

**ISOLATION AND TOXIN TYPING OF *CLOSTRIDIUM PERFRINGENS*
FROM SUNGAI SELANGOR AND SUNGAI BERNAM**

FLORENCE LEE CHI HIONG

**SUBMITTED IN FULFILLMENT
OF THE REQUIREMENT
FOR THE DEGREE OF MASTER OF SCIENCE**

**FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR**

AUGUST 2009

ABSTRACT

Clostridium perfringens (CP) is an anaerobic, spore forming bacterium that is emerging as a preferred faecal pollution indicator. In the local scene, little is known about the density and toxinotype of CP in river water, and how does river discharge affects the density. Therefore, water samples were taken from three Malaysian rivers, Sungai Bernam, Sungai Selangor and Tengi Canal between April 2007 and January 2008 to examine the CP densities and toxinotypes. The rivers were selected because of their differences in adjacent land usage. Sungai Selangor reported lower CP isolation rate ranging between 0 to 25% but higher CP densities of <1 to 2695 cfu/100ml. In contrast, CP isolation rates in Sungai Bernam were higher at 0 to 35% but the densities were lower at <1 to 763 cfu/100ml. Tengi Canal showed the lowest CP isolation rate of 0 to 10% and CP densities of <1 to 212 cfu/100ml. Sulphite reducing Clostridia (SRC) were consistently present, and the densities were significantly different temporally in each of the study sites. The highest CP and SRC densities were in the downstream of Sungai Selangor. No significant correlation was found between CP densities and river discharge in all the study sites. Toxinotyping was performed with Polymerase Chain Reaction (PCR) using published primer sequences, and multiplex primers designed specifically to include also the detection of enterotoxin (CPE) gene. All 142 CP isolates found in this study belonged to *Clostridium perfringens* Type A. Five of the isolates also harboured CPE gene.

ABSTRAK

Clostridium perfringens (CP) ialah sejenis bakteria anaerobik dan penghasil spora yang kini semakin kerap dijadikan sebagai petunjuk pencemaran najis. Namun, kepekatan CP dan jenis toksin CP dalam air sungai negara kita, serta kesan luahan sungai terhadap kepekatan CP adalah kurang diketahui. Maka, sampel air telah diambil daripada Sungai Bernam, Sungai Selangor dan Terusan Tengi dalam tempoh April 2007 hingga Januari 2008 untuk mengkaji kepekatan dan jenis toksin CP. Tiga sungai tersebut dipilih kerana kegunaan tanah sekeliling yang berbeza. Sungai Selangor melaporkan kadar isolasi CP yang lebih rendah dalam lingkungan 0 hingga 25% tetapi kepekatan CP sungai tersebut adalah lebih tinggi iaitu <1 hingga 2695 cfu/100ml. Sebagai perbandingan, kadar isolasi CP di Sungai Bernam adalah lebih tinggi dengan 0 hingga 35% tetapi kepekatan CP adalah lebih rendah iaitu <1 hingga 763 cfu/100ml. Terusan Tengi mencatatkan kadar isolasi dan kepekatan CP yang paling rendah, iaitu 0 hingga 10% dan <1 to 212 cfu/100ml. Clostridia penurun sulphite (SRC) didapati sentiasa wujud. Dalam setiap tapak kajian, kepekatan SRC sepanjang tempoh kajian menunjukkan perbezaan yang nyata. Kepekatan CP dan SRC yang tertinggi dicatatkan di hilir Sungai Selangor. Tiada hubungan yang nyata wujud di antara kepekatan CP dan luahan sungai di kesemua tapak kajian. Jenis toksin ditentukan melalui PCR dengan menggunakan turutan primer yang telah diterbitkan, dan juga primer multipleks yang direka khas untuk turut mengesan kehadiran gen enterotoksin (CPE). Kesemua 142 koloni CP yang diperolehi dalam kajian ini tergolong kepada CP jenis toksin A. Lima daripada jumlah koloni itu juga mempunyai gen toksin CPE.

ACKNOWLEDGEMENTS

I sincerely thank the Director General of Ministry of Health Malaysia, the Director General of Department of Irrigation and Drainage (DID) Malaysia, and Dean of the Faculty of Science, University of Malaya for their permission to perform this study. This research was supported by the Ministry of Health Malaysia (Grant no. JPP-IMR 07-030, NMRR ID 08-212-1367) and University of Malaya (Grant no. FR177/2007A and P0237/2007A).

I would like to express my deepest gratitude to the DID Selangor and Afftech Automation System Sdn Bhd for their great support in acquiring water samples for this study. I am also grateful to the Information Unit of DID Malaysia for helping me in study site selection and providing the supporting data. I wish to thank various personnel from the Molecular Pathology, Parasitology, Entomology, Bacteriology, Animal House and Epidemiology Units of the Institute for Medical Research; and Biomedical Science & Molecular Typing Lab (A407) of the Institute of Postgraduate Studies, University of Malaya for their generosity in facilities sharing and kind help.

