenem; CST, colistin
*, XDR-*Acinetobacter* spp. (resistant to all except colistin);
Non-ICUs (high care unit, vascular unit, labour ward, neurosurgery unit, Orthopedic unit)

Discussion

All tested *Acinetobacter* spp. (n=44) were MDR, defined in the current study as resistant to three or more classes of drugs or carbapenem,[27] and inclusive of XDR, defined as MDR *Acinetobacter* spp. resistant to all agents except colistin. Previous studies confirmed that *Acinetobacter* spp. are resistant to many antibiotics [2-4, 12, 13, 38] and susceptibility tests revealed that the strains displayed

409 the colistin-only-sensitive (COS) profile. Multidrug-resistance of *Acinetobacter* spp. is a major
410 challenge and treatment options for these infections are limited.
411 This study has demonstrated that carbapenem drugs are no longer active against selected MDR
412 *Acinetobacter* spp. at local setting. The data revealed isolates with high susceptibility to amikacin
413 (86%) and colistin (100%) among the forty-four tested MDR *Acinetobacter* spp. isolates.
414 Therefore, these agents are the mainstay, last resort antibiotics for MDR *Acinetobacter* spp. in the
415 local setting. In this current situation, there are no standardised guidelines for the management
416 for MDR- and XDR- *Acinetobacter* spp. infection.
417 *Acinetobacter* spp. isolates with the same sensitivity pattern in the antibiograms were selected for
418 molecular investigation, namely four XDR and fourteen MDR isolates. BlaOXA-23 encoded-
419 OXA-23-carbapenemase was detected in MDR and XDR but not sensitive *Acinetobacter* spp. at
420 intensive care units in an academic complex.
421 The $bla_{\text{OXA-23}}$ gene is believed to be responsible for the mechanism of carbapenem antibiotic
422 resistance in *Acinetobacter* spp.[39] The OXA-23 gene of carbapenem resistant *Acinetobacter* spp.
423 was first reported in 1985 in Scotland[40] and subsequent outbreaks of OXA-23-producing *A.*
424 *baumannii* occurred in various locations around the world. [39, 40]
425 MDR *Acinetobacter* spp. represents a high-risk global and local infection control challenge.[40] In
426 one study, the gene encoding OXA-23 was found in plasmids, facilitating its spread among
427 *Acinetobacter* species.[14] In this study, the spread of $bla_{\text{OXA-23}}$ carrying *Acinetobacter* spp. was
428 demonstrated by PFGE typing. These findings suggest horizontal transfer between the pairs of
429 patients in ICUs and the vascular unit (Table 4). Moreover, it should be noted that an isolate in
430 the neurology ward adjacent to the ICU unit showed possible relatedness. Thus, it is possible that
431 the establishment of clones in different wards does not account for clonal transmission in
432 hospitals.
433 The OXA-23 producing MDR *Acinetobacter* spp. isolates were cultured from both sterile and
434 non-sterile clinical specimens, hence the recommendation for aseptic handling of specimen
435 collection. In addition, hand washing practices need to be audited due to this investigation
436 confirming the dramatic rise of multiple clones with $bla_{\text{OXA-23}}$ producing MDR *Acinetobacter* spp.
437 in the local setting. Awareness of the development and existence of drug resistant organisms plays
438 a crucial role in optimising infection control practices, establishing antimicrobial stewardship
439 programs, and establishing active regional surveillance systems. This study also showed a
440 correlation between the MDR phenotype and genes related to carbapenem resistance. MDR
441 *Acinetobacter* spp. with resistant MICs to either of the carbapenem agents may be associated with
442 isolates producing the plasmid-mediated or chromosome-mediated gene encoding $bla_{\text{OXA-23}}$.
443

During this study time, the antibiograms of all these isolates were phenotypically identical for more than three strains in ICUs. However, the PFGE typing demonstrated a diversity in the MDR *Acinetobacter* spp. clones, which suggests that isolates were related from not more than two patients per cluster, and therefore, no outbreak had occurred based on the PFGE typing interpretation. Therefore, isolates with the same antibiogram nevertheless need to be typed genotypically, and as a minimum, their MIC values should be checked.

According to data analysis of clinical characteristics, patient outcomes and laboratory data, the clinical outcomes had no association with the following factors: resistance patterns of *Acinetobacter* spp. cultured, ICU *versus* non-ICU wards, sterile sites *versus* non-sterile sites, and monotherapy *versus* combination therapy. There was no statistical significance (p >0.05) in each analysis (Table 3). This may be due to the small sample size, which is the main limitation of our study. The selected small representative strains were subjected to molecular methods of analysis due to the financial limitation and lack of facilities at the molecular laboratory in the local setting. Continuous surveillance of antibiotic resistance genes in MDR *Acinetobacter* spp. is crucial for epidemiological purposes and to prevent further dissemination of these genes. In addition, it is necessary to monitor the clinical prevalence and spread of antibiotic resistance genes associated with *Acinetobacter* spp. Future research should include the detection of other resistance genes, as well as determining the genetic relatedness of *Acinetobacter* spp. isolates in other hospitals in KZN.

In conclusion, this study discovered that the main carbapenem resistance mechanism of *Acinetobacter* spp. was due to OXA-23 carbapenemase activity. Although the isolates were spread in ICUs and other ICU related units, there did not seem to be an outbreak according to the demographic clinical data, MICs and PFGE typing (Table 4).

This is the first report on epidemiological and molecular observations of *Acinetobacter* spp. with the detection of the $bla_{\text{OXA-23}}$ gene in MDR *Acinetobacter* species. Molecular typing of the selected isolates showed that MDR *Acinetobacter* species carried the $bla_{\text{OXA-23}}$ gene responsible for resistance to carbapenems (MICs 8 to >16 µg/mL). The outcomes provided support for a local infection prevention and control management guidelines as part of the antibiotic stewardship programme. Continued molecular surveillance of local epidemiological information and antibiotic resistance surveillance are crucial for infection prevention and control and also for an essential part of standard management at the hospital.

Contribution by first author

Poster presentation at UKZN Research day conference -2016 [2nd winner awarded]

Author's contributions as details

First author's (K. Swe Swe –Han) contributions:

(1) The conceptualisation and design of the study, ordering of the laboratory reagents for identification, susceptibility testing and molecular work.

(2) As a pathologist (microbiologist), interpretation of laboratory results and regular ward rounds. Based on the collaboration with clinicians and clinical characterisation and laboratory results of patients, the isolates of *Acinetobacter* spp. were selected and stored for the study.

(3) Subculture of the isolates, identification, confirmation of susceptibility by repeating the Vitek 2 and MICs with E- test.

(4) DNA extraction (in-house method) and PCR after training and assistance by the Medical Scientist.

(5) Arranged collaboration and transportation of the stored isolates to the molecular laboratory, NHLS, Johannesburg (JHB) for PFGE typing.

(6) Data acquisition, analysis and interpretation.

(7) Drafted the manuscript, critical revision for intellectual content, communication with the English editor and final approval of the version to be submitted.

Prof. M. Pillay, Prof. K. P Mlisana and Dr K. Baba helped design the study and edited the manuscript.

Prof. M. Pillay also provided scientific input into the PCR, and critically reviewed the drafts and final revised manuscript.

Melendhran Pillay, Medical Scientist, assisted with the molecular work such as DNA-extraction, PCR and sequencing, contributed the methodology of the in- house methods. He also reviewed the methods section of the manuscript.

At molecular laboratory in Johannesburg, PFGE typing and the report were performed by Karren le Roux, Medical scientist.

All authors read and approved the final manuscript.

Funding

The studies was funded by MEPI, NHLS and UKZN.

Acknowledgement

Medical Education Partnership Initiative (MEPI- 5R24TW008863), Medical Microbiology Department, NHLS, Inkosi Albert Luthuli Academic Complex,
University of KwaZulu-Natal.

Conflict of Interest

None declared.

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CHAPTER 6: ORIGINAL ARTICLE

Colistin resistant clinical *Acinetobacter* species may be mediated by the absence of the *Ipx*A gene at an academic complex hospital in Durban, KwaZulu-Natal, South Africa

African Journal of Laboratory Medicine: (Manuscript Reference number: No.AJLM: 597).

ORIGINAL RESEARCH ARTICLE

Title: **Colistin resistant clinical *Acinetobacter* species may be mediated by the absence of the *Ipx*A gene at an academic complex hospital in Durban, KwaZulu-Natal, South Africa**

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Abstract

Introduction: Drug resistant *Acinetobacter* species (*Acinetobacter* spp.) presents a serious therapeutic and infection control policy challenge globally. This study investigated the relationship between the minimum inhibitory concentrations (MICs) of standard drugs against *Acinetobacter* spp. and genes associated with colistin and amikacin resistance. The association between drug resistance and clinical outcomes of patients with *Acinetobacter* spp. in a central academic hospital was also determined.

Method: Case information from 107 patients cultured with *Acinetobacter* spp. was recorded during clinical wards rounds, including clinical outcomes, history of antibiotics prescribed and microbiological investigations. The 107 *Acinetobacter* spp. isolates were investigated for susceptibility to antimicrobial agents in use at local hospitals. Resistant genes related to colistin (*Ipx*A) and amikacin (*aph*A6) were investigated by polymerase chain reaction (PCR) and sequencing. Analysis was performed on the relationship between clinical outcomes and antimicrobial resistant patterns, as well as on the MICs of amikacin (n=6) and colistin (n=6) in resistant isolates *versus* their PCR results.

Results: Amikacin and colistin resistance were observed in six isolates each. All six amikacin resistant isolates were extensively drug resistant (XDR). The MICs were >16 μg/mL for the six colistin resistant isolates and 32 and ≥64 μg/mL for the amikacin resistant isolates. The *Ipx*A gene was absent in colistin resistant isolates and correlated with high MICs. The *aph*A6 gene was detected in all amikacin resistant isolates. While the majority (63%) of cases were discharged, mortality rates were high (21.5%). No underlying clinical factors were significantly associated with clinical outcome.

Conclusion: Colistin resistance may be associated with the absence of the *Ipx*A gene and is not a surrogate marker for MDR *Acinetobacter* species. The emergence of colistin resistance is of serious concern, highlighting the urgency for standardised guidelines for the treatment and management of *Acinetobacter* species.

Key words: molecular characterisation, *Ipx*A gene, *aph*A6 gene, phenotypic antibiogram, clinical outcome

Introduction

Acinetobacter species (*Acinetobacter* spp.) have emerged as major hospital-associated pathogen, which have developed into multidrug-resistant (MDR) and extensively drug-resistant (XDR) isolates in the past decade.[1] *Acinetobacter* spp. have the capacity to acquire resistance to antimicrobial agents through genetic factors, such as plasmids and pathogenicity islands,[2] resulting in resistant strains that are difficult to treat.[3] Therefore, the Infectious Diseases Society of America (IDSA) has included *Acinetobacter* spp. among six antimicrobial-resistant pathogens responsible for high morbidity and mortality.[3,4]

Although *Acinetobacter* spp. are common coloniser that may lead to community-acquired infection, also an opportunistic pathogen often found in immunocompromised patients with prolonged hospitalisation.[5] Immunosuppressive therapy places cancer patients at risk of developing *Acinetobacter* spp. infections which may result in sepsis, respiratory infections, wound infections and urinary tract infections. [3, 6-8]

Extensively drug- resistant (XDR) *Acinetobacter* spp. are defined as being resistant to all the tested antimicrobials, except for colistin, while pandrug-resistant (PDR) isolates are resistant to all agents.[9] A rise in infections from XDR *Acinetobacter* spp. has been reported. [10,11.] The global rise of multidrug-resistant (MDR) *Acinetobacter* spp. and the emergence of XDR and PDR *Acinetobacter* spp. therefore poses a major challenge to current treatment options and infection control.[12,13]

Until recently, amikacin was the most active aminoglycoside in the treatment of infections caused by *Acinetobacter* spp., especially in our local academic complex hospitals. They remain the drugs of choice for treatment of MDR *Acinetobacter* infections, yet resistance has increased in recent years.[14]

Acinetobacter spp. have several mechanisms of aminoglycoside resistance.[15,16] In general, the major mechanism in Gram-negative bacteria is enzymatic modification of the amino or hydrolol groups of the agent through aminoglycoside modifying enzymes (AMEs), most commonly acetyltransferases (AAC), nucleotidyl transferases (ANT) and phosphotransferases (APH). The enzymes alter the amino and hydroxyl groups of the agent, resulting in reduced binding to the ribosome. [15-17] AACs and APHs produce high levels of resistance. Amikacin resistance in *Acinetobacter* spp. is facilitated by APH (3')-VI, corresponding with the *aph*A6 gene.[14, 17] In addition, the *aac*A4 gene, which encodes AAC (6')-Ib, confers resistance of amikacin, netilmicin, and tobramycin, [15] while *aad*B is associated with resistance of kanamycin, gentamicin and tobramycin.[14]

Therapeutic agents for XDR *Acinetobacter* spp. infection often include colistin methansulfonate (CMS), a bactericidal agent used as a last resort.[14] However, there has been an increased use of colistin for treating MDR infections, leading to the emergence of colistin resistance.[18-23]

Colistin interacts with the lipid A components of lipopolysaccharide (LPS), disrupting the outer membrane of Gram-negative bacteria.[18] Colistin resistance in *Acinetobacter* spp. is mediated by a range of mutations affecting the structure and production of LPS. Colistin resistance in *Acinetobacter* spp. is mediated by a range of mutations affecting the structure and production of LPS. Mutations in the *lpx*A, *lpx*C and *lpx*D genes result in loss of LPS production. Mutations in the *pmr*A and *pmr*B genes of the two-component regulatory system, as well as in the *pmr*C gene coding for a lipid A phosphoethanolamine transferase, result in the modification of LPS.[18-23]

Fortunately, the local data showed that amikacin sensitivity was high (59% to 90%), and 99% of *Acinetobacter* spp. were still sensitive to colistin during the period 2008 to 2014. These two drugs are commonly used in our local academic complex hospital due to the increasing prevalence of MDR and XDR *Acinetobacter* species.

Despite the possible future risk, data in local academic complex hospital is scarce regarding the clinical, microbiological and molecular characteristics of *Acinetobacter* spp., including resistance mechanisms of amikacin and colistin-resistant infections. Such information will facilitate a better understanding of the pathogen, in order to formulate guidelines for a standardised approach to management.

This study aims to characterise *Acinetobacter* spp. isolates at academic complex central hospital in Durban, South Africa: by i) evaluating their susceptibility to colistin and amikacin and determining the minimum inhibitory concentrations (MICs); and ii) comparing the clinical outcomes of infected patients with phenotypic and genotypic characteristics of XDR and colistin resistant *Acinetobacter* species.

Methods

The study received ethical approval from the Biomedical Research Ethics Committee, College of Health Sciences, University of KwaZulu-Natal (Reference No BE 283/12).

Patients and bacterial isolates

Non duplicate *Acinetobacter* spp. isolates (n=60) were selected and stored from the specimens of 107 patients at Inkosi Albert Luthuli Central Hospital (IALCH) from 2013 to 2014.

107 patients were included for the analysis of clinical outcomes and antimicrobial resistant patterns: Information, including clinical outcomes, prescribed antibiotic history and microbiological results of 107 patients cultured with *Acinetobacter* spp. were recorded during clinical wards rounds. The 107 *Acinetobacter* spp. were analysed for the susceptibility to antimicrobial agents in use at local hospitals. The association of the clinical outcomes *versus* antimicrobial resistant patterns of the isolates from the 107 patients was analysed. These isolates were identified and tested for resistance to antimicrobial agents in use at local hospitals for routine management.

Amikacin and colistin-resistant *Acinetobacter* spp. were stored for further phenotypic and genotypic characterisation, at the Microbiology Laboratory, National Health Laboratory Service (NHLS), Durban.

Based on the antibiogram of the isolates from the 107 patients and MICs of 60 isolates, the six colistin resistant, another six amikacin resistant and seventeen susceptible isolates were identified. The presence of the genes related to colistin (*Ipx*A), amikacin (*aph*A6) and (*aac*A4) resistance were further investigated by using polymerase chain reaction (PCR). The MICs of amikacin (n=6) and colistin (n=6) were compared to the PCR results of these resistant isolates.

The *Ipx*A gene detected isolates, as well as the *aph*A6 gene detected isolates were sequenced.

