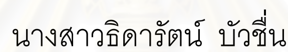
เชื้อ *Serratia marcescens* ที่แยกได้จากหออภิบาลผู้ป่วยทารกแรกเกิด โรงพยาบาลศิริราช:
การจำแนกสายพันธุ์โดยวิธี Pulsotype และการทดสอบความไวรับต่อยาต้านจุลชีพ



นางสาวธิดารัตน์ บัวชื่น

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จุฬาลงกรณ์มหาวิทยาลัย

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SERRATIA MARCESCENS FROM A NEONATAL INTENSIVE CARE UNIT AT SIRIRAJ HOSPITAL: PULSOTYPING AND ANTIMICROBIAL SUSCEPTIBILITY TEST

Miss Thidarat Buachuen

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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จุฬาลงกรณ์มหาวิทยาลัย

ธิดารัตน์ บัวชื่น: เชื้อ *Serratia marcescens* ที่แยกได้จากหออภิบาลผู้ป่วยทารกแรกเกิด โรงพยาบาลศิริราช: การจำแนกสายพันธุ์โดยวิธี Pulsotype และ การทดสอบความไวรับต่อยาต้านจุลชีพ. (*SERRATIA MARCESCENS* FROM A NEONATAL INTENSIVE CARE UNIT(NICU) AT SIRIRAJ HOSPITAL: PULSOTYPING AND ANTIMICROBIAL SUSCEPTIBILITY TEST) อ. ที่ปรึกษา : รศ. ดร. พิณทิพย์ พงษ์เพ็ชร, อ.ที่ปรึกษาร่วม : อ. เพ็ญพรรณ แน่นหนา จำนวนหน้า 133 หน้า. ISBN 974-03-1657-3.

เชื้อ *Serratia marcescens* เป็นสาเหตุที่สำคัญหนึ่งในการก่อให้เกิดการติดเชื้อในโรงพยาบาล โดยเฉพาะอย่างยิ่งในผู้ป่วยทารกแรกเกิด และผู้ป่วยที่มีความผิดปกติในระบบภูมิคุ้มกัน การศึกษาครั้งนี้ได้ทำการเก็บตัวอย่างจากหออภิบาลผู้ป่วยทารกแรกเกิด โรงพยาบาลศิริราช เป็นระยะเวลา 4 เดือน ตั้งแต่เดือนพฤษภาคม-สิงหาคม พ.ศ. 2543 และสามารถแยกเชื้อ *S. marcescens* ได้จากผู้ป่วย 36 ราย จาก 104 ราย คิดเป็น 34.62% สิ่งส่งตรวจที่นำมาศึกษาจากผู้ป่วยคือ สำลีป้ายคอ, ของเหลวจากคอหอย, ปัสสาวะ และชุดให้ยาผสมสำหรับสูด สิ่งส่งตรวจจากสิ่งแวดล้อมคือ สำลีป้ายจากอ่าง น้ำเกลือเช็ดตาและปาก และยาผสม นอกจากนี้ได้นำเชื้อ *S. marcescens* ที่แยกได้จากผู้ป่วยในหอผู้ป่วยอื่นในช่วงเวลาเดียวกันมาศึกษาร่วมด้วยอีก 10 isolates

เชื้อ *S. marcescens* ที่แยกได้และนำมาศึกษา 125 isolates มีแบบแผนความไวรับต่อยาต้านจุลชีพ 10 รูปแบบ และจำแนกสายพันธุ์โดยวิธี pulsed-field gel electrophoresis (PFGE) ได้ 12 สายพันธุ์ ซึ่งเชื้อที่แยกได้จากหออภิบาลผู้ป่วยทารกแรกเกิดมีเพียง 2 สายพันธุ์ คือ A และ B โดยสายพันธุ์หลักคือสายพันธุ์ A มีเชื้อเพียง 1 isolate ที่เป็นสายพันธุ์ B ส่วนเชื้อที่แยกได้จากหอผู้ป่วยอื่นๆ มีสายพันธุ์ที่แตกต่างออกไป 10 สายพันธุ์ได้แก่ C, D, E, F, G, H, I, J, K และ L จากผลการทดสอบความไวรับปรากฏว่าเชื้อส่วนใหญ่ดื้อต่อยาปฏิชีวนะกลุ่ม beta lactams รวมทั้ง third generation cephalosporins แบบแผนความไวรับต่อยาต้านจุลชีพกับการจำแนกสายพันธุ์โดยวิธี PFGEไม่มีความสัมพันธ์กัน

จากการศึกษาครั้งนี้สรุปได้ว่ามีการระบาดของเชื้อ *S. marcescens* pulsotype A ในลักษณะ colonization ใน NICU ในช่วงเวลาที่ศึกษา และเชื้อที่แยกได้ส่วนใหญ่มีการดื้อต่อยาที่ใช้ในการรักษาโรคติดเชื้อแบคทีเรียแกรมลบที่โรงพยาบาลศิริราช

ภาควิชาสหสาขาจุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต..
สาขาวิชาจุลชีววิทยาทางการแพทย์ ลายมือชื่ออาจารย์ที่ปรึกษา............................
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KEY WORD: *SERRATIA MARCESCENS* / NEONATAL INTENSIVE CARE UNIT (NICU) / NOSOCOMIAL INFECTION / EPIDEMIOLOGICAL TYPING / PULSED-FIELD GEL ELECTROPHORESIS (PFGE)

THIDARAT BUACHUEN: *SERRATIA MARCESCENS* FROM A NEONATAL INTENSIVE CARE UNIT (NICU) AT SIRIRAJ HOSPITAL : PULSOTYPING AND ANTIMICROBIAL SUSCEPTIBILITY TEST. THESIS ADVISOR : ASSOC. PROF. DR. PINTIP PONGPECH, THESIS COADVISOR : INSTRUCTOR PENPUN NAENNA, 133 pp. ISBN 974-03-1657-3.

Serratia marcescens is one of the important causative agents of nosocomial infections, particularly in critically ill neonates and immunocompromised patients. During the 4 months of specimen collection (May to August, 2000) in the Neonatal Intensive Care Unit (NICU), it was found that there were 36 out of 104 neonatal patients (34.62%) who had *S. marcescens* isolates in their throat swabs, tracheal secretion, urine and swabs from set of drugs mixture for inhalation. The organisms were also isolated from environments in the same patient unit. The environmental specimens included swabs from sinks, normal saline for wiping eyes/mouth and drugs mixture for inhalation. *S. marcescens* isolates from 10 patients admitted in the other different wards also included in this study.

One hundred and twenty-five *S. marcescens* isolates were discriminated into 10 antimicrobial susceptibility patterns (antibiograms) and 12 pulsotypes. The isolates in NICU were in only 2 pulsotypes; A and B. Except for one type B isolate, all the isolates were type A. The 10 isolates from the other different wards were 10 different pulsotypes; C, D, E, F, G, H, I, J, K, and L. The results from antimicrobial susceptibility test indicated that most of the isolates were resistant to beta lactam antibiotics including third generation cephalosporins.

It could be concluded that there was an outbreak of *S. marcescens* pulsotype A colonization in NICU during the time of study and the isolates resisted to most of the antimicrobial agents used in the treatment of the gram negative bacteria at Siriraj hospital.

Department Medical Microbiology	Student's signature..
Field of study Medical Microbiology	Advisor's signature...
Academic year 2001	Co-advisor's signature………………………………

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ABBREVIATIONS

ATCC	=	American Type Culture Collection
cm	=	Centimeter
°C	=	Degree Celsius
g	=	Gram
L	=	Liter
M	=	Molar
mM	=	Millimolar
mg	=	Milligram
ml	=	Milliliter
mm	=	Millimeter
NCCLS	=	National Committee for Clinical Laboratory Standards
µg	=	Microgram
µl	=	Microliter
s	=	Second
v	=	Volt
%	=	Percent

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CHAPTER I

INTRODUCTION

Serratia marcescens, a gram-negative bacillus classified as a member of the family Enterobacteriaceae, has been recognized as a cause of hospital-acquired infection for the last two decades (1). Outbreaks of nosocomial *S. marcescens* infections, including urinary tract infection, wound infection, pneumonia, respiratory tract infection, septicemia, meningitis, and bacteremia, have been frequently reported (2-9).

Environmental sources of *S. marcescens* in the hospital are widely described (10-14). This microorganism has a predilection for a moist environment. Some of the environmental sources mentioned previously were sponges, fiberoptic bronchoscopes, adhesive tape, distilled water, plastic caps of saline bottles, catheters, floors, inhalation therapy equipment, electrocardiogram bulbs, transducers, intravenous solution, irrigating fluids, hand lotions, shaving brushes, soap, and sink taps.

Traditional methods used for the typing of *S. marcescens* are often based on phenotypic characteristics including biotyping, serotyping, antibiogram analysis, bacteriocin typing, phage typing, and plasmid typing. Most of these techniques are not sufficiently sensitive to distinguish different strains or are affected by physiological factors. In recent reports, ribotyping and Polymerase Chain Reaction (PCR) have been used for typing *S. marcescens* and have shown a high degree of discriminatory potential and reproducibility. However, the evolution of DNA-regulating rRNA is very slow and sometime the genetic mutations among clonally related strains cannot be detected by ribotyping. The reproducibility of PCR may

altered by operating conditions, such as the use of different thermal cycles, the annealing temperature, and the concentrations of Taq polymerase, magnesium, template, and primer. Pulsed-Field Gel Electrophoresis (PFGE) typing is highly effective in molecular epidemiologic studies of bacterial isolations and is superior to ribotyping techniques in discriminating among isolates of *Escherichia coli*, *Staphylococcus aureus*, and many other species. Hence, PFGE typing can be used to evaluate the clonal relateness among bacterial isolates and to investigate outbreak (15-24).

To our knowledge, there have been several cases of infections due to *S. marcescens* in neonate in the Neonatal Intensive Care Unit (NICU) at Siriraj hospital for a long period of times. The investigation of nosocomial outbreak of *S. marcescens* is very important for controlling and prevention the spread of this pathogen. In order to perform the epidemiological study, the organism must be proved that it come from the same clone by typing method. Thus, an investigation of a nosocomial *S. marcescens* outbreak by PFGE has never been reported before in Thailand. This study will be one of the very first report about pulsotyping of *S. marcescens* for epidemiology study in our country.

In addition, multiple resistance of *S. marcescens* to antibiotics can cause many problems in treating infected patients and controlling outbreak, this study also included the antimicrobial susceptibility test for all *S. marcescens* isolates. By combining all the results from this study, the physicians and other medical personals could have informations about the sources and the occurrence of *S. marcescens* in the neonatal intensive care unit at Siriraj hospital along with the picture of antimicrobial susceptibility patterns of the isolates. Successful prevention and control of this pathogen as well as the treatment of the infections should be obtained.

Purpose of the study

The overall objectives of this study were to perform the pulsotyping of *S. marcescens* isolated from the Neonatal Intensive Care Unit (NICU) using Pulsed-Field Gel Electrophoresis (PFGE) and to determine the antimicrobial susceptibility patterns of the isolated *S. marcescens*.

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CHAPTER II

LITERATURE REVIEWS

I. Historical reviewed

In 1819, Bartolomeo Bizio, a pharmacist from Pauda, Italy, discovered and named *S. marcescens* when he identified the bacterium as the cause of a miraculous bloody discoloration in a cornmeal mush called polenta. Bizio named *Serratia* in honor of an Italian physicist named Serrati, who invented the steamboat, and Bizio chose *marcescens* from the Latin word for decaying because the bloody pigment was found to deteriorate quickly. During the year 1906 to 1950, physicians have used *S. marcescens* as a biological marker for studying the transmission of microorganisms, because this bacterium generally was considered as a harmless saprophyte (25). The first description of nosocomial infection cause by *S. marcescens* was Wheat's report of 11 cases over a 6-month period in 1951 at Stanford University Hospital. Infections caused by this organism have been reported with increasing frequency since 1960.

In 1966, McCormack and Kunin (26) reported a nursery epidemic involving 27 babies, although only 15 cases of *S. marcescens* bacteremia had been recorded by 1968. It's ability to cause infection was once thought to be limited to patients with chronic debilitating disorders, but *S. marcescens* has now been implicated as an aetiological agent in every conceivable kind of infection, including respiratory tract infection, urinary tract infection (UTI), septicemia, meningitis and wound infections. *S. marcescens* has been reported to cause infective endocarditis acquired in the community and in hospitals. In contrast to other gram-negative bacteria, it usually

affects the left side of the heart. *S. marcescens* endocarditis acquired in the hospital is usually an exogenous infection associated with cardiac surgery. Today, *S. marcescens* has attained the status of a fully fledged pathogen that causes infections particularly in two dispirate groups: heroin addicts and hospitalised patients (27).

