STRUCTURAL, OPTICAL AND DIELECTRIC PROPERTIES OF LITHIUM FLUORO AND CHLORO AND MAGNESIUM CHLORO PHOSPHATE GLASSES

LOH YEN NEE

FPSK(M) 2005 26
PREVALENCE OF GENETIC DIVERSITY BETWEEN GROUP A AND GROUP B OF RESPIRATORY SYNCYTIAL VIRUS ISOLATED FROM HOSPITALIZED PATIENTS IN A TEACHING HOSPITAL IN KUALA LUMPUR.

By

VINOMARLINI A/P GUNASAGARAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirement for the Degree of Master of Science

August 2005
DEDICATION

To my mom and dad
Respiratory Syncytial virus (RSV) is one of the most important causes of lower viral respiratory tract infection resulting in hospital admission among infants and early childhood worldwide. The virus is a seasonal virus, with annual outbreaks occurring during the winter in temperate climates and during the rainy season in tropical climates. The main objective of this study was to determine the prevalence of RSV group A and B in the Klang Valley area by using Polymerase Chain Reaction (PCR) as a rapid and simultaneous detection of RSV. In this study, Polymerase Chain Reaction (PCR) method was used to detect the RSV and seminested PCR was used to subtype RSV into groups. This method is more sensitive and reliable compared to the current method used for detecting RSV which is by using direct immunofluorescence immunoassay. The
detection and subtyping of RSV both used the amplification of F and the G genes of RSV. The primer from the F gene region was used as the antisense primer for both detecting and subtyping while primer from different parts of the G gene region were used as the sense primer for detecting and subtyping of RSV respectively.

Random Amplified Polymorphic DNA (RAPD) technique was performed in this research to study the diversity of twenty RSV isolates. Four out of fifteen primers that were screened for reproducible band yielded clear multiple bands. According to the dendrogram generated from the RAPDistance software program, RSV isolates were distinctly separated into their own groups based on the year of isolation. The percentage of similarity among these isolates ranged from 33% to 95% while the Nei and Li’s genetic distance obtained ranged from 0.0333 to 0.471.

The data obtained from this study only covers the Klang Valley area as all of the samples were collected from patients that were admitted in the pediatric ward of University Malaya Medical Center, Kuala Lumpur. All the samples were obtained from September 2002 until March 2004. The samples were collected from patients age ranging from one month to one year. Out of the twenty RSV samples obtained, thirteen of the RSV isolates were from the male patients and seven were from the female patients. The highest rate of infection occurred in the Malay community followed by the Chinese and the Indian. The chi-square test was done in order to determine whether the clinical data such as age, gender and the ethnicity was significant with the RSV infection. This study shows that,
the age, gender and the ethnicity of the patients were not significant and therefore, no relationship could be observed between the demographic data and the RSV infection.

From this study, RSV was successfully detected in 20 samples by PCR method. The F and G genes were amplified to detect and subtype RSV into groups A and B. The incidence of RSV A was much higher from September 2002 until March 2004 compared to the RSV B. Therefore, RSV A is prevalent in the Klang Valley area.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PREVALENS KEPELBAGAIAN GENETIK DI ANTARA KUMPULAN A DAN B VIRUS RESPIRASI SINSITIUM DIKALANGAN PESAKIT DI SEBUAH HOSPITAL PENGAJARAN DI KUALA LUMPUR.

Oleh

VINOMARLINI GUNASAGARAN

September 2005

Pengerusi : Zamberi Sekawi, M.D.

Fakulti : Perubatan dan Sains Kesihatan

Virus Respirasi Sinsitium (RSV) merupakan penyebab utama kepada jangkitan virus bahagian bawah sistem pemasaran di kalangan kanak-kanak. Jangkitan virus ini adalah bermusim dan kadar jangkitan meningkat pada musim sejuk dan hujan. Objektif utama projek ini adalah untuk menentukan prevalens RSV kumpulan A dan kumpulan B di Lebuah Klang dengan menggunakan kaedah tindakan rantai polymerase (PCR) sebagai satu kaedah yang mudah, cepat dan sensitif. Kaedah ini lebih sesuai dan sensitive berbanding kaedah imunofluresens untuk mengenalpasti RSV. Penyaringan dan pengenalpastian kumpulan RSV dilakukan dengan mengamplifikasikan gen-gen F dan G dari RSV. Primer yang spesifik dari gen F digunakan sebagai primer "antisense" untuk
penyaringan dan penentuan kumpulan RSV manakala gen G dari bahagian yang berlainan digunakan untuk tujuan yang sama.

