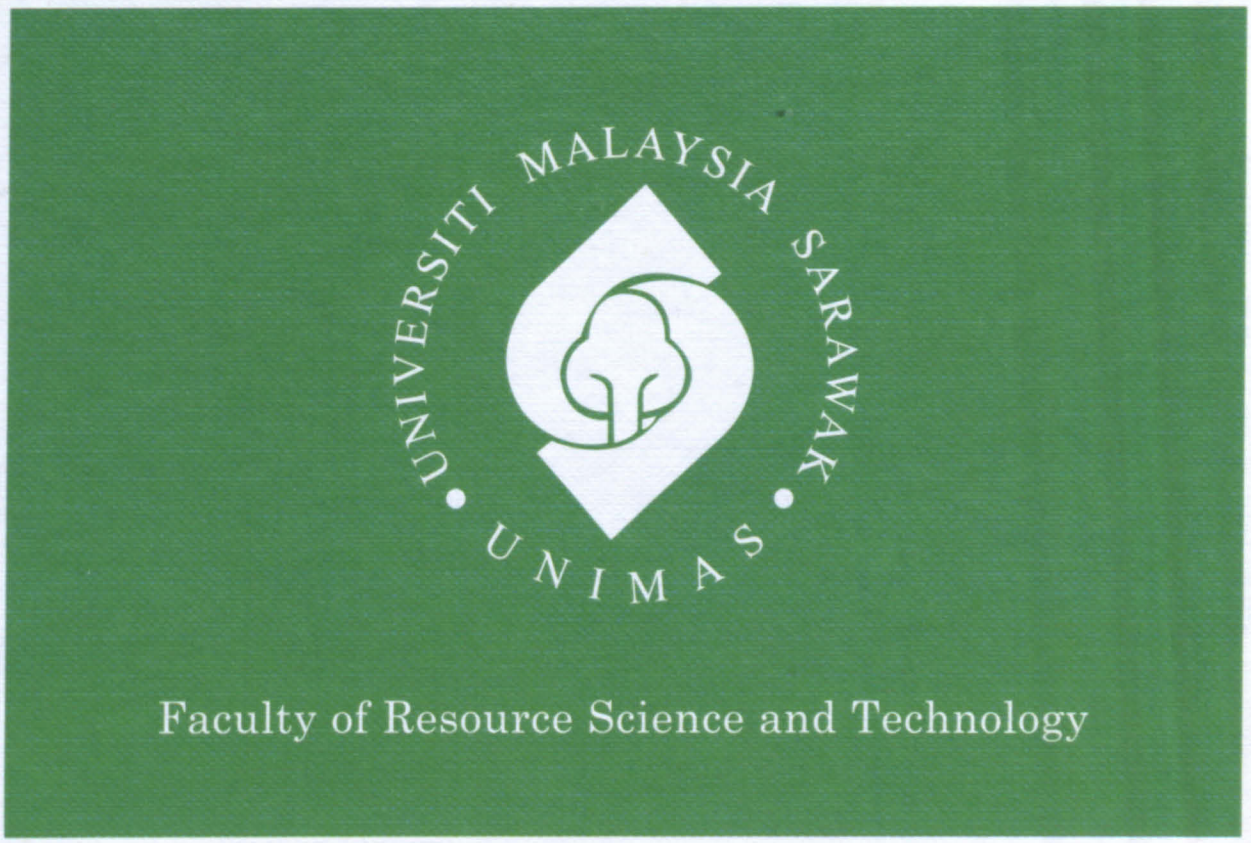

Antibiotic susceptibility of environmental isolates *Burkholderia* species

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(45582)

Bachelor of Science with Honours
(Resource Biotechnology)
2017

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This project is submitted in partial fulfillment of the requirements for the Degree of Bachelor of Science with Honours (Resource Biotechnology)

Supervisor: Dr. Yuwana Podin

Resource Biotechnology
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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

%	Percent
°C	degree celcius
AZM	Azithromycin
Bsa TTSS	*Burkholderia* secretion apparatus of Type-Three Secretion System
CAZ	Ceftazidime
GN	Gentamicin
LPS	Lipopolysaccharide structure
MH	Mueller Hinton's
ml	Millilitre
mm	Millimetre
MSMB	Menzies School of Health Research Miscellaneous Bacteria
NaCl	Sodium chloride
nm	Nanometre
OD	Optical density
SXT	Sulphamethoxazole/Trimethoprim