II. Bacterial characteristics

Serratia marcescens is a gram-negative rod shaped bacteria, 0.5-0.8 μm in diameter and 0.9-2.0 μm in length. It belongs to a group of bacteria classified in the large family Enterobacteriaceae (28), which also includes *Escherichia coli* and *Klebsiella* species. *Serratia* species are motile, by means of peritrichous flagella. They are facultatively anaerobic. Colonies are most often opaque, somewhat iridescent, and either white, pink or red in color. Almost all strains can grow at the temperatures between 10-36°C, at pH 5-9, and in the presence of 0-4% (w/v) NaCl. The catalase reaction is strongly positive. Acetoin is produced from pyruvate. Reducing compound(s) are produced from gluconate. D-Glucose is fermented in the presence (and in the absence) of 0.001 M iodoacetate and do not ferment lactose (or only very slowly) or produce H_2S on a triple-sugar-iron slant. Maltose, mannitol and trehalose are fermented and utilized as sole carbon sources. D-Alanine, L-alanine, 4-aminobutyrate, caprylate, citrate, L-fucose, D-glucosamine, kynurenate, L-proline, putrescine and tyrosine are utilized as sole carbon sources. Dulcitol and tagatose are neither femented nor utilized as sole carbon sources. Butyrate and 5- amino-valerate are not utilized as sole carbon sources. Extracellular enzymes hydrolyze DNA, lipids (tributyrin, corn oil) and proteins (gelatin, casein), but not starch (in 4 days), polygalacturonic acid or pectin. Phenylalanine (and tryptophan) deaminase and thiosulfate reductase (H_2S from thiosulfate) are not produced. O-Nitrophenyl-β-D-

galactopyranoside (ONPG) is hydrolyzed by most strains. Chlorate is reduced anaerobically by *Serratia* nitrate reductase (anaerobic growth does not occur with chlorate). Growth factors are generally not required by *Serratia* strains. They are Voges-Proskauer positive. The genus *Serratia* contain multiple species but only one, *S. marcescens,* has been consistently associated with human disease. There are currently nine other members of the genus, including *S. odifera, S. liquifaciens,* and *S. rubidaea,* but these have rarely caused infections in human. *S. marcescens* dose not ferment L-arabinose, which can differentiate it from those other species with exception of *S. entomophila,* which is not found in human clinical specimens (1). The organism is found in soil, water and plants. It often colonizes the gastrointestinal tract of humans. *Serratia* thrives in moist environments and frequently contaminates solutions and hospital equipment (10).

Some strains of *S. marcescens* are capable of producing a pigment called prodigiosin, a nondiffusible, water-insoluble pigment bound to the cell envelope, which ranges in color from dark red to pale pink, depending on the cultural conditions (e.g. amino acids, carbohydrates, pH, inorganic ions, temperature)(28) and age of the colonies. Prodigiosin is best produced on peptone-glycerol agar [Peptone glycerol agar: Bacto-peptone (Difco), 5.0 g; glycerol, 10.0 ml; Bacto-agar (Difco), 20.0 g; distilled water, 1000 ml.] at 20-35°C. The temperature range for pigment production is 12-36°*C.* It is not produced anaerobically. Chemically, prodigiosin is 2-methyl-3-amyl-6-methoxyprodigiosene (prodigiosene is 5-(2-pyrryl)-2-2′-dipyrrylmethene). In the cell, prodigiosin is formed by condensation of a volatile 2-methyl-3-amyl-pyrrol (MAP) and a nonvolatile 4-methoxy-2-2′-bipyrrole-5-carboxaldehyde (MBC). Several classes of nonpigmented mutants have been isolated that are either blocked on the MAP pathway or the MBC pathway (28). *S. marcescens* has a predilection for growth

on starchy foodstuffs, where the pigmented colonies are easily mistaken for drops of blood (25). The pigmented *S. marcescens* is found in various ecological niches, including soil, water, air, plants and animals. The ability to from prodigiosin is characteristic of *S. marcescens,* but the function of this red pigment remains unclear because clinical isolates are rarely pigmented (27). Factors, such as medium composition and oxygen supply, affect the production of prodigiosin and the incubation at 37°C may inhibit the pigmentation. In addition, non-pigmented *S. marcescens* are more resistant to antibiotics (most plasmid encoded) than pigmented isolates (29).

III. Diseases caused by *S. marcescens*

1. Sepsis

Patients with ***S. marcescens*** sepsis may present with fever, chills, shock, and respiratory distress. Risk factors include hospitalization, with intravenous, intraperitoneal, or urinary catheters, or prior instrumentation of the respiratory tract and the other factors include cardiac valve replacement and the use contaminated intravenous infusions or transfusions.

2. Urinary tract infection

Approximately 30-50% of patients are asymptomatic. When symptoms are present, patients may have fever, frequent urination, dysuria, pyuria, or pain upon urination

In 90% of cases, patients have a history of recent surgery or instrumentation of the urinary tract.

Important risk factors include diabetes mellitus, urinary tract obstruction, and renal failure.

3. Respiratory tract infection

These patients usually are colonized with ***S. marcescens*** after instrumenlation (e.g. ventilation, bronchoscopy), especially those patients with chronic obstructive pulmonary disease.

Patients may have pneumonia, but this development is rare. If pneumonia develops, patients may have fever, chills, productive cough (sometimes with pseudohemoptysis), hypotension, dyspnea, or chest pain.

4. Meningitis or cerebral abscess

Meningitis or cerebral abscesses resulting from ***S. marcescens*** infection may develop in premature children and neonates with prior sepsis or in patients who have experienced head trauma, neurosurgery, or lumbar puncture.

The symptoms are those of gram-negative meningitis (e.g. headache, fever, vomiting, stupor, coma)

5. Intraabdominal infection

Patients with intraabdominal infections resulting from ***S. marcescens*** infection may present with biliary drainage, hepatic abscess, pancreatic abscess, and peritoneal exudate.

6. Bone and joint infection

Patients with ***S. marcescens*** infection may have osteomyelitis or arthritis, which can be hematogenous in people addicted to intravenous drugs or may be caused exogenously by surgery, open trauma, or intraarticular injection.

7. Endocarditis

Patients with endocarditis resulting from ***S. marcescens*** infection may present fever petechiae, and occasionally, embolic complications (e.g. stroke, arterial emboli).

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8. Ocular infection

Patients with *Serratia*-related ocular infections will have keratitis or endophthalmitis. ***S. marcescens*** infection frequently causes nonulcerating bacterial keratitis, which is associated with soft and rigid contact lens wear. Endophthalmitis usually occurs after surgery.

9. Solf tissue infection

Patients with soft tissue infections resulting from ***S. marcescens*** may have surgical scars, cellulitis, phlebitis, or skin infections.

10. Otitis media

Patients with ***S. marcescens*** -related otitis media have earaches, hearing loss, and ear discharge. Bacterial parotitis associated with ***S. marcescens*** infectionis very rare.

Mortality/Morbidity

Crude mortality for nosocomial bloodstream infection with ***S. marcescens*** is 26% (25).

Mortality is very high in patients with meningitis and endocarditis caused by ***S. marcescens*** infection.

IV. Nosocomial infections

Nosocomial infections are a significant hazard in the health care facilities; they affect the patients, their families and the health care system. The term nosocomial comes from the Greek “nosokomeian”. This word could be separated into 2 parts; ‘nosos’ which meaned hospital, and word ‘komian’ meaned disease.

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Therefore a nosocomial infection is an infection associated with a hospital or a health care facility (30).

Outbreaks of nosocomial infections continue to occur among patients in a variety of healthcare settings. Although fungi, viruses, and parasites can cause nosocomial infections, bacterial agents remain the most commonly recognized cause of the disease outbreaks. In hospitals, the use of indwelling catheters, ventilators, and a variety of other medical devices often serve as sources and conduits for bacteria and make efforts to control outbreaks more difficult. In addition, the decreased effectiveness of resistant bacteria complicates infection control efforts (31).

In many instances, infection control personnels want to confirm that the outbreak-associated isolates are identical to each other and to those recovered from the implicated source. Microbiologic molecular techniques are available for typing a wide variety of nosocomial organisms, including typical bacterial pathogens, mycobacteria species, fungi, and viruses.

Frequency

In the US: ***S. marcescens*** species cause 1.4% of nosocomial bloodstream infections (25).

Internationally: The prevalence of ***S. marcescens*** in nosocomial infections is diminishing, but these bacteria still are able to cause hospital outbreaks, especially in intensive care units.

V. Epidemiological studies of *S. marcescens* infection

Liu P. Yuk-Fong, et al. studied the epidemiology of *S. marcescens* isolates in nosocomial infection by PCR. Their results indicated that the outbreak was due to the

spread of two epidemic strains. This technique was validated by comparison with rRNA gene restriction analysis. Combination of the two sets of restriction enzyme digestion patterns gave 17 distinct ribotypes, which were completely correlated with the 17 types observed in PCR based fingerprinting. A total of 5 biochemical patterns and 11 antibiotic susceptibility profiles were observed for all the clinical isolates. Typing with both biochemical profile and antibiogram profile, though simple, was found to be less reliable than genotyping (8).

Miranda G, et al. used pulsed-field gel electrophoresis (PFGE) technique to analyze an outbreak of *S. marcescens* in a neonatal intensive care unit (NICU). They included 25 patients isolates form an outbreak (March to July 1995), and 10 patients isolates from different wards during the same time period. PFGE typing showed seven patterns (patterns A, B, C, D, E, F, G). The 20 isolates from NICU and 4 isolates from different wards were patterns A. The 2 isolates from NICU and 2 isolates from different wards were patterns E. The isolates from five other patients showed distinct patterns (patterns B, C, D, F, G). All the isolates were resistant to aztreonam, trimethoprim/sulfamethoxazole, and ampicillin 100% (10).

Van Ogtrop ML, et al. studied an outbreak of *S. marcescens* in a neonatal intensive care unit (NICU). *S. marcescens* was isolated from five preterm infants. Two infants developed septicemia, which were both fatal, and one infant had conjunctivitis due to *S. marcescens.* Two infants were colonized without clinical signs of infection. All infants treated with antibiotic regimens including ciprofloxacin and gentamicin. The DNA fingerprints of isolates were determined by enterobacterial repetitive intergenic consensus primers by the polymerase chain reaction (PCR). This showed that a single strain had spread in the NICU. An extensive investigation

pointed to the infants born from a mother with an intra-uterine infection after prolonged rupture of foetal membranes as a presumed source of the outbreak (32).

In the study by Hejazi A, et al. 1997, the random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) was shown to be a convenient typing method for *S. marcescens*. The combinations of primer HLWL-74 and 1254 gave distinguishable patterns for different serotypes and proved to be the most satisfactory typing method. By applying this combination to 175 isolates of *S. marcescens,* which could be classified into 38 groups on the basis of serotyping and phage typing, 73 different RAPD patterns with good reproducibility were obtained (27).

Pulsed-field gel electrophoresis (PFGE) typing was applied to the epidemiological investigation of 20 *S. marcescens* isolates collected from urine specimens of 17 patients and three urinals over a 2-month period in the study by Shi ZY, et al. 1997. Twenty-five epidemiological unrelated strains were also tested to determine the discriminatory power of PFGE. They concluded that PFGE served as a highly discriminatory and reproducible method for the epidemiological investigation of the outbreak of *S. marcescens* infections (15).

Herra CM, et al. described a serious outbreak of infection caused by a strain of *S. marcescens* in two Dublin hospital which occurred over 11 week period and affected a total of 15 patients. All isolates of *S. marcescens* involved in the outbreak had the same antimicrobial susceptibility pattern, with reduced susceptibility to gentamicin, cefotaxime and ciprofloxacin. Epidemiological typing revealed that the strains of *S. marcescens* isolated in the outbreak were of an uncommon serotype, O21:K14, and using pulsed-field gel electrophoresis, *Xba*I DNA macrorestriction profiles clustered at 90% similarity. These results suggested the unique spread of a single strain of *S. marcescens* in Dublin hospital (33).

Berthelot P, et al. investigated an outbreak of *S. marcescens* in a maternity hospital (November 1994 to May 1995). Twenty-seven strains collected during the main outbreak were typed by AP-PCR using two different primers. A major epidemic profile shared by 22 strains (18 from babies of the neonatal unit, 2 from babies of other units, and 2 from breast milk) and another profile shared by 5 strains (2 from transducers of internal tocographs, 2 from babies, and 1 from a mother). The outbreak stopped only when infection control measures were reinforced in the delivery rooms, including the nonreuse of internal tocographs (34).