Untuk mengetahui kepelbagaian RSV dalam penyelidikan ini, “Random Amplified Polymorphic DNA (RAPD) telah dilakukan pada kesemua 20 sampel RSV. Empat daripada lima belas primer yang digunakan telah menghasilkan banyak “bands” yang terang. Berdasarkan kepada dendogram yang dihasilkan daripada program perisian RAPDistance, sampel-sampel RSV berpisah dengan jelas kepada kumpulan masing-masing. Peratus persamaan di antara sampel-sampel RSV ini adalah dari 33% hingga 95% manakala jarak genetik Nei and Li’s yang diperolehi adalah dari 0.0333 hingga 0.471.

mempengaruhi kadar jangkitan. Tiada sebarang hubungan telah dikenalpasti diantara jangkitan RSV dengan data hip could be observed between the data demografik.

ACKNOWLEDGEMENTS

I owe my respectful gratitude to my spiritual master, **His Divine Grace Yoga Jnana Sitthar Om Sri Rajayoga Guru**, whose blessings have accompanied me every step of the way in finishing this thesis work and made the impossible possible.

I am very grateful to Professor Dr. Shamala Devi Sekaran for providing me positive RSV isolates, without which the RSV study would not have been possible.

My appreciation goes to Dr. Zamberi Sekawi and Associate Professor Dr. Mariana Nor Shamsudin for their effort in helping to make this thesis a success. I would also like to thank Associate Professor Dr. Rozita Rosli, for providing me space and allowing me to use the equipments in the Human Genetic Laboratory.

Sincere thanks are due to Dr. Thilakavathy and Kak Wan Sormany for teaching me molecular methods and guiding me without fail throughout the whole project.

I would like to express my heartfelt thanks to my parents and my sisters for their endless encouragement, patience, care and sacrifices that helped me in my undertakings and to complete this research study successfully.

This study was supported by a grant (09-02-04-0891) and fellowship from the Ministry of Science, Technology and Environment, Malaysia.
I certify that an Examination Committee met on 19th August 2005 to conduct the final examination of Vinomarlini Gunasagaran on her Master of Science thesis entitled “Prevalence of Genetic Diversity between Group A and Group B of Respiratory Syncytial Virus Isolated from Hospitalized Patients in a Teaching Hospital in Kuala Lumpur” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

**Farida @ Fatema Jamal, PhD**  
Professor  
Faculty of Medicine and Health Science  
Universiti Putra Malaysia  
(Chairman)

**Abdul Rahman Omar, PhD**  
Associate Professor  
Faculty of Veterinary Medicine  
Universiti Putra Malaysia  
(Internal Examiner)

**Nor Amalina Emran, PhD**  
Lecturer  
Faculty of Medicine and Health Science  
Universiti Putra Malaysia  
(Internal Examiner)

**Ilina Isahak, PhD**  
Professor  
Faculty of Medicine  
Universiti Kebangsaan Malaysia  
(External Examiner)

**GULAM HUSUL RAHMAT ALI, PhD**  
Professor/Deputy Dean  
School of Graduate Studies  
Universiti Putra Malaysia

Date: **25 OCT 2005**
The thesis submitted to the Senate of Universiti Putra Malaysia and was accepted as fulfillment of the requirements for the degree of Master of Science. The members of the Supervisory Committee are as follows:

**Zamber Sekawi, M.D.**  
Faculty of Medicine and Health Sciences  
Universiti Putra Malaysia  
(Chairman)

**Mariana Nor Shamsudin, PhD**  
Associate Professor  
Faculty of Medicine and Health Sciences  
Universiti Putra Malaysia  
(Member)

---

**AINI IDERIS, PhD**  
Professor/Dean  
School of Graduate Studies  
Universiti Putra Malaysia

17 NOV 2005
DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

VINOMARLINI A/P GUNASAGARAN

Date: 1/10/05.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEDICATION</td>
<td>2</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>3</td>
</tr>
<tr>
<td>ABSTRAK</td>
<td>6</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>9</td>
</tr>
<tr>
<td>APPROVAL</td>
<td>10</td>
</tr>
<tr>
<td>DECLARATION</td>
<td>12</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>16</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>17</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>19</td>
</tr>
</tbody>
</table>