LIST OF TABLES

LIST OF FIGURES

Antibiotic susceptibility of environmental isolates *Burkholderia* species

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ABSTRACT

Antibiotics are antibacterial drugs that have been used for more than a 100 years to treat patients infected by bacterial infections where the use of antibiotics has successfully reduced illnesses and deaths. However, excessive and misuse of antibiotics has caused many bacteria to develop resistance against antibiotics. The *Burkholderia* species comprising of more than 70 members co-exist in the same ecological niche and this gives rise to interspecies competitions. It raises the question of whether their reaction to antibiotics is similar to that of *Burkholderia pseudomallei,* a member of the *Burkholderia* species which is pathogenic to human and animals. In this project, *Burkholderia* species, *Ralstonia* species and *Achromobacter xylosidians* were tested for their antibiotics susceptibility against gentamicin, ceftazidime, sulphamethoxazole/trimethoprim and azithromycin by using the disk diffusion test. The antibiogram profiles of the majority of *Burkholderia spp.* isolates tested in the study were consistent with that *B. pseudomallei.* This suggests that there may be similarities in the drug susceptibility of the mechanism of *Burkholderia spp.* and *B. pseudomallei* which is more well described due to its clinical importance.

Keywords: *Burkholderia spp.,* disk diffusion test, antibiotic susceptibility, infectious diseases

ABSTRAK

Antibiotik adalah ubat anti-bakteria yang telah digunakan lebih seabad untuk merawat pesakit yang dijangkiti oleh bakteria di mana penggunaan antibiotik telah berjaya mengurangkan penyakit dan kematian akibat pelbagai penyakit berjangkit. Walau bagaimanapun, penyalahgunaan antibiotik telah menyebabkan banyak bakteria untuk mewujudkan rintangan terhadap antibiotik. Spesies *Burkholderia* yang terdiri daripada lebih 70 anggota wujud dalam persekitaran ekologi yang sama dan ini menimbulkan interaksi antara spesis. Ia menimbulkan persoalan sama ada reaksi mereka terhadap antibiotik adalah sama dengan *Burkholderia pseudomallei* iaitu spesis *Burkholderia* yang meyebabkan penyakit kepada manusia dan haiwan. Dalam projek ini, spesies *Burkholderia*, spesies *Ralstonia* dan *Achromobacter xylosidians* telah dikaji sifat antibiotik mereka terhadap gentamicin, ceftazidime, sulphamethoxazole / trimethoprim dan azithromycin dengan menggunakan ujian cakera resapan. Profil antibiogram majoriti spesies *Burkholderia*. diuji dalam kajian ini adalah konsisten dengan *B. pseudomallei*. Ini menunjukkan bahawa mungkin ada persamaan dalam kecenderungan dadah mekanisme spesies *Burkholderia* dan *B. pseudomallei* yang lebih baik digambarkan kerana kepentingan klinikal.

Kata kunci: Spesis *Burkholderia*, cakera resapan, sifat antibiotik, penyakit berjangkit

CHAPTER 1

INTRODUCTION

The *Burkholderia* species is Gram-negative and aerobic bacilli bacteria. *Burkholderia* species consists of around 70 subspecies. It is one of the pathogenic bacteria which can be found in soil or water in the environment. They are present in a wide range of ecological niches (Coenye and LiPuma, 2006). *Burkholderia cepacia* is the first described of *Burkholderia* spp. to be described as a plant pathogen in year 1940s (Stoyanova *et al.*, 2007).

Some of the *Burkholderia* species are pathogenic to human such as *Burkholderia pseudomallei*, the causative agent that causes melioidosis in both humans and animals. The symptoms of melioidosis are similar to other respiratory infections which is often misdiagnosed. Besides, the other type of *Burkholderia* species that is pathogenic is *B. mallei* which affect the horse's glands and it can be transmitted to both humans and animals. However, there is a lack for dependable treatment of melioidosis and glanders hence *B. pseudomallei* and *B. mallei* are known as bio-threat agents. Apart from that, *Burkholderia cepacia* can infect patients with cystic fibrosis and chronic granulomatous disease (Coenye and LiPuma, 2006).