Hoyen C, et al. investigated the *S. marcescens* isolated from nine patients in an intensive care nursery during an 8-week period. Initial PFGE analysis performed after identification of the first eight patients, when closure of the nursery was imminent, revealed that the epidemic was caused by two groups of four isolates each. In both instances, the group was geographically contained, and the nursery remained open. A second PFGE analysis indicated that a ninth *S. marcescens* isolates, recovered at week 8th, was genetically unrelated to the other two. Surveillance during an additional 6 weeks revealed no new cases, and the epidemic was declared over. Real-time PFGE determined that an apparent nursery outbreak of *S. marcescens* infection was, in fact, caused by three genetically distinct strains (35).

Aucken HM, et al. produced DNA fingerprints from 57 isolates of *S. marcescens* using the restriction enzyme *Xba*I and pulsed-field gel electrophoresis (PFGE). The isolates were selected on the basis of their epidemiology, serotype and phage-typing patterns to include 28 unrelated strains and 29 representatives from 2 distinct outbreak. Twenty-six of the unrelated strains had unique profiles with over 10 band differences from all other strains, while 27 of the outbreak representatives could be assigned to the appropriate outbreak with confidence. The majority of the

outbreak isolates had none or 2 band differences from the index profile, although 3 isolates differed by 5-7 bands. The 2 exceptions among the unrelated strains differed by 4 bands, and 3 phage typing reaction. The results of this studied together with those of the 6 previous reports of PFGE for *S. marcescens* (which used either enzymes *Xba*I or *Spe*I) confirmed that this technique was of value for this species and that within *Xba*I at least, most epidemiologically related strains will only differ by 3-4 bands (36).

Knowles S, et al. studied an outbreak of multi-resistant *S. marcescens* involving 24 patients occurred in a bone morrow transplant and oncology unit, from September 1998 to June 1999, of whom 14 developed serious infection. All isolates demonstrated the same antimicrobial susceptibility pattern and were the same unusual serotype O21:K14. The antimicrobial susceptibility profile showed reduced susceptibility to ciprofloxacin, gentamicin, and piperacillin-tazobactam (37).

Hejazi A, Aucken HM and Falkiner FR. studied an epidemiology of *S. marcescens* over the 8 years period 1988-1995. The most common source of *S. marcescens* was sputum from patients. Strain identities were determined by serotyping and phage typing at least one isolate from each of 311 of the 582 patients. The results showed that a single epidemic strain of serotype O14:K14 was present in 69% of these patients, and persisted throughout the hospital for the whole of the eight-year period. This strain was recovered from a variety of clinical specimens, including blood cultures. A minor outbreak involving a serotype O16:K28 strain also occurred and this strain also persisted from at least 1989 to 1994. Extensive surveillance failed to reveal an environmental source or faecal carriage (38).

Dorsey G, et al. investigated an outbreak of invasive disease due to *Enterobacter cloacae* and *Serratia marcescens* in a surgical intensive care unit. The

pulsed-field gel electrophoresis (PFGE) analysis of restriction fragments was used to characterize the outbreak isolate genotypes. The sensitivity patterns of the *S. marcescens* isolates were variable. The results of the PFGE genotypic analysis revealed a highly heterogeneous pattern (39).

Jang TN, et al. used pulsed-field gel electrophoresis (PFGE) typing to analyse an outbreak in a neonatal intensive care unit (NICU). They included sample from nine patients, three hand-washes and ten environmental isolates from an outbreak (February to August 1999) in addition to four patient isolates from different wards of the same hospital during the same time period. Nine outbreak isolates exhibited an identical PFGE fingerprint, while the epidemiologically unrelated strains demonstrated distinct patterns. Epidemiological investigation failed to reveal a common source of the outbreak, although the epidemic *S. marcescens* strain was isolated from hand-washes and doors of incubators. They concluded that cross-transmission via transient contamination of hands was the major route for this outbreak. This PFGE method was highly discriminatory for the thorough epidemiological investigation of an outbreak of *S. marcescens* (40).

VI. When to use strain typing

Bacterial strain typing data are most effective when they are collected, analyzed, and integrated into the results of an epidemiological investigation. The hospital epidemiologist should initiate strain typing studies in consultation with the hospital infection control laboratory or the hospital microbiology staff when investigating a potential outbreak of an infectious disease (41). This may be triggered by a noticeable increase in the rate of isolation of a particular pathogen, a cluster of infections on a particular ward, or the recognition in the clinical microbiology

laboratory of multiple isolates with an unusual biotype or antibiogram. Strain typing data should supplement, and not replace, a carefully conducted epidemiological investigation. In some cases, typing data can effectively rule out an outbreak and thus avoid the need for an extensive epidemiological investigation. In other cases, strain typing data may reveal the presence of outbreaks caused by more than one strain. However, undue reliance on strain typing in the absence of epidemiological data is an inefficient use of laboratory resources.

VII. Typing methods in the epidemiologic study of *S. marcescens*

Typing methods fall into broad categories: phenotypic methods and genotypic methods. Phenotypic methods are those that characterize the products of gene expression in order to differentiate strains. Properties such as biochemical profiles; bacteriophage types, antigens present on the cell's surface, and antimicrobial susceptibility profiles all are examples of phenotypic properties that can be determined in the laboratory. Because they involve gene expression, these properties all have a tendency to vary, based on changes in growth conditions, growth phase, and spontaneous mutation.

Genotypic methods are those that are based on an analysis of the genetic structure of an organism and include polymorphisms in DNA restriction patterns based on cleavage of the chromosome by enzymes that cleave the DNA into 10 to 30 fragments (infrequent cutters), and the presence or absence of extrachromosomal DNA. Genotypic methods are less subject to natural variation, although they can be effected by insertions or deletions of DNA into the chromosome, the gain or loss of extrachromosomal DNA, or random mutations that may create or eliminate restriction endonuclease sites.

All typing systems can be characterized in terms of typeability, reproducibility, discriminatory power, and ease of interpretation. The characteristics of a number of typing methods are presented in Table 2.1. (42). Typeability refers to the ability of a technique to assign an unambiguous result (type) to each isolate. Although nontypeable isolates are more common with phenotypic methods, they have been recognized with most methods. For example, with PFGE, a technique that is almost uniformly applicable to bacteria, some strains of *Clostridium difficile* remain nontypeable because the chromosomal DNA is degraded, presumably by endogenous nuclease, before it can be cleaved properly by the restriction endonucleases used in the PFGE protocol.

A reproducible method is one that yields the same results upon repeat testing of a bacterial strain. In the context of an epidemiological study, this means that the same strain recovered from epidemiologically linked patients will give the identical (or nearly identical) typing result. Poor reproducibility may reflect technical variation in the method or biologic variation occurring during *in vivo* or *in vitro* passage of the organisms to be examined. Over time (a few weeks to years, depending on the species), the typing patterns produced by DNA-based methods, such as PFGE and AP-PCR, will show some minor, natural variation. Thus, when analyzing results, it is important to consider the length of time over which the bacterial isolates were collected.

The discriminatory power of a technique refers to its ability to differentiate among epidemiologically unrelated isolates, ideally assigning each to a different type. Traditional phenotypic methods, such as antibiogram typing, serotyping, and

Table 2.1. Characteristics of typing systems.

Typing systems	Proportion of strains typeable	Reproducibility	Discriminatory power	Ease of performance
Biotyping	All	Poor	Moderate	Easy
Antimicrobial susceptibility patterns	All	Good	Easy	Easy
Serotyping	Most	Good	Moderate	Moderate
Plasmid fingerprinting	Most	Good	Moderate	Moderate
REA of cDNA with conventional electrophoresis	All	Good	Difficult	Moderate
RFLP analysis with DNA probes	All	Excellent	Moderate	Difficult
PFGE	All	Excellent	Moderate	Moderate
AP-PCR	All	Good	Moderate	Moderate

Data from Arbeit, R.D. Manual of Clinical Microbiology, 1995.

Abbreviations: REA, restriction endonuclease analysis; cDNA, chromosomal DNA; RFLP, restriction fragment-length polymorphism; PFGE, pulsed-field gel electrophoresis; AP-PCR, arbitrarily primed polymerase chain reaction.

Ease of performance reflects the cost of specialized reagents and equipment the technical complexity of a method, and the effort required to learn and to implement the technique in the laboratory. Most molecular methods required the purchase of new equipment, some of which is costly ($4,000-$20,000)(31). However, these methods are learned easily and are widely applicable to a variety of species. Many traditional methods also involve considerable costs in labor and materials, but are restricted to a single or relatively few species. For example, bacteriophage typing, which is used primarily for *S. aureus* and a few other bacterial species, requires the maintenance of bacteriophage stocks that constantly must be replenished and titered, a process that is both time-consuming and labor-intensive.

Finally, ease of interpretation refers to the effort and experience required to obtain useful, reliable typing information using a particular method. At present, the interpretation of the results of molecular methods remains an area of active discussion. However, this is contrast to methods such as bacteriophage typing and pyocin typing, which require significant expertise to perform and interpret and often still yield ambiguous results.

1. Phenotypic methods

Typing methods that assess phenotypic differences are inherently limited by the capacity of microorganisms to alter the expression of the underlying genes. Such changes may occur unpredictably or in response to various environmental stimuli. In addition, point mutations representing a single nucleotide in the entire chromosome can result in the abnormal regulation or function of the gene responsible for a particular phenotype. Thus, isolates that represent the same strain

and that are genetically indistinguishable (or almost so) can vary in the phenotype detected.

1.1. Biotyping

In the 1960s and early 1970s, identification of bacterial species was frequently undertaken using racks of tubes representing a variety of biochemical tests, and the variability of certain tests, such as indole, H_2S, or pigment production, served as markers for particular strains. Thus, biotyping emerged as a useful tool for the epidemiological investigations. At present, the identification of bacterial species normally is accomplished by using a combination of biochemical and immunologic tests of which now are performed using commercial kits or automated devices. However, biotyping using automated methods relies on a variety of novel substrates, and some of these tests, such as carbohydrate fermentations, are highly variable even within isolates of the same strain. Thus, biotyping, like most phenotypic methods, has only modest reproducibility, because microorganism can alter unpredictably, the expression of many cellular products. Moreover, contemporary biotyping typically has poor discriminatory power and cannot differentiate among some of the current nosocomial problem pathogens, such as enterococci, where biochemical diversity is uncommon. Occasionally, outbreaks occurred when many cases of infections caused by bacterial strains that represent unusual species or unusual biotypes of common species, for example, H_2S-producing isolates of *Escherichia coli* were observed. In such situations, additional typing, techniques may not be needed. However, even clusters of unusual isolates may not always indicate a common-source outbreak, as indicated by a recent report in which four isolates of *Leptotrichia buccalis*, an unusual anaerobic gram-negative bacillus, recovered from blood cultures of four

different bone marrow transplant patients, were found to be unrelated by PFGE and fatty-acid profile analysis. The investigation of the suspected outbreak revealed that each of the patients had undergone dental manipulations prior to developing bacteremia. In addition, all of the patients had been placed on prophylactic antimicrobial agents to which the *L. buccalis* isolates were resistant. Thus, each neutropenic patient developed bacteremia with his own endogenous strain of *L. buccalis*, which served as an opportunistic pathogen. Nonetheless, it should be noted that outbreaks can, in some cases, be caused by multiple pathogens.

1.2. Antimicrobial susceptibility patterns

Antimicrobial susceptibility patterns also have relatively poor discriminatory power, because antimicrobial resistance is under tremendous selective pressure in healthcare institutions and often is associated with mobile genetic elements (e.g. transposons and plasmid). Changes in antibiograms also may reflect spontaneous point mutations, such as seen with fluoroquinolones. Thus, isolates that are epidemiologically related and otherwise genetically indistinguishable may manifest different antimicrobial susceptibilities due to acquisition of new genetic material over time or the loss of plasmids. Conversely, unrelated isolates may have indistinguishable resistance profiles, which may represent acquisition of the same plasmid by multiple species (a “plasmid outbreak”).

1.3. Serotyping

Serotyping, a nonmolecular method, uses a series of antibodies to detect different antigenic determinants on the surface of the bacterial cell. Serotyping is the one of the classic strain typing techniques that has been used over the years for

the epidemiological studies of many species of bacteria. It remains a key method for typing isolates of *Salmonella, Shigella*, and pneumococci. However, maintaining stocks of typing sera (including the >2,200 antisera required for definitive *Salmonella* typing) is a major limitation of this method. Because of the association of certain *Salmonella* serotypes with foodborne disease, and the association between specific pneumococcal serotypes and invasive disease, particularly in children, serotyping continues to be a valuable typing technique. Nonetheless, PFGE has been shown to resolve distinct clonal strains within individual serotypes of both *Samonella* and pneumococci, thus indicating that it is a more discriminatory typing tool.