Susceptibility Testing

Susceptibility testing was performed using the Vitek 2 automated system (BioMérieux, France) with the VITEK® 2 GN ID card and the VITEK®2 AST-N255 card. The MICs of the appropriate antimicrobial agents in use were determined for sixty *Acinetobacter* spp. isolates using the Epsilometer test (E-test®) (BioMérieux, France). The MIC_{90} and MIC_{50} were determined for each tested antibiotic agent against the sixty isolates. The antibiotics included amikacin, cabapenems (imipenem, meropenem), ceftazidime, ciprofloxacin, colistin and piperacillin-tazobactam. *Acinetobacter* ATCC 19606 was used as the quality control strain. The results were interpreted according to the Clinical and Laboratory Standards Institute.[24] MIC >32 μg/mL for amikacin and >0.5 μg/mL for colistin were considered to be resistant.[24]

Molecular methods (PCR and Sequencing)

Genomic DNA, from each of twenty-seven isolates, comprising six clinical colistin resistance strains, six amikacin resistance strains, three controls and twelve sensitive clinical isolates, was extracted from an overnight culture using a loopful of colonies suspended in 500 μL of 1x TE buffer (Tris EDTA) (10 mM Tris hydrochloride-1 mM EDTA (pH 8.0), (Capital Laboratory Supplies, Durban, South Africa). Cell lysis and protein digestion were performed using a

combination of 10% Sodium Dodecyl Sulfate and 10 mg/mL (1%) proteinase K (Capital Laboratory Supplies, Durban, South Africa). Proteins were precipitated with 10% Cetyltrimethyl ammonium bromide (CTAB)/4% NaCl and polysaccharides were extracted by the addition of 24:1 chloroform-isoamyl alcohol (Capital Laboratory Supplies, Durban, South Africa). The DNA was precipitated with isopropanol, washed with 70% cold ethanol (Capital Laboratory Supplies, Durban, South Africa) and dissolved in an appropriate volume of 1xTE buffer. The DNA was electrophoresed in a 1% agarose gel (Capital Laboratory Supplies, Durban, South Africa) to determine quality and quantity. The extracted genomic DNA was stored at -20°C.[25, 26] (Defy Ltd, Multimode, SA) until further analysis.

PCR to detect the *Ipx*A gene was performed on six colistin resistant and seventeen colistin susceptible *Acinetobacter* spp. isolates. The amikacin resistant genes *aph*A6 and *aac*A4 were also investigated on twelve isolates (including controls). PCR was performed using primer sets shown in Table 1 and *Taq* DNA polymerase (ThermoFisher Scientific, USA). The thermal cycling conditions were set for initial denaturation at 5 min at 95°C followed by 40 cycles of 20 s at 95°C, 1 min at 55°C and 30s at 72°C. This was followed by a final extension step of 5min at 72°C. A negative template free control and an ATCC19606 control were included. Amplification was repeated at least twice for strains with negative PCR results. The expected PCR product sizes of 1179 bp for *Ipx*A, 797 bp for *aph*A6 and 489 bp for *aac*A4 were detected on a 1.5% agarose gel (Capital Laboratory Supplies, Durban, South Africa).

Mutations in the amplified genes were determined by purification of amplicons with Shrimp Alkaline Phosphatase and exonuclease 1 as per manufacturer's instructions followed by sequencing using the ABI Big Dye Terminator v 3.1 Cycle Sequencing kit (Applied Biosystems, USA) in an ABI 3500 Genetic Analyser (ThermoFisher Scientific, Waltham, Massachusetts). Sequences were analysed, aligned and compared using the Basic Local Alignment Searching Tool (BLAST). http://www.ncbi.nlm.nih.gov/blast/

Comparison colony morphology of colistin resistant and sensitive *Acinetobacter* spp. (n: 10)

Six colistin resistant and four sensitive isolates were subcultured on MacConkey agar plates to examine colony morphology. Plates were inoculated with a bacterial suspension of optical density equivalent to the 0.5 McFarland standardand incubated aerobically at 37°C for 24 hrs and the colony morphology was examined.

Clinical and laboratory data collection

The clinical and laboratory data of 107 patients were analysed. The data included demographics, underlying medical condition, type of specimen, exposure to antimicrobial agents before and after isolation of *Acinetobacter* spp. isolates, admission to intensive care units or other units and clinical outcomes. The type of infection was defined by the clinicians. Patients who did not receive specific treatment for *Acinetobacter* spp. were classified as colonised. Clinical response to treatment was classified as successful in patients whose infection-defining signs and symptoms resolved, and as failed for patients who deteriorated or whose signs and symptoms persisted.

Statistical analysis of the data

The data was captured, standardised and analysed using the Statistical Package for Social Sciences (SPSS version 19). The association between underlying conditions and outcome was analysed using the Pearson chi-square test. Logistic regression was used to test for factors associated with the survival status of patients. This analysis represents *p* values mentioned under "Demographic features, clinical characteristics and outcomes of all patients with infections due to *Acinetobacter* spp. (n=107).

Results

Susceptibility of *Acinetobacter* spp. strains (n=107)

Six isolates (5.6%) that were resistant to amikacin were defined as extensively drug-resistant (XDR) based on their antibiograms. Another six (5.6%) were resistant to colistin. Eighty isolates (75%) were MDR. The rest were resistant to less than three different class tested agents and therefore not classified as MDR (Table2). Table 3 shows the antimicrobial MICs of sixty *Acinetobacter* species. The MIC_{50} and MIC_{90} of imipenem differed at 24 and >32 μg/mL respectively. Both MIC values were the same for ciprofloxacin and piperacillin-tazobactam, at >32 and >256 μg/mL respectively. The MIC_{50} and MIC_{90} of amikacin (8 and 16 μg/mL) and colistin (0.25 and 0.5 μg/mL) were within the sensitive range among the tested antibiotics.

Among the six colistin resistant isolates, the MICs of colistin were >16 μg/mL, while among the other six amikacin resistant isolates, the MICs of amikacin ranged between 32 and ≥ 64 μg/mL (Table 4).

Colony morphology of colistin resistant species on MacConkey plates

No difference was observed in the appearance of the colonies of the resistant and susceptible strains cultured on McConkey agar plates. The strains looked like those that can be considered wild-type.

Detection of the *Ipx*A, *aph*A6 and *aac*A4 genes

Six patients with colistin-resistant and six with amikacin-resistant *Acinetobacter* spp. were identified in the study period between 2013 and 2014 (Table 5).

From the twenty-three (clinical isolates and the ATCC19606 control isolate), the *Ipx*A gene was not detected by PCR in the six colistin resistant isolates. In contrast, the remaining seventeen isolates, phenotypically sensitive to colistin with MICs <0.5 µg/mL, harboured the *Ipx*A gene. (Table 4, Figures 1A, 1B).

PCR amplification allowed for detection of the *aph*A6 gene (797 bp) from the six amikacin resistant *Acinetobacter* spp. clinical isolates [Figure1C]. However, the *aac*A4 gene (489 bp) was not present in these isolates [Figure 1.D]. Sequencing of the *Ipx*A in susceptible isolates and *aph*A6 amplicons revealed 100% identity with the genes specifically related to *Acinetobacter* spp. listed in the GenBank database.

Phenotypic and genotypic analysis of the colistin resistant and amikacin resistant *Acinetobacter* spp.

Twenty-three *Acinetobacter* spp. isolates comprising colistin resistant (n=6) and colistin sensitive (n=17), were characterised phenotypically and genotypically. The MICs of colistin and other drugs against these isolates are shown in Table 4.

One of the six colistin resistant isolates was resistant to piperacillin-tazobactam (MIC >256 µg/mL) and sensitive to the other agents. However, the remaining five isolates were sensitive to appropriate drugs, including amikacin, carbapenems (imipenem, meropenem), ceftazidime, ciprofloxacin and piperacillin-tazobactam.

Correlation of antibiogram with *Ipx*A gene

From the selected strains, colistin-susceptible *Acinetobacter* spp. isolates (n=17) that were identified by MIC values (Table 4) also showed amplification of the *Ipx*A gene. Fourteen [82% (17)] were resistant to meropenem and imipenem (MDR AB), and three [18%) (17)] were sensitive to only colistin (XDR AB) (Table 4).

All seventeen colistin-susceptible *Acinetobacter* spp. isolates harboured the *Ipx*A gene. All six colistin-resistant *Acinetobacter* spp. isolates showed an absence of the *Ipx*A gene (Figure 1 A-B).

Seventeen strains out of the selected twenty-three [74% (23)] had colistin-susceptible *Acinetobacter* spp. isolates which correlated phenotypically and genotypically (Table 4, Table 5).

Correlation of antibiogram with *aph*A6 and *aac*A4 genes

The MICs of amikacin and other tested drugs are shown in Table 4. The six amikacin resistant strains were sensitive to only colistin and thereby defined as XDR *Acinetobacter* spp. (Table 4, Table 5). These six strains were phenotypically resistant and showed the presence of the *aph*A6 gene but not the *aac*A4 gene (Figure 1C, 1D).

Demographic features, clinical characteristics and outcomes of all patients with infections due to *Acinetobacter* spp. (n=107)

Clinical data was analysed by using simple descriptive data analysis. The demographic data of patients with *Acinetobacter* spp. (n=107) are shown in Table 6. More males than females were infected, at a ratio of 3:1 in children and 3:2 adults, with the predominant age group 25 to 60 (Table 6). *Acinetobacter* spp. were more commonly isolated from adult patients in non-ICU wards and in neonates among pediatric patients.

Underlying diseases

Acinetobacter spp. were cultured more commonly in adults presenting with trauma and injury, and in pediatric patients with abnormal congenital organs. Trauma was predominant overall. Retroviral disease (RVD), oncology and other conditions showed little risk of colonisation and infection (Table 6). No statistically significant difference [P >0.05 (0.151)] were observed between children and adults with medical and surgical conditions and the presence of *Acinetobacter* spp. infections.

Antibiotic usage

Tazocin (piperacillin–tazobactam), ciprofloxacin and meropenem were used in the majority of cases. Colistin monotherapy and colistin combinations were not commonly used. This analysis revealed that *Acinetobacter* spp. isolates were treated mostly with a piperacillin-tazobactam and amikacin combination, while XDR strains were treated with colistin monotherapy or other combinations according to individual cases (Table 6). The usage of colistin, combinations and amikacin showed a significant statistical difference between adult and pediatric patients [P <0.05; (0.018)].

Clinical outcome

The majority of cases, 67 (63%) of the 107, were discharged but mortality was high at 23 (21.5%) (Table 6). Clinical outcome was not significantly associated with age [P >0.05; (0.942)].

Clinical characteristics and outcomes of patients with infections due to colistin resistant *Acinetobacter* spp. (n=6)

The types of infection in patients harbouring colistin-resistant *Acinetobacter* spp. included bacteraemia and suspected line sepsis (Table 5). Five of the six isolates were cultured from blood, one from a catheter tip and all samples from patients with clinical sepsis. However, none of the patients with the colistin-resistant isolates were from the ICUs. Four patients were from labour ward and two patients had underlying cardiac disease. Five patients were discharged and one patient from the oncology unit demised after eight days in hospital (Table 4).

Clinical characteristics and outcomes of patients with infections due to amikacin resistant *Acinetobacter* spp. (n=6)

All six patients with amikacin resistant *Acinetobacter* spp. were hospitalised in different units for longer than two weeks (21 to 43 days) with chronic illness (Table 5). Two isolates were obtained from blood culture, three from pus swabs and one from an ETA. Two patients were treated with colistin while two received no antibiotics. Two out of the six demised while four recovered and were discharged (Table. 5).

Discussion

Despite *Acinetobacter* spp. being classified by the Infectious Diseases Society of America a decade ago as one of six most important MDR microorganisms in hospitals worldwide, [3, 4, 27] drug resistant *Acinetobacter* spp. still presents a serious therapeutic and infection control challenge. Increasing antimicrobial resistance among *Acinetobacter* isolates resulting in the evolution of XDR and PDR strains has been documented globally.[12]

This study revealed amikacin and colistin resistant *Acinetobacter* spp. isolates, with six (5.6%) of 107 of the isolates being amikacin resistant and sensitive only to colistin, defined as XDR *Acinetobacter* species. Interestingly, the other six (5.6%) of 107 colistin-resistant strains were not PDR, i.e. resistant to all appropriate tested drugs,[9] as they were sensitive to other appropriate antibiotics such as the carbapenems, ceftazidime, ciprofloxacin, amikacin and piperacillin-tazobactam, with the exception of one isolate that was resistant to piperacillin-tazobactam. These findings suggest that colistin resistance, therefore, is not a surrogate marker for MDR *Acinetobacter* spp. and even for PDR *Acinetobacter* spp. The rates of colistin resistance in the current study (5.6%) are slightly higher than that of another surveillance study (5.3%), [27] while much lower than rates those of study in Asia (28%). [28]

In this study, colistin-resistant *Acinetobacter* spp. was found mostly in patients who had not received prior colistin therapy. In contrast, a previous study determined the distinguishing factor of colistin resistance in *Acinetobacter* spp. as prior drug exposure.[29] This conclusion is similar to a report on colistin-resistant *Acinetobacter* spp. from the US military health system.[20]

According to Moffatt *et al.*, the complete loss of lipopolysaccharides (LPS) is responsible for polymyxin resistance in *Acinetobacter* species.[30] Mutations in either *Ipx*A, *Ipx*C or *Ipx*D were responsible for this complete loss of LPS production, resulting in high-level colistin resistance.[31-33] The current study revealed the complete loss of the *Ipx*A gene that encodes the initial binding target, the lipid A component of LPS in all colistin-resistant *Acinetobacter* spp. isolates, but in none of the colistin-susceptible isolates. Furthermore, sequencing revealed no polymorphisms were observed in the *Ipx*A gene in all 17 of the colistin sensitive isolates tested. These findings could possibly indicate a complete loss of the Lipid A motif due to the complete absence of *Ipx*A gene that encodes the Lipid A domain. However, it is possible that the *Ipx*A gene was not amplified due to the specifity of the primers for *A. baumannii* and not other *Acinetobacter* species. However, further research is necessary to confirm this, such as the inclusion of internal controls to exclude amplification inhibition, a second primer set to exclude mispriming, amplicon sequencing to detect for mutations in the *pmr*A and *pmr*B genes, whole genome sequencing and southern hybridisation. In addition, future research is essential to understand the mechanism by which the gene is lost.

The findings in this study also provides motivation for implementing enhanced infection control measures in patients colonised and infected with polymyxin resistant *Acinetobacter* spp., with the view of preventing its continued spread.

In this local setting, amikacin is commonly used with piperacillin-tazobactam as a second line treatment option in general antibiotic policy. Fortunately, 101 (94%) of 107 *Acinetobacter* spp. isolates were highly sensitive to amikacin. In the past, aminoglycosides have played a crucial role in the treatment of MDR *Acinetobacter* spp. However, recent reports indicated that *Acinetobacter* isolates are developing resistance to aminoglycosides around the globe.[33] Modifying enzymes such as acetyl transferases, phosphotransferases, and adenylyl transferases result in inactivation of aminoglycosides, leading to resistance,[14] and a range of resistance genes have emerged in recent times.[33] The current study showed that amikacin resistant *Acinetobacter* spp. isolates carry the *aph*A6 gene but not the *aac*A4 gene in the local academic complex hospitals in KwaZulu-Natal, South Africa. The prevalence at 5.6% was significantly lower in the local setting compared

to the study **i**n Korea, according to the KONSAR Study 2009, where amikacin-resistant *Acinetobacter* spp. increased to 48%.[33]

Our data analysis identified a potential emerging challenge to treatment and clinical management that was elucidated by phenotypic and genotypic characterisation of *Acinetobacter* species. Due to the MIC_{50} and MIC_{90} of imipenem, ciprofloxacin, ceftazidime and piperacillin-tazobactam were within the highly resistant range, while the MIC_{50} and MIC_{90} of amikacin and colistin were within the sensitive range among the tested isolates (Table 3), this study highlights the crucial role of amikacin and colistin usage standardly.

Therapy for MDR *Acinetobacter* spp. infection usually requires the use of other appropriate drugs based on the local antibiogram or individualised microbiological results. *Acinetobacter* spp. isolates were mostly treated with piperacillin-tazobactam plus amikacin, while XDR *Acinetobacter* spp. were treated with colistin monotherapy or combinations according to the individual case. However, there was no standard criteria and guideline for colistin therapy during the study period.

Previous studies have reported MDR *Acinetobacter* spp.-associated sepsis as more common in ICU patients.[1, 34, 35] *Acinetobacter* spp. in our study showed that the isolates were more common in both non-ICUs wards and, among ICU patients, were associated more with trauma cases. The prevalence was significantly lower in our study than in the literature.[33] All isolates were cultured from the specimens after 21 to 43 days of hospitalisation and prior to amikacin exposure.

