Identification and Differentiation of *Acinetobacter calcoaceticus-Acinetobacter baumannii* Complex by Multiplex PCR: Common Nosocomial Pathogens Among Children

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Project Report

Session: Summer 2019

Shuborno Islam

ID: 2015-3-77-001

Department of Genetic Engineering and Biotechnology

East West University

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Common Nosocomial Pathogens Among Children

A project report submitted to the Department of Genetic Engineering and Biotechnology, East West University in the partial fulfillment for the Degree of Bachelor of Science in Genetic Engineering and Biotechnology

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August 25th, 2019.

Declaration

We certify that we carefully read and recommend to the Department of Genetic Engineering and Biotechnology, East West University, Dhaka for the approval of the project report entitled:

"Identification and Differentiation of Acinetobacter calcoaceticus-Acinetobacter baumannii Complex by Multiplex PCR: Common Nosocomial Pathogens among Children"

Submitted by Shuborno Islam for the partial fulfillment of the requirements for the Degree of Bachelor of Science in Genetic Engineering and Biotechnology.

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Abbreviation

Short Form	**Full Form**
ACB	*Acinetobacter calcoaceticus-Acinetobacter baumannii* complex
AST	Antibiotic Susceptibility Test
BCC	*Burkholderia cepacia*
bp	Base pair
et al,	And others
ICU	Intensive Care Units
ITS	Internal Transcribed Spacer
MIU	Motility Indole Urease
PCR	Polymerase Chain Reaction
RT-PCR	Real Time Polymerase Chain Reaction
STGG	Skim milk Tryptone Glucose Glycerin
TSI	Triple Sugar Iron
WHO	World Health Organization

Abstract

Acinetobacter baumannii is one of the most common pathogens causing nosocomial infections worldwide involving a range of infections such as surgical site infections, pneumonia, urinary tract infections and others. They are also an important pathogen to study due to multidrug resistant worldwide, for which identification is very crucial for effective treatment. *Acinetobacter* is a complex genus which contains multiple species, most of which have similar morphological characteristics and biochemical properties leading to a complicated analysis process in a regular routine care. This study was based on clinical isolates where *Acinetobacter baumannii* and other closely related species of *Acinetobacter,* commonly called *Acinetobacter calcoaceticus-Acinetobacter baumannii* (ACB) complex, were confirmed by conventional multiplex PCR using primers targeting the ITS region followed by confirmation of *Acinetobacter baumannii* only by RT-PCR using "bla$_{\text{OXA-51-LIKE}}$" primers, which helped us to validate the different kinds of primers as well as help us to compare the identification results between these multiple methods. At the same time, the antibiotic susceptibility test (AST) results from the routine labs were analyzed using these isolates to understand their resistance pattern. The results indicated that PCR methods were able to confirm *Acinetobacter baumannii* more accurately compared to conventional biochemical methods, and that both the primers can be used simultaneously without hesitation. We also found the presence of other *Acinetobacter species* from the ACB complex which was not possible to identify by conventional biochemical methods. On the other hand, *Burkholderia cepacia* was found among these isolates indicating that, these isolates showed a conflicting biochemical results with *Acinetobacter species* that might lead to misidentification. From the AST results, we found out that *Acinetobacter baumannii* isolates showed high resistance pattern in almost all of the available antibiotics. To conclude, conventional multiplex PCR method was a much better option for identification due to low cost expenses which can be performed in a regular diagnostic routine lab practices.

Introduction

Bangladesh is a low-middle income country and according to the World Bank, it will be graduating towards middle-income by 2024 (Risse, 2018). Despite all there great achievements, Bangladesh is still lacking in some certain issues such as health services which is one of the fundamental rights of any country. One of the major issues yet to be solved in the health sector of Bangladesh is maintaining and practicing the hygiene conditions in hospitals to avoid the emergence of nosocomial infections among the existing patients. In Bangladesh, there has been very little study about the burden of common nosocomial *Acinetobacter* pathogens on clinical samples, based on molecular methods in health-care facilities. The purpose of the study is based on the detection at a molecular level which will help us to understand the occurance of nosocomial *Acinetobacter baumannii* and other closely related species which are ubiquitous in our community and also their antimicrobial resistance trend in Bangladesh that will help us to take necessary steps in the future.

Nosocomial infections are commonly known as Health Care-Associated Infections which includes opportunistic viral, bacterial or fungal pathogens. These infections develop in a patient during the course of stay in a hospital and even from a community. The pathogens mostly enters the body through the invasive supportive measures such as endotracheal intubation, mechanical ventilation and also though urinary tract catheters which were being used for more the 7 days (Richards, et al., 1999) and further leads to various types of severe diseases in patients such as pneumonia, sepsis, urinary tract infections, respiratory tract infections and even meningitis (Laure & Guillou, 2005) (Khan, et al., 2017). Nosocomial infections deals further damage to the patients in both physical and economical aspects. Such infections act as a risk to other patients who will be admitted in the same facility such as ICUs and the risk is higher in densely populated areas (Lamarsalle, et al., 2013).

Such nosocomial infections cases are worldwide and have a high mortality and morbidity rate which sometimes get unnoticed. In the near future, nosocomial infections will bring greater public health damage followed by additional use of drugs and cost all because of rapid increasing population and more illness, new microorganism and most importantly due to antibacterial resistance trend (WHO, 2002).

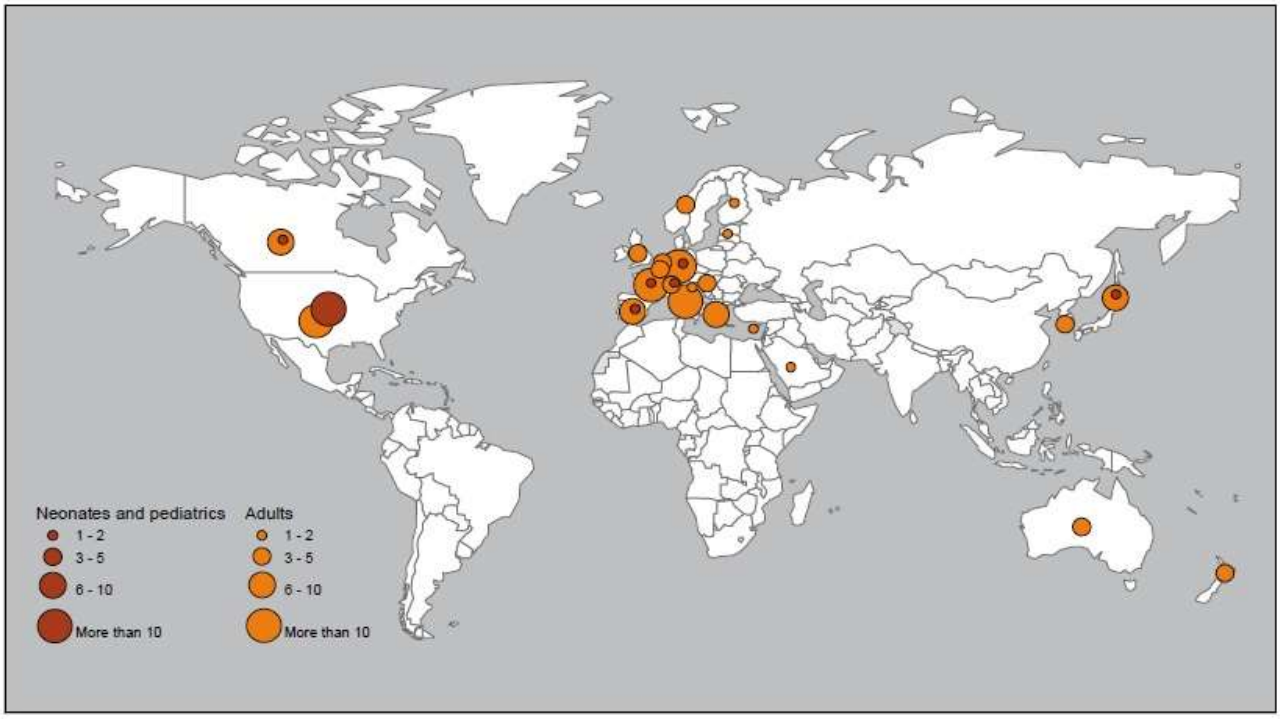


Figure 1: Number of studies reporting health care-associated infection in low- and middle-income countries, 1995-2010 (Allegranz, 2011).

