Faculty of Resource Science and Technology

Identification and Characterisation of Bacteria Isolated (During a Cholera Outbreak) in Limbang, Sarawak

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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ABSTRACT

Cholera epidemics have occurred in Malaysia since 1991 till 2003 which can be proved from the records by the Infectious Diseases Division of the Ministry of Health. Additionally, from 1994 to 2003, Sarawak saw a series of cholera epidemics. Cholera outbreaks in Malaysia are primarily caused by the El Tor O1 *Vibrio cholerae* serogroup. The aims of this study were to identify bacterial species from clinical and environmental samples (n=28) from Limbang, Sarawak by collaboration with Sarawak Government Hospital by the approach of active cased detection (ACD) and passive case detection (PCD), and to detect the toxin genes of the *V. cholerae* from the isolates. Apart from that, to determine the genetic relatedness of the isolates, to detect antibiotic susceptibility, and to determine the isolates' minimal biofilm eradication concentration (MBEC). All the isolates were sub-cultured in alkaline peptone water (APW). The boiled-cell method was used for DNA extraction. The total DNA extracted was amplified by polymerase chain reaction (PCR). In this work, two forms of PCR were used: 16S rRNA PCR and multiplex PCR. The results obtained from the study found out that 16 out of 28 (57.14 %) samples were confirmed to be *V. cholerae* species. Four primers specific for *V. cholerae* were used in multiplex PCR (O1 type, O139 type, *ctx*A and *ctx*AB) to confirm the species type and the toxin genes. All samples shown positive for *V. cholerae* O1 serotype and 100% positive to all genes for the identification of *ctx*A and *ctx*AB genes. Following that, all 16 isolates were subjected to molecular typing using the random amplified polymorphic DNA (RAPD) PCR and enterobacterial repetitive intergenic consensus (ERIC) PCR techniques. Antimicrobial testing was also performed using the disk-diffusion method and the MBEC method with chloramphenicol antibiotic. Sixteen confirmed *V. cholerae* O1 were successfully fingerprinted and demonstrated to be strain discriminating. Although all isolates were sensitive to Norfloxacin, they exhibited six distinct forms of

antimicrobial resistance. Finally, all isolates were eradicated at concentrations of 50 mg/mL and 100 mg/mL. Multiplex PCR was demonstrated in this work to be useful for research purposes in the field of molecular genetics including cholera outbreaks. Then, both RAPD-PCR and ERIC-PCR are suitable for profiling, and Norfloxacin can be utilised for future therapy because it shown 100 % susceptibility to all isolates in this research.

Keywords: *Vibrio cholerae* O1, *ctx*AB genes, polymerase chain reaction, antibiotic, MBEC

Kajian Molekul Wabak Vibrio cholerae di Limbang Sarawak

ABSTRAK

Wabak taun telah berlaku di Malaysia sejak tahun 1991 hingga 2003 yang boleh dibuktikan daripada rekod oleh Bahagian Penyakit Berjangkit Kementerian Kesihatan. Selain itu, terdapat juga wabak taun dari tahun 1994 hingga 2003 yang telah berlaku di Sarawak. Wabak taun di Malaysia kebanyakannya disebabkan oleh serogroup El Tor O1 Vibrio cholerae. Matlamat kajian ini adalah untuk mengenal pasti spesies bakteria daripada sampel klinikal dan persekitaran (n=28) dari Limbang, Sarawak dengan kerjasama Hospital Kerajaan Sarawak melalui pendekatan pengesanan kes aktif (ACD) dan pengesanan kes pasif (PCD), dan untuk mengesan gen toksin V. cholerae daripada isolat. Selain itu, kajian ini juga adalah untuk mengenal pasti perkaitan genetik antara sampel bakteria, mengesan kerentanan terhadap antibiotik dan menentukan minima kepekatan pembasmian biofilem (MBEC) bakteria. Kesemua isolat bakteria telah dibiakkan dalam air pepton beralkali (APW). Kaedah sel rebus digunakan untuk pengekstrakan DNA. Jumlah DNA yang diekstrak telah dikuatkan oleh tindak balas rantai polimerase (PCR). Dua jenis PCR telah digunakan dalam kajian ini iaitu PCR 16S rRNA dan PCR multipleks. Keputusan yang diperolehi daripada kajian mendapati 16 daripada 28 (57.14 %) sampel disahkan sebagai spesies V. cholerae. Empat primer khusus untuk V. cholerae telah digunakan dalam multipleks PCR (jenis O1, jenis O139, ctxA dan ctxAB) untuk mengesahkan jenis spesies dan gen toksin. Semua sampel menunjukkan keputusan positif untuk V. cholerae O1 serotype dan 100% positif kepada semua gen untuk pengenalpastian gen ctxA dan ctxAB. Kesemua 16 isolat bakteria kemudiannya diteruskan ke kaedah menaip molekul iaitu DNA polimorfik yang diperkuat secara rawak (RAPD)-PCR dan konsensus intergenik berulang bakteria (ERIC)-PCR. Ujian antimikrob dengan kaedah resapan cakera diuji berserta kaedah MBEC