2. Genotypic methods

Over the last several years, various molecular techniques have emerged as the methods of choice for typing bacterial isolates. They are plasmid fingerprinting; restriction endonuclease analysis (REA) of plasmid DNA; REA of chromosomal DNA using frequent cutting enzymes and conventional electrophoresis; restriction fragment-length polymorphism (RFLP typing) analysis using DNA probe; AP-PCR and other related nucleic acid amplification-based typing methods; and PFGE. The detail of each method was described as followed.

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2.1. Plasmid fingerprinting

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Plasmid fingerprinting was the first molecular method to be used as a bacterial typing tool. Plasmids are extrachromosomal DNA elements that are present in most clinical isolates and can be identified readily by simple cell lysis procedures followed by agarose gel electrophoresis of the lysates (Figure 2.1)(43). The number and size of the plasmid present is used as the basis for strain identification. This

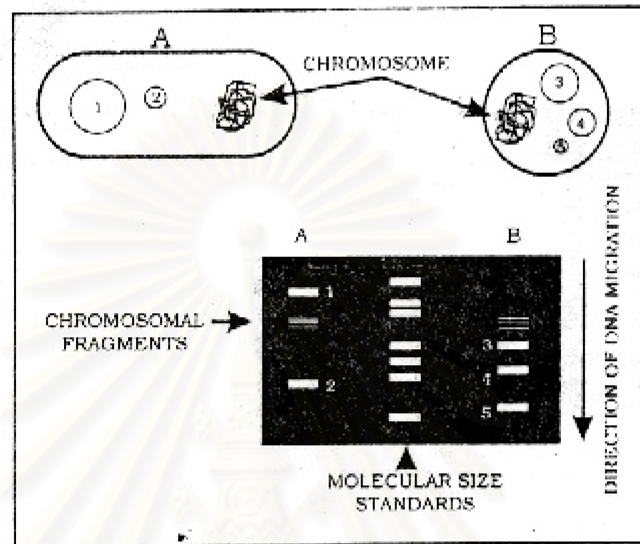

Figure 2.1. Schematic drawing of the plasmid fingerprinting technique using agarose gel electophoresis. The oval on the left signifies a typical gram-negative rod, and the circle on the right signifies a typical gram-positive coccus. Cell are lysed using detergents at high pH, the chromosomal DNA is removed, and the plasmid DNA is applied to an agarose gel that is stained and photographed. Some chromosomal fragments usually are visible on the gel and serve as an internal molecular size standard (approximately 12-15). Plasmid DNA within the bacteria is shown in circular form. Each plasmid is numbered within the cell, and its corresponding position in the agarose gel is indicated by the same number (43).

strain typing technique has been used successfully for analysis of the outbreaks of nosocomial infections and community-acquired infections caused by a variety of species of gram-negative rods.

2.2. REA of plasmid DNA

Some strains of bacteria contain only a single large plasmid, often in the size range of 100 to 150 kilobases (kb). Because it is difficult to differentiate plasmids in this size range, especially those that vary by only 10 kb to 15 kb, some investigators have added a restriction endonuclease digestion step to try to increase the discriminatory power of agarose gel electrophoresis (Figure 2.2)(44). While this can be helpful, large plasmids produce many restriction fragments, which can make interpretation more difficult, especially when the multiple large plasmids are present. Thus, for gram-negative rods, the REA step is no longer performed in most laboratories. However, for analysis of staphylococci, where the plasmids typically are <50 kb, REA appears to increase the discriminatory power of the analysis, because the number of restriction fragments generated usually is <20 fragments. Digestion also makes the patterns of the restriction fragments produced from staphylococcal plasmids easier to analyze than the undigested profiles, which often show multiple forms for plasmids of less than 15 kb, because circular and linear forms of the plasmids migrate at different rates than the covalently closed circular form. Plasmid fingerprinting is technically simple to perform and requires relatively inexpensive equipment ($1,500-$3,000)(31). At this time, the method is used primarily as an alternative technique for staphylococcal isolates, which frequently carry multiple plasmids, and for selected species of *Enterobacteriaceae*, which often have large distinctive plasmids.

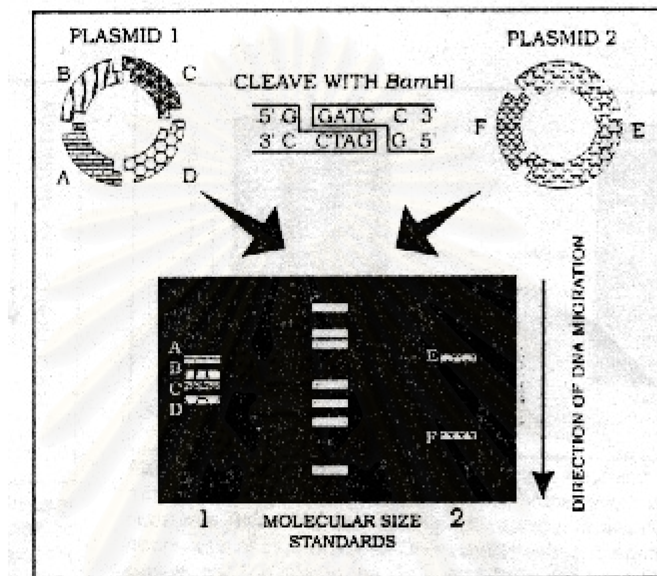

Figure 2.2. Schematic drawing of restriction endonuclease digestion of two unique plasmid, followed by agarose gel electrophoresis. Different-sized restriction fragments are denoted by the different patterns (44).

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When applying the plasmid fingerprinting technique, investigators must be aware of two confounding factors. First, it is possible that plasmids can spread to multiple species of bacteria, causing a plasmid outbreak in which unusual antibiograms are recognized in multiple species. This has been recognized both in gram-negative rods and in staphylococci. Second, it is important to appreciate that the structure of individual plasmids and the plasmid content of a particular strain may vary over time. This variability reflects two factors: over time, plasmids can be lost spontaneously or acquired from other organisms, and plasmids frequently carry smaller mobile genetic elements (transposon and insertion sequences) that promote duplications and deletions of DNA segments. Both plasmids and transposon often include antimicrobial resistance determinants and thus are subject to considerable selective pressure within hospitals due to antimicrobial agent use. In general, plasmid fingerprinting is most useful for epidemiological studies that are limited both temporally and geographically. In selected instances, plasmid fingerprinting may complement other techniques, such as PFGE analysis, by providing a basis for differentiating isolates that are related genotypically but are separated epidemiologically by moderate time periods, such as several months.

2.3. Gel electrophoresis techniques for analysis of chromosomal DNA

There are two methods of typing organisms based on fragment patterns produced by cleaving chromosomal DNA with restriction endonucleases. The first method, often referred to as conventional electrophoresis, uses a restriction enzyme that cuts the chromosome into hundreds of pieces (frequent cutter), followed by standard agarose gel electrophoresis. Fragments that are 25 kb to 0.5 kb are resolved into a discernible banding pattern, although a single band may contain fragments of

similar size from several different areas of the chromosome. Larger fragments coalesce at the top the gel or do not migrate into the gel. The second method, PFGE, uses enzymes that cut chromosomal DNA infrequently, generating from 10 to 30 bands, followed by a novel from of electrophoresis that can separate fragments from 1 kb up to 1,000 kb (1 megabase). Each method, and a variation of the conventional electrophoresis method, is described in greater detail below.

2.3.1. REA of chromosomal DNA with frequent cutting enzymes and conventional electrophoresis

Each restriction endonuclease cleaves DNA at a particular sequence of nucleotides that may be repeated numerous times around the chromosome. The number and size of the restriction fragments generated by digesting a given piece of DNA reflects the frequency and distribution of the restriction sites. In conventional REA, endonucleases with frequently occurring sites in the bacterial genome are used to digest total DNA (plasmid and chromosome), thereby generating hundreds of fragments ranging from approximately 0.5 to 50 kb in length (Figure 2.3)(45). Such fragments can be separated by size using agarose gel electrophoresis, and the pattern can be detected by staining the gel with ethidium bromide (or other dyes) and photographing under ultraviolet light. Different strains of the same bacterial species have different REA profiles (depicted as a series of bands on agarose gels) because of variations in their DNA sequences. All isolates are typeable by REA; however, it can be very difficult to interpret the complex profiles, which consist of hundreds of bands that may be indistinct or overlapping. Although the approach has been applied to many species, at this time, its primary use is as an alternative technique for analyzing *C. difficile*.

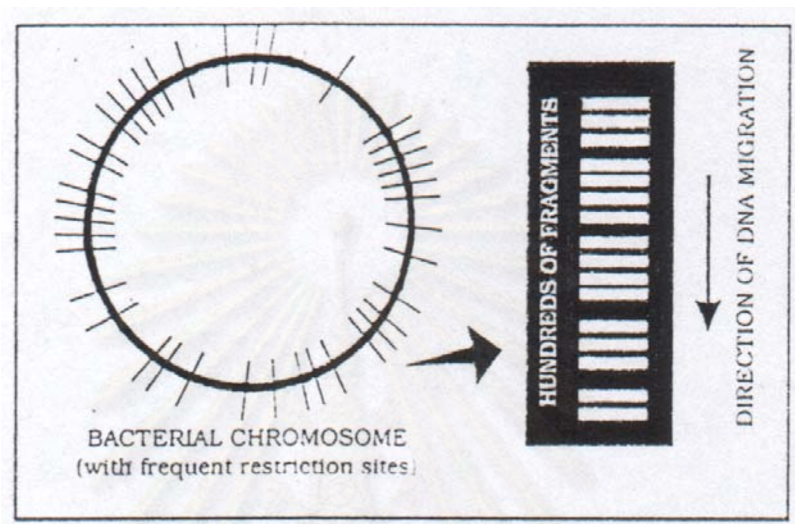

Figure 2.3. Schematic drawing of restriction endonuclease analysis of chromosomal DNA using conventional electrophoresis. The box on the right represents the banding pattern of hundreds of fragments after conventional agarose gel electrophoresis. Each band may contain a number of unique chromosomal fragments of similar size (45).

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2.3.2. PFGE

Pulsed-field gel electrophoresis was first described in 1984 as a tool for examining the chromosomal DNA of eukaryotic organisms (Figure 2.4)(46). Subsequently, PFGE has proven to be a highly effective molecular typing technique for many different bacterial species.

Types of PFGE

PFGE size resolves DNA molecules of a millimeter in length through the use of pulsed-field, which selectively modulate mobilities in a size-dependent fashion. The pulsed electrophoresis effect has been utilized by a variety of instrument (FIGE, TAFE, CHEF, OFAGE, PACE, and rotating electrode gel) to increase the size resolution of both large and small DNA molecules (47). It is important when choosing a PFGE system to evaluate cost and performance in the light of projected use. There are different types of PFGE. These are:

Field-Invertion Gel Electrophoresis (FIGE): In 1986, Carle, Frank and Olson developed a simpler system, FIGE, in which the two fields were 180° apart. Electrode polarity was reversed at intervals, with a longer forward than reverse pulse time to generate a net forward sample migration. Net forward migration is achieved by increasing the ratio of forward to reverse pulse times to 3:1. To improve the resolution of the bands by FIGE, the duration of pulse times is increased progressively during a run. This is called "switch time ramping". By changing pulse durations continually during the course of an experiment, FIGE has the advantages of straight lanes and simple equipment. All that is needed are standard gel boxes and a pulse controller. Today, FIGE is very popular for smaller fragment separations. FIGE provides acceptable resolution up to 800 kilobases (600-750 kb).