Major four types of nosocomial infections (Khan, et al., 2017):

1. Central Line-Associated Bloodstream Infections (CLABSI): Catheters are used in the bloodstream to supply fluid and medicines and long usage duration can cause infections in the bloodstream as well. This is one of the deadly nosocomial infections with a death rate of 12%-25%.
2. Catheter Associated Urinary Tract Infection (CAUTI): This is a common type of infection and has an occurrence rate of 12% worldwide. These infections are caused by the endogenous pathogens found on the normal microflora of the patients. When the catheters are placed on the urinary tract, they create a pathway for the bacterial entry and due to the improper drainage system by the catheter, bacteria stabilizes along with the retained urine in the catheter and further causing the infections. CAUTI can cause meningitis, cystitis in all patients and prostatitis in most males.

3. Surgical Site Infection (SSI): This is a common infection of around 2-5% occurrence to patients who went through surgery, involving under the skin only. *Staphylococcus aureus* pathogens are the cause behind such infections mainly when the patients are admitted in a hospital for a long duration and can further risk death. Normal microflora bacteria also has the ability to contribute to SSI. The reason behind SSI is mainly due to the systems/procedures and equipment which will be used during surgical treatment and the occurrence rate might even increase to 20% if not properly maintained.
4. Ventilator Associated Pneumonia (VAP): 9-27% of the patients suffers from VAP after they used mechanically assisted ventilator whereas 86% VAP occurrence rate is found with normal ventilation system. Bacteria such as *Acinetobacter species* and *Pseudomonas aeruginosa* are considered are common causative agents behind VAP.

Sites and Distribution of Nosocomial Infections:

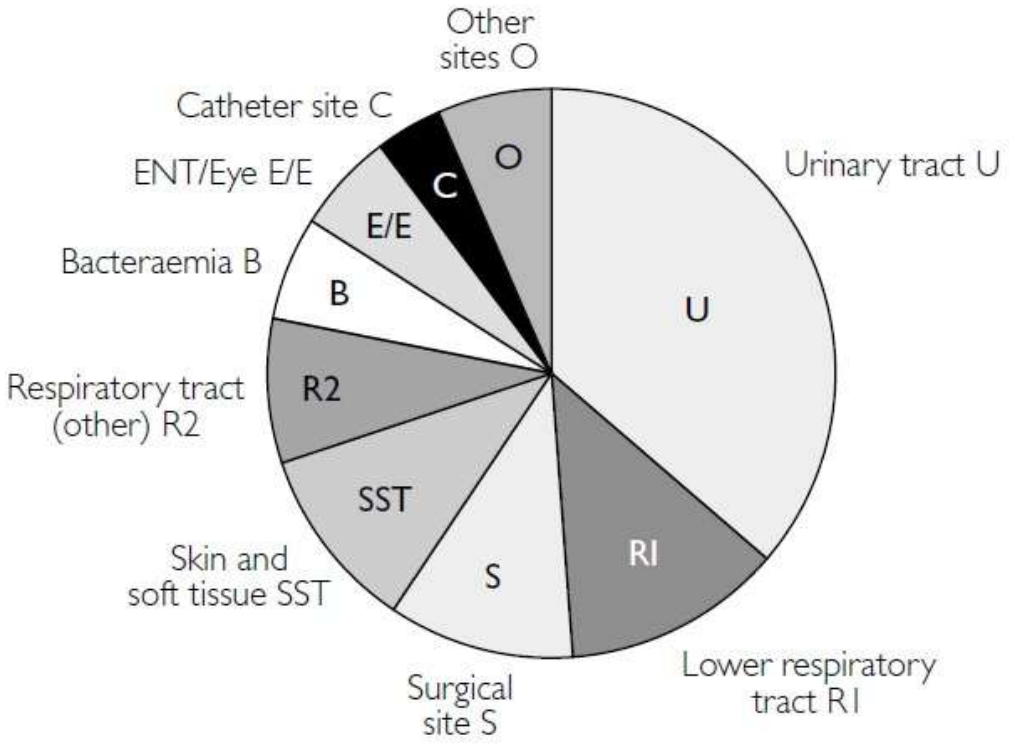


Figure 2: Sites and Distributions of Nosocomial Infections. (WHO, 2002)

Background of the Study

Acinetobacter species are one of the most successful pathogens which has been considered as a source of nosocomial infections in hospitals. Before the 1980s, there were very few cases involved with *Acinetobacter species*, causing urinary tract infection. The species were believed to have a low virulence factor pathogen which were ignored before the 1980s but over the years, *Acinetobacter species* infections increased in number and also in their disease patterns which includes pneumonia, sepsis and can further cause meningitis, mostly in children and admitted patients. One of the reasons behind why *Acinetobacter species* have become an important pathogen to study is because of the increased antibiotic resistant strains involved in admitting patients (Lee, et al., 2017) (Falagas & Karveli, 2007). Among the species, *Acinetobacter baumannii* is the most frequent case of infections worldwide. In 2017, World Health Organization released a list of antibiotic-resistant pathogens and listed them in a category of three different sets based on importance for the introduction of new antibiotics: Priority 1 (Critical), Priority 2 (High), and Priority 3 (Medium). *Acinetobacter baumannii* has been enlisted in the Priority 1 (Critical) category, stating that it is highly Carbapenems-resistant (WHO, 2017). The transmission of *Acinetobacter baumannii* is common due to the environment surfaces and bacteria colonization from the hands of a health care workers. Infection through aerosolized *Acinetobacter baumannii* has also been reported as the health care workers developed pneumonia while treating a ventilated patient undergoing endotracheal suctioning. A new study based on the presence of aerosolized *Acinetobacter baumannii* showed that, the air samples were contaminated with Carbapenem-resistant *Acinetobacter baumannii* (CRAB) in a health care facility (Wong, et al., 2016).

Since *Acinetobacter baumannii* is a multidrug resistant associated with nosocomial infection, can cause a range of diseases, it is important to detect the pathogen precisely and accurately, which will help a community or an institution to understand the burden and also predict the future outcomes such as an outbreak of nosocomial infection (Bergogne-Berezin & Towner, 1996). Conventional biochemical method is one way to perform and detect *Acinetobacter baumannii* from clinical samples but there are a few limitations when it comes to such biochemical methods. The *Acinetobacter* genus has went through lots of changes over the past few years with new species

and taxonomy (Nemec, et al., 2011). There are some species among the *Acinetobacter* genus that have a high degree of similar phenotypically characteristics such as morphological characteristics as well as their biochemical properties which often makes it difficult to differentiate and identify them individually in a clinical routine diagnostic laboratories, and these species are *Acinetobacter baumannii, Acinetobacter pittii* (formerly *Acinetobacter genomic species 3*), *Acinetobacter nosocomialis* (formerly *Acinetobacter genomic species 13TU*), *Acinetobacter calcoaceticus* and two more unnamed species. *Acinetobacter baumannii* is the leading cause of many infection while the latter is rarely pathogenic (Pourabbas, et al., 2016). Due to the high degree similarity among these certain species, all these together are termed and are denoted in many clinical diagnostic labs as *Acinetobacter calcoaceticus-Acinetobacter baumannii complex* (ACB) (Laure & Guillou, 2005).