Acinetobacter spp. were most prevalent in patients aged 25 to 60 in wards commonly including non-ICU, trauma and post-op pediatric units. Trauma cases were predominant overall, since *Acinetobacter* spp. is part of the skin flora and an environmentally acquired organism. Moreover, in this study, retroviral disease, oncology and other clinical conditions were not prone to colonisation and infection in the local academic hospital, possibly because infection prevention control measures are enhanced in all high care units.

The majority of the 107 patients were treated with antibiotics such as piperacillin-tazobactam, amikacin, ciprofloxacin and meropenem as per generalised local protocol. There was no standard criteria and guidelines for colistin therapy during study period. However, colistin monotherapy, drug combinations and amikacin with tazocin combination, were used significantly more in adult patients than pediatric patients [p <0.05; (0.018)]. XDR *Acinetobacter* spp. isolates were treated with colistin monotherapy or combinations according to the individual case, based on consultation

between the clinician and microbiologist. Our study highlighted that colistin is a key therapeutic option for the treatment of XDR *Acinetobacter* species. In addition, colistin-resistant *Acinetobacter* spp. is not necessarily MDR- or PDR-*Acinetobacter* spp. in our clinical setting.
While the majority of cases, 67/107 (63%) resulted in discharge, the high mortality rates 23/107(21.5%) are a serious cause for concern and interventions are urgently required to reduce this. According to the patient data, the usage of antibiotics should be standardised with appropriate guidelines that should be implemented as an antibiotic guideline policy for *Acinetobacter* spp. infections.

Colistin-resistant *Acinetobacter* spp. isolates were sensitive to other appropriate antibiotics from the academic hospital, suggesting that colistin resistance is not a surrogate marker for MDR *Acinetobacter* species. However, the emergence of colistin resistance in *Acinetobacter* spp. isolates in our local setting is of great concern and highlights the urgent need for standardised antibiotic guidelines, including colistin usage and antibiotic combinations specifically for the management of patients with MDR-, XDR-, and PDR-*Acinetobacter* spp. at academic complex hospitals in Durban and the wider KwaZulu-Natal. In another interesting observation, colistin resistant strains were sensitive to other appropriate antibiotics and colistin-resistant *Acinetobacter* spp. occurred mostly among patients who had not received previous colistin therapy. It is possible as these isolates were contracted from the hospital environment as wild-type. Four out of six isolates were isolated from the blood culture specimens of sepsis patients. This finding highlights the need to enhance infection prevention and control measures.

As far as we are aware, this study is the first to describe detailed clinical and molecular characteristics of colistin- and amikacin-resistant *Acinetobacter* spp. at local academic complex hospitals in KwaZulu-Natal. Molecular analysis suggested a potential mechanism of colistin resistance may be associated with absence of the *Ipx*A gene (requires confirmation) and for amikacin to be the presence of the *aph*A6 gene. However, additional molecular methods (Southern blotting and genome sequencing) to test the veracity of our *Ipx*A findings, will be planned for the near future, as funding is not available to perform this within the scope of the PhD.
The underlying clinical diseases were not significantly associated with clinical outcome in *Acinetobacter* spp. infections.
Molecular epidemiological studies are required when investigating transmission dynamics, which will in turn inform intervention strategies to prevent spread of drug resistant strains. Further studies should also focus on the best use of colistin to minimise the risk of developing increased

449 resistance. There is a need for continuous surveillance of antibiotic resistance genes and their
450 association with antibiotic resistance profiles. Infection prevention and control should also aim to
451 identify reservoirs and sources of infection in an attempt to recognise and prevent further spread
452 of MDR, XDR, and PDR *Acinetobacter* species.
453
454
455 **Acknowledgements**
456 The authors would like to express their gratitude to Fikile Nkwanyana for the assistance with
457 statistical analysis;
458
459 **Author's contributions**
460
461 **First author's contributions as details**
462 **First author's (K. Swe Swe –Han) contributions:**
463 (1) The conceptualisation and design of the study, ordering of the laboratory reagents for
464 identification, susceptibility testing and molecular work.
465 (2) As a pathologist (microbiologist), interpretation of laboratory results and regular ward rounds.
466 Based on the collaboration with clinicians and clinical characterisation and laboratory results of
467 patients, the isolates of *Acinetobacter* spp. were selected and stored for the study.
468 (3) Subculture of the isolates, identification, confirmation of susceptibility tests.
469 (4) DNA extraction (in-house method), primer selection and PCR after training and assistance by
470 the Medical Scientist.
471 (5) Data acquisition, analysis and interpretation.
472 (6) Drafted the manuscript, critical revision for intellectual content, communication with the
473 English editor and final approval of the version to be submitted.
474 Prof. M. Pillay, Prof. K. Mlisana and Dr. K. Baba helped design the study and edited the
475 manuscript.
476 Prof. M. Pillay also provided scientific input into the PCR, and critically reviewed the drafts and
477 final revised manuscript.
478 Melendhran Pillay, Medical Scientist, assisted with the molecular work such as DNA-extraction,
479 PCR and sequencing, contributed the methodology of the in- house methods. He also reviewed
480 the methods section of the manuscript.
481 All authors read and approved the final manuscript.
482
483

Funding information:

The study was funded by Medical Education Partnership Initiative (MEPI), UKZN.

Conflicts of interest

All authors declared no conflicts of interest.

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Table 1: PCR primer sequences of *Ipx*A, *aph*A6 and *aac*A4 Genes

Primers	Sequence (5' to 3')	References
***Ipx*A forward**	ACGCCAGGATCCGGTTCATTATTCCTGTTTGCT	**(18)**
***Ipx*A reverse**	ATTCAAGGATCCCACCTCGAGCATTGTACCA	**(18)**
***aph*A6 forward**	ATGGAATTGCCCAATATTATTC	**(36)**
***aph*A6 reverse**	TCAATTCAATTCATCAAGTTTTA	**(36)**
***aac*A4 forward**	ATGACTGAGCATGACCTTGCG	**(36)**
***aac*A4 reverse**	TTAGGCATCACTGCGTGTTCG	**(36)**

Table 2: Drug resistance patterns of *Acinetobacter* spp. (n= 107)

Antibiotic susceptibility patterns	n= 107 (%)
MDR *Acinetobacter* spp.	80 (75)
XDR *Acinetobacter* spp. *	6 (6)
PDR *Acinetobacter* spp.	0
Amikacin resistance*	6 (6)
Colistin resistance	6 (6)
Resistance to < 3 tested agents (Not MDR)	15 (14)
Total (80+6+6+15)	107

Key: * same *Acinetobacter* spp.

MDR *Acinetobacter* spp.: multidrug resistant *Acinetobacter* spp.;

XDR *Acinetobacter* spp.: extensively drug resistantly *Acinetobacter* spp.;

PDR *Acinetobacter* spp.: Pandrug resistant *Acinetobacter* spp.

Tabe 3: MIC_{50} and MIC_{90} value of the *Acinetobacter* spp. (n= 60)

	n=60		MICs (CLSI)	
Antibiotics	**MIC_{50}**	**MIC_{90}**	**Sensitive**	**Resistant**
	µg/mL	**µg/mL**	**µg/mL**	**µg/mL**
CST	0.25	0.5	<0.5	>0.5
IMP	24	>32	<1	>4
MEM	24	>32	<1	>4
TZP	>256	>256	16	>32
AK	8	16	16	>64
CIP	>32	>32	0.5	4
CAZ	>16	>16	16	>16

Key: Antimicrobial MICs of *Acinetobacter* spp. isolates from clinical specimens (n= 60)

CST, colistin; IMP, Imipenem; MEM, Meropenem; TZP, Piperacillin-tazobactam;

AK, Amikacin; CIP, Ciprofloxacin; CAZ, Ceftazidime

Table 4: Patients' clinical characteristics and outcome, and MICs of other tested antibiotics against colistin and amikacin resistant and sensitive *Acinetobacter* spp. (n=26). [n = no. of patients]

	MIC (µg/ mL)											
Isolates	**IMP**	**MEM**	**AK**	**TZP**	**CAZ**	**CIP**	**CST**	**Wards**	**Specimen**	**Days in hospital**	**Treat with**	**Outcome**
CST-R	0.5	0.5	<2	<4	8	<0.25	>16	LW	BC	21	TZP+AK	DC
CST-R	2	4	<2	>128	16	0.5	>16	D2W	BC	42	TZP+AK	DC
CST-R	<0.25	<0.25	4	<4	4	<0.25	>16	LW	BC	35	TZP+ AK	DC
CST-R	<0.25	<0.25	<2	<4	16	<0.25	>16	Onco	BC	8	MEM+VAN	D
CST-R	<0.25	1	4	16	16	<0.25	>16	LW	BC	22	MEM	DC
CST-R	0.5	0.5	<2	<4	8	<0.25	>16	LW	CT	20	TZP+AK	DC
AK-R	>16	>16	>64	>128	64	>4	<0.5	LW	BC	15	TZP+AK/ MEM+CST	DC
AK-R	>16	>16	>64	>128	64	>4	<0.5	ICUT	PUS	23	TZP+AK	D
AK-R	>16	>16	>64	>128	64	>4	<0.5	ICU2A	ETA	28	CST	DC
AK-R	>16	>16	32	>128	64	>4	<0.5	C4E	BC	35	CST	DC
AK-R	8	>16	32	>128	64	>4	1	D1E	PUS	43	None	DC
AK-R	>16	>16	32	>128	64	>4	0.5	D1W	PUS	29	None	DC
CST, AK-S	>16	>16	16	>128	64	>4	<0.5	ICU2A	CT	32	TZP+AK	DC
CST, AK-S	<0.25	<0.25	<2	<4	4	<0.25	<0.5	NS	ETA	32	None	DC
CST, AK-S	<0.25	0.5	<2	16	8	0.5	<0.5	A4E	BC	27	CST	DC
CST, AK-S	<0.25	1	<2	<4	16	1	<0.5				ATCC	
CST, AK-S	>16	>16	16	>128	64	>4	<0.5	ICU2A	BC	35	CST	DC
CST, AK-S	>16	>16	16	>128	64	>4	<0.5	LW	BC	14	None	DC
CST, AK-S	>16	>16	4	>128	64	>4	<0.5	NICU	FL	33	MEM	DC
CST, AK-S	>16	>16	8	>128	8	>4	<0.5	ICUT	PUS	25	CST +MEM	DC
CST, AK-S	>16	>16	4	>128	8	>4	<0.5	BU	BC	113	None	DC
CST, AK-S	>16	>16	16	>128	8	<0.25	<0.5	NHC	ETA	48	TZP+AK	DC
CST, AK-S	>16	>16	16	>128	64	>4	<0.5	NS	CSF	10	CST	DC
CST, AK-S	>16	>16	8	>128	32	>4	<0.5	NS	CSF	35	CST	D
CST, AK-S	>16	>16	16	>128	64	>4	<0.5	ICU2B	BC	37	CST	D
CST, AK-S	>16	>16	16	>128	64	>4	<0.5	N-ICU	BC	24	TZP+AK	DC

Key: None: No antibiotics given

CST-R, colistin resistant (n= 6); AK-R, amikacin resistant (n= 6);

CST, AK-S: colistin and amikacin sensitive isolate (n= 14); BC: blood culture;

CT, catheter tip; WS, wound sepsis; FL, Fluid; ETA, Endotracheal aspirate;

MVA, motor vehicle accident; TB, tuberculosis; RVD, retroviral disease;

Non-ICU, (LW: labour ward; A4E, Rheumatology unit; C4E, high care unit;

D1W, Vascular Unit (VU); D2E, Plastic unit; BMTU, bone marrow transplant unit;

D2W, Surgery unit; NS, Neurosurgery);

CST, colistin; AK, amikacin; MEM, meropenem; TZP, piperacillin-tazobactam;

VAN, vancomycin; DC, Discharged; D, Deceased.

Table 5: Phenotypic, genotypic and clinical characteristics and outcomes of patients with colistin and amikacin resistant *Acinetobacter* species

Antimicrobial resistance profile								Molecular results		Cinical characteristics and outcome					
Strain no.	CAZ	AK	TZP	CIP	IMP	MEM	CST	PCR Results	Sequencing	Wards	Days in hospital	Clinical dx	Specimens	Treatment	Outcome
1	S	S	S	S	S	S	R	*ipxA* -	-	LW	21	AS	BC	TZP+AK	DC
2	S	S	R	S	S	S	R	*ipxA* -	-	D2W	42	MVA	BC	TZP+AK	DC
6	S	S	S	S	S	S	R	*ipxA* -	-	LW	35	TB,RVD	BC	TZP+ AK	DC
46	S	S	S	S	S	S	R	*ipxA* -	-	Onco	8	A-A	BC	MEM+VAN	D
47	S	S	S	S	S	S	R	*ipxA* -	-	LW	22	Cardiac	BC	MEM	DC
60	S	S	S	S	S	S	R	*ipxA* -	-	LW	20	A-P	CT	TZP+ AK	DC
9	R	R	R	R	R	R	S	*aphA6* +	+	LW	21	Sepsis	BC	TZP+AK MEM+CST	D
11	R	R	R	R	R	R	S	*aphA6* +	+	ICUT	30	MVA	PUS	TZP	D
15	R	R	R	R	R	R	S	*aphA6* +	+	ICUA	24	VAP	ETA	TZP+AK	DC
31	R	R	R	R	R	R	S	*aphA6* +	+	C4E	35	Sepsis	BC	CST	DC
42	R	R	R	R	R	R	S	*aphA6* +	+	D1E	43	WS	PUS	Colonisation	DC
51	R	R	R	R	R	R	S	*aphA6* +	+	D1W	29	WS	PUS	Colonisation	DC

Key: BC, Blood culture; CT, Catheter tip; ETA, Endotracheal aspirate;
WS, wound sepsis; MVA, motor vehicle accident; TB, tuberculosis; RVD, retroviral disease; A-S, aortic stenosis; A-P, abruptio placenta; A-A, aplastic anaemia
Non –ICUs [LW, labour ward; D2W, surgery unit; Onco, oncology unit; C4E, high care unit; D1E, Ortho unit ; D1W, vascular unit (VU)];
R, resistannce; S, sensitive
CST, colistin; AK, Amikacin; MEM, Meropenem; VAN, vancomycin;
TZP, Piperacillin-tazobactam; DC, Discharged; D, Deceased

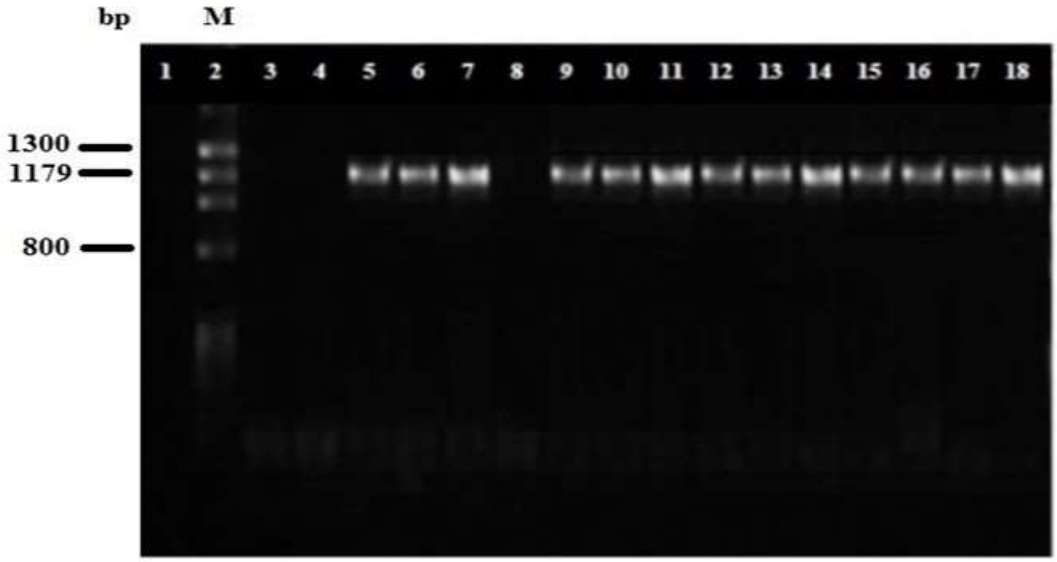
655 **Table 6: Demographic and clinical data of patients cultured with *Acinetobacter***