To Prof Thong Kwai Lin, my supervisor, Dr Lokman Hakim Sulaiman, my co-supervisor, and Dr Muhammad Amir Kamaluddin, my boss; your guidance, support and trust throughout this study are the key to its completion – thank you!

To my colleagues in EHRC, my friends and my dearest family members, your cherish and encouragements have always keep things pleasant. Dear Lord our Father in heaven, “My grace is sufficient for you” - thanks for every single thing that you have prepared me for and lead me through, Amen.

TABLE OF CONTENTS

Page

Chapter One : Introduction

1.1	Research Background	1
1.2	Research Objectives	3
1.3	Research Rationales and Limitations	4
1.4	Significance of Study	4

Chapter Two : Literature Review

2.1	Advantages of <i>Clostridium perfringens</i> as an indicator	6
2.2	<i>Clostridium perfringens</i> In Terrestrial and Marine Environment	8
2.3	<i>Clostridium perfringens</i> As Faecal Pollution Indicator and Surrogate Indicator In the Environment	9
2.4	<i>Clostridium perfringens</i> As Surrogate and Real Indicator In Drinking Water and Waste Water Treatment Plants	11
2.5	<i>Clostridium perfringens</i> In Healthy Individuals	14
2.6	<i>Clostridium perfringens</i> In Food Poisoning and Antibiotic Associated Diarrhea	14
2.7	<i>Clostridium perfringens</i> Pathology	15
2.8	<i>Clostridium perfringens</i> Toxins	15
2.8.1	Alpha Toxin	16
2.8.2	Beta Toxin	16
2.8.3	Epsilon Toxin	17
2.8.4	Iota Toxin	17
2.8.5	CPE Toxin	18

TABLE OF CONTENTS	Page
Chapter Three : Materials and Methods	
3.1 Study Sites	19
3.2 <i>Clostridium perfringens</i> and Sulphite Reducing Clostridia Detection	23
3.3 Water Sampling	23
3.4 Water sample analysis and culture work	
3.4.1 Water sample analysis	24
3.4.2 Presumptive CP enumeration, subcultures and biochemical confirmation tests	24
3.5 Mean Sulphite Reducing Clostridia (MBCC) and Mean <i>Clostridium perfringens</i> (MCPC) Densities Calculation	
3.5.1 Mean sulphite reducing Clostridia densities (MBCC) in cfu/100ml	25
3.5.2 <i>Clostridium perfringens</i> isolation rate (IRt), mean <i>Clostridium perfringens</i> density (MCPC) in cfu/100ml and <i>Clostridium perfringens</i> prevalence	25
3.6 Data Analysis	26
3.7 <i>Clostridium perfringens</i> Toxin Gene Detection	
3.7.1 DNA Extractions	27
3.7.2 Toxin Gene Primers	27
3.7.3 Polymerase Chain Reaction (PCR) and Gel Electrophoresis	28
3.7.4 Alpha Toxin Gene Detection and Optimization	31
3.7.5 Beta, Epsilon, Iota and CPE Toxin Gene Detection	32
3.7.6 Duplex PCR of Alpha and CPE Toxin Gene Detection	32
3.7.7 PCR Product Sequencing	33

TABLE OF CONTENTS		Page
Chapter Four : Results		
4.1	Presumptive <i>Clostridium perfringens</i> (Sulphite Reducing Clostridia) Colonies Isolation from TSC and OPSP Selective Media	34
4.2	Pooling Method in Presumptive <i>Clostridium perfringens</i> (Sulphite Reducing Clostridia) Isolates Selection	34
4.3	Comparison Among Rivers and Study Sites: Mean Sulphite Reducing Clostridia Densities (MBCC), <i>Clostridium perfringens</i> Prevalence and Mean <i>Clostridium perfringens</i> Densities (MCPC)	35
4.4	Correlation Between Mean <i>Clostridium perfringens</i> Densities, Mean Sulphite Reducing Clostridia Densities, and Mean River Discharge	41
4.5	Correlation of Mean <i>Clostridium perfringens</i> Densities (MCPC), Mean Sulphite Reducing Clostridia Densities (MBCC), and Mean River Discharge (Q) Along Sungai Bernam and Sungai Selangor	51
4.6	Physico-chemical Parameters of River Water	53
4.7	DNA Quantification	53
4.8	Detection of Alpha, Beta, Epsilon, Iota and CPE Toxin Gene in <i>Clostridium perfringens</i> by Polymerase Chain Reaction (PCR)	54
4.9	Duplex PCR of Alpha and CPE toxin gene Using Set 2 Primers	55
4.10	Alpha and CPE Toxin Gene Sequencing Results	55