Primers for both Real time PCR and Conventional PCR for detection have been designed based on the presence of a natural intrinsic Carbapenemase gene that is found in the chromosome of *Acinetobacter baumannii*. The primers for both types of PCR were constructed separately targeting the "*bla*$_{\text{OXA-51-LIKE}}$" genes, a collection of closely related variants of the first reported *bla*$_{\text{OXA-51}}$ gene that is the Carbapenem-resistant gene unique to this *Acinetobacter baumannii* species (Turton, et al., 2006). A study in Taiwan in 2011 showed that, the genetic structure of "*bla*$_{\text{OXA-51-LIKE}}$" genes has been integrated in the plasmids through transposition and was further inserted into the genome of the Non-*baumannii* species, indicating that there is a possibility of a small rate of misidentification for *Acinetobacter baumannii* detection (Lee, et al., 2011). New sets of Conventional PCR primers were designed to detect the ITS region of *Acinetobacter baumannii* that will help to identify *Acinetobacter baumannii* from the complex followed by other primers which will differentiate other species among the ACB (Chen, et al., 2014).

One of the main concerns of this study was to detect *A. baumannii* and differentiate the other pathogenic species among the ACB complex by conventional PCR methods and further RT-PCR using "*bla*$_{\text{OXA-51-LIKE}}$" primers to detect *A. baumannii* to validate the primers.

Aims and Objectives.

- Identification of *Acinetobacter baumannii* and differentiation of other *Acinetobacter species* in the ACB complex using conventional molecular methods.
- Validation of the different kinds of primers based on their target sequences
- Analysis of antimicrobial resistance pattern by Antibiotic Disc Diffusion.
- Understanding the disease burden of these common nosocomial pathogens.

Methodology

Sample Collection and Selection:

Blood samples were collected from the patients who were admitted in Dhaka Shishu Hospital and send to the Department of Microbiology for routine-care of these patients. Presence of bacteria in these blood samples was confirmed by BD BACTEC^{TM} Automated Blood Culture System which was further processed by culturing them in Blood agar, Chocolate agar and MacConkay agar followed by the identification analysis by conventional biochemical tests from the culture positive results and Antimicrobial Susceptibility Test in Muller Hilton agar. The common biochemical tests includes TSI, MIU, Simmon's Citrate test and Kovac's Oxidase test. During this blood culture procedure and analysis, bacteria having similar biochemical properties and colony morphology are recorded as "suspected *Acinetobacter species*". These "suspected *Acinetobacter species*" clinical samples showed negative MIU test results (no change), positive alkaline reaction for both slant and butt (*K/K*) in TSI agar, positive Citrate result (blue) and negative Oxidase result (no change). Although some of these samples showed weakly positive oxidase test results (blue/purple) but it took a long time for the change making it difficult to differentiate, as a result they were reported as Oxidase negative. These organisms recorded as suspected *Acinetobacter species* are further selected for differentiation among the *Acinetobacter species* and also identification of other organism such as *Burkholderia cepacia* among them as it also happens to have similar biochemical properties like the *Acinetobacter species*.

Bacterial isolation and preservation:

After sample collection and blood culture for routine care, these "suspected *Acinetobacter species"* samples were further lawned in Mueller-Hilton agar from a single colony and stored in STGG medium in -80°C.

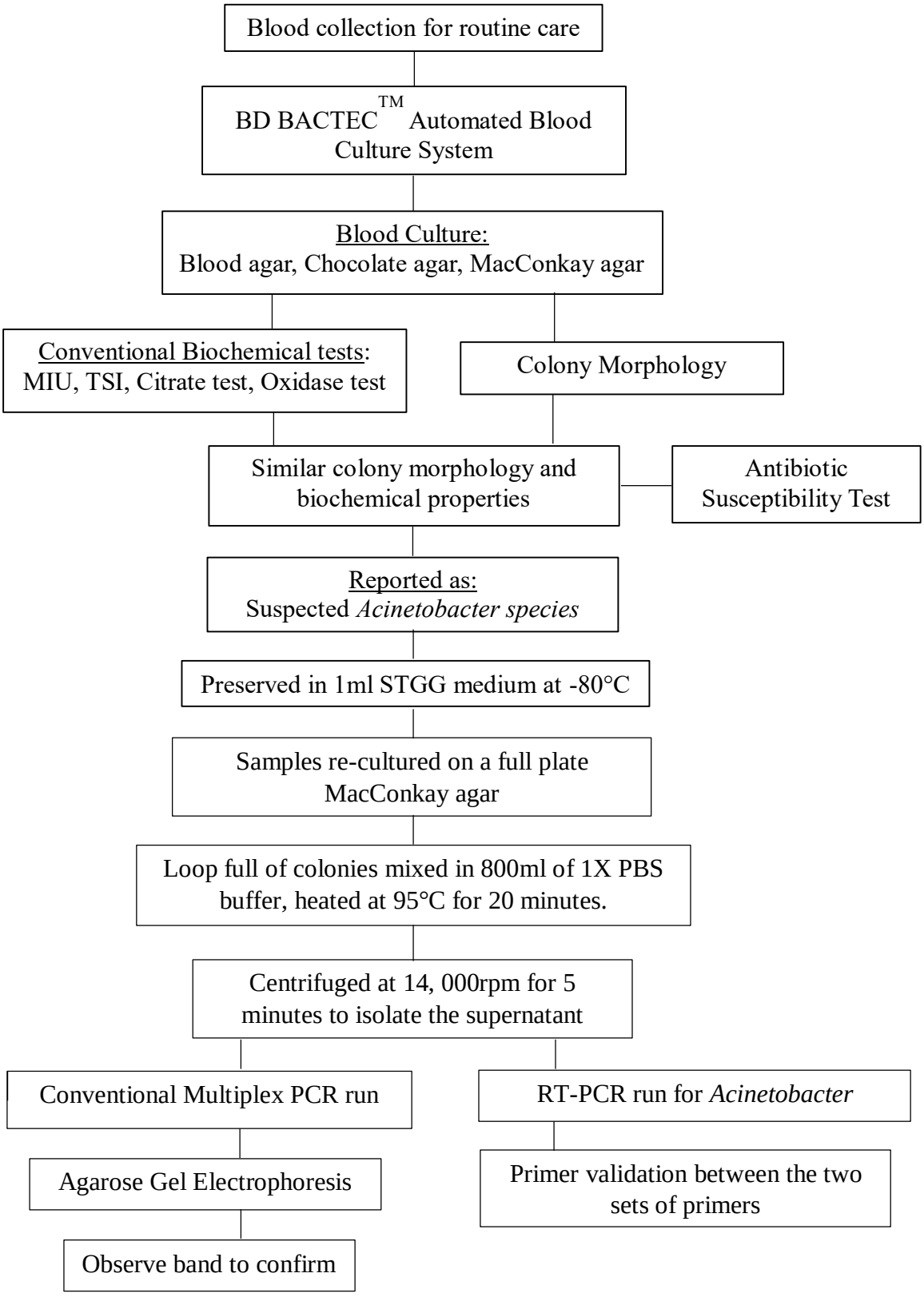
DNA Preparation:

A total of 423 clinical isolates were selected according to the "suspected *Acinetobacter species"* reports were again cultured in a MacConkay agar from the STGG medium from which a single

colony was selected and was re-cultured on a full plate MacConkay agar. From the freshly re-cultured MacConkay agar, loop full of bacterial colonies were transferred and mixed in 800ml of 1X PBS buffer in separate eppendorf tubes and heated at 90°C for 20 minutes. Each tubes were then centrifuged at 14, 000 rpm for 5 minutes. The supernatant which contains the isolated DNA was used for PCR reactions.

All these 423 clinical isolates were screened to detect *Acinetobacter baumannii* and other common pathogenic *Acinetobacter species* among the ACB complex using the conventional PCR primers. After screening, Non-*Acinetobacter species* samples were further tested for the detection of *Burkholderia cepacia,* if any *(Table 1, Table 2).* Primers for *Pseudomonas aeruginosa* was used as a negative control to check for any misidentifications during the routine care process *(Table 3)*. Additionally, a subset of the confirmed *Acinetobacter baumannii* samples were screened again using the RT-PCR primers for confirmation *(Table 5)*.