656 **spp. (n=107)**

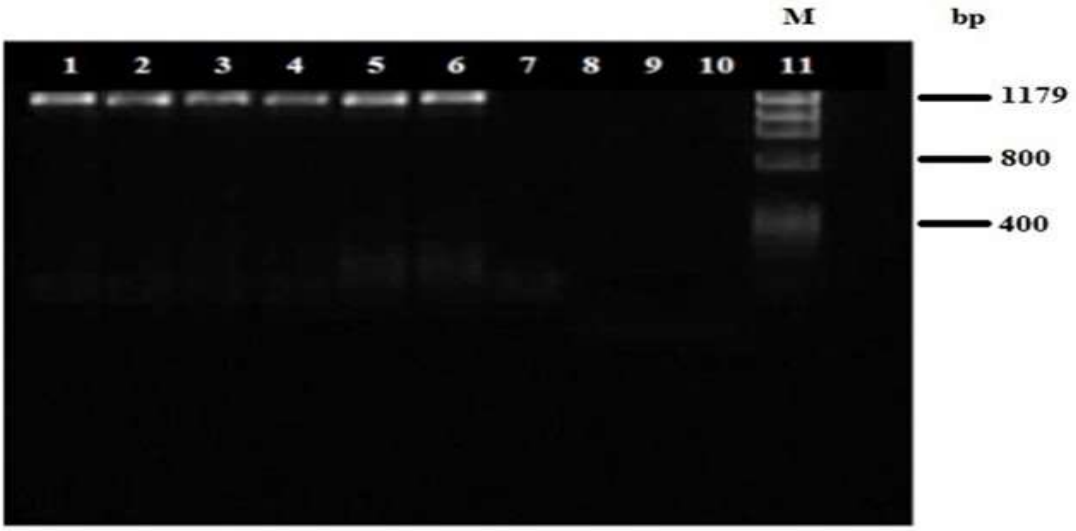
(n=107 patients)			
	<1year (n)	**>1 year /paed (n)**	**Adults (n)**
Gender/Sex-			
Male	12	6	46
Female	5	1	31
NA	3	1	2
Total	20	8	79
Ward			
ICU pediatrics	5	1	
Pedriatric surgery	1	1	
Neonatology	14		
Pedriatric Oncology		2	
Pedriatric Medical unit		1	
Trauma		2	
NA		1	6
ICUs adults			18
Non-ICU			55
Underlying disease			
RVD	5		7
abnormal organ	10		
Respiratory Disease	2	1	
Sepsis	3		
Oncology		2	3
Surgery			17
Medical cases		2	20
Injury / Trauma		3	32
Antibiotic History			
Colistin	1		11
Colistin + combination			1
Amikacin (inhalation)	1	2	11
Others (TZP, Cip, MEM)	17	4	30
No antibiotics given	1	2	26

Outcomes	<1year (n)	>1 year /paed (n)	Adults (n)
Discharged [67/107 (63%)]	10	8	49
Deceased [23/107 (21.5%)]	6		17
NA [17/107 (16%)]	4		13

Key: NA, Not available;
TZP, piperacillin –tazobactam; Cip, ciprofloxacin; MEM, meropenem



1A: PCR detection of *Ipx*A gene
Lanes 3- 17: Isolates 1-15
Lanes 3, 4, 8: Colistin resistant *Acinetobacter* species 1, 2, and 6 (*Ipx*A gene absence :)
Lane 1: negative control (NC); Lane 18: positive control (PC) ATCC19606
Lane 2: molecular weight marker (MWM);



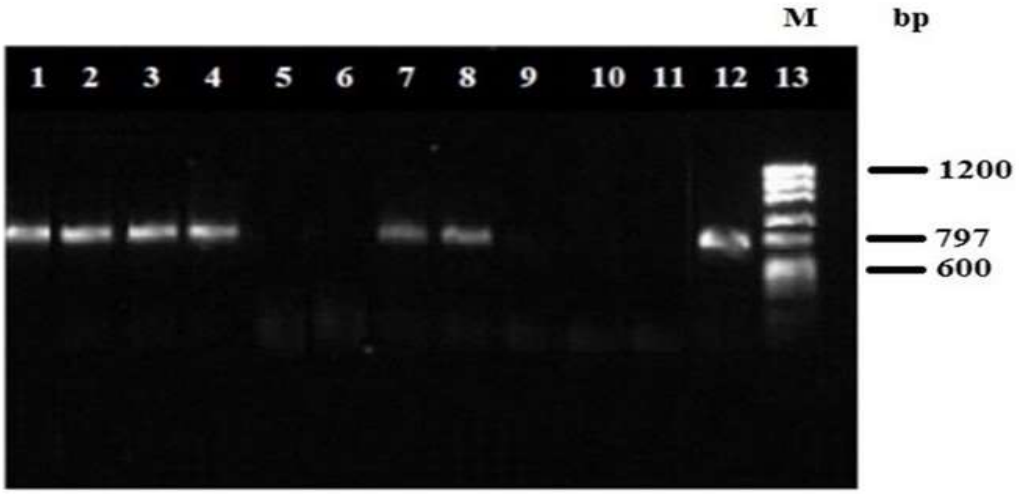
1B: *Ipx*A gene absence in lanes 7, 8, 9 and 10

1B: PCR detection of *Ipx*A gene

Lanes 7-9: Colistin resistant *Acinetobacter* species isolates 46, 47 and 60 (*Ipx*A gene absence)

Lanes 1-5: Isolates 17- 21

Lane 6: positive control; Lane 10: negative control; Lane 11, MWM.

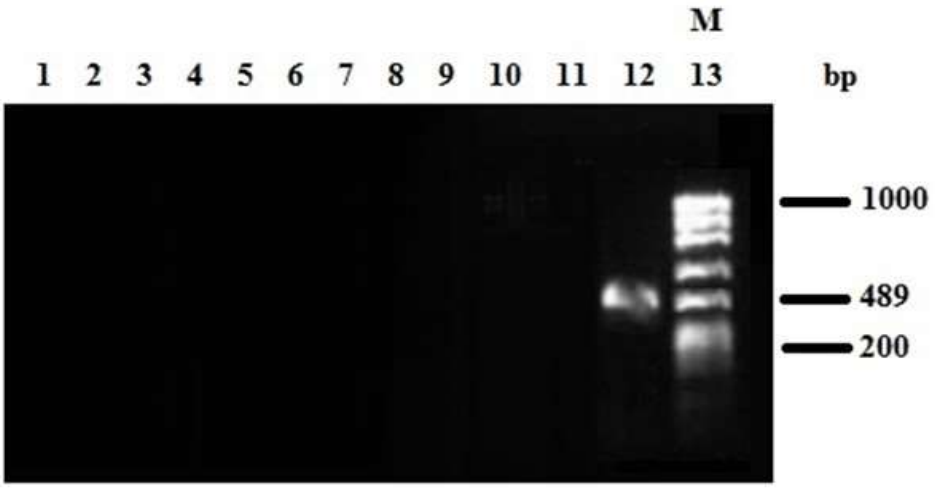


1C: PCR for detection of *aph*A6 gene

Lanes 1-4 and 7-8: *Acinetobacter* species resistant strains 9, 11, 15, 31, 42, 51
(*aph*A6 gene detected);

Lanes 5, 6, 9, 10: amikacin sensitive strains 8, 20, 25, and 60,
(*aph*A6 gene bands absence);

Lane 11: negative control; Lane 12: positive control; Lane 13, MWM.



686

687 1**D:** *aac*A4 gene absence in all tested *Acinetobacter* species isolates

688 Lanes 1-11, Isolates; Lane 12, positive control; Lane 13, MWM

689

690 **Figure 1A, 1B, IC and 1D.** PCR detection of the colistin resistant *Ipx*A, and amikacin resistant

691 *aph*A6 and *aac*A4 genes of *Acinetobacter* species (n=27) (including control isolates).

CHAPTER 7: MANAGEMENT GUIDELINE

A standardised approach to the treatment and management of significant *Acinetobacter* species infection at academic complex hospitals in KwaZulu-Natal

Submitted to IALCH Management and ASWP Committee

MANAGEMENT GUIDELINE

A standardised approach to the treatment and management of significant *Acinetobacter* species infection at academic complex hospitals in KwaZulu-Natal, South Africa

SUMMARY

Acinetobacter species (*Acinetobacter* spp.) are known important nosocomial pathogen whose resistance patterns result in significant challenges for clinicians and microbiologists. Despite the prevalence of infection, there is limited scientific data to help the clinician select optimal empirical and subsequent targeted therapy. One of the problems identified in local settings was the absence of a standardised algorithm for patients with significant *Acinetobacter* spp. infection and a flow chart of definitions to differentiate between significant sepsis and mere colonisation. In this standard management guideline, we review the currently available antimicrobial agents and discuss local data supporting the use of various agents.

According to the first one year study, multidrug resistant *Acinetobacter* species were found to be significant cause of sepsis at the intensive care unit of a regional hospital in Durban. Further studies determined the prevalence of *Acinetobacter* spp. over seven years (2008 to 2014) with analysis of clinical and microbiological criteria on isolates that assist the preauthorisation of antibiotics at the patient level for an effective antibiotic stewardship programme. The synergy effect of colistin with seven combinations against *Acinetobacter* spp. isolates was tested to determine the effectiveness of combination therapy. Molecular methods such as; pulsed field gel electrophoresis (PFGE) typing, polymerase chain reaction (PCR) detection, sequencing of resistant genes in *Acinetobacter* spp. isolates were performed. In addition, clinical data and laboratory data of 107 patients in 2013 were analysed using with clinical and microbiological criteria, as well as a review of previous antibiotic guidelines for multidrug-resistant Gram-negative bacteria (MDR GNB) and *Acinetobacter* species. Based on the outcomes, a flow chart of definitions for *Acinetobacter* spp. sepsis and colonisation was developed, as well as a standardised management algorithm for clinicians and synergy test protocol for the microbiology laboratory. This standard approach recommends individual specific antibiogram as the best approach for treatment in KZN, South Africa.

A proposed standardised algorithm for treatment guideline specifically for *Acinetobacter* spp. Infections in academic complex hospitals in Durban, KwaZulu Natal, South Africa, is recommended and implemented.

Key words: Individual specific antibiogram approach; standardised algorithm for management; significant *Acinetobacter* species infections

7.1. Introduction

Antibiotic resistance is a major challenge in the healthcare sector. Pathogens display changing resistance patterns while antibiotic costs are rising for management. This results in difficult decisions for clinicians selecting optimal treatment. Furthermore, if the optimal usage of antibiotics guideline are not developed and overusing and underusing of antimicrobial agents are not determined, the efficacy of antibitic usage will be compromised.

In response to these challenges, the Antimicrobial Stewardship Program (ASWP) was created in 2015 and firstly we need to develop a standardised algorithm for the management of patients with significant *Acinetobacter* species (*Acinetobacter* spp.) infections that is part of the urgent essential antibiotic policy in Inkosi Albert Luthuli Central Hospital (IALCH), KwaZulu-Natal, South Africa.

The general guidelines were initially developed by unit specific antibiograms and have been revised and expanded annually. However, the standardised management algorithm for *Acinetobacter* spp. infections will be the new guideline, based on literature review, national guidelines, consensus statements and current microbiologic data from the National Health Laboratory Service (NHLS), IALCH Academic complex.

Acinetobacter spp. isolate are opportunistic nosocomial pathogen and one of the six most important multidrug-resistant microorganisms in hospitals worldwide [1]. The organism leads to a range of infections, most commonly ventilator-associated pneumonia and bloodstream infections, with mortality rates reaching 35% [1]. Community-acquired infections have also been observed but few isolates have been recovered from environmental sources and infection reservoirs external to the hospital have not yet been identified [2, 3].

Acinetobacter spp., is an important pathogen whose resistance patterns result in significant treatment challenges for the clinician. Despite its increasing prevalence and improved research, there is limited scientific data to help the clinician select optimal empirical and subsequent targeted therapy for a variety of infections. We will review the available antimicrobial agents supporting the use of the various agents and discuss clinical and local data.

How to use this guide

Dose of antibiotics for a particular infection.

All doses in the text are for pediatric and adult patients with normal renal and hepatic function.

Some important treatment notes explain why particular antibiotics were chosen and provide some important tips on diagnosis and management.

Please refer to the sections on antibiotic dosing to determine the correct dose.

PLEASE glance at these notes when you are treating infections, as we think the information will prove helpful. All references are on file in the office of the Antimicrobial Stewardship Program (ASWP).

Contacting us

Dr Khine Swe Swe-Han (draft the standard approach guideline)

Approved by Prof. Koleka P Mlisana [Head of Department]

Precaution: The recommendations given in this guide are meant to serve as treatment guidelines. They should NOT supplant clinical judgment or Infectious Diseases consultation when indicated. The recommendations were developed for use at the IALCH Academic complex, Durban, KZN, South Africa and thus may not be appropriate for other settings. We have attempted to verify that all information is correct. However, since research is ongoing, please contact the Microbiologists and Infectious Diseases Specialists for the latest information.

Also, please note that copies of the book should not be distributed outside of the institution without permission.

7.2. Inkosi Albert Luthuli Central Hospital formulary and restriction status

7.2.1 Obtaining ID (Infectious diseases), approval

The use of restricted and non-formulary antimicrobials requires preapproval from Microbiologists. This approval can be obtained by any of the following methods:

7.2.1.1 Approval method

The clinicians (consultants) sign the prescription form, providing the reason for prescription, and send the form to the specific Microbiologist in charge for signature. Then the signed prescription forms along with the microbiology result form will be sent to the pharmacy for release of the specific restricted antimicrobial agents.

7.2.2 Selected formulary antimicrobials and restriction status

The following list applies and includes the appropriate antimicrobial agents for *Acinetobacter* species infection (Table 1).

Table 1. Appropriate antimicrobial agents for *Acinetobacter* spp. infection

Unrestricted anti- *Acinetobacter* spp. infection	**Restricted anti- *Acinetobacter* spp. infection**
Ceftazidime	Colistin
Piperacillin –tazobactam	
Amikacin	
Ciprofloxacin	
Imipenem	
Meropenem	

7.3 Antimicrobial agents –specific guidelines

7.3.1 Appropriate for Acinetobacter spp. *infections*

Medications to which *Acinetobacter* is usually sensitive include the following;

Ceftazidime
Piperacillin-tazobactam
Amikacin
Ciprofloxacin
Meropenem
Imipenem
Colistin
Minocycline
Tigecycline

In general, first-, second-, and third-generation cephalosporins, macrolides, and penicillins have little or no anti-*Acinetobacter* activity, and their use may predispose to *Acinetobacter* colonisation [4]. Monotherapy for nebuliser medication and combination therapy has been used successfully [(e.g. amikacin), and minocycline, or colistin ± rifampincin)] [5] with synergy tests on individual isolates [6].

7.3.2 Antibiotics

7.3.2.1. Colistin

Colistin is a polymixin antibiotic. It has in *vitro* activity against *Acinetobacter* spp. and *Pseudomonas* spp. but does NOT have activity against *Proteus, Serratia, Providercia, Burkholderia, Stenotrophomonas,* Gram-negative cocci, Gram-positive organisms, or anaerobes.

Acceptable uses

Management of infections due to MDR *Acinetobacter* and *Pseudomonas* is on a case by case basis.

Unacceptable uses

Monotherapy for empirical treatment of suspected Gram negative infections.

Dose

- Renal function and dialysis (see Table 2. For dose adjustment recommendation).

Toxicity

- Neurotoxicity & renal toxicity

Colistin methanesulfonate (CMS) dosages and dosing interval terminology:

1 Vial = 1 MU = 80 mg CMS= 30 mg colistin base

Colistin dosing instructions:

- Reconstitute each vial with 5 mL 0.9% sodium chloride (normal saline), further dilute to 100 mL for loading dosage and 50 mL for maintenance

Dosage:

- Infuse loading dose over 60 minutes.
- Infuse maintenance dose over 15 to 30 minutes.
- Must be given with a second agent (either rifampicin or a carbapenem) – never on its own.