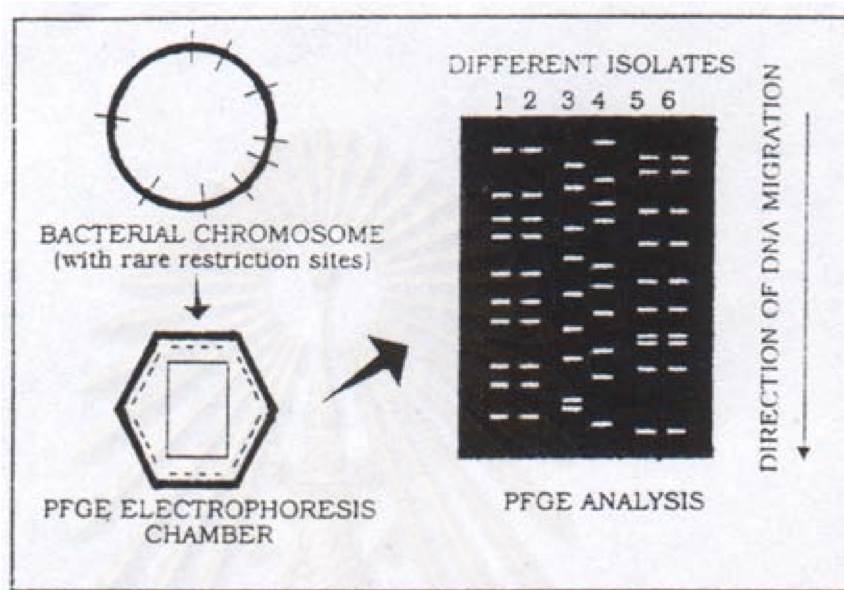

Figure 2.4. Schematic drawing of pulsed-field gel electrophoresis, in which chromosomal DNA is cleaved with a rare cutting enzyme followed by electrophoresis, using a unique chamber and current switching protocol. The box on the right is the agarose gel showing the very large DNA fragments derived from the unique electrophoresis chamber (46).

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Transverse-alternating Field gel Electrophoresis (TAFE): This form of PFGE allows separation of large DNA fragments in a simple, convenient format without the drawbacks of earlier pulsed-field techniques. In TAFE, the gel is oriented vertically and a simple four-electrode array is placed not in the plane of the gel, but in front and at the back of it. Sample molecules are forced to zigzag through the thickness of the gel, and all lanes experience the same effects so the bands remain straight. As the molecules move down the gel, they are subjected to continual variations in field strength and reorientation angle, but to all lanes equally. However, the angle between the electric fields varies from the top of the gel (115°) to the bottom (approximately 165°) and hence molecules still do not move at a constant velocity over the length of the gel. TAFE technology, with regular and sharp separation of DNA bands, will be of special advantage in the study of genetics of many pathogenic protozoans, where such analysis was impossible before. TAFE has been used for the separation of fragments up to 1,600 kilobase fragments.

Contour-Clamped Homogeneous Electric Fields (CHEF): CHEF is the most widely used apparatus. The CHEF apparatus provides a more sophisticated solution to the distorting effects of both the edges of the chamber and the passive electrodes. CHEF has twenty-four point electrodes equally spaced around the hexagonal contour. In the CHEF system, there are no “passive” electrodes. All the electrodes are connected to the power supply via an external loop of resistor, all of which have the same resistance. This loop is responsible for setting the voltages of all the electrodes around the hexagonal contour to values appropriate to the generation of uniform fields in each of the alternate switching position. The CHEF system sets the voltages at these 24 points. This apparatus produces electric fields that are sufficiently uniform so that all lanes of the gel run straight. CHEF uses an angle of

reorientation of 120° with gradiations of electropotential radiating from the positive to the negative pores. Molecules up to 7,000 kb can be separated by CHEF.

Orthogonal-Field Alternating Gel Electrophoresis (OFAGE):

A similar apparatus that used two nonhomogeneous electric fields was reported by Carle and Olson in 1984. The major drawbacks of these apparatuses were that because the electric fields were not uniform, and the angle between the electric field varied across the gel and DNA molecules migrated at different rates depending on their location in the gel. This is especially problematic in mammalian genome mapping, where a continuous distribution of fragment sizes is generated. Lane-to-lane comparisions and size estimations for digested genomic DNA are less straightforward when fewer discrete bands are being separated, as with the chromosomes of lower organisms like yeast. The angle between the electric fields varies from less than 180° and the more than 90°. DNA molecules from 1,000 to 2,000 kb can be separated in OFAGE.

Rotating Gel Electrophoresis (RGE): In England in 1987, Southern described a novel PFGE system that rotates the gel between two set angles while the electrodes are off. In RGE, the electric field is uniform and bands are straight because only one set of electrodes is used. RGE makes it easy to perform time and voltage ramping. It also enables users to study the effects of different angles, and even to vary these, during an experiment-angle ramping. RGE uses a single homogeneous field and changes the orientation of the electric field in relation to the gel by discontinuously and periodically rotating the gel. Switch times are too long in RGE. The DNA molecules from 50 kb to 6,000 kb can be separated by adjusting the frequency of the gel rotation. In addition, the angle of reorientation can be easily altered simply by changing the angle of rotation.

Programmable Autonomously-Controlling Electrodes (PACE): The PACE electrophoresis system offers precise control over all electric field parameters by independent regulation of the voltages on 24 electrodes arranged in a closed contour. The flexibility of the PACE system derives from its ability to generate an unlimited number of electric fields of controlled homogeneity, voltage gradient, orientation and duration. The PACE system can perform all previous pulsed field switching regimens (i.e. FIGE, OFAGE, PHOGE, unidirectional pulsing), as well as generate voltage clamped homogeneous static fields. The PACE system separates DNA fragments from 100 bp to over 6 Mb. The ability to alter the reorientation angle between the alternating fields permits an increased speed of separation for large DNA molecules. A computer-driven system known as PACE, designed by Lai et al. may be the ultimate PFGE device. It is an extremely useful tool for studying variables such as pulse time, temperature, agarose concentration, voltage and angles between fields affecting DNA migration in PFGE.

Pulsed-Homogeneous Orthogonal Field Gel Electrophoresis (PHOGE): The major difference between this instrument and other gel boxes with homogeneous electric fields is that the field reorientation angle is 90°. PHOGE uses a 90° reorientation angle, but the DNA molecules undergo four reorientations per cycle instead of two. The DNA lanes in PHOGE do not run straight, a phenomenon which has been described for gel runs involving multiple electric fields in this manner. This system separates DNA fragments of up to 1 Mb.

2.4. RFLP analysis using DNA probes

In this technique, chromosomal restriction digests produced by frequent cutting enzymes are separated by conventional agarose gel electrophoresis,

as described above (section 2.3.1), and then the DNA fragments are transferred onto a nitrocellulose or nylon membrane (Figure 2.5)(48). The DNA on the membrane then is hybridized with a specific chemically or radioactively labeled piece of DNA or RNA (a probe), with binds to the relatively few fragments on the membrane that have complementary nucleic acid sequences. Variations in the number and size of the fragments detected by hybridization are referred to as RFLPs.

One common typing method that uses chromosomal DNA preparations and a ribosomal RNA probe is ribotyping. Because all bacterial isolates have one or more chromosomal rRNA operons distributed around the chromosome, and because those sequences are highly conserved, essentially all bacterial isolates can be typed using probes directed to the DNA sequences that encode the rRNA loci using a single rRNA probe. However, enthusiasm for this system has diminished because the approach has proven to be only moderately discriminatory.

2.5. Typing methods using PCR

Polymerase chain reaction, which has been used for several years for the direct detection of many types of infectious agents in clinical samples, has been adapted for use as a typing tool. The hallmark of PCR is the ability to produce literally millions of copies of a particular DNA segment with high fidelity within 3 to 4 hours' time. The procedure requires template DNA (or RNA if a reverse transcriptase step is used initially), which may be present in the sample in minute quantities; two oligonucleotide primers, which flank the sequences on the template DNA to be amplified (thus defining the starting points for DNA polymerase activity); and a heat-stable DNA polymerase. Efficient amplification is accomplished readily for templates of less than 2,000 base pairs, although templates as large as 35 kb now

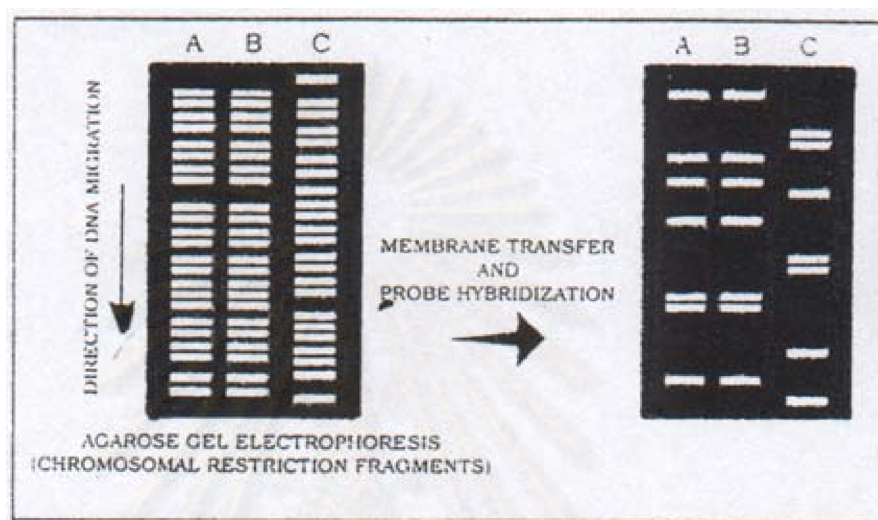

Figure 2.5. Schematic drawing of restriction fragment-length polymorphism analysis using a DNA or RNA probe, such as IS6110 or ribosomal RNA. The box on the left represents agarose gel electrophoresis of chromosomal DNA cleaved with a restriction endonuclease, and the box on the right represents the nylon filter to which the DNA has been attached and hybridized with a specific probe. Only the DNA fragments on the nylon filter that bind the probe can be visualized. The organisms represented in lanes A and B are indistinguishable, whereas the isolate represented in lane C is a different strain (48).

can be amplified by using newer polymerases. A typical PCR assay requires approximately 3 hours to complete 30 cycles, where each cycle consists of a heat denaturation phase, in which double-stranded DNA is melted into single strands; an annealing phase, in which the primers bind to the target sequences on the single strands; and an extension phase, in which DNA synthesis proceeds from the primers along each strand of the template DNA, thereby generating two new double-stranded copies of the original template. After 30 such cycles, a single initial copy of template DNA theoretically can be amplified to 1 billion copies.

VIII. Molecular typing of specific organism

In practice, the majority of hospital-based epidemiologic analyses involve a relatively limited set of organisms. Virtually every method described has been applied to these species, and consideration of all available reports is outside the scope of this review. A recent position paper by the Society of Hospital Epidemiologists of America has identified the preferred techniques for common bacterial pathogens (Table 2.2)(31,49).

IX. Interpretation

Interpretation of strain typing results is facilitated greatly by an appreciation of the molecular basis of genetic variability of bacteria and the technical factors that can affect results. Three assumptions usually are made: such isolates will have the same genotype; and, epidemiologically unrelated strains will have different genotypes. Ideally, strain typing will provide a clear, objective basis for identifying the outbreak strain and distinguishing it from epidemiologically unrelated isolates.

Table 2.2. Preferred strain typing techniques for common bacterial pathogens.

Species	Reference method	Alternative methods
Staphylococcus aureus	PFGE	AP-PCR, Plasmid analysis
Coagulase-negative staphylococci	PFGE	Plasmid analysis
Streptococcus pneumoniae	PFGE	Serotyping
Enterococci	PFGE	AP-PCR
Escherichai coli, Citrobacter, Proteus, Providencia	PFGE	AP-PCR
Klebsiella, Enterobacter, Serratia	PFGE	Plasmid analysis
Samonella, Shigella	Serotyping	PFGE
Pseudomonas aeruginosa	PFGE	-
Clostridium difficile	rep-PCR, AP-PCR	REA, PFGE
Mycobacterium tuberculosis	IS6110 RFLP	rep-PCR
Mycobacteria other than *M. tuberculosis*	PFGE	-

Data from Tenover, F.C.; et al. Infect. Control Host. Epidemiol, 1997 and Murray, P.R.; et al. Manual of Clinical Microbiology seven edition.

Abbreviations: PFGE, pulsed-field gel electrophoresis; AP-PCR, arbitrarily primed polymerase chain reaction; REA, restriction endonuclease analysis; IS6110 RFLP, RFLPs detected on southern blots probed with IS6110; rep-PCR, PCR with primers directed toward repetitive chromosomal elements (e.g., ribosomal sequences and repetitive oligonucleotide sequences).