B. pseudomallei is resistant to a wide range of antibiotics such as β-lactams, aminoglycosides and macrolides. But, in Sarawak, over 80% *B. pseudomallei* strains are susceptible towards gentamicin *in vitro* (Podin *et al.*, 2014). Apart from a previous report of *B.cepacia* being resistance to tobramycin. There is a little knowledge on the antibiotics susceptibility profile of other *Burkholderia* spp. Having found to be co-existing in the same ecology as *B. pseudomallei* in the environment, knowledge of antibiotics susceptibility profile of other *Burkholderia* spp. may help in understanding the interactions of the various

Burkholderia spp. in the natural habitat. Moreover, there is lack of data regarding the drug susceptibility of *Burkholderia* spp. Hence, the purpose of this research is to test for antibiotic susceptibility of *Burkholderia* species isolated from the environment.

The main objective of this study is;

1. To characterize the antibiotics susceptibility profile of *Burkholderia* spp. using disk diffusion method strains against clinically relevant antibiotics. (Eg: Gentamicin, Azithromycin, Trimethoprim/Sulphamethoxazole, Ceftazidime)

There is significance of these four types of antibiotics. For gentamicin, *B. pseudomallei* is naturally resistant towards gentamicin except in Sarawak. Besides, in the previous study as described by Podin *et al.*, azithromycin shares similar drug susceptibility mechanism with gentamicin in *B. pseudomallei* (Podin *et al.*, 2014). Ceftazidime and sulphamethoxazole/trimethoprim, on the other hand, are antibiotics that are used to treat melioidosis in which ceftazidime is for intensive phase, while sulphamethoxazole/trimethoprim is for eradication phase.

CHAPTER 2

LITERATURE REVIEW

2.1 *Burkholderia* species

The *Burkholderia* species is commonly found in environmental soil and water. It is a Gram-negative and motile bacteria with the measurement of 1-5μm in length and 0.5 to 1.0 μm in width (Choh *et al.*, 2013). Researchers have shown that some of the *Burkholderia* species are beneficial to the biotechnology and agricultural aspects. For example, *Burkholderia* species can be involved in the biological control of plant disease, plant growth stimulation, the nitrogen fixation improvement and bioremediation (Stoyanova *et al.*, 2007). Pathogenic bacteria such as *Burkholderia pseudomallei, Burkholderia mallei* and *Burkholderia cepacia* may cause life-threatening infections which are difficult to be treated due to multiple antibiotics resistance and the infections in the hosts are often chronic (Choh *et al.*, 2013).

2.2 The population of *Burkholderia* species

2.2.1 *Burkholderia pseudomallei*

B. pseudomallei does not form any spores and it is saprophytic. This bacterium infects both humans and animals through the respiratory system, ingestion and open wounds causing a disease called melioidosis. In certain cases, the bacteria may stay latent in the body for a long period resulting in patients not realizing of an on-going infection until severe symptoms occur. This bacterium infects the lungs which are involved in respiratory system resulting in bronchitis and pneumonia (Wiersinga *et al.*, 2012). Apart from that, this bacterium's growth favours slightly in acidic condition. *B. pseudomallei* used to be known

as the older names such as *Pseudomonas pseudomallei, Bacillus whitmori, Malleomyces pseudomallei, Pfeiferella pseudomallei* and *Whitmorella pseudomallei* (Pringle, 2010).

2.2.2 *Burkholderia* cepacia

B. cepacia is a Gram-negative bacterium that is found in the aquatic environment. It is non-pathogenic to healthy individuals and it is the frequent colonizer of fluids that has been used in the hospital such as irrigation solutions and intravenous fluid (Coenye *et al.*, 2001). In addition, this bacterium has been reportedly responsible in opportunistic respiratory infections of cystic fibrosis or bronchiectasis patients (Cunha, 2015).

2.2.3 *Burkholderia cepacia* complex

B. cepacia complex is a set of *Burkholderia* spp. namely *B. cenocepacia, B. multivorans, B. vietnamiensis, B. dolosa, B. cepacia* and *B. gladioli* which are found in natural environment (Coenye *et al.*, 2001). This type of bacteria will cause a serious risk to lung if the person has cystic fibrosis.