Additional comments:

- Very nephrotoxic.
- Need blood results to apply for a Section 21 approval – Very important!!
- Store below 25°C.
- Cannot be stored once mixed – therefore discard any unused portion

Table 2: Recommended adult dosages of IV colistin methanesulfonate (CMS) in critically-ill patients [7]

Normal renal function:	**Loading dose: 12 million units- Then:** **3 million units every 8 hours [or]** **4.5 million units every 12 hours**
Renal impairment:	
CrCl* 40-60 ml/min	2 million units every 12 hours
CrCl* 10-40 ml/min	2 million units every 24 hours
CrCl* <10 ml/min	1.5 million units every 36 hours
Renal replacement therapy:	
Haemodialysis	As per CrCl*, with an additional 2 million units after dialysis
CVVHD**	Dosing as for normal renal function

***Creatinine clearance (CrCL) based on Cockcroft-Gault equation;**

****Continuous veno-venous hemodialysis**

Table 3: Recommended paediatric dosages for colistin methanesulfonate (CMS) [7]

	Dosage based on colistin methanesulfonate (CMS)
Neonates	Neonates 50 000 - 75 000 IU/kg/day in three divided dosages 75 000 – 120 000 IU/kg/day in three divided dosages have been used in this population
Infants and children	75 000 - 150 000 IU/kg/day in three divided dosages.
Inhalation	CMS < 40kg: 500 000 IU every 12 hours

Table 4: Colistin methanesulfonate (CMS) reconstitution outline information [7]

Dosage	Final Volume	Diluent	Infusion time
12 MU loading dose	100 mL	0.9% sodium chloride (normal saline)	60 minutes
3 MU 8 hourly	50-100 mL	0.9% sodium chloride (normal saline)	15-30 minutes
4.5 MU 12 hourly	50-100 mL	0.9% sodium chloride (normal saline)	15-30 minutes

Inhalational use of colistin

Inhalational use of colistin prevents systemic side effects, while providing high concentrations in the airways represents a significant advantage. Studies have suggested that the lung concentrations of colistin obtained following the inhalational route of administration may reach levels adequate to eradicate the susceptible *Acinetobacter* spp. isolates [8].

Table 5: The dosing of aerosolised colistin [7]

Body weight	Dosing recommendation
<40 kg	500 000 IU 12-hourly
>40 kg	1 000 000 IU 12-hourly
Recurrent/severe pulmonary infections	2 000 000 IU 8-hourly

NB: The use of colistin must be restricted in hospitals in South Africa. A hospital's consumption of colistin is a surrogate marker of the efficacy of its antimicrobial stewardship and infection prevention efforts, which should be considered standard care [9].

Table 6. Colistin dosing: [7]

Table 6 A. Colistin dosing in normal patients

Dosing in normal patients	
Loading dose	12 MU
Maintainance dose	3 MU tds (8-hourly) OR 4-5 MU bd (12 hourly)

Table 6 B. Colistin dosing in renal impairment patients [7]

Renal impairment:	Colistin dosing
CrCl* 40-60 ml/min	2 million units, 12-hourly
• CrCl* 10-40 ml/min	2 million units, 24-hourly
• CrCl* <10 ml/min	1.5 million units, 36-hourly
Renal replacement therapy:	
Haemodialysis	As per CrCl*, with an additional 2 million units after dialysis
• CVVHD**	Dosing as for normal renal function

7.3.2.2 Tigecycline

Tigecycline is a tetracycline derivative called a glycylcycline. It has in *vitro* activity against most isolates of *Staphylococci* and *Streptococci* (including MRSA and VRE), anaerobes, and many Gram-negative organisms including *Acinetobacter* species, with the exception of *Proteus* spp. and *Pseudomonas aeruginosa.* It is Food and Drug Administration (FDA) approved for skin and skin-structure infections and intra-abdominal infections.

- NOTE: Peak serum concentrations of tigecycline do not exceed 1 mcg/mL which limits its use for treatment of bacteraemia.

Acceptable uses

Management of intra-abdominal infections in patients with contraindications to both β-lactams and fluoroquinolones.

Management of infections due to organisms including *Acinetobacter* species and *Stenotrophomonas maltophilia* on a case by case basis.

Salvage therapy for MRSA, VRE infections on a case by case basis.

Dose

- 100 mg IV once & 50 mg IV 12Hrly
- 100 mg IV once & 25 mg IV 12 hrly

Toxicity: Nausea & vomiting

7.4 Organism-specific guidelines

Background and Literature review of *Acinetobacter* spp.

The following people served as section/topic reviewers

Dr K Baba

Prof. K P Mlisana

Headed by ASWP Committee, an Infectious Disease physician and an Infectious Disease clinician, the mission of the program is to ensure that every patient on antibiotics at IALCH Academic complex KZN, SA receives optimal therapy. These guidelines are a step in that direction.

Academics from various departments have reviewed and approved these guidelines. As you will see, in addition to antibiotic recommendations, the guidelines also contain information about how to interpret the microbiology results and clinical data and other useful management tips.

We want to learn about new approaches and new data as they become available so that we may update the guidelines as needed.

You should also document the reasons for departure in the patient's chart.

The study was conducted in the Department of Microbiology, National Health Laboratory Service (NHLS), IALCH, Durban, KwaZulu- Natal, South Africa, between January 2013 and December 2013. Four-hundred-and-four isolates of *Acinetobacter* spp. from different sources (both sterile and non-sterile specimens), from unique patients, were subjected to antimicrobial sensitivity testing by (Kirby–Bauer method as recommended by Clinical and Laboratory Standards Institute,(CLSI, USA) [10] and the Vitek 2 (bioMérieux, France) automated method for the following antimicrobial agents: ceftazidime (30 μg), cefepime (30 μg), ciprofloxacin (5 μg), amikacin (30 μg), piperacillin (100 μg), piperacillin-tazobactam (100/10 μg), and imipenem (10 μg) .

These isolates were subsequently divided into three groups based on their resistance patterns to different classes of antimicrobials—i.e., aminoglycosides (amikacin), β-lactams with inhibitors (piperacillin–tazobactam), fluoroquinolones (ciprofloxacin), carbapenem (imipenem) and colistin; as follows:

Group 1 consisted of those isolates that were resistant to carbapenem or any three groups of antimicrobials and defined as multidrugs- resistant isolates (MDR). Multidrug- resistant isolates were defined according to the (European Centre of Disease Control and Prevention/ Centers of Diseases Control and Prevention (ECDC/CDC). Multidrug-resistant isolates are resistant to at least one agent in three or more antimicrobial categories [11].In this study, MDR *Acinetobacter* spp. are those isolates that showed resistance to carbapenems or any three groups of antimicrobials. [11].

Group 2 consisted of those isolates that were resistant to four groups of antimicrobials except colistin and defined as extensively drug- resistant isolate (XDR).

Group 3 consisted of those isolates that were resistant to all five groups of antimicrobials and defined as pandrug-resistant isolate (PDR).

Group 4 Sensitive *Acinetobacter* spp. (Community acquired *Acinetobacter* spp.)

Group 5 Polymicrobial infection *Acinetobacter* spp. with Gram- positive bacteria

Group 6 Polymicrobial infection *Acinetobacter* spp. with Gram –negative bacteria

7.4.1 Resistant patterns of Acinetobacter species

7.4.1.1 MDR Acinetobacter species *(Carbapenem-resistant Acinetobacter* spp.*)*

Acinetobacter species have emerged as important nosocomial pathogens worldwide, capable of accumulating multiple antibiotic resistance genes, including β-lactamases, alterations in membrane permeability, and efflux pumps, leading to the emergence of isolates resistant to all commercially available antibiotics [12]. As with other carbapenem-resistant organisms, prolonged infusion carbapenem therapy in combination with a second agent is a reasonable therapeutic approach [13]. Polymyxins have been used with variable success for the treatment of *Acinetobacter* spp. pneumonia, bacteraemia, and meningitis [14-15].

- **Prolonged infusion meropenem PLUS aminoglycoside OR fluoroquinolone OR colistin** (dosed as above)

The sulbactam component of ampicillin-sulbactam may retain activity against highly drug-resistant *Acinetobacter* spp. [16] and remains a treatment option for carbapenem-resistant *Acinetobacter* species when susceptible in *vitro* [17-19].

7.4.1.2 XDR Acinetobacter species

Despite the limitations of existing data, we believe intravenous polymyxins remain an option for patients infected with *Acinetobacter* spp. resistant to β-lactam agents.

When no other options are available, tigecycline should be considered [17].

- **Tigecycline** (dosed as above) [not available in local hospital]

7.4.1.3 PDR Acinetobacter species *(Acinetobacter* spp.*)*

Consisted of those isolates that were resistant to all five groups of antimicrobials and defined pan drug-resistant isolate (PDR).

Colistin with carbapenem or piperacillin-tazobactam or ciprofloxacin;

However, the synergy test will be done for confirmation of synergy effect [6].

7.4.2 Sensitive Acinetobacter species *(Community acquired Acinetobacter* spp.*)*

Although the sensitive strain can be treated with appropriate antimicrobial agents, the clinical and microbiological indicators of sepsis need to be confirmed to avoid over- and under-treatment for *Acinetobacter* spp. infections. The treatment chosen will depend on the site of infection, underlying risk of patients, and type of unit (ICU or other high risk areas).

- Ceftazidime
- Piperacillin –tazobactam with amikacin or

- Ciprofloxacin or
- Carbapenem

7.4.3 Polymicrobial infection Acinetobacter species *with Gram- positive bacteria*

- Piperacillin –tazobactam with amikacin or carbapenem or ciprofloxacin with cloxacillin for sensitive Gram-positive polymicrobial infections.
- Colistin with vancomycin or rifampicin for resistant Gram-positive polymicrobial infection (synergy test should be done) [6].

7.4.4 Polymicrobial infection Acinetobacter species *with Gram-negative bacteria*

- Carbapenem (imipenem or meropenem)
- Carbapenem (imipenem or meropenem) with amikacin
- Colistin with Carbapenem (imipenem or meropenem) or ciprofloxacin or Piperacillin –tazobactam
- Colistin with rifampicin

  If PDR with polymicrobial infections
- Colistin with carbapenem with rifampicin or
- Colistin with fosfomycin

NOTE: synergy test should be done.

Patients with infection or colonisation with the resistant *Acinetobacter* species (MDR-, XDR-, and PDR- *Acinetobacter* species) sould be placed on CONTACT precautions.

7.5. Microbiology report information

7.5.1 Interpreting the microbiology report

Acinetobacter spp. isolates were identified using standard laboratory techniques [API], antimicrobial susceptibility testing (Kirby–Bauer method as recommended by CLSI, USA [10] and the Vitek 2 automated system. Their antibiograms were studied to categorise isolates as MDR-, XDR- and PDR.

The clinical findings were correlated with laboratory data to assess their pathogenic status, and pure and mixed growth of *Acinetobacter* spp. were compared in patients with clinically defined sepsis and colonised groups [supplementary Figure 1 flow chart].

7.5.2 Classification and Treatment

The isolation of *Acinetobacter* spp. from each site was considered one episode of infection.

If it was isolated on more than one occasion from the same site (at least 7 days apart), or from a different site, these events were considered separate infection episodes.

Infections for which the first isolation of *Acinetobacter* spp. was sent for analysis at least 48 h after admission to the ICU were considered hospital-acquired infections. It was reported as community-acquired if the patient was admitted directly from the emergency room or an outpatient department.

All initial antibiotic prescriptions and any changes to existing medication were made practically. The appropriateness of changes to the antibiotic regimen was determined by analysing the microbiological sensitivity patterns and this local policy guideline will be used.

The Microbiology laboratory utilises standard reference methods for determining susceptibility. The majority of isolates are tested by the automated system.

The minimum inhibitory concentration (MIC) value represents the concentration of the antimicrobial agent required at the site of infection for inhibition of the organism. The MIC of each antibiotic tested against the local organism was reported (Table 10)

NOTE: MIC values vary from one drug to another and from one bacterium to another, and thus MIC values are NOT comparable between antibiotics or between organisms.

7.5.3 Spectrum of antibiotic activity

The spectrum of activity table is an approximate guide of the activity of commonly used antibiotics against frequently isolated bacteria. It takes into consideration IALCH Academic complex specific resistance rates, in vitro susceptibilities and expert opinion on clinically appropriate use of agents. For antibiotic recommendations for specific infections refer to relevant specific unit of the IALCH antibiotic guidelines.

7.5.4 IALCH Antibiogram of Acinetobacter **spp.**

In our local hospital, the prevalence (proportion) of MDR *Acinetobacter* spp. was 53 to 60% during a seven year period (2008 to 2014). Among these MDR isolates, the prevalence of *Acinetobacter* spp. in patients diagnosed with sepsis ranged from 37% and 51% during the study period [20]. Therefore, clinical and microbiological indicators of sepsis need to be confirmed to avoid over- and under-treatment for MDR *Acinetobacter* spp. infections.

In 2013, the local antibiogram showed that 45 (75%) of the 60 *Acinetobacter* spp. were MDR and 54 (90%) were still sensitive to amikacin [6, 20]. This local data highlighted that direct therapy is a good approach rather than empirical therapy for *Acinetobacter* spp. infections.

In addition**,** MIC was estimated for 60 representative isolates of differing levels of drug resistance for the mentioned drugs, proving that drug-resistant *Acinetobacter* spp. presents an increasing challenge to health care. Although previous surveillance studies reported that resistance is increasing among carbapenems, they are still considered as the primary treatment against these bacteria [21]**.**

Similarly, although this study also showed that *Acinetobacter* spp. display lower sensitivity to carbapenems 15 (25%), the carbapenems are still be considered to be the primary treatment if the organism is sensitive according to susceptibility results [20].

Other studies showed that β-lactams, aminoglycosides, ceftazidime, fluoroquinolones and colistin have been the mainstay for the treatment of *Acinetobacter* spp. infections. However, the intensive use of antimicrobials inevitably leads to the appearance of isolates resistant to these drugs [21]. Although the study indicated that *Acinetobacter* spp. showed a low prevalence of sensitivity to imipenem and ciprofloxacin (25%), penicillins with inhibitors (piperacillin-tazobactam) (20%), these drugs are still be used as appropriate direct therapy. Individual specific antibiogram approach is the best way of treatment in KZN, South Africa [20].

7.6 Guidelines for the treatment of various infections (Table.7)

Table 7. Antimicrobial Agents for the Treatment of *Acinetobacter* Infections [22]

Medication	Dosage	Route	Toxicity	Comments
Imipenem-cilastatin	500 mg every 6 h up to 1 g every 6–8 h	IV	Phlebitis, GI, anaphylaxis, seizures, nephritis	Extended infusions have been used,
Meropenem	500 mg to 1 g every 8 h	IV	GI, headache, dermatologic, hematologic, angioedema, seizure	Extended infusions have been used, limited data
Amikacin				
Regimen 1	15 mg/kg daily	IV	Nephrotoxicity, ototoxicity, neuromuscular blockade	
Regimen 2	30 mg	IVent		

Continue;				
Medication	**Dosage**	**Route**	**Toxicity**	**Comments**
Colistin (colistimethate)				
Regimen 1	5 mg/kg/day, 2–4 divided doses	IV	Nephrotoxicity and neurotoxicity	
Regimen 2	1–3 million IU every 8 h	IH	Must be used immediately after reconstitution to prevent accumulation of colistin–lung toxicity	1 million IU is 80 mg of colistimethate. A variety of doses used in studies(see in colistin)
Polymyxin B	50,000 units daily (5 mg)	IT	Meningeal irritation	Has been used as intraventricular injection but not FDA labeled as approved by this route
Polymyxin E (colistin)	10 mg daily	IT/ IVent	Meningeal irritation	Has not been FDA approved for either route of administration
Tigecycline	100 mg once then 50 mg every 12 h	IV	GI, shock, pancreatitis, anaphylaxis	Avoid use in blood stream infections due to large volume of distribution and low mean maximum. steady-state levels
Minocycline	100 mg every 12 h	IV	GI, hepatic, dermatologic	MIC_{90} lower than that of doxycycline; limited data on use in severe infections; most active of all the tetracyclines

Key: FDA, US Food and Drug Administration;
GI, gastrointestinal (eg, nausea, vomiting, and diarrhea); H, inhalational; hepatic, jaundice and hepatitis; IT, intrathecal; IV, intravenous; IVent, intraventricular

7.6.1 Community-acquired infections

Pure growth of *Acinetobacter* spp. from sterile specimens with clinical significance infection. Sensitive to all appropriate antibiotics *Acinetobacter* spp. isolates are mostly from community (Table 8).

Table 8. Appropriate antibiotics for community-acquired *Acinetobacter* spp. infection.

Prescribe antibiotics	Indication for infections
Ceftazidime	CNS infection , CVS infection
Ciprofloxacin	Skin & soft tissue infection , local infection ,UTI
Piperacillin-tazobactam	Intra-abdominal infections, systemic sepsis
Amikacin	PD Peritonitis , nebulizer for respiratory tract infection
Imipenem	Intra-abdominal infections, systemic sepsis
Meropenem	Intra-abdominal infections, systemic sepsis , CNS infection

7.6.2 Hospital-acquired infections

Direct therapy with individual specific antibiogram approach for medication of *Acinetobacter* spp. infections, is the best way of treatment in IALCH, KZN, South Africa.

Appropriate antibiotics for *Acinetobacter* spp.