In practice, the interpretation of typing data is complicated by the fact that isolates from an ongoing outbreak may demonstrate some, albeit typically limited, genetic variability. The purpose of interpretive criteria is to establish a guide for distinguishing true differences in strains from the natural genetic variation that occurs over time within a given strain. For illustration, assume that a set of up to 20 putative outbreak isolates has been typed and that the analysis has detected a subset of isolates with a common (modal) type, which is presumed to represent the outbreak strain. Typically, among the other isolates in the set, some have similar types (as represented, for example, by a few band changes in a PFGE pattern), and some distinctly different types (distinctive PFGE patterns)(50). The interpretative criteria should provide consistent, objective guidelines for correlating the level of variation observed between an individual isolate and the putative outbreak strain with an estimate of the likelihood that the isolate is, in fact, part of the outbreak (Table 2.3)(50). To provide a generally applicable approach, this correlation focuses on the number of genetic events required to generate the observed typing variation, rather than on the types of specific changes observed in a particular typing system. In the example cited, there is a group of isolates that produce identical typing patterns, ie, the presumed outbreak strain. Because only a small portion of the organisms' genetic complement is undergoing analysis, isolates that give identical results are classified as "indistinguishable", not "identical." A more detailed analysis theoretically could uncover differences in the isolates that appeared to give identical patterns but that were epidemiologically unrelated. However, when a set of epidemiologically linked isolates are analyzed, this is unlikely to occur.

Table 2.3. General principles for the interpretation of molecular typing.

Microbioilogic interpretation based on typing results	No. of genetic differences compared with outbreak strain	Typical No. of fragment difference compared to outbreak pattern	Epidemiological correlation
Indistinguishable	0	0	Isolate is part of the outbreak
Closely related	1	2-3	Isolate probably part of the outbreak
Possibly related	2	4-6	Isolate possibly part of the outbreak
Different	3	≥7	Isolate is not part of the outbreak

Data from Tenover, F.C.; et al. Infect. Control Host. Epidemiol, 1997 and Murray, P.R.; et al. Manual of Clinical Microbiology seven edition.

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CHAPTER III

MATERIALS AND METHODS

1. Specimens All the specimens in this study were obtained from the Neonatal Intensive Care Unit (NICU) at Siriraj hospital.

Grouping of specimens.

1.1. Clinical specimens

1.1.1. Specimens from neonates included:

397 throat swabs, 185 tracheal secretion and 4 specimens from other infective sites included: 2 pus from stump of umbilical cords, 1 discharge from eyes and 1 urine.

1.1.2. Specimens from fluid administered to neonates included:

32 normal saline for wiping eyes/mouth of the patients, 32 drugs mixtures for inhalation and 240 fluid from humidifier or nebulizer.

1.1.3. Specimens from 5 swabs from inhalation set

1.1.4. Specimens from environments in the NICU included:

Swabs from 96 sinks and 96 sink taps.

1.1.5. Specimens from the other different wards: Ten *S. marcescens* isolates obtaining from the clinical specimens from the other different wards at Siriraj hospital during the same period of the specimen collection in the NICU. The type of specimens where they were isolated were as follow: 2 blood samples, 7 tracheal secretion and 1 urine sample.

The total number of specimen collection was 1,097 isolates.

2. Specimen collection

The specimen collection was performed by the nurses who were in charge of the neonatal intensive care unit (NICU). The frequency of specimen collection were as followed.

1.1 The throat swab and tracheal secretion from each neonatal was collected twice a week.

1.2 The drugs mixtures or the other fluid administered to the neonates were collected once a week.

1.3 The swabs from the environments in the unit were collected once a week.

The duration of the specimen collection was 4 months.

3. Isolation and identification of *Serratia marcescens*

Each specimen was streaked on a blood agar and a MacConkey agar and incubated at 37°C for 24 hours. The identification of *S. marcescens* was performed according to Bailey and Scott's (51). The tests and the results were shown in Table 3.1(51).

4. Antibiotic susceptibility test: Paper disk susceptibility test was performed according to disk diffusion method by Kirby-Bauer (52) and NCCLS (53). *Escherichia coli* ATCC 25922 was also included in this test as the control strain. The test was performed as followed.

4.1. Preparation of media

Twenty-five millimeters of Mueller-Hinton agar (MHA)(Becton dickinson, USA) were poured into 10 cm-diameter petri dish to yield an agar depth of 4 mm. The medium was then stored at 4°C and used within 2 weeks. Before performing the test, the petri dishes were placed in an incubator at 35°C for 30 minutes with their lids slightly open to permit the evaporation of the surface mixture.

4.2. Preparation of inoculum and standardization of inoculum

The well-isolated colonies of each 18 hour tested *S. marcescens* culture and *E. coli* ATCC 25922 as the control strain were selected from Tryptic Soy Agar (TSA)(Merck, Germany) and transfered to a tube containing 5 ml sterile normal saline solution (NSS). The suspension was adjusted with sterile NSS to 0.5 McFarland standard solution to obtain approximately 1.5 x 10^8 cells/ml.

4.3. Inoculation of standard inoculum

A sterile cotton swab was dipped into the adjusted suspension and excess suspension was removed by pressing and rotating the swab against the inside wall of the tube. Dried surface of the MHA plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum.

4.4. Antimicrobial agents

4.4.1. Ampicillin 10µg (AMP)

4.4.2. Amoxicillin/Clavulanic acid 30µg (AMC)

Table 3.1. Biochemical tests of *S. marcescens*.

Test	result
Triple Sugar Iron (TSI)	acid butt/alkaline slant (K/A)
Indole	negative
Lysine deaminase	negative
Lysine decarboxylase	positive
Motility	positive
Urease	negative
Citrate	positive
Methyl red	negative
Voges - Proskauer	positive
Manitol	positive
Malonate	negative
DNase	positive
Fermentation of arabinose	negative
Fermentation of sorbitol	positive
Fermentation of sucrose	positive
Fermentation of raffinose	negative

Data from Bailey and Scott's. Diagnostic Microbiology, 1998.

4.4.3. Cefotaxime 30 µg (CTX)

4.4.4. Ceftriaxone 30 µg (CRO)

4.4.5. Cefoperazone/Sulbactam 30 µg (CEF/SUL)

4.4.6. Ceftazidime 30 µg (CAZ)

4.4.7. Cefepime 30 µg (FEP)

4.4.8. Imipenem 10 µg (IPM)

4.4.9. Piperacillin 100 µg (PIP)

4.4.10. Piperacillin/Tazobactam 100 µg (PIP/TAZ)

4.4.11. Amikacin 30 µg (AN)

4.4.12. Gentamicin 10 µg (GM)

4.4.13. Netilmicin 30 µg (NET)

4.4.14. Ciprofloxacin 5 µg (CIP)

4.4.15. Sulfamethoxazone 21.75 µg/Trimethoprim 1.25 µg (SXT)

4.5. Application of disks

The unopened disk containers were be removed from the refrigerator or freezer one to two hours before use in order to minimizes amount of condensation that occurred when warm air contacts cold disks. Immediately or not later than 15 minutes after the inoculation of the plates, the antibiotic disks were applied to the surface of the medium with sterile forceps in order that diffusion and growth proceeded simultaneously. The disks were then slightly pressed down to ensure complete contact of the disks to the agar surface.

The disks were arranged at least 15 mm apart from the edge of the plate and 15 to 20 mm aparted from each other.

The plates were inverted and placed in an incubator set to 35°C within 15 minutes after the disks were applied.

4.6. Interpretation of the disk susceptibility test

After 18 hours of incubation, each plate was examined. The diameter of each zone of inhibition was measured with digital vernier caliper. Zone diameter interpretation chart for *S. marcescens* according to the standard of NCCLS was used as shown in Table 3.2 (53).

5. Analysis of restricted fragments of chromosomal DNA from *S. marcescens* by Pulsed-Field Gel Electrophoresis (PFGE)

Chromosomal DNA analysis by Pulsed-Field Gel Electrophoresis was performed according to the method recommended by Maslow *et al*. (54) as followed:

5.1. Sample preparation

Each isolate of *S. marcescens* was streaked onto Tryptic Soy Agar (TSA)to yield a single colony which was then inoculated into 0.5 ml Tryptic Soy Broth (TSB) and incubated for 2 hours at 37°C. The culture was streaked out onto a TSA plate and incubated for 20 hours at 37°C. A single colony was picked and inoculated into 5 ml TSB and then incubated for 20 hours at 37°C. Dispensed 1.5 ml of the culture into 5 ml cold PIV buffer and then centrifuged at 1100xg for 15 minutes at 4°C. The PIV buffer was removed from the cell pellet. The cell was resuspended and mixed thoroughly in 1.5 ml cold PIV buffer and was placed on ice.

The 1.3% of low melting point agarose (Promega, USA) in PIV buffer was prepared. The agarose was melted by placing the flask into the beaker of boiling

Table 3.2. Zone diameter interpretation chart.

Antimicrobial agents	Control zone diameter limit (mm) of *E. coli* ATCC 25922	Zone diameter interpretive standards (mm) susceptible of *S. marcescens*
Ampicillin 10μg (AMP)	16-22	≥17
Amoxicillin/Clavulanic acid 30μg (AMC)	19-25	≥18
Cefotaxime 30 μg (CTX)	29-35	≥23
Ceftriaxone 30 μg (CRO)	29-35	≥21
Cefoperazone/Sulbactam 30 μg (CEF/SUL)	28-34	≥21
Ceftazidime 30 μg (CAZ)	25-32	≥18
Cefepime 30 μg (FEP)	29-35	≥18
Imipenem 10 μg (IPM)	26-32	≥16
Piperacillin 100 μg (PIP)	24-30	≥21
Piperacillin/Tazobactam 100 μg (PIP/TAZ)	24-30	≥21
Amikacin 30 μg (AN)	19-26	≥17
Gentamicin 10 μg (GM)	19-26	≥15
Netilmicin 30 μg (NET)	22-30	≥15
Ciprofloxacin 5 μg (CIP)	30-40	≥21
Sulfamethoxazone 21.75 μg /Trimethoprim 1.25 μg (SXT)	24-32	≥16

Data from The National Committee for Clinical Laboratory Standard, 2000.

water. One ml of melted agarose was dispensed into 5 ml snap-top tube and was then placed in 50°C waterbath. One ml of *S. marcescens* cell in PIV buffer was added into each tube then slightly vortexed. Three hundred microliters of the mixture was immediately dispensed into each well of the plug molds that had already placed in the ice-tray for 15 minutes before used. The molds were then placed at 4°C for 30 minutes to solidify the agarose plug.

Fresh lysis solution was prepared by adding 80 µl RNase (10 mg/ml) and 800 µl lysozyme (50 mg/ml) into 40 ml lysis buffer. Four ml lysis solution were dispensed into each 15 ml snap-top tube. When the plug were solidified, each of them were pushed out from the molds into each lysis solution tube and then incubated for 20 hours at 37°C on a tube roller. The tubes were then chilled on ice for at least 15 minutes to harden the plugs. The lysis solution was carefully aspirated, then 4 ml of ESP solution was dispensed into each tube. Each plug were incubated overnight at 50°C with gently shaking. The tubes were again chilled. The ESP solution was changed one more and then stored at 4°C.

5.2. Restriction enzyme digestion

The plugs were washed in 7 ml 1xTE buffer at 37°C on a tube roller four times at 2 hours, 2 hours, 1 hour and overnight, respectively. A labeled microcentrifuged tube containing restriction enzyme *Spe*I, 10x restriction enzyme buffer, bovine serum albumin (final concentration, 100 µg/ml) and water to a final volume of 250 µl were prepared for each strain. Each washed plug was sliced into a small piece about 1 mm thick using a glass coverslip. A sliced plug was added to the labeled microcentrifuged tube. The restriction enzyme solution was added and then incubated overnight at 37°C in waterbath. Each sliced plug was washed using 1000 µl

1xTE buffer and placed on ice for 30 minutes. The 1xTE was removed and 1000 μl 0.5xTBE was dispensed into each plug. The plugs were placed on ice for 15 minutes.

5.3. Gel preparation and preelectrophoresis

The running gel was prepared by dissolving 0.9 gram of ultrapure high-melting temperature agarose (1%wt/vol) in 90 ml 0.5xTBE buffer. The agarose was melted until completely dissolved and was cooled down to approximately 50°C, and then poured into the gel casting. The 10-well comb was placed in the gel to make 10-well running agarose gel. The gel was placed in the gel casting until solidified. The gel was then transferred to the electrophoresis tank (CHEF-DRIII system, BioRad, USA). The running gel was preelectrophoresed for 0.5 hour in 0.5xTBE buffer to improve the clarity and resolution of the gel using the following condition; V = 6 v/cm, initial switch time = 5 s, final switch time = 60 s, and the temperature was 14°C. The running gel was removed from the tank.

5.4. Sample loading and electrophoresis

Each sliced plug sample including a plug of λ ladder marker were loaded into each well of the preelectrophoresed gel. All the wells of the gel were filled with 1% low-melting point agarose to protect the sliced plug from floating out of the well. The gel was then placed in the PFGE tank with 0.5xTBE buffer and electrophoresed using the same condition as the preelectrophoresis condition except that the running time was 22 hours.