2.2.4 Other non-*Burkholderia pseudomallei* of *Burkholderia* species

B. ubonensis is an environmental bacteria which belongs to *B. cepacia* complex. It causes non-fatal infections in healthy individuals. Apart from that, *B. thailandensis* is also found in the environment which is similar to *B. pseudomallei.* But, *B. thailandensis* is able to assimilate L-arabinose as compared with *B. pseudomallei* which lacks the L-arabinose operon and it is rarely pathogenic to humans (Haraga *et al.,* 2008). Besides, *B. multivorans*is a species that causes human disease for example the colonisation of the lung in cystic fibrosis similar to *B. cepacia* complex. In addition, *B. pyroccinia* is one of the species from *B. cepacia* complex. This species found from the forest soils that can be used as biocontrol agent in agriculture (Song *et al.*, 2012). Apart from that, *Ralstonia* species is one of the bacteria that infect cystic fibrosis patients similar with *B. cepacia* complex

(Coenye, 2002). *Achromobacter xylosodians* is an aerobic and Gram-negative bacteria that comes from natural environment. Besides, *A. xylosodians* is pathogenic to human that can cause infections for instance bacteraemia, meningitis, pneumonia and peritonitis. Apart from that, this bacteria may cause disease towards the immunocompromised hosts and patients with other underlying diseases. *A. xylosodians* may also cause diseases cystic fibrosis patients similar to *Burkolderia cepacia* complex (Lambiase *et al.,* 2011).

2.3 Interaction between *Burkholderia* species in the environment

B. ubonensis is widespread in the surrounding areas and found from the same soil samples as the other species such as *B. cepacia* complex and *Burkholderia* spp. near neighbour such as *Ralstonia* where they may be found within the same ecological niche. There are some species that are non-pathogenic to the human that are found in the same environmental as *B. pseudomallei* such as *B. ubonensis, B. cepacia* complex, *B. humptydooensis, Cupriavidus* spp. and *Pandoraea* spp. This suggests that the *Burkholderia* species living in the same environmental niche that might be sharing their genes through lateral genes transfer (Ginther *et al.*, 2015).

2.3.1 *Burkholderia thailandensis* vs *Burkholderia pseudomallei*

B. thailandensis is closely related to *B. pseudomallei* because of the similar characteristics that have shown by the both species. However, there are some differences shown from the ability of *B. thailandensis* is able to assimilate L-arabinose whereas *B. pseudomallei* lacks the arabinose-assimilation operon (Haraga *et al.*, 2008). *B. pseudomallei* and *B. thailandensis* are similar structurally and immunologically but there are differences in the nucleotide sequences, the profile of biochemical and in their ability to cause severe diseases. In other words, *B. thailandensis* and *B. pseudomallei* are closely related even though *B. thailandensis* is relatively avirulent (Brett *et al.*, 1998)

2.4 Antibiotic sensitivity testing method of *Burkholderia* species

The antibiotic susceptibility testing of *Burkholderia* species is important to detect the possibility of antibiotic resistance in *Burkholderia* species. Besides, the goal of this testing method is to determine melioidosis patient's response towards the antibiotic treatments to ensure that infections are managed well. Disk diffusion method is the most widely used for the antibiotic resistance in clinical diagnostic laboratories. The reason is because the method is convenient and yet efficient method (Reller *et al.*, 2009). However, the other type of testing method such as e-Test will provide more accurate antibiotics susceptibility profiles with the quantitative results by providing more definite minimal inhibitory concentration (MIC) values as compared with disk diffusion tests (Hendrikson, 2003).

2.5 Mechanism of antibiotics susceptibility or resistance of *Burkholderia* spp.

Different types of bacterial species have different antibiotics susceptibility patterns. The antibiotic susceptibility mechanism may be related to the role of limited permeability such as lipopolysaccharide structure, porins, efflux and biofilm-related resistance mechanism (Burns, 2006).

2.5.1 Lipopolysaccharide structure (LPS)

B. cepacia unique structure was determined that the binding site for the antibiotics within the purified LPS of the *B. cepacia* complex where the outer membrane of the cation binding sites is concealed (Burns, 2006). Besides, the resistant of *B. cepacia* complex towards trimethoprim, chloroamphenicol and quinolones was associated together with the changes of LPS. These changes include the lack of high molecular weight O-antigen and the variations in size (Rajyaguru and Muszynski, 1997).