Overall Flowchart:



Conventional Multiplex PCR for ACB Complex Differentiation and *B. cepacia* detection:

Primer Sequences and Targets for Conventional Multiplex PCR:

Table 1: PCR primer and target sequence for *Acinetobacter species*

Organism	Targeted region	primer name	Sequence 5'-3'	Appr product size	Reference
All *Acinetobacter species* (control)	*recA* gene	P-rA1	CCTGAATCTTCTGGTAAAAC	425 bp	(Chen, et al., 2014)
		P-rA2	GTTTCTGGGCTGCCAAACATTAC		
Acinetobacter nosocomialis/ Acinetobacter baumannii	*gyrB* gene	sp4F	CACGCCGTAAGAGTGCATTA	294 bp	(Chen, et al., 2014)
		sp4R	AACGGAGCTTGTCAGGGTTA		
Acinetobacter baumannii	Known ITS region	P-Ab-ITSF	CATTATCACGGTAATTAGTG	208 bp	(Chen, et al., 2014); (Chen, et al., 2007)
		P-Ab-ITSR	AGAGCACTGTGCACTTAAG		
Acinetobacter pittii	ITS region	P-AGS3-F	CTCAAGAGTTTAGATTAAGCAAT	150 bp	(Chen, et al., 2014)
		P-AGS3-R	GTCCGTGCGATTCTTCATCG		

Table 2: PCR primer and target sequence for *Burkholderia cepacia*

Targeted region	Primer name	Sequence 5'- 3'	Appr. Product size	Reference
recA gene	REC-IN-5c	CATGATCGTCATCGACTCGGTC	620 bp	(Drˇvínek, et al., 2002)
	BCRBM2b	TCCATCGCCTCGGCTTCGT		

Table 3: PCR primer and target sequence for *Pseudomonas aeruginosa*

Targeted region	primer name	Sequence 5'-3'	Appr. product size	Reference
O-antigen acetylase gene	PA431CF	CTGGGTCGAAAGGTGGTTGTTATC	232 bp	(Choi, et al., 2013)
	PA431CR	GCGGCTGGTGCGGCTGAGTC		

Conventional Multiplex PCR Master Mix components:

25µl of Master Mix was used for the reaction for each tube:

Table 4: Components for the conventional PCR Master Mix

Master Mix Buffer	5 µl
Primer F	1 µl (200nM)
Primer R	1 µl (200nM)
dH_2O	16 µl (DNase/RNase-Free)
Template	2 µl
Total	**25 µl**

Solis BioDyne Master Mix Buffer Components:

- FIREPol® DNA polymerase
- 5x Reaction Buffer B (*0.4 M Tris-HCl, 0.1 M $(NH_4)_2SO_4$, 0.1% w/v Tween-20)*
- 7.5 mM $MgCl_2$ *1x PCR solution (1.5 mM $MgCl_2$*)
- 1 mM dNTPs of each *1x PCR solution (200 µM dATP, 200 µM dCTP, 200 µM dGTP and 200 µM dTTP)*

Multiplex PCR Cycling-Condition Development:

Two sets of conventional multiplex PCR was designed to differentiate the *Acinetobacter species* followed by the identification of *Burkholderia cepacia*. The calculations for the PCR components for both sets of multiplex PCR are the same, as given in the previous page.

1. First set for conventional multiplex PCR includes the differentiation of ACB complex using specific primers.

	Steps	**Temperature**	**Time (seconds)**
Stage 1	Initial Denaturation	94.0 ºC	15 minutes
Stage 2 (30x cycle)	Denaturation	94.0 °C	1 minute
	Annealing	57.0 ºC	30 seconds
	Extension	72.0 °C	30 seconds
Stage 3	Final Extension	72.0 °C	10 minutes

2. Second set for conventional multiplex PCR included the detection of *Burkholderia cepacia* using specific *Burkholderia cepacia* primers and *Pseudomonas aeruginosa* primers.

	Steps	**Temperature**	**Time (seconds)**
Stage 1	Initial Denaturation	94.0 ºC	15 minutes
Stage 2 (35x cycle)	Denaturation	94.0 °C	30 seconds
	Annealing	62.0 ºC	45 seconds
	Extension	72.0 °C	90 seconds
Stage 3	Final Extension	72.0 °C	10 minutes

Real Time PCR for *Acinetobacter baumannii* detection:

Primer Sequences and Targets for RT-PCR:

Table 5: RT-PCR primer, probe and target sequence for *Acinetobacter baumannii*

Organism	Target region	Sequence 5`-3`	Reference
Acinetobacter baumannii	bla_{OXA-51}	F, TATTTTTATTTCAGCCTGCTCACCTT	(Diaz, et al., 2013)
		R, AAATACTTCTGTGGTGGTTGCCTTA	
		P, Cy3-TGACTGCTAATCCAAATCACAGCGCTTCA- BHQ1	

RT-PCR Master Mix components:

20µl of Master Mix was prepared for each reaction tube and the components were as follows:

Table 6: Components for the Real-Time PCR Master Mix

Master Mix Buffer	10 µl
Primer F	0.8 µl (400nM)
Primer R	0.8 µl (400nM)
Probe	0.4 µl (200nM)
dH_2O	6 µl (DNase/RNase-Free)
Template	2 µl
Total	**20 µl**

PerfeCTa qPCR ToughMix, Low ROX (2X) Components (Master Mix):

- 2X Reaction Buffer
- Optimized concentration of $MgCl_2$
- Optimized concentration of dNTPs *(dATP, dTTP, dGTP, dCTP)*
- **Hot-start DNA polymerase**
- ROX reference dye
- AccuVue blue qPCR dye
- Stabilizers

Table 7: RT PCR Cycling Condition Development for *Acinetobacter baumannii*:

	Steps	**Temperature**	**Time (seconds)**
Stage 1	Initial Denaturation	94.0 ºC	10 minutes
Stage 2	Denaturation	94.0 ºC	30 seconds
(40x cycle)	Annealing	60.0 ºC	60 seconds

Results

Total of three sets of bacterial genus were observed in this experiment from a sample of 423 samples according to the "suspected *Acinetobacter species"* diagnosis. Three different subsets were also observed from the ACB complex to understand the presence of the pathogenic species. The sub-sets of *Acinetobacter species* included *Acinetobacter baumannii*, *Acinetobacter nosocomialis* and *Acinetobacter pittii*.

Table 8: Positivity among the "suspected *Acinetobacter species*" and comparison between multiple methods.

Organism	**Biochemical tests**	**Conventional PCR**	**RT-PCR**
Acinetobacter baumannii	7.09% (30)	44.2% (187)	187
Acinetobacter pittii	0	2.8% (12)	N/A
Acinetobacter nosocomialis	0	0.5% (2)	N/A
Acinetobacter species	92.9% (393)	5% (21)	0
Burkholderia cepacia	0	20.5% (87)	N/A
Pseudomonas aeruginosa	0	0	N/A
Undetected	N/A	27% (114)	236
Total	**423**	**423**	**423**

Colony Morphology and Comparison:

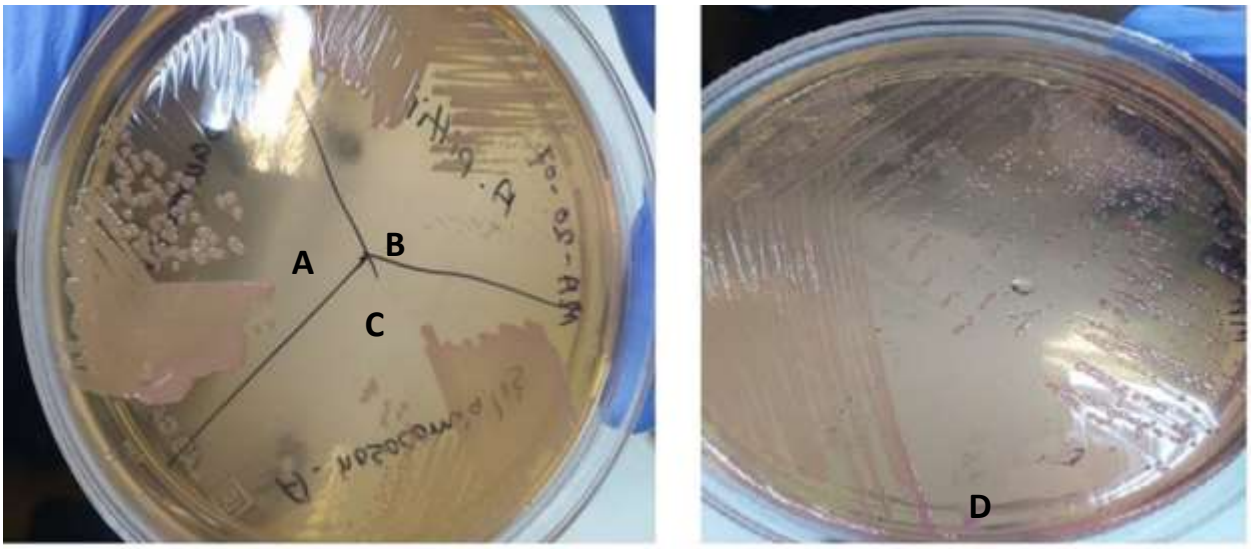
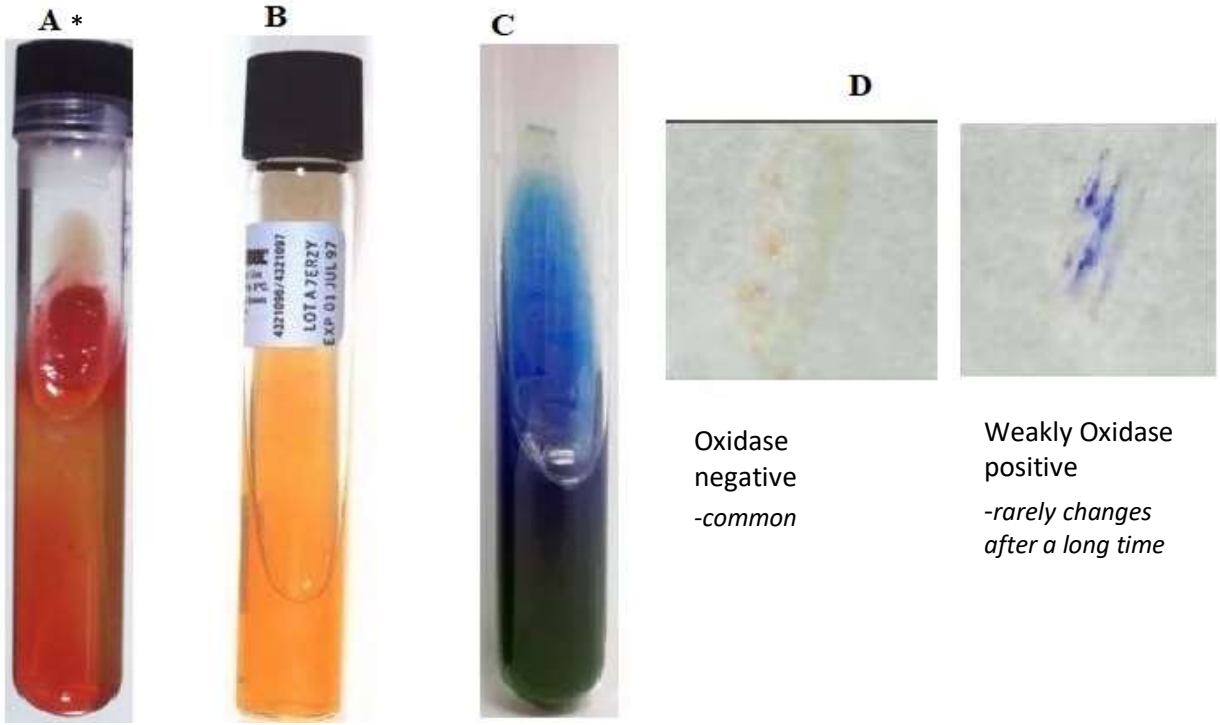


Figure 3: Colony Morphology and Comparison of *Acinetobacter baumannii* (A), *Acinetobacter pittii* (B) and *Acinetobacter nosocomialis* (C) among the ACB complex and *Burkholderia cepacia* (D)



***ALKALINE (RED) REACTION FOR BOTH SLANT AND BUTT (K/K)**

Figure 4: Conventional Biochemical Test Results of all these Isolates:
TSI (**A**)*, Negative MIU (**B**), Positive Citrate test (**C**), Oxidase Test (**D**)

Conventional Multiplex PCR images:

Set 1: Detection of *A. baumannii* and other species among the ACB complex

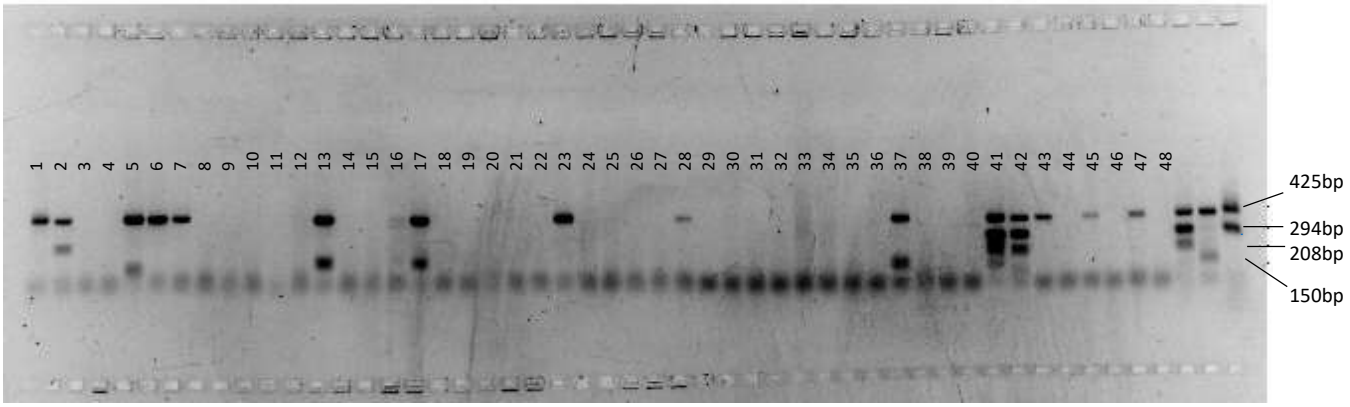


Figure 5.1: Set 1 Multiplex PCR (A)*

Acinetobacter baumannii: lane 41, 42
Acinetobacter pittii: lane 5, 13, 17, 37
Acinetobacter nosocomialis: lane 2
Acinetobacter species: lane 1, 6, 7, 23, 28, 43,45, 47

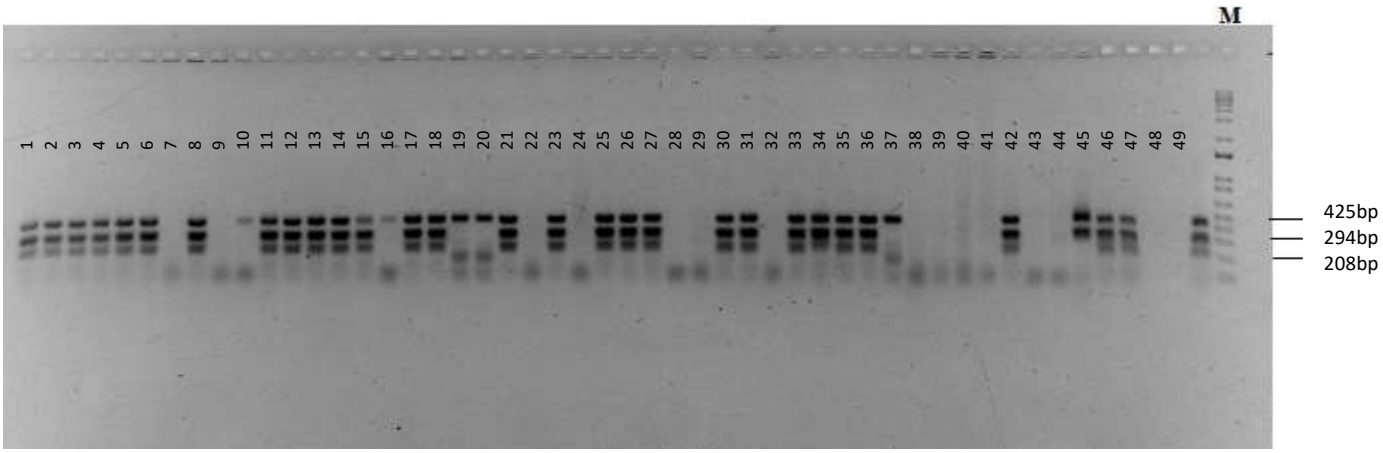


Figure 5.2: Set 1 Multiplex PCR (B)*

Acinetobacter baumannii: lane 1-6, 8, 11-15, 17, 18, 21, 23, 25-27, 30, 31, 33-36, 43, 46, 47
Acinetobacter pittii: lane 19, 20, 37
Acinetobacter nosocomialis: lane 37
Acinetobacter species: lane 10, 16.
Ladder DNA: Lane M