menggunakan antibiotik kloramfenikol. Enam belas V. cholerae O1 berjaya diprofilkan dan menunjukkan diskriminasi antara strain. Semua isolat bakteria terdedah kepada Norfloxacin tetapi membentuk enam corak Profil Rintangan Antimikrob. Akhirnya, semua isolat telah dibasmi pada konsentrasi 50 mg/mL dan 100 mg/mL. Daripada kajian ini, ia menunjukkan bahawa PCR multipleks boleh digunakan untuk tujuan penyelidikan dalam bidang genetik molekul yang melibatkan wabak kolera. Kemudian, RAPD-PCR dan ERIC-PCR kedua-duanya sesuai untuk pemprofilan dan Norfloxacin boleh digunakan untuk terapi masa hadapan kerana ia menghasilkan 100 % kerentanan kepada semua isolat bakteria.

Kata kunci: *Vibrio cholerae O1, ctxAB gen, tindak balas rantai polimerase, antibiotik, MBEC*

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LIST OF ABBREVIATIONS

°C	Degree of Celsius
µL	Microlitres
16S rRNA PCR	16S ribosomal ribonucleic acid
Ace	Accessory cholera enterotoxin
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine monophosphate
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
cGMP	Cyclic guanosine monophosphate
CGS	Centre for Graduate Studies
Cl-	Chloride ion
CLCA	Chloride channel
CT	Cholera Toxin
ctx	Cholerae toxin gene
DNA	Deoxyribonucleic acid
EPS	Extracellular polymeric substances
ERIC	Enterobacterial Repetitive Intergenic Consensus
ESBL	Extended Spectrum Beta-lactamase
HA/P	Haemagglutinin/protease
LB	Luria-Bertani
MGE	Mobile Genetic Elements
MISSR	Modified inter simple sequence repeat
Na^{+}	Sodium ion

NAG	Non-agglutinable
NHE	Sodium-hydrogen exchanger
OMP	Outer Membrane Protein
PCR	Polymerase Chain Reaction
PKA	Protein Kinase A
RAPD	Random Amplified Polymorphic DNA
REP	Repetitive-element PCR
RFLP	Restriction Fragment Length Polymorphism
RLS	Resource-limited Settings
RTX	Repeat in toxin
sp.	Species
UNIMAS	Universiti Malaysia Sarawak
V. cholerae	*Vibrio cholerae*
Zot	Zonula occludens toxin

CHAPTER 1

INTRODUCTION

1.1 Study Background

Cholera disease is characterised by the passing of big stools resembling rice water and vomiting that results in dehydration as a result of underlying deficiencies caused by the loss of significant amounts of extracellular fluid. The critical therapy is appropriate rehydration, which must be maintained in the absent of antibacterial treatment. Antibiotic microbial agents provide a secondary but critical role in therapy by reducing the severity of sickness and the organism's excretion time (Singh & Mohapatra, 2008).