2.5.2 Porins

Porin-mediated action which was discovered in species of *Burkholderia* and *Pseudomonas* decreases antibiotic permeability. This action is related to the pore physical size that formed by porin proteins or channel conductance (Burns, 2006).

2.5.3 Efflux

Efflux system is a unique mechanism for high-level of aminoglycoside resistance and it has also been described to confer macrolide resistance in *B. pseudomallei* (Cheng and Curie, 2005).

2.5.4 Biofilm-related resistance mechanisms

The biofilm formation which confers antibiotic resistance has been observed in *B. cepacia* complex, *B. thailandensis* and *B. pseudomallei* which are associated with the production of capsular exopolysaccharide. The biofilm formation has been demonstrated on the plastic surface for *B. cepacia* (Conway *et al.*, 2002).

2.6 Antibiotic susceptibility of *Burkholderia pseudomallei*

B. pseudomallei is intrinsically gentamicin resistant worldwide but it was discovered that over 80% of strains in Sarawak, Malaysian Borneo is gentamicin sensitive (Podin *et al.*, 2014). For the large range of antibiotic *B. pseudomallei* was found to be resistant to β-lactam antibiotics, aminoglycosides and macrolides through inactive enzyme, cell exclusion and broad-range efflux pump (Podin *et al.*, 2014). However, the type of antibiotic in which *B. pseudomallei* is susceptible to tigercycline, ampicillin or sulbactam and piperacillin or tazobactam which can be used in the treatment for melioidosis (Ahmad *et al.*, 2013).