*All *Acinetobacter species*: 425 bp (P-rA1/P-rA2)
Acinetobacter baumannii: 425 bp (P-rA1/P-rA2) + 294 bp (sp4F/sp4R) + 208 bp (P-Ab-ITSF/P-Ab-ITSR).
Acinetobacter pittii: 425 bp (P-rA1/P-rA2) + 150 bp (P-AGS3-F/P-AGS3-R)
Acinetobacter nosocomialis: 425 bp (P-rA1/P-rA2) + 294 bp (sp4F/sp4R)

Set 2: Detection of *Burkholderia cepacia*

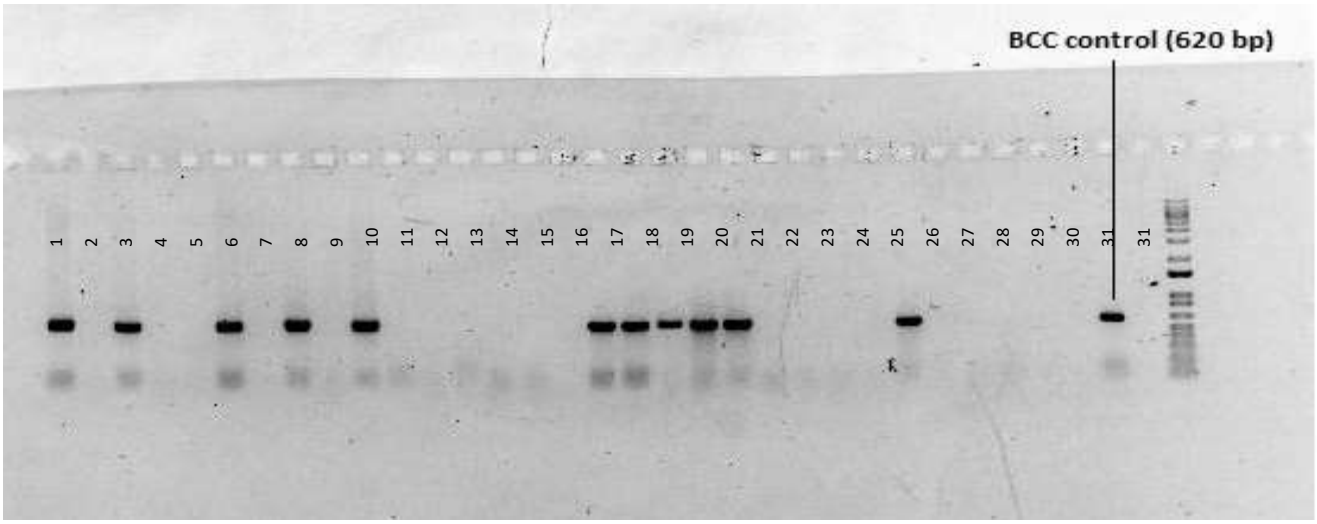


Figure 6.1: Set 2 Multiplex PCR (A)*

Burkholderia cepacia (BCC): Lane 1, 3, 6, 8, 10, 17-21, 26
Positive Control: Lane 31

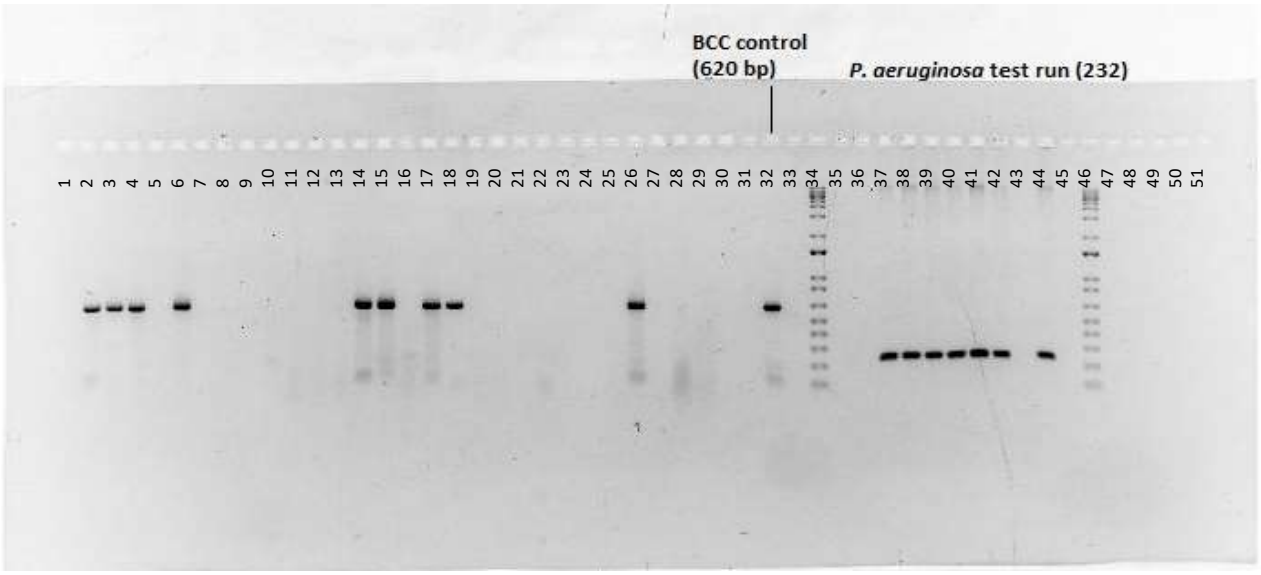


Figure 6.2: Set 2 Multiplex PCR (B)*

Burkholderia cepacia (BCC): Lane 2, 3, 4, 6, 14, 15, 17, 18, 26
Positive Control: Lane 32
Ladder DNA: Lane 34
Pseudomonas aeruginosa control test: lane 37-42, 45

**Burkholderia cepacia:* 620bp (Rec-IN-5C/ BCRBM2b)

Antibiotic Resistance Chart (2009 – 2018):