## CHAPTER

### I INTRODUCTION

Objective 24

### II LITERATURE REVIEW

History 25
Viral properties 26
Growth Characteristics 28
Attachment and entry 28
Viral Genome and Proteins 29
- F protein 30
- G protein 32
- SH protein (Small hydrophobic region) 34
- Nucleocapsid-associated protein N, P, and L 34
- Matrix proteins (M and M2) 35
- Nonstructural proteins NS1 and NS2 35
RNA Replication 36
Classification 36
RSV subgrouping 37
Transmission 38
Polymerase Chain Reaction 39
Random Amplified Polymorphic DNA (RAPD) 43
Applications of RAPD 45
Epidemiology Studies of RSV 47
RSV In Malaysia 56

### III METHODOLOGY

Study Location 58
Cells for RSV cultivation
Mycoplasma Detection In Cells
Virus
Culturing of the virus
RNA extraction
Cell Lysis
Precipitation of Total RNA
Removal of Contaminating DNA from RNA Preparations
Electrophoresis Gel for RNA confirmation
Quantification of Total Viral RNA
cDNA synthesis
Polymerase Chain Reaction
Detection of RSV by PCR
PCR Amplification
Agarose Gel Electrophoresis of PCR Product
Detection of RSV subgroup by PCR
PCR Product Purification
DNA Sequencing
Random Amplified Polymorphic DNA (RAPD)
RAPD Analysis
Statistical and Demographic Data Analysis

IV RESULTS

Cells
Virus culturing
RNA Extraction
Detection of RSV by Polymerase Chain Reaction (PCR)
RSV Subgroup by PCR
DNA sequencing
Random Amplified Polymorphic DNA (RAPD)
RAPD Analysis
Dendrogram
Demographic Data
Age Distribution
Gender Distribution
Ethnicity
Symptoms of Patients with RSV infection

V DISCUSSION

Viruses and cells
RNA extraction
Detection and subtyping of RSV by Polymerase Chain Reaction (PCR)
RAPD analysis
Demographic Data
**LIST OF TABLES**

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Identity of RSV isolates which were used in this study</td>
<td>60</td>
</tr>
<tr>
<td>2</td>
<td>Sequences of primers screened for random amplification, RAPD procedure</td>
<td>71</td>
</tr>
<tr>
<td>3</td>
<td>Duration of the syncytial formation of each RSV isolates</td>
<td>76</td>
</tr>
<tr>
<td>4</td>
<td>The concentrations and purities of the genomics RNA for the total viral RNA isolates</td>
<td>79</td>
</tr>
<tr>
<td>5</td>
<td>Detection and subtyping of 20 samples used in this study</td>
<td>83</td>
</tr>
<tr>
<td>6</td>
<td>Value of genetic distance (above diagonal) and percentage of similarity (below diagonal) between 20 RSV isolates based on RAPD data</td>
<td>92</td>
</tr>
<tr>
<td>7</td>
<td>Age distribution of RSV infection</td>
<td>95</td>
</tr>
<tr>
<td>8</td>
<td>Gender distribution between gender and RSV infection</td>
<td>97</td>
</tr>
<tr>
<td>9</td>
<td>Association between ethnic and RSV infection</td>
<td>98</td>
</tr>
<tr>
<td>10</td>
<td>Comparison of symptoms of a subset of 30 children hospitalized for RSV infection</td>
<td>99</td>
</tr>
</tbody>
</table>
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vero cells growth 1st day (400X)</td>
<td>74</td>
</tr>
<tr>
<td>2</td>
<td>Confluent Vero (90% growth) cells growth 4th day (400X)</td>
<td>75</td>
</tr>
<tr>
<td>3</td>
<td>Vero cells (A) Uninfected Vero cells (400X); (B) Vero cells that have been infected with RSV (400X)</td>
<td>77</td>
</tr>
<tr>
<td>4</td>
<td>Lane M represents the RNA marker while lanes 1, 2, 3 and 4 are RSV samples respectively. Total viral RNA extracted using MasterPure™ RNA Purification Kit. Lane 5 was a negative control. Two bands were observed at the 3500 and at the 1383 bases</td>
<td>78</td>
</tr>
<tr>
<td>5</td>
<td>Detection of RSV by PCR: The F and G genes were amplified by using F1 and G1 primers. Lane M represents 1 kb molecular weight marker, lanes 2, 4, 5, 7, 8, 10 and 11 are amplified RSV while lane 1 was a positive control of RSV and lane 9 was a negative control</td>
<td>80</td>
</tr>
<tr>
<td>6</td>
<td>Amplification of RSV A genes: Agarose gel showing the results of amplified RSV A. Lane M represents 100bp molecular weight marker, lanes 1, 2, 3 and 5 was amplified RSV A with the size of 0.9kb while lane 4 was a negative control indicating no contamination</td>
<td>82</td>
</tr>
<tr>
<td>7</td>
<td>Amplification of RSV B genes: Agarose gel showing the results of amplified RSV B. Lane M represents 100bp molecular weight marker, lanes 1, 2 and 3 were amplified RSV B with the size of 0.78kb. Lane 4 was a negative control</td>
<td>82</td>
</tr>
<tr>
<td>8</td>
<td>RSV A sequence analysis</td>
<td>85</td>
</tr>
<tr>
<td>9</td>
<td>RSV B sequence analysis</td>
<td>86</td>
</tr>
<tr>
<td>10</td>
<td>RAPD patterns obtained from primer OPAE 6: Lane M represents 1kb molecular weight marker while lanes 1-20 are RSV isolates. Lane 21 was negative control</td>
<td>88</td>
</tr>
</tbody>
</table>
11 RAPD pattern obtained from primer OPAE 10: Lane M represents 1kb molecular weight marker while lanes 1-20 are RSV isolates. Lane 21 was negative control