- Imipenem 1 g IV q6h **or**
- Meropenem 1 g IV q8h **or**
- Doripenem 500 mg IV q8h **or**
- Ampicillin-sulbactam 3 g IV q6h **or (not use in IALCH)**
- Tigecycline 100 mg IV in a single dose, then 50 mg IV q12h **or (NOT available at IALCH)**
- Colistin 5 mg/kg/day IV divided q12h – combination therapy
- Duration of therapy: 14-21d

7.6.2.1 Ventilator-associated pneumonia

Indication for Empirical therapy: Patients with a history of risk factors for *Acinetobacter* and other resistant Gram-negative organisms, example: bronchiectasis, broad-spectrum antibiotics for >7 days in the past month; prolonged hospitalisation>7 days; immunocompromised due to organ

transplant, debilitated nursing home resident; recent mechanical ventilation >48 hours; haematologic malignancy, bone marrow transplantation, active chemotherapy; prednisolone treatment >20 mg daily for >3 weeks; treat as severe illness with tailoring of antibiotics based on past culture data [23].

7.6.2.2 Line sepsis/Catheter-related blood stream infections. [24]

Diagnosis: if the catheter is infected (> minimum erythema or any purulence at the exit site) - it should be removed and replaced at a different site.

If catheter related blood stream infection (CR-BSI) is suspected, two sets of blood cultures (>1 from peripheral sites) should be sent.

If the blood culture is negative and tip has *Acinetobacter* spp., should not be treated. If meet with the criteria for line sepsis, (CR-BSI), should be treated with direct therapy.

7.6.2.3 Catheter-related urinary tract infection

- Remove the catheter whenever possible
- Replace catheters that have been in ≥2 weeks if still indicated
- Prophylactic antibiotics at the time of catheter removal or replacement are NOT recommended due to low incidence of complications and concern for development of resistance.
- Catheter irrigation should not be used routinely [25].

7.6.2.4 Wound sepsis / surgical related post operative wound sepsis.

- Exposure to soaking, whirlpool, hot tub: usually polymicrobial, may involve *Acinetobacter* and *Pseudomonas*
- Chronic wound with prolonged exposure to antibiotics
- Necrosis or gangrene (Treat according to IDSA guideline) [26]

7.6.3 Other anatomical sites-infections

(Examples: Eye infection, ear infection, joint infection etc.)

Direct therapy with individual specific antibiogram approach for *Acinetobacter* spp. infections, is the best way of treatment in IALCH, KZN, South Africa.

NB: The flow chart (Figure 1) and algorithm for the management: (Figure 2) have to be applied.

7.7 Prognosis of *Acinetobacter* spp. infections

The prognosis of *Acinetobacter* infection depends on the underlying health of the host and the extent of organ involvement; it is the same as for other aerobic Gram-negative bacillary infections.

7.8 Infection control

7.8.1 Hospital Epidemiology and infection Control

The presence of OXA-23 in MDR *Acinetobacter* spp. correlated with resistant MICs. Molecular analysis suggested horizontal transmission in ICUs and vascular unit. Six percent of isolates showed amikacin and colistin resistance. Resistance to colistin in *Acinetobacter* spp. may be associated with absence of *Ipx*A gene (this will be confirmed using further molecular sequencing method in future) at academic complex Hospital, KwaZulu-Natal, South Africa. The presence of the *aph*A6 gene in amikacin resistant *Acinetobacter* spp. isolates was detected. Continuous surveillance for the prevalence of resistance genes in MDR-, XDR- *Acinetobacter* spp. is crucial for epidemiological and infection control purposes (local data-submitted).

7.8.2 Infection control precautions (Table. 9)

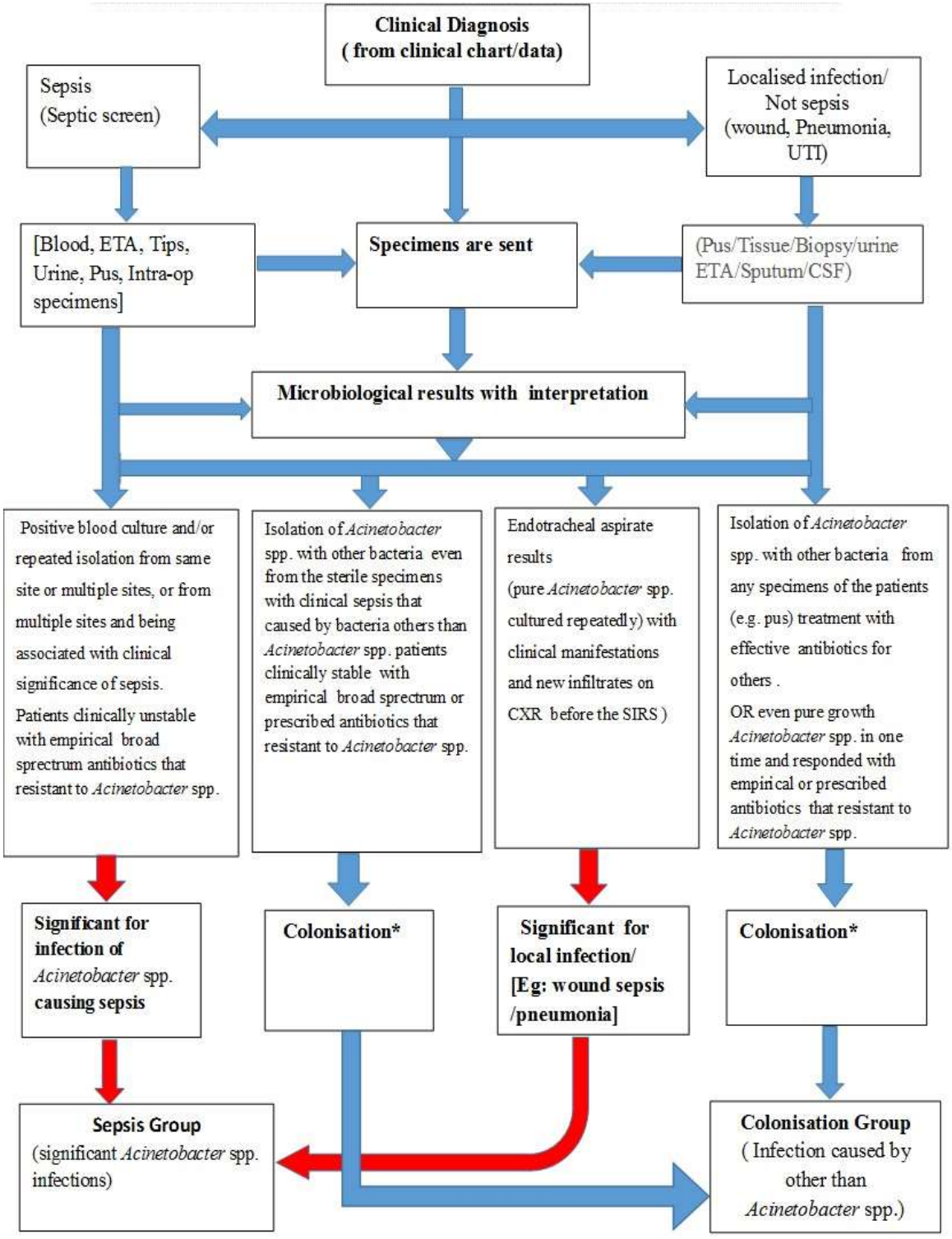
Drug-resistant *Acinetobacter* spp. presents a serious global infection control challenge. The increasing rates of resistance of *Acinetobacter* spp. to the available antimicrobial drugs means that outbreaks should be identified and controlled early. The high prevelence of MDR *Acinetobacter* spp. hightlighted to enhence the infection prevention and control (IPC) measures as below;

Table 9. Methods for control and prevention of multidrug-resistant *Acinetobacter* infection [19].

Method	Comments
Point source control	Effective in the outbreak setting when a point source is identified
Standard precautions	Includes hand hygiene, correct and consistent glove use, and appropriate use of gowns and eye protection; reported compliance among healthcare personnel is often poor
Contact barrier precautions	Includes dedicated patient care equipment and gowns and gloves for health care personnel on entry to an isolation room

Environmental cleaning and disinfection	Widespread environmental contamination is often reported in the epidemic setting, and environmental reservoirs likely play a role in the endemic setting as well
Cohorting of patients	Grouping colonised and infected patients into a designated unit or part of a unit
Cohorting of health care personnel	Designating staff to care for only patients colonised or infected with the organism
Clinical unit closure	Required in some outbreak settings to interrupt transmission and allow for thorough environmental disinfection
Antimicrobial stewardship	Programs to promote judicious antimicrobial use and prevent emergence of resistance
Surveillance	Passive or active surveillance can identify infected or colonised patients so that interventions can be implemented

7.9 Flow charts, supplementary table and definitions



7.9.1 Figure 1. Flow chart of clinical and microbiological criteria of sepsis and colonisation

7.9.2 Definitions

***Colonisation criteria**

Microbiological criteria for Colonisation

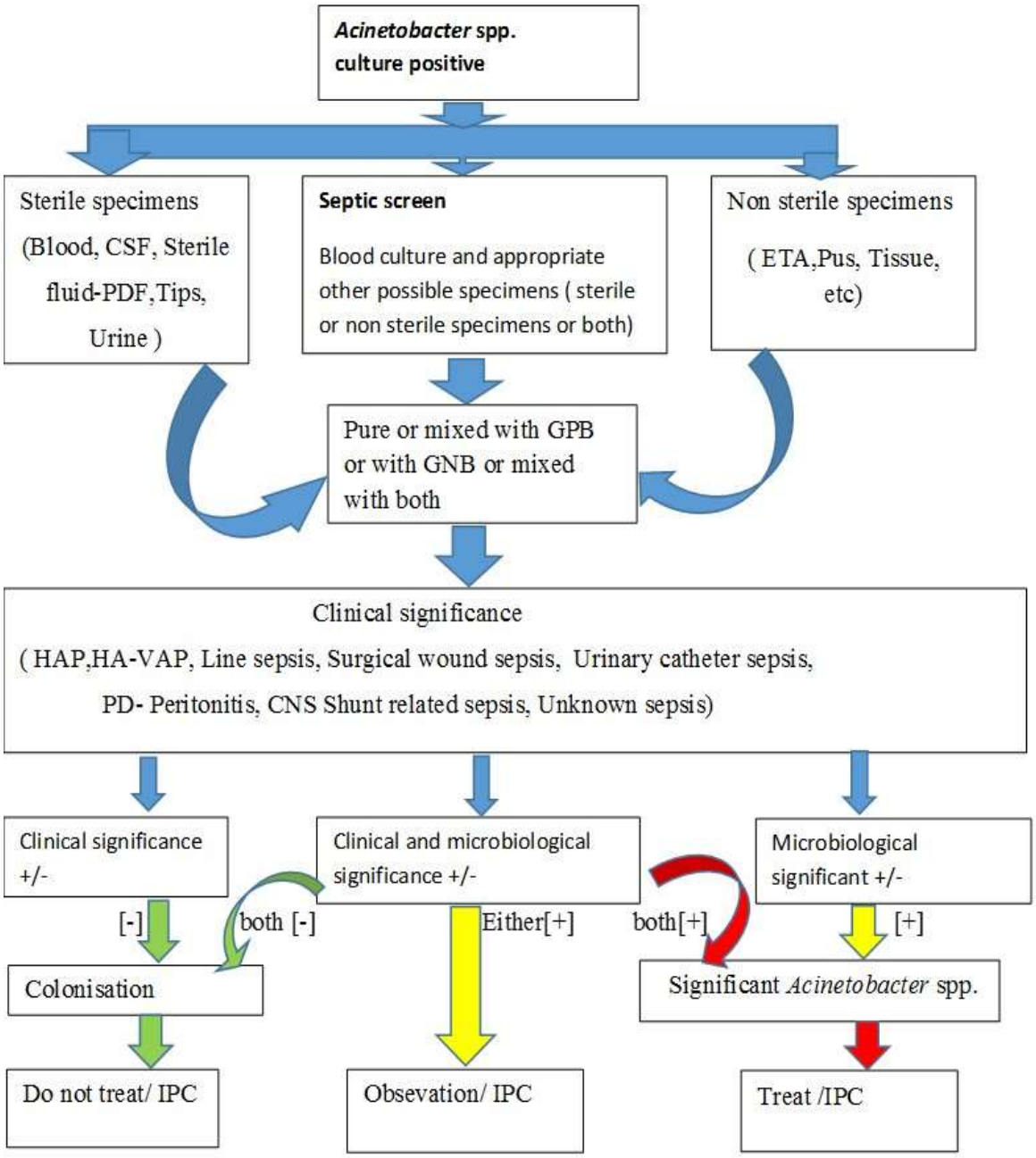
- *Acinetobacter* spp. isolated from positive blood culture once; repeated blood culture with other bacteria or no growth.
- *Acinetobacter* spp. isolated from ETA once and other organisms without *Acinetobacter* spp. or no bacterial growth from repeated ETA.
- Mixed growth in any one site of specimens of septic screen.
  Isolation of *Acinetobacter* spp. with other bacteria even from the sterile specimens with clinical sepsis caused by bacteria others than *Acinetobacter* spp.

Clinical criteria for colonisation

- Patients responded to the empirical or current broad spectrum antibiotics that are resistant to *Acinetobacter* spp. isolate.
- Clinically stable (no signs and symptoms of infections)

Criteria for microbiological significance:

Acinetobacter spp. cultured in pure growth from blood culture bottle and/or other appropriate specimens, sterile specimens or repeated specimens from the same or multiple sites, with patients not responsive to empirical or current broad-spectrum antibiotics; *Acinetobacter* spp. cultured in mixed growth cultures from repeated specimens from the same site with clinical symptoms.



7.9.3 Figure 2. A standardised algorithm for the management of patients with significant Acinetobacter* species *infections; Individual specific antibiogram approach antibiotics guideline in KwaZulu- Natal, South Africa.

7.9.4 Table 10: MIC_{50} and MIC_{90} value of the Acinetobacter spp. *(n=60)*

n=60		
Antibiotics	**MIC_{50}**	**MIC_{90}**
CST	0.25	0.5
IMP	24	>32
IMP+CST	6	32
TZP	>256	>256
TZP+CST	96	>256
AK	8	16
AK+CST	6	16
CIP	>32	>32
CIP+CST	>32	>32
VAN	>256	>256
VAN+CST	16	>256
LIZ	>256	>256
LIZ+ CST	96	>256
RIF	4	>32
RIF+CST	0.32	8

Table 10: Antimicrobial MICs of *Acinetobacter* spp. isolates from clinical specimens (n=60)

Key: The MIC_{50} of amikacin was 8 µg/mL and MIC_{90} was 16 µg/mL Both the MIC_{50} and MIC_{90} of imipenem was 32 µg/mL, and both MIC values were 32 µg/mL for ciprofloxacin and >25 µg/mL for pipercillin-tazobactam. MIC_{50} was 0.25 µg/mL and MIC_{90} was 0.5 µg/mLfor colistin.

The MIC_{50} of vancomycin, linezolid and rifampicin was: >256 µg/mL, >256µg/mL and 4 µg/mL respectively, while the MIC_{90} was >256 µg/mL, >256 µg/mL and >32 µg/mL respectively.

The MIC_{50} and $MIC_{90\ of}$ amikacin and colistin were within the sensitive range among the tested appropriate antibiotics.

SYNERGY TEST

Future Plan: NHLS SOP

DRUG SUSCEPTIBILITY TESTING

Media preparation

Iso-Sensitest agar plate (without colistin/drug free agar media) and with a fixed concentration of colistin 0.5 µg/mL were prepared [27].

Minimum inhibitory concentration (MIC)

Acinetobacter spp. susceptibility results were identified using a Vitek®2 (bioMerieux, France) with the VITEK® 2 GN card and the VITEK® 2 AST-N255 card (bioMerieux, France) according to the manufacturer's instructions.

The Epsilometer test (E-test®) (bioMerieux, France) was used to test the minimum inhibitory concentration (MIC) for each of the isolates that exhibited differing levels of drug resistance for the colistin in combination with different drugs. MIC results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) breakpoint criteria for *Acinetobacter* spp. [28]. Since there are no criteria relevant to *Acinetobacter* spp. for some agents such as vancomycin, linezolid and rifampicin, the susceptibility breakpoints for these antibiotics were based on the MIC interpretive standards of CLSI for Gram-positive bacteria [27]. An *Acinetobacter* spp. control strain (ATCC 19606) was included.

Synergy testing by the Epsilometer test (E-Test®) method

The synergy test was performed using by the 'E-test®' method [27, 29].

Colistin was incorporated into Iso-Sensitest agar at a fixed concentration of 0.5 µg/mL. Plates were inoculated with a bacterial suspension of optical density equivalent to 0.5 McFarland standard. Thereafter, the drug Etest® strip (bioMerieux) was applied on the colistin containing plate and the colistin free plate.