TABLE OF CONTENTS	Page
Chapter Five : Discussion	
5.1 Dynamics of Mean <i>Clostridium perfringens</i> Densities (MCPC) and Mean Sulphite Reducing Clostridia Densities (MBCC)	56
5.2 Correlation Between Mean Sulphite Reducing Clostridia Densities (MBCC), Mean <i>Clostridium perfringens</i> Densities (MCPC) and River Discharge	58
5.3 <i>Clostridium perfringens</i> And Sulphite Reducing Clostridia : Possibility of Indicating Faecal Pollution and Other Types of Pollution Simultaneously	58
5.4 Toxinotypes of Environmental <i>Clostridium perfringens</i>	59
5.5 Relationship between <i>Clostridium perfringens</i> Prevalence in the Environment and <i>Clostridium perfringens</i> Food Poisoning	60
Chapter Six : Conclusion and Recommendations	61
Appendix A1 to Appendix A6	63 - 68
Study Site water samples : Sulphite Reducing Clostridia CFU of two water sample replicates on TSC media, water sample replicates MBCC, and Site MBCC (Average)	
Appendix A7 to Appendix A12	69 - 74
Site A water samples (A1, A2 & A3) : Number of presumptive CP selected for biochemical confirmation tests, Number of confirmed CP by alpha toxin gene detection, CP IRt and CP Prevalence	
Appendix B Mean River Discharge Normality and Statistics	75

TABLE OF CONTENTS	Page
Appendix C Statistical Difference of Water Sample Replicates	76 - 81
Appendix D MBCC Difference Among First, Mid and Third Quarter Point Sampling	82 - 84
Appendix E Temporal difference of Sulphite Reducing Clostridia CFU in First, Mid and Third Point Sampling	85 - 87
Appendix F Nonparametric Correlations	88 – 93
Appendix G1 MBCC, MCPC and River Discharge Correlations Along Sungai Selangor	94
Appendix G2 MBCC, MCPC and River Discharge Correlations Along Sungai Bernam	95
Appendix H1 to H4 Photographs of CP and Sulphite Reducing Clostridia	96 - 97
Appendix H5 Photograph of sewage discharge outlets in Tanjung Malim (Site B)	98
Appendix H6 Photographs of sampling site in Jambatan Mergastua	98
Appendix I1 to I5 Sequencing Results	99 - 103
References	104 - 121

LIST OF FIGURES

- Fig. 3.1** Study Site Locations
- Fig. 4.1** Sites Comparison of Arithmetic Mean Sulphite Reducing Clostridia Density, Arithmetic Mean *Clostridium perfringens* Density and *Clostridium perfringens* Prevalence
- Fig. 4.2** Sites Comparison for Mean *Clostridium perfringens* Densities (MCPC) and Mean Sulphite Reducing Clostridia Densities (MBCC)
- Fig. 4.3** Sungai Bernam and Tenggi Canal *Clostridium perfringens* Isolation Rate (IRt)
- Fig. 4.4** Sungai Selangor *Clostridium perfringens* Isolation Rate (IRt)
- Fig. 4.5** Sites Comparison for *Clostridium perfringens* Densities (MCPC) and Mean River Discharge
- Fig. 4.6** Sites Comparison for Mean Sulphite Reducing Clostridia Densities (MBCC) and Mean River Discharge
- Fig. 4.7** Site A River Discharge and First, Mid & Third Point Sampling Mean Sulphite Reducing Clostridia Densities (MBCC)
- Fig. 4.8** Site F River Discharge and Grand Mean Sulphite Reducing Clostridia Densities (MBCC)
- Fig. 4.9** Site G River Discharge and First, Mid & Third Point Sampling Mean Sulphite Reducing Clostridia Densities (MBCC)
- Fig. 4.10** Site D1D2E Grab Sampling Mean Sulphite Reducing Clostridia Densities (MBCC)
- Fig. 4.11** Site B River Discharge and First, Mid & Third Point Sampling Mean Sulphite Reducing Clostridia Densities (MBCC)

LIST OF FIGURES

- Fig. 4.12** Site C River Discharge and First, Mid & Third Point Sampling
Mean Sulphite Reducing Clostridia Densities (MBCC)
- Fig. 4.13** DNA quantification
- Fig. 4.14** Representative gel of monoplex PCR detection for beta, epsilon, alpha
and iota toxin gene using Set 1 Primers
- Fig. 4.15** Duplex PCR of alpha and CPE toxin gene

LIST OF TABLES

- Table 1.1** *Clostridium perfringens* toxinotype categorization
- Table 3.1** Primers Set 1
- Table 3.2** Primers Set 2
- Table 4.1** Mean Sulphite Reducing Clostridia Density (MBCC), *Clostridium perfringens* Isolation Rate (IRt) and Mean *Clostridium perfringens* Density (MCPC): Comparison Among Rivers
- Table 4.2** Sulphite Reducing Clostridia : Replicates Normality, Statistical Differences and Temporal Difference
- Table 4.3** Correlation Between Mean *Clostridium perfringens* Densities (MCPC), Mean Sulphite Reducing Clostridia Densities (MBCC) and River Discharge (Q)
- Table 4.4** Sulphite Reducing Clostridia Densities Correlations in the First, Mid and Third Quarter Point Across River
- Table 4.5** Correlations of Mean River Discharge, Mean Sulphite Reducing Clostridia Densities (MBCC) and Mean *Clostridium perfringens* Densities (MCPC) Along Sungai Bernam and Sungai Selangor