12 RAPD pattern obtained from primer OPAE 17: Lane M represents 1kb molecular weight marker while lanes 1-20 are RSV isolates. Lane 21 was negative control

13 RAPD pattern obtained from primer OPAE 10: Lane M represents 1kb molecular weight marker while lanes 1-20 are RSV isolates. Lane 21 was negative control

14 Dendrogram from cluster analysis on RSV isolates based on Nei and Li's (1979) genetic distance demonstrating relationship among 20 isolates using combined data from all the primers

15 Age distribution of RSV positive cases

16 Ethnic distribution of positive RSV cases in percentage
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>[T]</td>
<td>test statistics</td>
</tr>
<tr>
<td>BRSV</td>
<td>bovine respiratory syncytial virus</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>COPD</td>
<td>chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleoside triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetatic acid</td>
</tr>
<tr>
<td>EMEM</td>
<td>Eagle's minimal essential medium</td>
</tr>
<tr>
<td>F protein</td>
<td>fusion protein F</td>
</tr>
<tr>
<td>G protein</td>
<td>glycoprotein G</td>
</tr>
<tr>
<td>HHV</td>
<td>Human Herpesvirus</td>
</tr>
<tr>
<td>HRSV</td>
<td>human respiratory syncytial virus</td>
</tr>
<tr>
<td>kb</td>
<td>kilo base pair</td>
</tr>
<tr>
<td>MAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>magnesium chloride</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>NJTREE</td>
<td>neighbour joining tree</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PDA</td>
<td>Patent Ductus Arteriosus</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PIV</td>
<td>Parainfluenza Virus</td>
</tr>
<tr>
<td>RAPD</td>
<td>Random amplified polymorphic DNA</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RSV</td>
<td>respiratory syncytial virus</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcribed- PCR</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-Acetate-EDTA</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet</td>
</tr>
<tr>
<td>Vero</td>
<td>African Green Monkey Kidney Cell</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>μl</td>
<td>microliter</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

Respiratory Syncytial virus (RSV) is one of the most important causes of lower viral respiratory tract infections resulting in hospital admission among infants and early childhood worldwide. It is also considered as a serious problem in the elderly. The spectrum of infection ranges from mild upper respiratory tract disease to bronchiolitis and pneumonia. Most children recover from illness in 8 to 15 days (Collins et al., 1996).