5.5. Gel visualization

The gel was stained with 40 μl ethidium bromide (1 mg/ml) in 300 ml ultrapure water for 30 minutes. After that, it was destained with 300 ml ultrapure water for 2 hours and photographed under UV illumination.

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CHAPTER IV

RESULTS

1. The prevalence of *S. marcescens*

The prevalence of *S. marcescens* isolated from the neonates in NICU during the time of the specimen collection was shown in Table 4.1. It was found that there were 36 neonates (34.62%) haboured *S. marcescens* from the total 104 neonates.

The type of specimens and number of *S. marcescens* isolated from the neonates and the environments in NICU were summarized in Table 4.2. The organisms were isolated from 124 out of 397 (31.23%) throat swabs and 61 out of 185 (32.97%) tracheal secretion. The number of *S. marcescens* isolated from the other specimens were also shown in Table 4.2. The types of specimens and number of *S. marcescens* isolations from the other different wards were shown in Table 4.3.

The frequency of *S. marcescens* isolated from all types of specimens from each of the 36 neonates and the environments in the NICU during 4 months of the study as shown in Fig. 4.1. The 190 isolates of *S. marcescens* were obtained from the 36 neonates and the environments in the NICU were shown in Fig. 4.2. *S. marcescens* could be isolated from both the patients and the environments throughout the time of the study. For example patient NO1 had the organisms in the throat swabs and tracheal secretion throughout the time of his hospital stay.

The prevalence of *S. marcescens* isolated from the neonates in the NICU was shown in Table 4.4. None of *S. marcescens* was isolated from the discharge from eyes, pus from stump of umbilical cords and fluid from the humidifier/nebulizer.

The prevalence of *S. marcescens* isolated from the environments in the NICU was shown in Table 4.5. The organisms were isolated from only 3 out of 96 swabs from the sinks and each 1 out of 32 of the normal saline for wiping eyes/mouth and drugs mixture for inhalation used for the treatment of the patients.

2. The antimicrobial susceptibility patterns of *S. marcescens*

2.1. The antimicrobial susceptibility patterns of the 202 *S. marcescens* isolates from the neonates and the environments in the NICU and the other different wards.

The 202 *S. marcescens* isolates from the neonates and the environments in the NICU and the other different wards were tested against the 15 antimicrobial agents in order to study the susceptibility patterns of the organism. The results in Table 4.6 showed that there were 10 susceptibility patterns or antibiograms found among all of the tested isolates.

The antimicrobial susceptibility patterns and changing of the patterns of *S. marcescens* from the neonates in the NICU was shown in Table 4.7.

The summary results of antibiograms of the isolates were shown in Table 4.8. Most of the isolates (144 isolates) were antibiogram pattern 1 which were susceptible to cefoperazone/sulbactam, cefepime, imipenem, piperacillin/tazobactam, amikacin, netilmicin and ciprofloxacin. Thirty-nine *S. marcescens* isolates were antibiogram pattern 2 and were susceptible to cefoperazone/sulbactam, cefepime, imipenem, piperacillin/tazobactam, amikacin and netilmicin. There were only few *S. marcescens* isolates in each of the other antibiogram patterns. The resistance patterns were also shown in Table 4.9.

2.2. Percentage of the antimicrobial susceptible *S. marcescens* from the neonates and the environments in the NICU

The percentage of the susceptibility of *S. marcescens* which were the first isolates from each of the neonates and the environments in the NICU against all 15 antimicrobial agents were shown in Table 4.10. All isolates were susceptible to cefoperazone/sulbactam, cefepime, imipenem, piperacillin/tazobactam. Most of the isolates were susceptible to amikacin and netilmicin. The isolates were moderate susceptible to ciprofloxacin and were resistant to ampicillin, amoxicillin/clavulanic acid, cefotaxime, ceftriaxone, ceftazidime, piperacillin, gentamicin and sulfamethoxazone/trimethoprim.

2.3. The antimicrobial susceptibility of the isolates from the other different wards

The number of susceptible of the 10 isolates against each antimicrobial agent from the other different wards were shown in Table 4.11. The isolates from the other different wards showed slightly more susceptible to some antimicrobial agents such as cefotaxime, ceftriaxone, piperacillin, gentamicin and sulfamethoxazone/trimethoprim than the NICU isolates.

3. Profiles of *S. marcescens* using restricted-fragments of chromosomal DNA patterns from PFGE (Pulsotype)

3.1. The *S. marcescens* isolates from the NICU

PFGE analysis of *Spe*I restricted fragments of chromosomal DNA from 190 *S. marcescens* isolates from the neonates and the environments in the NICU revealed only one pattern as shown in Fig. 4.3. About half of the isolates from each patient and environment (115 isolates) were typed by PFGE. All of them were type A except for one isolate from one out of the 2 patients from whom the specimens were collected after 6 months after the specimen collection had finished was type B as shown in Table 4.12 and 4.13. The PFGE patterns of *S. marcescens* isolated from these two new cases shown in Fig 4.4(b) lanes 9 was pulsotype A and lane 10 was pulsotype B.

3.2. The *S. marcescens* isolates from the other different wards

Pulsotypes of 10 *S. marcescens* isolates from the other different wards during the same period time were shown in Table 4.14. There were 10 different patterns (C, D, E, F, G, H, I, J, K and L) of *S. marcescens* were identified. The comparison of these pulsotypes to the type A isolates from the NICU were shown in Fig. 4.4. [lane 10(a) and 2-6(b)]

The summary of the pulsotypes and the number of isolates in each type were also shown in Table 4.15.

4. Comparison of the pulsotypes and the antibiograms

The correlation between the pulsotypes and the antibiograms were summarized in Table 4.16, 4.17 and 4.18. It was shown that pulsotype A *S. marcescens* which most prevalence type in this study show various different antibiogram patterns. There was no correlation between the pulsotypes and the antibiograms.

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Table 4.1. Prevalence of *Serratia marcescens* isolated from the neonates in the NICU during the 4 months of the study.

Source	Total Patient number.	Number of *S. marcescens* positive	Percentage of isolation
Neonates	104	36	34.62

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Table 4.2. Prevalence of *Serratia marcescens* isolated from each specimen of the neonates and the environments in the neonatal intensive care unit (NICU).

Wards	Type of specimens	Number	Number of *Serratia marcescens* isolates	Percentage of isolation
Neonatal in NICU	Throat swab (T)	397	124	31.23
	Tracheal secretion (S)	185	61	32.97
	Pus from stump of umbilical cord (Pus)	2	0	0
	Discharge from eyes (D)	1	0	0
	Urine (U)	1	1	100
	Set of drugs mixture for inhalation (P)	5	1	20
Environments in NICU	Sinks (C)	96	3	3.12
	Sink taps (K)	96	0	0
	Normal saline for wiping eyes/mouth (N)	32	1	3.12
	Drugs mixture for inhalation (D)	32	1	3.12
	Fluid from humidifier/nebulizer (H)	240	0	0
Total		1087	192	17.66

Table 4.3. Type and number of specimens which *S. marcescens* were isolated from the other different wards.

Type of specimens	Number of specimens
Blood	2
Tracheal secretion	7
Urine	1
Total	10

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S = Tracheal secretion H = Fluid from humidifier/nebulizer Red and black colors letters indicated positive and negative *S. marcescens* isolation respectively

T = Throat swab P = Swab from inhalation set *S. marcescens* = *Serratia marcescens* NICU = neonatal intensive care unit

N07	H S T	H S T																					
N06	P H T	H T																					
N05	H T																						
N04	P H S T	H S T																					
N03	P H S T	H S T	H S T	H S T	H S T	H S T	H S T	H S T	H S T	H S T	H S T	H S T	H S T	H S T	H S T	H S T	T	H T					
N02	P H S T	H S T	H S T	H S T																			
N01	H S T	H T	H S T	H S T	H S T	H S T	H S T	H S T	H S	H S T	H S T	H S T	H S T	H S T	H S T	H S T	H S T	H S T	H S T	H S T	H S T	H S T	H T

APRIL MAY JUNE JULY AUGUST

Figure 4.1. The types of specimens as compared to the specimens with *S. marcescens* isolates from each of the 36 neonates and the environments in the NICU during 4 month of the study.

S = Tracheal secretion
T = Throat swab

H = Fluid from humidifier/nebulizer
P = Swab from inhalation set

Red and black colors letters indicated positive and negative *S. marcescens* isolation respectively

N15							T	H S T	H S T	H **T**	T						
N14										T	H T	**T**	**T**				
N13										H S T	H S T	H S **T**	H **S** T	**T**	**T**		H S T
N12							T	H **T**									
N11			H S T	H S T	H **S** T	H **S** **T**	H **S** **T**	H **S** **T**	H S T	H S T	H S T	H **S** **T**	H S T	H **S** **T**	H **S** T	H **S** **T**	T
N10			T	T	H **T**	**T**											
N09			H S T	**T**	H **T**	H **T**		H **T**	**T**	T	T	**T**					
N08	P H S T	H S T	H S T	H S T	H S T	H S **T**	H **S** **T**	H **S** **T**									
	APRIL	MAY	MAY	MAY	MAY	MAY	MAY	MAY	JUNE	JUNE	JUNE	JUNE	JUNE	JUNE	JUNE	JUNE	JUNE

JULY | AUGUST

Figure 4.1 (continue)

S = Tracheal secretion

T = Throat swab

H = Fluid from humidifier/nebulizer

Red and black colors letters indicated positive and negative *S. marcescens* isolation respectively

Patient	H	S	T
N23	H H H H H H	S S	T T T T T T
N22	H H H H H H	S S S S S S	T T T T T T U T T T T T T T T T T
N21	H H H H H	S S S S S	T T T T T T
N20	H H	S S	T T T T
N19	H H H H	S S S S	T T T T
N18	H H H H H H	S S S S S S	T T T T T T T
N17	H H H H H H H H H H H H H	S S S S S S S	T T T T T T T T T T T T T T
N16			T T T T T T T

APRIL MAY JUNE JULY AUGUST

Figure 4.1 (continue)

S = Tracheal secretion H = Fluid from humidifier/nebulizer

T = Throat swab Red and black colors letters indicated positive and negative *S. marcescens* isolation respectively

	APRIL	MAY	JUNE	JULY			AUGUST									
N32								H S T	H S T		H T	H S T	H S **T**	H S **T**	H **S** **T**	H **S** **T**
N31												H S T	**T**	H S **T**	H **S** **T**	H **S** **T**
N30													T			
N29												**T**				
N28				T	H S T	H S T	T	T	T	T	**T**	**T**		**T**	**T**	
N27							H S T	H S T	H T	H T	**T**	**T**				
N26											**T**	**T**		H **S** **T**	**T**	**T**
N25				H S T		H S T		H T	H **T**							
N24				H S T	H S T	H S T	H S T	H S **T**	H **T**		**T**	T				

Figure 4.1 (continue)

S = Tracheal secretion
T = Throat swab
C = Swab from sinks

N = Normal saline for wiping eyes/mouth
D = Drugs mixture for inhalation
H = Fluid from humidifier/nebulizer

K = Swab from sink taps
E = Environments in NICU
Red and black colors letters indicated positive and negative *S. marcescens* isolation respectively

	APRIL	MAY	MAY	MAY	JUNE	JUNE	JUNE	JUNE	JUNE	JULY	JULY	JULY	AUGUST	AUGUST	AUGUST	AUGUST	AUGUST	AUGUST	AUGUST
E		D N K C	D N K C	D N K C	D N K C	D N K C	D N K C	D N K C	D N K C	D N K C	D N K C	D N K C	D N K C	D N K C	D N K C		D N K C		D N K C
N36																H T	H S T	H S T	H S T
N35																	H S T	H S T	H S T
N34																H S T		T	
N33															S T	T	T	T	T

Figure 4.1 (continue)

C = Swab from sinks
D = Drugs mixture for inhalation
N = Normal saline for wiping eyes/mouth
P = Swab from inhalation set
S = Tracheal secretion
T = Throat swab
U = Urine
E = environments in NICU
Black letters = specimens with *S. marcescens*
Red letters = specimens with *S. marcescens* were selected for pulsotyping

E																				N			D		C								C		C				
N36																																						S,T	S,T
N35																																						S,T	S,T
N34																																						T	
N33																																					T	T	T
N32																																				T	T	S,T	S,T
N31																																				T	T	S,T	S,T
N30																																				T			
N29																																			T				
N28																																		T	T	T	T		
N27																																		T	T				
N26																																		T	T		S,T	T	T
N25																																T							
N24																															T	T		T					
N23																														S,T	T								
N22																										T	T	T	U	S,T	S,T	S,T	S,T	T	T	T	T	T	T
N21								T																															
N20																								T															
N19																								S	S,T														
N18																								S,T	S,T	S,T													
N17																								S,T	S,T	S,T	T	T		T	T	T	T	T	T				
N16																						T	T	T	T		T												
N15													T																										
N14															T	T																							
N16															T	S	T	T																					
N12											T																												
N11								S	S,T	S,T	S,T				S,T		S,T	S	S,T																				
N10								T	T																														
N09							T	T	T		T	T			T																								
N08						T	T		T	S,T	S,T																												
N07			S	T																																			
N06			T	T																																			
N05			T																																				
N04		T		S,T																																			
N03		S,T	P	S,T			S,T	S,T	S,T				S	S,T		S																							
N02		T		T			S,T	S,T																															
N01		T		T			S,T			S	T	S,T	S	S,T		T	S,T	S,T	S,T		S		S,T	S,T	S	S		S											
		APRIL		MAY									JUNE									JULY									AUGUST									

Figure 4.2. Positive isolation of *S. marcescens* from the neonates and the environments in the NICU and the duration of specimen collection.