Plates were incubated aerobically in incubator (Jouan aerobic incubator) at 37°C for 18-24 h. The MIC of each drug in the presence of colistin was compared to the MIC of each drug on colistin-free agar and all MIC values were recorded.

Interpretation I [30]

DEFINITIONS

MICA: MIC of drug A alone

MICB: MIC of drug B alone

MICAB: MIC of A in the presence of drug B

Synergy

MIC of combination is ≥ 2 dilutions LOWER than MIC of the most active drug alone.

e.g. MICA = 8, MICB = 16 (i.e. A= most active);

MICAB = 2. MIC of A is reduced from 8 to 2 in combination with B i.e. by 2 dilutions.

Antagonism

MIC of combination is ≥ 2 dilutions HIGHER than MIC of the most active drug alone.

e.g. MICA = 4, MICB = 16 (i.e. A= most active);

MICAB = 16. MIC of A is increased from 4 to 16 in combination with B i.e. by 2 dilutions.

Indifference/Additive

MIC of combination is within +/- 1 dilution compared to the most active drug alone.

E.g. MICA= 1, MICB = 2 (i.e. A= most active);

MIC of A or B in combination = 1.

Combination of A with B shows no change in MIC of A, the most active drug (Indifference)

ACKNOWLEDGEMENTS

HOD Prof. K P Mlisana, Dr K Baba, Prof. M Pillay:

For the guidance to complete the synergy test manuscript and standard guideline.

Medical Education Partnership Initiative, National Health Laboratory Service and the University of Kwa-Zulu Natal:

For facilitating and financial assistance.

Mrs Fikile Nkwanyana, (Biostatistician):

For expertise in statistics.

Medical technologists, Medical scientist, clinicians, Units-HODs, Management of academic complex hospitals:

For all verified clinical and laboratory data crucial part of my work.

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CHAPTER 8: SYNTHESIS

8. SYNTHESIS

This thesis comprises a study with various components, all aimed at developing a standardised approach to the management of patients with *Acinetobacter* species (*Acinetobacter* spp.) infection at academic complex hospitals in Durban, KwaZulu-Natal. The problem identified in local settings was the absence of a standardised algorithm for patients with significant *Acinetobacter* spp. infections and the lack of definitions to differentiate between significant sepsis and mere colonisation. At the time of this study, which includes intensive care units (ICUs) in the regional hospital and ICUs and non-ICUs in academic complex hospitals in Durban, there was institutional knowledge of management strategies for categories of infection, appropriate antimicrobial agents, prevention of multidrug- resistant (MDR) *Acinetobacter* spp. and characterisation of MDR *Acinetobacter* spp. isolates. However, it was observed that *Acinetobacter* spp. were more commonly managed as a coloniser, while targeted treatment was reserved for MDR *Acinetobacter* spp.

The study demonstrated the use of clinical, microbiological, molecular and epidemiological data to develop a standard laboratory and clinical approach to the management of MDR *Acinetobacter* spp. infection. Included in this approach is synergy testing on individual isolates and definitions of the criteria for clinical and microbiologically significant sepsis. Based on this work, steps for developing a standard guideline are recommended, which may be adapted to suit administrative structures in the KwaZulu-Natal (KZN) areas of implementation. For academic complex hospitals, a specific guideline for standard approach of *Acinetobacter* spp. infections needs to be implemented urgently in order to assist the antibiotic stewardship program (ASWP). The study outcomes revealed that local *Acinetobacter* spp. was a significant cause of sepsis, with the emergence of horizontal transfer of carbapenem resistant genes, XDR- and colistin resistant *Acinetobacter* species in ICUs and non-ICUs, and that the results of synergy testing are species dependent. Therefore, the integration of a standard approach to treatment and prevention is valuable and applicable. Moreover, this study discovered that *Acinetobacter* spp. resistance to colistin was potentially mediated by the absence of the *Ipx*A gene which encodes the lipid A component of lipopolysaccharide (LPS). This could possibly indicate a complete loss of the Lipid A motif. This is contradictary with other reports and therefore, it is essential to perform further molecular techniques to confirm the absence of the *Ipx*A gene in colistin resistant *Acinetobacter* species.

Due to budget constraints, the study did not include the molecular identification of *Acinetobacter baumannii* using multiplex PCR, screening of the other resistant genes and whole genome sequencing/southern hybridisation/new primer sequences for confirmation of absence *Ipx*A gene. Further research at the post-doctoral level is recommended to determine the mechanisms

associated with drug resistance and for continuous molecular surveillance. More immediate research should focus on the extent to which the proposed standardised guideline is utilised in academic complex hospitals, province of KZN. Similar studies on MDR *Acinetobacter* spp. are recommended for other locations in KZN. Moreover, in local academic hospitals, research on clinical outcomes, including toxicity of colistin combination therapy and mechanisms of resistance during therapy, is recommended.

The key findings of this study and the conclusions are outlined below.

8.1 Multidrug-resistant *Acinetobacter* species: A significant cause of sepsis in an intensive care unit in a regional hospital, Durban

In order to address the overall study objectives, the prevalence of multidrug-resistant *Acinetobacter* species (MDR- *Acinetobacter* spp.) causing significant sepsis in an ICU was first established, along with differentiation criteria and clinical outcomes. According to the results of this study, MDR *Acinetobacter* spp. causing significant sepsis was generally high, with a high mortality rate compared to mere colonisation in the one year study period (SweSwe-Han and Pillay, 2015). This is the first study of its kind in a local hospital and consistent with those conducted elsewhere (Peleg *et al*., 2008; Morrow *et al*., 2009; Ahmed *et al.*, 2012; Leao *et al.*, 2016). The data revealed a picture of MDR *Acinetobacter* spp. leading to sepsis and high mortality rates among patients in surgical ICU in a local academic complex hospital. These results support the view that nosocomial sepsis due to *A. baumannii* is associated with increased mortality in other areas (Weinstein *et al.*, 2005; Robenshtok *et al*., 2006; Peleg *et al*., 2008; Turkoglu *et al*., 2011; Punpanich *et al*., 2012; Özgür *et al*., 2014; Basri *et al*., 2015; Uwingabiye *et al*., 2016).

The findings highlighted the importance of antibiotic stewardship and the urgent need for the development of standardised guidelines for the treatment of *Acinetobacter* species. Patients with MDR *Acinetobacter* spp. sepsis presented with advanced disease and suffered a great deal of morbidity and mortality in ICUs and non-ICUs. This is a continuing problem.

It is still difficult to differentiate between colonisation and sepsis caused by *Acinetobacter* spp. for treatment decisions at the hospital level (Almasaudi, 2016.). This is an important part of the ASWP, since *Acinetobacter* spp. are recognised as common hospital and community acquired pathogens and colonisers, while a significant number of nosocomial isolates, including MDR isolates, are present in the academic complex hospitals of KZN. However, the prevalence of resistant patterns, the significance of differentiation between sepsis and colonisation, and the criteria of clinical and microbiological sepsis *versus* colonisation, are lacking. In addition, it was necessary to explore the prevalence of significant sepsis in order to establish the study aim of developing a recommended standard management guideline.

8.2 Analysis of clinical and microbiological data on *Acinetobacter* species assist the preauthorisation of antibiotics at the patient level for an effective antibiotic stewardship programme (Seven years study from 2008- 2014)

The integration of antibiotic preauthorisation at the patient level into the antibiotic stewardship programme (ASWP) requires standard criteria for the analysis of clinical and microbiological data (Barlam *et al*., 2016). This study developed definitions of the criteria for clinical and microbiological sepsis and colonisation based on laboratory and clinical data of a large sample of patients during the seven-year study period.

Previous literature mentions the high polymicrobial bacteraemic rate of *Acinetobacter* spp., which may reflect potential polymicrobial sources of infection (Siau *et al*., 1996; Begum *et al.*, 2013; Dash *et al*., 2013). This study revealed a high percentage of polymicrobial infection in the sepsis group. Pure growth of *Acinetobacter* spp. was not always interpreted as a significant pathogen for sepsis, while correlation of the clinical and microbiological data showed a statistically significant difference between the presence of the pathogen and colonisation. Therefore, interpretation of both clinical and microbiology data is essential before prescribing appropriate medication for *Acinetobacter* spp., in order to reduce over and under medication.

Management of MDR-, XDR- and PDR- *Acinetobacter* spp. infection poses a great challenge for physicians and clinical microbiologists (MacDougall and Polk, 2005; Manchanda *et al.*, 2010). This study highlighted the impact of infection prevention control in reducing MDR *Acinetobacter* spp. infection. The high prevalence of MDR *Acinetobacter* spp. and the rates of resistance to polymyxins are concerning, since polymyxins are generally considered the last option due to the lack of new antimicrobial agents (Mathaia *et al*., 2012).

This study uncovered XDR and PDR *Acinetobacter* spp., highlighting the need to use broad-spectrum antibiotics with caution. The study also revealed a high prevalence of *Acinetobacter* spp., specifically MDR strains, in both ICU and non-ICU settings. The specific concern was XDR and PDR *Acinetobacter* spp., which emerged in both the significant sepsis and colonised patient groups. Findings from this research may aid clinicians in using early and appropriate antibiotic regimens, particularly in patients at risk of more virulent MDR infection.

Clinical and microbiological indicators of sepsis in patients should be analysed by clinical microbiologists, clinicians and infectious diseases specialists by using the flow chart in chapter 3 (Swe-Han SK *et al.*, 2017) and management guideline (Chapter 7). The urgent development of standardised management for patients with significant *Acinetobacter* spp. infection is

recommended. Further recommendations include research on the effectiveness of synergy tests on colistin therapy and clinical outcomes, continuous surveillance for modification of local, unit specific antibiograms, as well as phenotypical and genotypical investigation of MDR *Acinetobacter* spp. and its spread.

8.3 Colistin exhibits diverse and species dependent synergy in combination with different antibiotics against *Acinetobacter* spp. isolates

Several studies have investigated synergistic combinations for treating multidrug resistant pathogens (Bonapace *et al*., 2000; Tatman-Otkun *et al*., 2004; Wareham and Bean, 2006; Pankey and Ashcraft, 2009; Falagus *et al*., 2010; Cai *et al*.,2012; Vidaillac *et al*.,2012; Daoud *et al.*,2013; Durante *et al*., 2013; lee *et al.*, 2013; Temocin *et al*., 2015; Zafar *et al.*, 2015). However, there were no studies involving colistin with seven different combinations on the same *Acinetobacter* spp. isolates during the same time.

This study therefore investigated the effectiveness of colistin combination therapy with different commonly used appropriate agents, observing the effects of the combinations used on each isolate at the same time. The results of synergy testing were highly diverse and there is no evidence to use these combinations for empirical treatment of *Acinetobacter* spp. infections in the academic complex hospitals in Durban.

Our findings suggest that synergy testing should be performed for individualised direct therapy and a synergy protocol is recommended for the laboratory.

Due to the emergence of extensively drug resistant **(**XDR)-*Acinetobacter* spp. and the discovery of colistin resistant *Acinetobacter* spp., synergistic drug combinations were a suitable alternative to carbapenems. Moreover, colistin combinations provide effective, first-line drug treatment options, and may be used for XDR-, PDR-*Acinetobacter* spp. and mixed polymicrobial infection. Optimal treatment and the role of combination therapy should be explored in a future prospective clinical trial. This study provided a recommendation for the standard operation procedure (SOP); namely that synergy testing should be part of a standard management guideline for XDR-, PDR-*Acinetobacter* spp. and polymicrobial infections.

8.4 Horizontal transfer of OXA-23-carbapenemase- producing *Acinetobacter* species in intensive care units at an academic complex hospital, Durban, KwaZulu-Natal, South Africa

The production of carbapenemase is one of the mechanisms of carbapenem resistance in MDR *Acinetobacter* species. The gene encoding the enzyme OXA-23 of carbapenem resistant *Acinetobacter* spp. has been identified in both chromosomally and on plasmids, which allows it to spread among various *Acinetobacter* spp., and outbreaks have been observed globally (Dalla-Costa *et al*., 2003; Carvalho *et al*., 2009; Chang *et al*., 2015). Genetic elements may be responsible for the spread of *Acinetobacter* spp., particularly in ICUs and high risk areas.

This study reported on epidemiological and molecular observations of *Acinetobacter* spp. in the academic complex central hospitals in KwaZulu- Natal. The existence of MDR *Acinetobacter* spp. with the $bla_{\text{OXA-23}}$ gene was discovered by polymerase chain reaction (PCR) and confirmed in an academic complex hospital in Durban. The presence of OXA-23 in MDR *Acinetobacter* spp. strains correlated with their phenotypical antibiograms.

The results showed that amikacin and colistin are the mainstay antibiotics for MDR *Acinetobacter* spp. in the local setting. There was a correlation between the MDR phenotype and genes related to carbapenem resistance, as well as with $bla_{\text{OXA-23}}$. Molecular analysis suggested horizontal transmission of MDR *Acinetobacter* species. Molecular typing of the selected MDR *Acinetobacter* species carried the $bla_{\text{OXA-23}}$ gene responsible for resistance MICs to carbapenems (8 to >16µg/mL). In addition, the PFGE typing of a diverse collection of MDR *Acinetobacter* spp. clones showed that strains were related from no more than two patients. This suggests, therefore, no outbreak had occurred during the study period.

The outcomes provided support for a local infection prevention and control management guidelines as part of the antibiotic stewardship programme. Continued molecular surveillance of local epidemiological information and antibiotic resistance surveillance are crucial for infection prevention and control purposes and an essential part of standard management at the hospital.

Strict infection prevention control measures must be urgently implemented to prevent the spread of infection. Continuous surveillance of antibiotic resistance genes in MDR *Acinetobacter* spp. is crucial for epidemiological purposes and to prevent further dissemination of these resistance genes. In addition, it is also necessary to monitor the prevalence and spread of resistance genes linked to other antibiotics associated with *Acinetobacter* spp. (namely amikacin and colistin) in clinical settings, other units at hospitals and community health clinics in KZN.

8.5. Colistin resistant clinical *Acinetobacter* species may be mediated by absence of *Ipx*A gene at academic complex hospitals in Durban, KwaZulu-Natal, South Africa

Acinetobacter spp. are recognised as community, hospital acquired pathogen and classified by the Infectious Diseases Society of America as one of six important MDR microorganisms in hospitals worldwide (Talbot *et al.*, 2006; Gonzalez-Villoria and Valverde-Garduno, 2016) while XDR and PDR- *Acinetobacter* spp have emerged globally (Manchanda *et al*., 2010; Gonzalez-Villoria and Valverde-Garduno, 2016). This study was set in a central academic hospital and investigated the relationship between the minimum inhibitory concentrations (MICs) of standard drugs against *Acinetobacter* spp. and resistant genes for colistin and amikacin. In addition, the association between drug resistance and clinical outcomes of patients with *Acinetobacter* spp. was examined. The results revealed amikacin and colistin resistant *Acinetobacter* spp. strains with high MICs correlated with the presence of the *aph*A6 gene and an absence of the *Ipx*A gene.

According to Moffatt *et al*., high level polymyxin resistance is the result of the complete loss of all lipopolysaccharides (LPS) (Moffatt *et al* 2010), caused by mutations in either *lpx*A, *lpx*C *or lpx*D (Li *et al*., 2006; Moffatt *et al*., 2011; Pogue *et al*., 2015).

In this study, *Acinetobacter* spp. resistance to colistin was potentially mediated by the absence of the *Ipx*A gene which encodes the lipid A component of lipopolysaccharide (LPS). This modification was observed in all colistin-resistant *Acinetobacter* spp. isolates, and in none of the colistin-susceptible isolates. Sequencing revealed no polymorphism, confirming that colistin sensitive isolates with the *Ipx*A gene harbor no mutations. In this study, *Ipx*A primer detection by polymerase chain reaction (PCR) was optimised and performed three times with susceptable isolates and resistant isolates at the same run. Therefore, in order to determine the type of modification associated with the loss of lipid A, further lipid analysis should be conducted. The cell wall may have a different appearance and should be investigated by electron-microscope. This can be achieved through mass spectrometry or alternatively primers associated with *Ipx* modifications (mutants), while the entire genome sequencing may be used to determine point mutations associated with colistin resistance.

The discovery of colistin resistant *Acinetobacter* spp. lacking the *Ipx*A gene is new in KZN, and described for the first time at an academic complex hospital in Durban, KwaZulu-Natal, South Africa. However, before any conclusions can be drawn, further investigation is needed to confirm our findings.

In another interesting observation, colistin-resistant strains were sensitive to other appropriate antibiotics. In addition, colistin-resistant *Acinetobacter* spp. occurred mostly among patients who had not received previous colistin therapy. It is likely that these isolates originated from the

hospital environment as a wild-type. Four out of six isolates were isolated from the blood culture specimens of sepsis patients. This finding highlighted the need to enhance infection prevention and control measures.