Cholera is spread by the faecal-oral route, and *V. cholerae* is particularly susceptible to the acidic environment of the human stomach. As a result, individuals with insufficient stomach acid production, such as infants, the elderly, and those who take antacids, are at an increased risk of contracting cholera. The deadly signs of the disease are caused by a potent enterotoxin (CT, also termed choleragen) produced by *V. cholerae* strains (Tabatabaei & Khorashad, 2015).

Antibiotic-resistance testing, biotyping, serotyping, and phage typing are increasingly being used for cholera epidemiological surveillance rather than phenotypic characteristics such as biochemical and antigenic qualities. Because of their low discriminatory strength, traditional typing approaches cannot reliably estimate the epidemic potential of *V. cholerae* strains (Bhowmick et al., 2009). The use of genome-based bacterial identification and typing would indicate an epidemic relationship as well as the link between specific clones and suspected contamination sources (Bhowmick et al., 2009).

Demographic changes are predicted to lead to increased *Vibrio* infection rates (Bier et al., 2015). The central region's supply of safe drinking water is insufficient as a result of increased urban population density, forcing people to drink water from any accessible source, treated or not. Other variables, such as floods, landslides, open defecation habits, and a breakdown in sanitation and the provision of potable water, all contribute to the cholera epidemic's frequency (Rijal et al., 2019).

1.2 Problem Statement

Since 1991 to 2003, cholera epidemics have occurred in Malaysia, as documented by the Infectious Diseases Division of the Ministry of Health (Patrick et al., 2012). Additionally, the course of cholera epidemics from 1994 to 2003 was recently detailed in Sarawak (Patrick et al., 2012). Numerous factors may play a role in the propagation of this cholera sickness. Inadequately treated water supply and inadequate sanitation may have contributed to and aided the spread of infections in Sarawak, particularly in rural areas where the toxigenic *V. cholerae* is prevalent (Patrick et al., 2012).

Teh et al. (2012) reported that cholera epidemics in Malaysia are predominantly caused by the El Tor O1 *V. cholerae* serogroup. The O139 serogroup occasionally causes most cases compare to the non-O1/non-O139 because the non-O1/non-O139 *V. cholerae* serogroup has not been incriminated in major outbreak. Transmission vehicles for the cholera outbreak are mostly contaminated drinking water, unwashed contaminated food, and undercooked seafood from *V. cholerae* endemic estuaries (Griffith et al., 2006; Teh et al., 2012). According to Reimer et al. (2011), in Asia and Africa, the endemic and epidemic cases that recently occurred are increasingly associated with the genetically atypical El Tor variants which share characteristics of classical and El Tor strains.

“It is unnecessary to create a quarantine centre at the hospital because the district's cholera outbreak is under control”, said Dr Abdul Rahman Ismail. Despite the fact that the district has reported fewer than ten occurrences of the sickness, Bukit Kota assemblyman Dr Abdul Rahman Ismail, a medical doctor by profession, recommended residents living in riverine areas to exercise additional caution when using river water (Borneo Post, 2016). Even if the condition is under control, prompt action/detection/prevention must be performed to avert a worsening outbreak.

As such, it is critical to understand the microbiological components of cholera epidemic containment. Sarawak General Hospital suspected that the bacterial species from Limbang, Sarawak were *V. cholerae* due to the symptoms observed during the outbreak. Confirmation of the bacterial species is necessary for the advancement of bacterial research. This study is therefore conducted to identify and confirm the bacterial species. This research also will provide the relationship between clinical and environmental samples from the Limbang outbreak. Apart from that, the data would be beneficial for comparing the efficacy of various molecular typing approaches and will allow for additional actions to be taken to contain the cholera outbreak.

1.3 Objectives

The objectives for this study are:

i. To identify bacterial species from clinical and environmental samples using 16S rRNA PCR.
ii. To detect the toxin genes of the *V. cholerae* using multiplex PCR.
iii. To determine the genetic relatedness of *V. cholerae* isolates.

iv. To determine the antimicrobial susceptibility of *V. cholerae* strains and to determine the minimum biofilm eradication concentration (MBEC) of the isolates.