In the United States of America (USA), 50,000 to 80,000 infants each year are hospitalized and about 500 infants die each year because of the RSV infection. RSV infections cause symptoms like those of a common cold, a stuffy nose or runny nose, sore throat, wheezing and coughing, low-grade fever and earache. Babies may have additional symptoms that include listlessness, lack of appetite, irritability (fretfulness), disrupted sleep and a decreased interest in things going around them. Rarely, some babies may also have apnea, a condition where breathing stops for about 15 to 20 seconds. The majority of admissions to hospitals are due to bronchiolitis, the common lower respiratory tract manifestation of RSV infection (McNamara & Smyth, 2002).

The virus is a seasonal virus, with annual outbreaks occurring during the winter in temperate climates and during the rainy season in tropical climates. It is extremely
infectious and the predictability is similar among respiratory viruses. The virus causes syncytial masses when grown in culture and this is where the virus gets its name. Infection with RSV does not cause life long immunity. Children and adults may be infected year after year because of the presence of intratypic strains (McNamara & Smyth, 2002).

RSV has a single stranded, negative-sense RNA. It is classified in the genus *Pneumovirus* of the Paramyxoviridae family. It is a medium sized enveloped RNA virus and has a nucleocapsid morphology that is different than other paramyxoviruses that leads to the separate sub-family classification. The different nucleocapsid morphology is because the RSV has a N-bound RNA (which is referred as the nucleocapsid), which is considered one of the defining features of the *Paramyxoviridae* and is described as having a 'herringbone' appearance when imaged under a transmission electron microscope (TEM). Its viral genome is composed of 15,200 nucleotides and encodes 10 proteins. The virion is variable in shape and size (average diameter of between 120 and 300 nm), is unstable in the environment (surviving only a few hours on environmental surfaces), and is readily inactivated by soap and water and disinfectants (Feldman, *et al*., 1999). The virus multiplies in the mucous membrane of the nose, throat and in the larynx. In infants, the virus may spread to the trachea, bronchi, bronchioles and alveoli. Fatal cases usually show extensive bronchiolitis and pneumonitis with scattered areas of atelectasis and emphysema resulting from bronchiolar obstruction (Chanock *et al*., 1968).
RSV has 10 genes and the gene order is NS1, NS2, N, P, M, SH, G, F, M2 and L. The infection is initiated with the G protein binding to a host cell receptor, possibly a heparin-like glycosaminoglycan, followed by F protein mediated fusion of the viral and cell membrane, and penetration of the nucleocapsid complex into the cytoplasm (Feldman et al., 1999).

RSV exists in two distinct subgroups, which is RSV group A and RSV group B. These two antigenic groups of RSV A and B have been identified based on monoclonal antibodies to the F gene (fusion protein) and the G gene (attachment protein). Both RSV subgroups are capable in infecting severe lower respiratory tract disease (Sullender, 2000). Even though, the clinical infection of both subgroups appears to be similar, infection with group A may produce disease with slightly greater severity (McConnachie et al., 1990).

The molecular epidemiology of RSV in Malaysia is largely unknown. Currently, in Malaysia, there are no published data available on the epidemiology and the prevalence of RSV groups. The information of prevalence of the RSV groups is important to relate and to indicate the severity of the viral infection.

To conduct this molecular epidemiology study, Polymerase Chain Reaction (PCR) was used as a rapid detection and to identify the prevalence of RSV group A and B. The most common method for detecting RSV, which is used in hospitals, is by using direct immunofluorescence assay. This rapid technique gives a less sensitive result and could
not be used to subgroup the RSV. The sensitivity and specificity of the interpretable immunofluorescence assay direct stains in comparison with shell vial cultures were 85.9% and 87.1% respectively (Matthey, et al., 1992). Thus, PCR is used as an alternative method because this method provides a sensitive tool for both detection and typing of RSV into groups. Identifying the prevalence of RSV groups will give extra advantage in preventing RSV infection. The information on prevalent of RSV subgroup would assist in the development of DNA vaccine.

OBJECTIVE

General Objective:

1. To establish baseline information on prevalence of RSV and the subgroups in the Klang Valley area.

The specific objectives of this research are:

1. To detect RSV by amplifying the F and G genes by PCR method.
2. To subtype RSV into subgroups A and B by seminested PCR.
3. To determine the diversity of the RSV strains based on the Random Amplified Polymorphic DNA (RAPD) technique.
4. To study the relationship between prevalence of RSV infection with the age, ethnicity and gender of patients with RSV infection.