Although colistin resistance is not a surrogate marker for MDR *Acinetobacter* spp., the frequent occurrence of colistin resistance in *Acinetobacter* spp. isolates at a local academic hospital is worrying. The researcher found no standard guidelines for colistin therapy in IALCH academic complex hospitals that highlighted a standard management antibiotics approach, including the recommended colistin therapy standard guideline.

Future studies should focus on how to best utilise colistin to minimize resistance. There is a need for continuous surveillance of antibiotic resistance genes, as well as need to recognise and trace pathogenic drug resistant *Acinetobacter* spp. isolates.

8.6 A standardised approach to the treatment and management of significant *Acinetobacter* species infection at academic complex hospitals in KwaZulu-Natal (Including a standardised algorithm: Individual specific antibiogram approach)

The treatment, prevention and prediction of MDR Gram-negative bacteria is included in the current local protocol, but without specific data on *Acinetobacter* species. Moreover, medication was mainly focused on general cultured microorganisms other than MDR *Acinetobacter* spp.

Based on this study, a standardised algorithm for a treatment guideline specifically for *Acinetobacter* spp. infections in academic hospitals was developed.

8.7 Limitation of this study

The study limitations relate primarily to financial and facility restrictions. We were not able to perform a number of additional requirements currently, although we expect to implement them in future.

The identification of individual *Acinetobacter* species by their phenotypic traits is difficult and the use of current automated or manual commercial systems will require further confirmatory testing. Although it may be facilitated by molecular methods such as 16S rDNA sequencing, DNA-DNA hybridisation, *gyr*B multiplex PCR and the *rpoB* gene sequencing, the local routine diagnostic laboratory is unable to perform these molecular methods. Therefore, the study is based on *Acinetobacter* spp. as a whole.

Further research is needed in the form of comprehensive studies with clinical evidence. Synergy mechanisms should be explored in order to facilitate understanding of our results and predict the effects of other antibiotic combinations. In addition, the validation and comparison of the new rapid synergy tests might be done in future (Van-Belkum *et al.,* 2015).

As far as we are aware, this study is the first to describe detailed clinical and molecular characteristics of colistin- and amikacin-resistant *Acinetobacter* spp. at a local academic complex hospital in KwaZulu- Natal.

A further limitation of this study is the lack of confirmation of the absence of the *Ipx*A gene using additional molecular techniques such as including internal amplification controls, new primer design to exclude mis-priming, southern hybridisation and genome sequencing. These have been planned for the near future, as funding is not available to perform this within the scope of the PhD. However, while the published primers that were used possess a *Bam*HI restriction site, and the annealing temperature was high, the *Ipx*A gene was successfully amplified in the positive control, ATCC19606 and susceptible clinical strains. This success confirms that conditions were optimal for the amplification of the *Ipx*A gene. Amplification of the resistant strains was performed at the same time as the susceptible and ATCC strain. The PCR was repeated more than once. Future research should include the detection of the exact mechanism if complete loss of the *Ipx*A gene in colistin resistant *Acinetobacter* species is confirmed. Should the confirmatory tests prove the complete absence of the *Ipx*A gene as a novel resistance mechanism, further lipid analysis through mass spectrometry should be conducted in order to determine the type of modification associated with the loss of lipid A. Cell walls may appear differently and should be investigated by electron microscope.

The final limitation of the study is that we were not able to perform PFGE at the local setting. PFGE analysis software is currently not used by at the Johannesburg (JHB) molecular laboratory because of financial limitations and lack of access to this software program. We hope to be able to access this valuable tool in the near future. The PFGE typing interpretation was correlated with the clinical demographic data and phenotypical MICs results of the *Acinetobacter* species in order to accurately facilitate user interpretation.

8.8 General conclusions

Significant findings in the one year study at ICU (Chapter 2) include a high mortality rate (60%) in sepsis patients with multi-drug resistant *Acinetobacter* species.

XDR-and MDR- *Acinetobacter* spp. infections were significantly associated with severe sepsis in ICU and non-ICU patients in an expanded study during a seven year period. Based on the analysis of clinical and microbiological information of the patients with *Acinetobacter* spp., strategies and criteria for differentiation of significant sepsis from colonisation were developed as a standard algorithm.

The results of specific effective combination drugs with colistin were diverse and species dependent. The synergy test protocol will be implemented at the local microbiology laboratory to make synergy testing a part of the standard guideline.

The presence of resistant genes *bla*$_{\text{OXA-23,}}$ and *aph*A6 were correlated with high level MICs of carbapemem and amikacin resistant *Acinetobacter* spp. isolates respectively.

Therefore, isolates with the same antibiogram nevertheless need to be typed genotypically. At the minimum, their MIC values should be checked. The selected MDR *Acinetobacter* species carried the *bla*$_{\text{OXA-23}}$ gene responsible for resistance to carbapenems (MICs 8 to >16 mg/L), while molecular and clinical data analysis suggested horizontal transmission in ICUs. In addition, the PFGE typing of a diverse collection of MDR *Acinetobacter* species clones showed that isolates from no more than two patients were related, suggesting that no outbreak had occurred.

The absence of the *Ipx*A gene was detected in colistin resistant (high MICs) *Acinetobacter* spp. isolates, while the gene was detected in colistin-susceptible *Acinetobacter* spp. isolates.

In addition, colistin resistance was not a surrogate marker of MDR- and PDR- *Acinetobacter* species. The frequent occurrence of colistin resistance in *Acinetobacter* spp. isolates at local academic hospital is worrying. Continued molecular surveillance of local epidemiological information and antibiotic resistance surveillance are crucial for infection prevention and control purposes and an essential part of standard management at the hospital.

The results of the study may help the clinician to select optimal therapy by providing a flow chart of definitions for *Acinetobacter* spp. sepsis and colonisation, a standardised management algorithm and a synergy test protocol.

8.9 Future Research

In order to further ideas explored in this thesis, a standard management antibiotic guideline is recommended and should be implemented urgently. Future studies should focus on how best to utilise colistin to minimise the risk of developing resistance.

Moreover, the synergy testing should be done with a new method, a novel two-dimensional antibiotic gradient technique named Xact^{TM}, and thereafter compared with the E-test method. This method may be applicable in routine microbiology in future and the new test has been shown to be diagnostically useful, easy to implement and less labour intensive than the classical method (Van Belkum *et al*., 2015). Currently, the synergy testing cannot be done using the new method and subsequently compared to the E-test due to funding limitations.

The absence of the *Ipx*A gene was detected in colistin resistant (high MICs) *Acinetobacter* spp. isolates, while the gene was detected in colistin-susceptible *Acinetobacter* spp. isolates.

Therefore, these results will be confirmed by other techniques such as the inclusion of an internal control to exclude amplification inhibition, PCR with a second primer set to exclude mispriming, sequencing and southern hybridisation. Furthermore, it is possible that the primers are specific for *A. baumannii* and not the other *Acinetobacter* species which may not have been identified through the current identification system. Moreover, future research should include the detection of the exact mechanism associated with the *Ipx*A gene in colistin resistant *Acinetobacter* spp. In order to determine the type of modification associated with the loss of lipid A, further lipid analysis should be conducted. Cell walls may appear differently and should be investigated by electron microscope. This can be achieved through mass spectrometry or alternatively primers associated with *Ipx* modifications (mutants) and the entire genome sequencing can be used to determine point mutations associated with colistin resistance.

An audit of pre-authorisation and prospective review of antibiotics as a measure to improve the efficacy of ASWP will be conducted based on the standard approach of management, in order to achieve a successful ASWP in a local academic complex hospital.

There is a need for continuous surveillance of antibiotic resistance genes compared to resistance profiles and the source of infection, with the aim of preventing further spread. The prevalence and spread of other resistance genes in clinical settings in KZN should be monitored.

A prospective study for the effect of the proposed interventions on morbidity and mortality will be planned. The results will be shared as recommendations to other tertiary referable hospitals. A Standard Operating Procedure (SOP) Synergy test using the National Health Laboratory Service (NHLS) Format will be planned for departmental use.

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APPENDIX: ETHICAL APPROVAL

The study was approved by the Ethics Committee of University of KwaZulu- Natal (Ethic approved: BE 283/12).

UNIVERSITY OF
KWAZULU-NATAL
INYUVESI
YAKWAZULU-NATALI
RESEARCH OFFICE
Biomedical Research Ethics Administration
Westville Campus, Govan Mbeki Building
Private Bag X 54001
Durban
4000
KwaZulu-Natal, SOUTH AFRICA
Tel: 27 31 2604769 - Fax: 27 31 2604609
Email: BREC@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

27 May 2016

Dr K Han
Department of Medical Microbiology
Nelson R Mandela School of Medicine
University of KwaZulu-Natal

PROTOCOL: A novel standardized approach to the treatment and management of significant Acinetobacter species infection in KwaZulu-Natal. REF: BE283/12.

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved: 18 June 2016
Expiration of Ethical Approval: 17 June 2017

I wish to advise you that your application for Recertification received on 12 May 2016 for the above protocol has been noted and approved by a sub-committee of the Biomedical Research Ethics Committee (BREC) for another approval period. The start and end dates of this period are indicated above.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

This approval will be **ratified** by a full Committee at its meeting taking place on **14 June 2016.**

Yours sincerely

Mrs A Marimuthu
Senior Administrator: Biomedical Research Ethics

health
Department:
Health
PROVINCE OF KWAZULU-NATAL

OFFICE OF THE HOSPITAL CEO
KING EDWARD VIII CENTRAL HOSPITAL
Private Bag X02, CONGELLA, 4013
Corner of Rick Turner & Sydney Road
Tel.031-3603853/3015; Fax.031-2061457;
Email.rejoice.khuzwayo@kznhealth.gov.za;
www.kznhealth.gov.za

Ref.: KE 2/7/1/ 16/2013)
Enq.: Mrs. R. Sibiya
Research Programming

2 April 2013

Dr. K. Han
Department of Medical Microbiology
Nelson R. Mandela -School of Medicine
UNIVERSITY OF KWAZULU-NATAL

Dear Dr. Dr. Han

Protocol: "A Novel Standardized approach to the treatment and management of significant acinetobacter species infection in KwaZulu-Natal" REF: BE283/12

Permission to conduct research at King Edward VIII Hospital is provisionally granted, pending approval by the Provincial Health Research Committee, KZN Department of Health.

Kindly note the following:-

- The research will only commence once confirmation from the Provincial Health Research Committee in the KZN Department of Health has been received.
- Signing of an indemnity form at Room 8, CEO Complex before commencement with your study.
- King Edward VIII Hospital received full acknowledgment in the study on all Publications and reports and also kindly present a copy of the publication or report on completion.

The Management of King Edward VIII Hospital reserves the right to terminate the permission for the study should circumstances so dictate.

Yours faithfully

SUPPORTED/~~NOT SUPPORTED~~

12/4/2013

DR. H. GOSNELL
CHIEF EXECUTIVE OFFICER

DATE

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

DEPARTMENT OF MEDICAL MICROBIOLOGY
NELSON R. MANDELA SCHOOL OF MEDICINE /
NATIONAL HEALTH LABORATORY SERVICE
LEVEL 4, Laboratory Building,
Inkosi Albert Luthuli Central Hospital
800 Bellair Road, Mayville, Durban, S.A. 4058
Tel : (031) 240 2770 Fax : (031) 240 2786

NATIONAL HEALTH LABORATORY SERVICE

30 October 2012

Biomedical Research Ethics Committee
Westville Campus
Govan Mbeki Building
Private Bag X54001
Durban
4000

Dear Prof Wassenaar

DR K. SWE SWE HAN - STUDENT NUMBER : 212561772

Title: A novel standardized approach to the treatment and management of significant *Acinetobacter species* infection in KwaZulu-Natal.

I Prof K. P. Misana have already applied for approval for the use of departmental stored isolates.
Approval No : BE085/12.

I confirm that isolates to be used in this study fall under the list of departmental stored isolates.

Yours sincerely,

Kind Regards

Prof Koleka P. Misana
MB ChB, MMedPath(Micro)
Head: Dept of Medical Microbiology
Nelson R Mandela School of Medicine & IALCH Academic Complex
Univ of KwaZulu Natal
Durban, 4001
tel: +27 31 2402787
fax: +2731 2402786
email: misanak@ukzn.ac.za
..."Your success brings me honour"...

Prof Koleka P. Misana *Head : Department of Medical Microbiology NHLS & UKZN*

PERMISSION TO CONDUCT A RESEARCH STUDY/TRIAL

This must be completed and submitted to the Medical Superintendent/s / Hospital Manager/s for signature.

For King Edward VIII Hospital (KEH) and Inkosi Albert Luthuli Central Hospital (IALCH) studies please submit the document together with the following:

1. Research proposal and protocol.
2. Letter giving provisional ethical approval.
3. Details of other research presently being performed by yourself if in the employ of KEH, (individually or as a collaborator).
4. Declaration of all funding applications / grants, please supply substantiating documentation.
5. Complete the attached KEH Form - *"Research Details"*

Once the document has been signed it should be returned to Mrs Patricia Ngwenya: Biomedical Research Ethics Administrator, Room N40, Govan Mbeki Building, Westville Campus, University of KwaZulu-Natal.

To: Chief Medical Superintendent / Hospital Manager

Permission is requested to conduct the above research study at the hospital/s indicated below:

Site 1 address:
Inkosi Albert Luthuli Central Hospital
Durban
KZN.

Investigator/s:
Principal: DR Khine Swe Swe-Han
Co-investigator: DR Manormoney Pillay
Co-investigator: DR K Mlisana and Dr K. Baba

Signature of Chief Medical Superintendent/Hospital Manager:
Date: 20/03/2013

Site 2 address:
King Edward VIII Hospital
Durban
KZN

Investigator/s
Principal: DR Khine Swe Swe-Han
Co-investigator: DR Manormoney Pillay
Co-investigator: DR K Mlisana and Dr K. Baba

Signature of Chief Medical Superintendent / Hospital Manager:

Date:

NB: Medical Superintendent/s / Hospital Manager/s to send a copy of this document to Natalia

PERMISSION TO CONDUCT A RESEARCH STUDY/TRIAL

This must be completed and submitted to the Medical Superintendent/s / Hospital Manager/s for signature.

For King Edward VIII Hospital (KEH) and Inkosi Albert Luthuli Central Hospital (IALCH) studies please submit the document together with the following:

1. Research proposal and protocol.
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To: Chief Medical Superintendent / Hospital Manager

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Inkosi Albert Luthuli Central Hospital
Durban
KZN

Investigator/s:
Principal: DR Khine Swe Swe-Han
Co-investigator: DR Manormoney Pillay
Co-Investigator: DR K Mlisana and Dr K Baba.

Signature of Chief Medical Superintendent/Hospital Manager:

_____ Date: _____

Site 2 address:
King Edward VIII Hospital
Durban
KZN

Investigator/s
Principal: DR KHINE SWE SWE HAN
Co-investigator: DR Manormoney Pillay
Co-Investigator: Prof KMlisana and Dr K.Baba

Signature of Chief Medical Superintendent / Hospital Manager:
X _____ Date: 12/6/2013

NB: Medical Superintendent/s / Hospital Manager/s to send a copy of this document to Natalia

health
Department:
Health
PROVINCE OF KWAZULU-NATAL

Inkosi Albert Luthuli Central Hospital
Ethekwini Health District
Office of the Medical Manager
Private Bag X 03, Mayville, 4058
800 Bellair Road, Mayville, 4058
Tel.: 031 240 1059,
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Email.:ursulanun@ialch.co.za
www.kznhealth.gov.za

Reference: BE283/12
Enquiries: Dr M E L Joshua

20 March 2013

Dr K Han
Department of Medical Microbiology
IALCH

Dear Dr Han

RE: PERMISSION TO CONDUCT RESEARCH AT IALCH

I have pleasure in informing you that permission has been granted to you by the Medical Manager to conduct research on: **A novel standardized approach to the treatment and management of significant Acinetobacter species infection in Kwa-Zulu- Natal**

Kindly take note of the following information before you continue:

1. Please ensure that you adhere to all the policies, procedures, protocols and guidelines of the Department of Health with regards to this research.
2. All relevant consent forms are to be completed.
3. This research will only commence once this office has received confirmation from the Provincial Health Research Committee in the KZN Department of Health.
4. Kindly ensure that this office is informed before you commence your research.
5. The hospital will not provide any resources for this research.
6. You will be expected to provide feedback once your research is complete to the Medical Manager.

Yours faithfully

.....
Dr M E L Joshua
Medical Manager