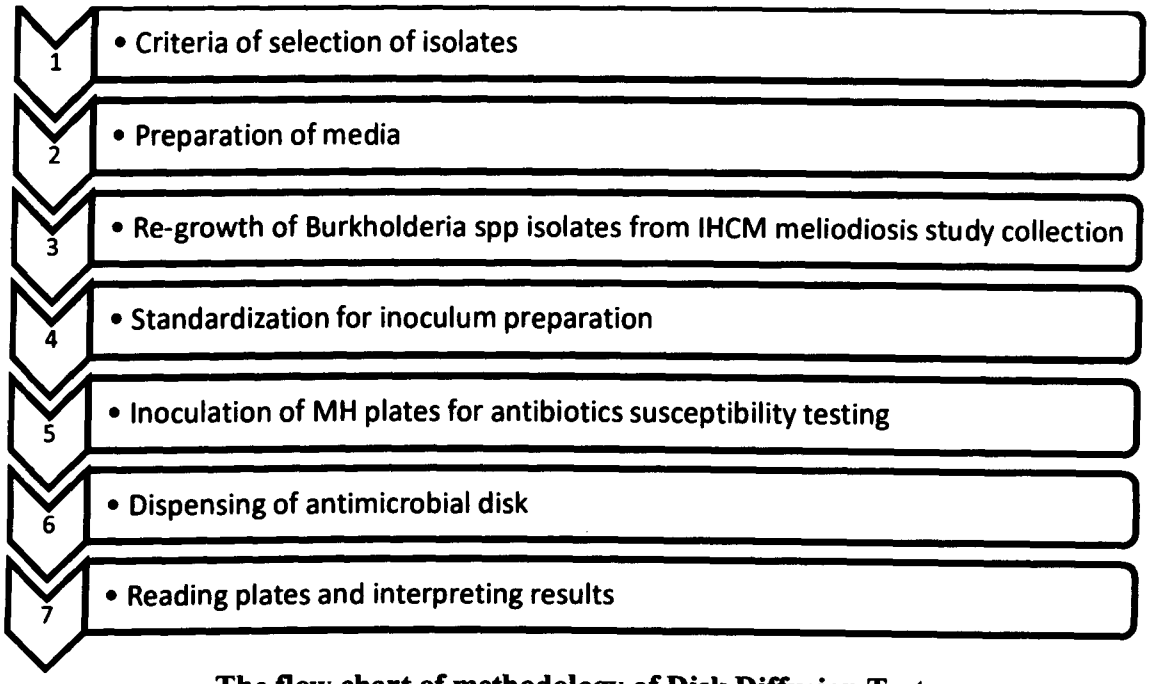
CHAPTER 3

MATERIALS AND METHOD

3.1 Materials

Materials	Brand name
Mueller-Hinton Agar	15.2g - Agar powder (Himedia, Himedia Labarotories, India) Up to 400 ml - UHQ water (Veolia Water Technologies, UK)
Luria-Bertani Agar	4 g - tryptone (Oxoid, Thermo Fisher Scientific Co., MA, USA) 2 g - yeast extract (AMRESCO, USA) 4 g - sodium chloride, NaCl (R&M Chemicals, UK) 6 g - bacteriological agar (Scarlau, Barcelona, Spain) Up to 400 ml - UHQ water (Veolia Water Technologies, UK)
Antibiotic (Disk diffusion)	Gentamicin (LIOFILCHEM, Italy) Ceftazidime (LIOFILCHEM, Italy) Sulphamethoxazole/Trimethoprim (LIOFILCHEM, Italy) Azithromycin (LIOFILCHEM, Italy)
Inoculum preparation	Spectrophotometer (BioPhotometer, Eppendorf, Germany) BRAND® Standard Disposable Cuvette (Sigma-Aldrich, USA) Sterile wooden cotton applicator 6"

3.2 Methodology



The flow chart of methodology of Disk Diffusion Test

3.2.1 Criteria of selection of isolates

Burkholderia spp identified based on recA gene sequencing;

i. *Burkholderia ubonensis*
ii. *Burkholderia thailandensis*
iii. *Burkholderia pyroccinia*
iv. *Burkholderia cepacia* complex
v. *Burkholderia multivorans*
vi. *Ralstonia* species
vii. *Ralstonia solanacearum*
viii. *Burkholderia spp.*
ix. *Achromobacter xylosodians*
x. *Burkholderia diffusa*

3.2.2 Preparation of media

3.2.2.1 Mueller-Hinton Agar

MH agar was re-suspended with UHQ water. The mixture was heat and shaken constantly to mix evenly. Then, the mixture was boiled at 100 °C for about one minute and autoclave for 15-20 minutes at 121 °C. The autoclaved mixture was allowed to cool down around 50-55 °C and poured into Petri dishes. After that, the agar was left to solidify at room temperature before being stored at 4 °C.

3.2.2.2 Luria-Bertani agar

The ingredients were mixed together and autoclaved at 121 °C for 15-20 minutes. The mixture was allowed to cool down to 50-55 °C and poured into Petri dishes. After that, it was left to solidify at room temperature before being stored at 4 °C.

3.2.3 Re-growth of *Burkholderia* species isolates from IHCM melioidiosis study collection

The *Burkholderia spp.* isolates were taken from the glycerol stock in -80°C. Then, the bacteria was taken to grow in Luria-Bertani agar into 2-3 days and incubated in the growth chamber of 37°C. After the growth of bacteria was stable in 2-3 days, one colony was picked to sub-culture again and was streaked in fresh Luria-Bertani agar overnight in growth chamber 37°C.

3.2.4 Standardization for inoculum preparation

1. On the next day, the cell suspension was prepared by picking one colony using a cotton bud.

2. The picked colonies were resuspended in 0.85% saline (NaCl) to optical density (OD) of 0.13 at 600 nm by using a spectrophotometer, BioPhotometer (Eppendorf, Germany).
3. A sterile cotton swab was used to dip into the inoculums cell suspension to prepare the bacterial lawn.
4. Then, the cotton swab is pressed against the inside of the tube wall in order to remove any of the excess liquid culture.
5. The entire surface of the Mueller-Hinton's (MH) agar plate was streaked gently by inoculating the plate three times with the plate being turned 60 degrees after each inoculation.

3.2.5 Dispensing of antimicrobial disk

A sterile forceps was used to dispense the disk on the agar surface. The forceps was sterile by using ethanol. The disk must have complete contact with the agar surface by touching using forceps at the top of the disk. After the disk was placed on the agar surface, it should not be moved or relocated. This is because, the diffusion of the antibiotics starts immediately upon placement. If the disk is moved, this will affect the unreliable results by producing distorted zones.