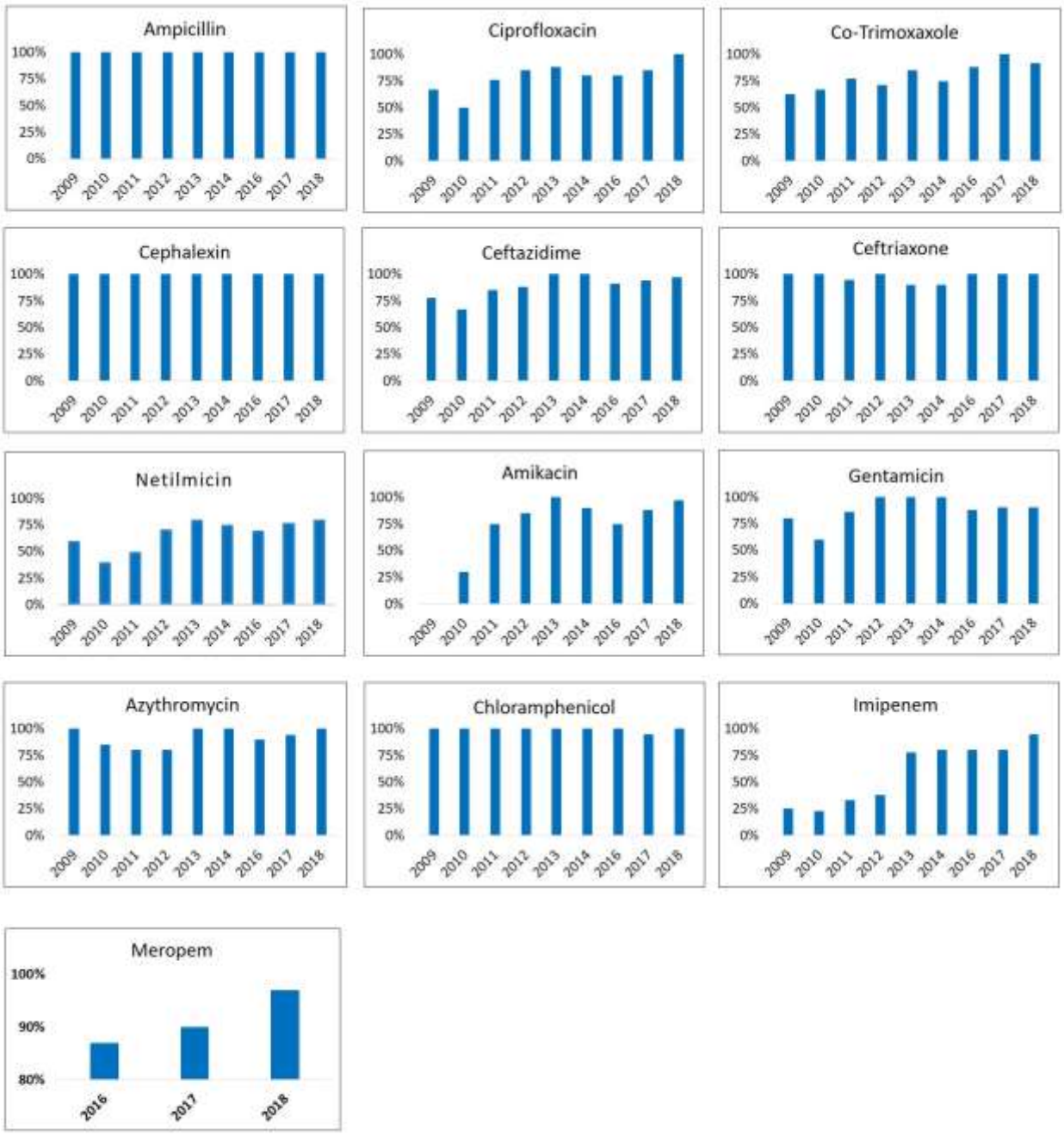


Figure 7: Antibiotic Resistance Pattern among *Acinetobacter baumannii* (2009-2018)

Discussion

The aim of this study was to identify *Acinetobacter baumannii* by molecular methods and also to differentiate other *Acinetobacter species* among the ACB complex, since conventional biochemical tests sometimes cannot be as accurate as possible. New set of conventional PCR primers targeting the ITS regions of *Acinetobacter baumannii* have been used in the study because the intrinsic Carbapenem-resistant gene, which was primarily targeted to detect the species, was found in some closely related *Acinetobacter species.* Therefore, the study will also help us to validate the two different primers. From a total of 423 clinical isolates which were preserved in STGG medium and were reported as "suspected *Acinetobacter species",* most of the isolates were confirmed as *Acinetobacter baumannii*. From the rest, some isolates were confirmed as *Acinetobacter pittii* and very few isolates were confirmed as *Acinetobacter nosocomialis*. Among them, many isolates have been confirmed as *Acinetobacter species* but the species were not yet determined, suggesting that other species from the same genus are also pathogenic. From this, it can be suggested that, *Acinetobacter baumannii* appears to be the most common species among the *Acinetobacter* genus to cause infections. Other species of this genus, *Acinetobacter pittii* and *Acinetobacter nosocomialis* has shown to have a clinical importance as well but did not have a high clinical significance like *Acinetobacter baumannii*. Similar findings was observed in previous studies, showing that *Acinetobacter baumannii* poses a high clinical significance compared to other species of the same genus (Laure & Guillou, 2005).

Acinetobacter baumannii is one of the common pathogens associated with nosocomial infections worldwide. It is also highly multidrug resistant to most clinically available antibiotics, which makes it difficult to treat patients affected by. That is why, accurate identification is also important to detect the presence and also prevent it from spreading. There are many tools which can be used to detect *Acinetobacter baumannii* accurately. Some of these tools are expensive and sometimes are not available in many routine diagnostic labs. Conventional biochemical tests and PCR are one of the most common tools which are convenient in many labs. In this study, we also simultaneously observed the detection accuracy by both the methods.

Multiple methods were performed to detect the organisms. Biochemical methods was able to only confirm 7.09% from a total of 423 isolates, but not was able to detect other organisms. Whereas,

conventional PCR method was able to confirm *Acinetobacter baumannii* at a large number followed by confirmation of *Acinetobacter pittii*, *Acinetobacter nosocomialis* and other *Acinetobacter species.* On the other hand, RT-PCR was performed to validate the two different sets of primers. The results showed concordance between the results from the two PCRs. Therefore, both RT-PCR primers and conventional PCR primers can be used to identify *Acinetobacter baumannii* as well as other species using primers instead of conventional biochemical tests without hesitation. Alternatively, conventional PCR can be used for regular use instead of RT-PCR due to its less expensiveness.

Additionally, another set of *Burkholderia cepacia* specific primers were used in this study as they also happen to have a similar biochemical properties and colony morphology. We also confirmed the presence of *Burkholderia cepacia* among these isolates which was not possible by biochemical tests. 20% isolates were confirmed to be *Burkholderia cepacia* using these specific primers, indicates that the results from the conventional biochemical results and colony morphology from the routine care were inconclusive and were further conflicting with the results of *Acinetobacter species*, for which these samples altogether were reported as "suspected *Acinetobacter species"*. Among them, *Pseudomonas aeruginosa* specific primers were also used for the same purpose but was not observed indicating that they were being identified correctly.

The Antibiotic Resistant Charts were prepared using the Antibiotic Susceptibility Test results which were done during the routine care. Based on the charts, we can see that these *Acinetobacter baumannii* isolates are 100% resistant to Ampicillin, Cephalexin and Chloramphenicol. Moreover, there is an increase in Antibiotic Resistance among *Acinetobacter baumannii* from 2009 to 2018 of other antibiotics, resulting in some of them to be 100% resistant and some being resistant more that 75%. Similar findings was found in previous results as described in this study (Falagas & Karveli, 2007). As a results, new treatment facilities and better hospital management is mandatory to avoid outbreaks or further damage to children.

Conclusion

This study provides the improved results for a possible outcome of identification of *Acinetobacter baumannii* using molecular methods which cannot be achieved by conventional biochemical methods for routine lab practices. Moreover, by studying specificity of the different sets of primers to identify the pathogen, we can suggest both the primers are good candidates to confirm *Acinetobacter baumannii*. As previous studies showed that, the "*bla*$_{\text{OXA-51-LIKE}}$" genes has been transferred to other closely *Acinetobacter species* in some countries, we need to arrange more studies in the future to understand the transfer of this gene in other species so that there won't be any sorts of misidentification. This study also gives us a small view about the presence of other *Acinetobacter species* among the ACB complex in Bangladesh. On the other hand, the study provides us the increasing pattern of antibiotic resistance of *Acinetobacter baumannii* for which new treatments and new drugs are necessary to introduce, as informed by WHO. If the resistance pattern continues to increase immensely, then it will be difficult to treat patients. Since the rate of other *Acinetobacter species* infections are quite low, we still cannot understand their burden or their antibiotic resistance pattern. Till now, very few study has been done based on burden of *Acinetobacter baumannii* infections in Bangladesh. Further study such as resistance mechanisms or pathogenicity will help us to understand the importance of new treatment facilities, hospital management facilities or even introduction of vaccines.