CHAPTER 2

LITERATURE REVIEW

2.1 Diarrhoea in General

Diarrhoea is a clinical symptom of disrupted intestinal ion transport channel proteins, channels, physical and chemical barriers, resulting in water and electrolyte transport abnormalities in the digestive tract (Li et al., 2021). Diarrhoea is defined as the passing of three or more loose or watery stools per day, or more frequently than is usual for the individual, according to the World Health Organization (WHO). It is usually a sign of an infection in the digestive tract, which can be caused by bacteria, viruses, or parasites. Infections can be passed from one person to another through contaminated food or water or because of poor hygiene. There are three clinical types of diarrhoea, and each has its own treatment: acute watery diarrhoea, which can last for hours or days and includes cholera; acute bloody diarrhoea, which is also called dysentery; and persistent diarrhoea, which lasts 14 days or longer (WHO).

Diarrhoea is also a symptom as well as an indicator. Chronic diarrhoea can be caused by a variety of factors. The activation of fluid and electrolyte secretion in one or more segments of the small intestine, large intestine, or both is central to practically all diarrhoeal diseases. It can be either osmotic or secretory in nature (Binder, 2006).

The majority of enteropathogens, both bacterial and nonbacterial, cause acute watery diarrhoea (DuPont, 2009). *V. cholerae* serotypes O1 and O139 have two primary virulence genomes: cholera toxin (CT) and pathogenic toxin colonies, which induce acute watery diarrhoea (Li et al., 2021). In the United States, it is estimated that the cause is recognised in

less than 3% of instances. Compounding the problem of low detection rates, a large number of potentially significant substances are undetectable using normal diagnostic techniques. Fluid and electrolyte supplementation are critical in all episodes of diarrhoea. Drugs used to treat symptoms may result in a decrease in the number of stools voided. For those with acute bacterial diarrhoea, a "BRAT" (bananas, rice, applesauce, and toast) diet is frequently prescribed. Antimicrobial therapy is critical usually of diarrhoea caused by invasive or inflammatory bacterial infections and is beneficial in other kinds of bacterial diarrhoea that are not invasive (DuPont, 2009).

2.2 Types of Diarrhoea

Non-inflammatory diarrhoeas are caused by microorganisms that infect the small intestine and attach to the mucosa, but do not cause significant acute inflammation or mucosal damage. Non-inflammator diarrhoea patients typically present with few systemic signs or symptoms. Meanwhile, inflammatory diarrhoea is caused by organisms that attack the lower gut, particularly the distal ileum and colon. These organisms induce sickness through the secretion of toxic cytotoxins or by penetrating the intestinal epithelium (Navaneethan & Giannella, 2008).

2.3 Mechanisms of Diarrhoea

Diarrhoea's pathogenesis is complex. Drugs and toxins can interfere with natural bodily functions and result in diarrhoea. For instance, Enteropathogenic *E. coli* (EPEC) infection rapidly lowered Serotonin Transporter (SERT) function, resulting in an increase in serotonin levels and increased diarrhoea (Surawicz, 2010).

Other than that, *V. cholerae* is one of the most prevalent causes of diarrhoea around the world. Cholera-associated diarrhoea is most prevalent in the Indian subcontinent, in

Southeast Asia, Africa, and South America. Each *Vibrio* strain produces the identical enterotoxin known as cholera toxin. While cholera toxin can infect the entire intestine, it is primarily caused by toxin activity in the proximal small intestine, which is characteristic of non-inflammatory diarrhoea (Navaneethan & Giannella, 2008).

The bacterium's adhesion to the intestinal epithelium is crucial. *V. cholerae* is attached via a fimbria colonisation component called the toxin-coregulated pilus. Additionally, other pilus structures, such as the fucose- and mannose-binding hemagglutinins, increase colonisation of *V. cholerae* O1 classical and El Tor biotypes, respectively. *V. cholerae* secretes cholera toxin following adhesion. Cholera toxin is composed of two subunits namely, subunit A (CTA) and subunit B (CTB) that control intestinal epithelial cells' toxin interactions (Navaneethan & Giannella, 2008). Figure 2.1 depicted the general mechanism of non-inflammatory diarrhoea.