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Appendices

Appendix 1: Culture Media Preparation

1.1 Blood agar:

1. Suspend the dehydrated powder (Oxoid CM0055, Hampshire, England) with required amount of distilled water according to manufacturer's instruction. In case of Oxoid product CM0055, suspend 40g of dehydrated media in 1 litre of distilled water.
2. Mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
3. Measure the pH of the media. It should be within the range of 7.2-7.6 at room temperature. Adjust the pH if required using 0.1 mol/l (N/10) sodium hydroxide when the medium is too acid, and 0.1 mol/l (N/10) hydrochloric acid when too alkaline.
4. Insert a dry, tight cotton plug on the top of the flask.
5. Autoclave the media at 121°C for 15 minutes.
6. Transfer the autoclaved media to a 50°C water bath.
7. When the agar has cooled to 50°C, **aseptically add 5%** sterile sheep blood and mix gently. During mixing, avoid forming air bubbles.
8. Dispense 20 ml onto a sterile petri dish inside the laminar flow cabinet. **Remove the bubbles** from the plate if formed after dispensing.
9. Place the plate inside the leveled surface of the laminar flow and keep at room temperature to solidify the media.
10. Label the plate with media name, **date and batch number**.
11. Send a randomly selected media for QA test. Store the rest at 2-8ºC in **sealed plastic bags** to prevent the loss of moisture.

1.2 Chocolate agar:

1. Suspend the dehydrated powder (Oxoid CM0055, Hampshire, England) with required amount of distilled water according to manufacturer's instruction. In case of Oxoid product CM0055, suspend 40g of dehydrated media in 1 liter of distilled water.
2. Mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
3. Measure the pH of the media. It should be within the range of 7.2-7.6 at room temperature. Adjust the pH if required.
4. Insert a dry, tight cotton plug on the top of the flask.
5. Autoclave the media at 121°C for 15 minutes.
6. Transfer the autoclaved media to a 50°C water bath.
7. When the agar has cooled to 50°C, **aseptically add 5%** sterile sheep blood and mix gently.
8. Heat the liquid media mixed blood at 80ºC for 15 minutes to lyse the blood. The red color of the media will turn into chocolate.
9. Dispense 20 ml onto a sterile petri dish inside the laminar flow. **Remove the bubbles** from the plate if formed after dispensing.
10. Place the plate inside the leveled surface of the laminar flow and keep at room temperature to solidify the media.
11. Label the plate with media name, **date and batch number**.
12. Send a randomly selected media for QA test. Store the rest at 2-8ºC in sealed plastic bags to prevent the loss of moisture.

1.3 MacConkay Agar:

1. Suspend the dehydrated powder (Oxoid CM0115, Hampshire, England) with required amount of distilled water according to manufacturer's instruction.
2. In case of Oxoid product CM0115, suspend 51.5 gram of dehydrated media in 1 litre of distilled water.
3. Mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
4. Measure the pH of the media. It should be within the range of 7.2-7.6 at room temperature. Adjust the pH if required as described above.
5. Insert a dry, tight cotton plug on the top of the flask.
6. Autoclave the media at 121°C at 15 PSI for 15 minutes.
7. Transfer the autoclaved media to a 50°C water bath.
8. When the agar has cooled to 50°C, dispense 20 ml onto a sterile petri dish inside the laminar flow cabinet. **Remove the bubbles** from the plate if formed after dispensing.
9. Place the plate inside the leveled surface of the laminar flow and keep at room temperature to solidify the media.
10. Label the plate with media name, **date and batch number**.
11. Send a randomly selected media for QA test. Store the rest at 2-8ºC in **sealed plastic bags** to prevent the loss of moisture.

1.4 Mueller-Hinton Agar:

1. Suspend the dehydrated powder (Oxoid CM0337, Hampshire, England) with required amount of distilled water according to manufacturer's instruction. In case of Oxoid product CM0337, suspend 38 g of dehydrated media in 1 liter of distilled water.
2. Mix thoroughly. Heat with frequent agitation, and boil for 1 minute to completely dissolve the powder.
3. Measure the pH of the media. It should be within the range of 7.2-7.6 at room temperature. Adjust the pH if required.
4. Insert a dry, tight cotton plug on the top of the flask.
5. Autoclave the media at 121°C for 15 minutes.
6. Transfer the autoclaved media to a 50°C water bath.
7. When the agar has cooled to 50°C, dispense 20 ml onto a sterile petri dish. **Remove the bubbles** from the plate if formed after dispensing.
8. Place the plate inside the leveled surface of the laminar flow and keep at room temperature to solidify the media.
9. Label the plate with media name, **date and batch number**.
10. Send a randomly selected media for QA test. Store the rest at 2-8ºC in **sealed plastic bags** to prevent the loss of moisture.

Appendix 2: Biochemical Test Media Preparation

2.1 Triple Iron Sugar Agar:

1. Suspend the dehydrated powder (Oxoid CM0277, Hampshire, England) of TSI with required amount of distilled water according to manufacturer's instruction. In case of Oxoid product CM0277, suspend 13 gm of dehydrated media in 200 ml of distilled water.
2. Mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
3. Measure the pH of the media. It should be within the range of 6.8-7.2 at room temperature. Adjust the pH if required.
4. **Dispense 7 ml** in each large size screw cap tubes (approx. 16×160 mm) before doing autoclave.
5. Loosen the cap of tubes containing media and **autoclave** at 121°C for 15 minutes.
6. Allow the medium to solidify in a **sloped position** to give a butt 25-30 mm deep and slope 20-25 mm long (**the butt should be longer than the slope**).
7. Label the plate with media name, date and batch number.
8. Send a randomly selected media for QC test. Store the rest at 2-8ºC in a **dark place**.

2.2 Motility Indole Urease Agar

1. Suspend the dehydrated powder (HIMEDIA M1076, Mumbai, India) with required amount of distilled water according to manufacturer's instruction. In case of HIMEDIA product M1076, suspend 3.6 gm of dehydrated media in 190 ml of distilled water.
2. Mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
3. Measure the pH of the media. It should be within the range of 6.6-7.0 at room temperature. Adjust the pH if required.
4. Insert a dry, tight cotton plug on the top of the flask.
5. Autoclave the media at 121°C for 15 minutes.
6. Transfer the autoclaved media to a 50°C water bath.
7. When the agar has cooled to 50°C, aseptically add 10 ml of sterile 40% urea solution in 190 ml media and mix gently. During mixing, avoid forming air bubbles.
8. **Dispense 5 ml** in each tube (approx. 16×150 mm).
9. Allow the media to solidify Place the tube in upright position and **DO NOT MAKE ANY SLOPE IN THIS MEDIA**. Cover with cap or cotton at the top.
10. Label the plate with media name, date and batch number.
11. Send a randomly selected media for QC test. Store the rest at 2-8ºC.

2.3 Simmon's Citrate Agar

1. Suspend the dehydrated powder (Oxoid CM0155, Hampshire, England) with required amount of distilled water according to manufacturer's instruction. In case of Oxoid product CM0155, suspend 4.6 gm of dehydrated media in 200 ml of distilled water.
2. Mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
3. Measure the pH of the media. It should be within the range of 6.8-7.2 at room temperature. Adjust the pH if required.
4. **Dispense 7 ml** in each large tube (approx. 16×150 mm) before doing autoclave.
5. Loose the cap of the tube containing media and **autoclave** at 121°C for 15 minutes.
6. Allow the medium to solidify in an oblique **position** to prepare only slant **(no butt is required)**.
7. Label the plate with media name, date and batch number.
8. Send a randomly selected media for QC test. Store the rest at 2-8ºC.

2.4 Kovac's Oxidase Test:

1. The reagent was allowed to reach room temperature before use.
2. Filter paper was moisture with 1 or 2 drops of Oxidase reagent and placed into a sterile petri dish.
3. Targeted colony to be tested was touched with the end of a sterile wooden applicator or platinum loop and a smear was formed onto the filter paper
4. Colour change was observed for 10 seconds.