Table 4.4. Number of *S. marcescens* isolates from each of the 38 neonates in the NICU.

No.	T/total	S/total	U/total	P/total	Dis/total	Pus/total	H/total
N01	12/23	14/22	-	-	-	-	0/24
N02	4/4	2/4	-	0/1	-	-	0/4
N03	6/19	8/17	-	1/1	-	-	0/18
N04	2/2	1/2	-	0/1	-	-	0/2
N05	1/1	-	-	-	-	-	0/1
N06	2/2	-	-	0/1	-	-	0/2
N07	1/2	1/2	-	-	-	-	0/2
N08	5/8	2/8	-	0/1	-	-	0/8
N09	6/9	0/1	-	-	0/1	-	0/4
N10	2/4	-	-	-	-	0/1	0/1
N11	6/15	8/14	-	-	-	-	0/14
N12	1/2	-	-	-	-	-	0/1
N13	3/7	1/5	-	-	-	-	0/5
N14	2/4	-	-	-	-	-	0/1
N15	1/5	-	-	-	-	-	-
N16	5/7	-	-	-	-	-	-
N17	11/14	3/7	-	-	-	-	0/13
N18	3/7	3/6	-	-	-	-	0/6
N19	1/4	2/4	-	-	-	-	0/4
N20	1/4	0/2	-	-	-	-	0/2
N21	1/6	0/5	-	-	-	-	0/5
N22	13/16	4/6	1/1	-	-	-	0/6
N23	2/6	1/2	-	-	-	-	0/6
N24	3/8	0/5	-	-	-	-	0/6
N25	1/4	0/2	-	-	-	-	0/4
N26	5/5	1/1	-	-	-	-	0/1
N27	2/6	0/2	-	-	-	-	0/4
N28	4/11	0/2	-	-	-	-	0/2
N29	1/1	-	-	-	-	-	-
N30	1/1	-	-	-	-	-	-
N31	4/5	2/4	-	-	-	-	0/4
N32	4/8	2/7	-	-	-	-	0/8
N33	3/5	0/1	-	-	-	-	-
N34	1/2	0/1	-	-	-	0/1	0/1
N35	2/3	2/3	-	-	-	-	0/3
N36	2/4	2/4	-	-	-	-	0/4
N37*	-	1/1	-	-	-	-	-
N38*	-	1/1	-	-	-	-	-
Total	124/234	61/142	1/1	1/5	0/1	0/2	0/169

T = throat swab, S = tracheal secretion, U = urine, N01-N38 = neonates number 1-38
P = swab from inhalation set, Dis = discharge from eyes, NICU = neonatal intensive care unit,
H = fluid from humidifier/nebulizer, *S. marcescens* = *Serratia marcescens,* No. = number,
* = isolates were collected from new cases 6 months after the specimen collection had finished

Table 4.5. Number of *S. marcescens* isolates from the environments in the NICU.

Source	Number	Number of positive *S.marcescens* isolates
Sinks	96	3
Sink taps	96	0
Normal saline for wiping eyes/mouth	32	1
Drugs mixture	32	1
Fluid from humidifier/nebulizer	240	0
Total	496	5

S. marcescens = Serratia marcescens, NICU = neonatal intensive care unit

Table 4.6. Antimicrobial susceptibility patterns of *S. marcescens* isolates from the neonates and the environments in the NICU and the other different wards.

	Antimicrobial agents and susceptibility patterns													
Patterns	AMP	AMC	CTX	CRO	CAZ	CEF/SUL	FEP	IPM	PIP	PIP/TAZO	AN	GM	NET	CIP	SXT
1	R	R	R	R	R	S	S	S	R	S	S	R	S	S	R
2	R	R	R	R	R	S	S	S	R	S	S	R	S	R	R
3	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S
4	R	R	R	R	R	S	S	S	R	S	R	R	R	R	R
5	R	R	R	R	R	S	S	S	R	R	S	R	S	R	R
6	R	R	R	R	R	S	S	S	R	S	S	R	R	R	R
7	R	R	R	S	S	S	S	S	R	S	S	R	S	R	R
8	R	R	R	R	R	S	S	S	R	R	R	R	R	R	R
9	R	R	S	S	S	S	S	S	R	S	S	R	S	S	R
10	R	R	S	S	R	S	S	S	R	S	S	R	R	R	R

S. marcescens = *Serratia marcescens,* AMP = ampicillin, AMC = amoxicillin/clavulanic acid, CTX = cefotaxime, CRO = ceftriaxone, CEF/Sul cefoperazone/sulbactam, CAZ = ceftazidime, FEP = cefepime, IPM = imipenem, PIP = piperacillin, PIP/TAZ = piperracillin/tazobactam, AN = amikacin, GM = gentamicin, NET = netilmicin, CIP = ciprofloxacin, SXT = sulfamethoxazone/trimethoprim, R = resistant, S = susceptible

Table 4.7. The antimicrobial susceptibility patterns and change of the patterns of *S. marcescens* isolated from the neonates in the NICU.

No.	Specimens	Antimicrobial Patternas	Changed patterns	Antimicrobial agents (susceptibility changed patterns)
N01	T	2	1	CIP(R→S)
	S	2	1	CIP(R→S)
N02	T	2	-	-
	S	2	-	-
N03	T	2	1	CIP(R→S)
	S	2	1	CIP(R→S)
	S	2	7	CAZ(R→S), CRO(R→S)
	P	2	-	-
N04	T	2	-	-
	S	2	-	-
N05	T	2	-	-
N06	T	2	-	-
N07	T	2	-	-
	S	2	-	-
N08	T	4	1	AN(R→S), NET(R→S), CIP(R→S)
	T	4	2	AN(R→S), NET(R→S)
	S	2	4	AN(S→R), NET(S→R)
N09	T	2	1	CIP(R→S)
	T	2	4	AN(S→R), NET(S→R)
N10	T	2	-	-
N11	T	5	1	PIP/TAZO(R→S), CIP(R→S)
	S	2	1	CIP(R→S)
	S	2	5	PIP/TAZO(S→R)
	S	2	8	PIP/TAZO(S→R), AN(S→R), NET(S→R)
N12	T	1	-	-
N13	T	1	-	-
	S	1	-	-

Table 4.7. (Continue)

No.	Specimens	Antimicrobial Patterns	Changed patterns	Antimicrobial agents (susceptibility changed patterns)
N14	T	1	-	-
N15	T	1	-	-
N16	T	1	4	AN(S→R), NET(S→R), CIP(S→R)
N17	T	1	-	-
	S	1	-	-
N18	T	1	-	-
	S	1	-	-
N19	T	1	-	-
	S	1	-	-
N20	T	1	-	-
N21	T	1	-	-
N22	T	1	-	-
	S	1	-	-
	U	1	-	-
N23	T	1	-	-
	S	1	-	-
N24	T	1	-	-
N25	T	1	-	-
N26	T	1	-	-
	S	1	-	-
N27	T	1	-	-
N28	T	1	-	-
N29	T	1	-	-
N30	T	1	-	-
N31	T	1	-	-
	S	1	-	-
N32	T	1	-	-

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Table 4.7. (Continue)

No.	Specimens	Antimicrobial Patterns	Changed patterns	Antimicrobial agents (susceptibility changed patterns)
	S	1	-	-
N33	T	1	-	-
N34	T	1	-	-
N35	T	1	-	-
	S	1	-	-
N36	T	1	-	-
	S	1	-	-
N37*	S	9	-	-
N38*	S	3	-	-

S. marcescens = *Serratia marcescens*, NICU = neonatal intensive care unit,

T = throat swab, S = tracheal secretion,

P = swab from inhalation set,

(S) = susceptible, (R) = resistant

- = no change

* = isolates were collected from new cases 6 months after the specimen collection had finished

N01-N38 = neonates number 1-38

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Table 4.8. Antimicrobial susceptibility patterns and number of isolates from the neonates and the environments in the NICU and the other different wards.

patterns	Antimicrobial agents	No. of suscep.	No. of isolates	Number of isolations
1	CEF/SUL FEP IPM PIP/TAZO AN NET CIP	7	144	N01(18) N03(5) N08(2) N09(4) N11(9) N12(1) N13(4) N14(2) N15(1) N16(4) N17(14) N18(6) N19(3) N20(1) N21(1) N22(18) N23(3) N24(3) N25(1) N26(6) N27(2) N28(4) N29(1) N30(1) N31(6) N32(6) N33(3) N34(1) N35(4) N36(4) N(1) D(1) C(3) OW02(1)
2	CEF/SUL FEP IPM PIP/TAZO AN NET	6	39	N01(8) N02(6) N03(9) N04(3) N05(2) N06(2) N07(2) N08(3) N09(1) N10(2) N11(1) OW07(1)
3	CTX CRO CEF/SUL CAZ FEP IPM PIP PIP/TAZO AN GM NET CIP SXT	13	6	N38(1) OW03(1) OW04(1) OW05(1) OW06(1) OW09(1)
4	CEF/SUL FEP IPM PIP/TAZO	4	4	N08(2) N09(1) N16(1)
5	CEF/SUL FEP IPM AN NET	5	3	N11(3)
6	CEF/SUL FEP IPM PIP/TAZO AN	5	2	OW01(1) OW08(1)
7	CRO CEF/SUL CAZ FEP IPM PIP/TAZO AN NET	8	1	N03(1)
8	CEF/SUL FEP IPM	3	1	N11(1)
9	CTX CRO CEF/SUL CAZ FEP IPM PIP/TAZO AN NET CIP	10	1	N37(1)
10	CTX CRO CEF/SUL FEP IPM PIP/TAZO AN	7	1	OW10(1)

suscep. = susceptible agents, No. = number, NICU = neonatal intensive care unit,
N01-N38 = *Serratia marcescens* isolation from the neonates number 1-38 in the Neonatal Intensive Care Unit, C = *Serratia marcescens* isolation from sinks
OW01-OW10 = *Serratia marcescens* isolation from the other different ward number 1-10, D = *Serratia marcescens* isolation from drugs mixture for inhalation
N = *Serratia marcescens* isolation from normal saline for wiping eyes/mouth, AMP = ampicillin, AMC = amoxicillin/clavulanic acid, CTX = cefotaxime, CRO = ceftriaxone,

CEF/SUL = cefoperazone/sulbactam, CAZ = ceftazidime, FEP = cefepime, IPM = imipenem, PIP = piperacillin, PIP/TAZ = piperracillin/tazobactam, AN = amikacin,

GM = gentamicin, NET = netilmicin, CIP = ciprofloxacin, SXT = sulfamethoxazone/trimethoprim

Table 4.9. Resistance patterns and the number of isolation from the neonates and the environments in the NICU and the other different wards.

patterns	Antimicrobial agents	No. of suscep.	No. of isolates	Number of isolations
1	AMP AMC CTX CRO CAZ PIP GM SXT	8	144	N01(18) N03(5) N08(2) N09(4) N11(9) N12(1) N13(4) N14(2) N15(1) N16(4) N17(14) N18(6) N19(3) N20(1) N21(1) N22(18) N23(3) N24(3) N25(1) N26(6) N27(2) N28(4) N29(1) N30(1) N31(6) N32(6) N33(3) N34(1) N35(4) N36(4) N(1) D(1) C(3) OW02(1)
2	AMP AMC CTX CRO CAZ PIP GM CIP SXT	9	39	N01(8) N02(6) N03(9) N04(3) N05(2) N06(2) N07(2) N08(3) N09(1) N10(2) N11(1) OW07(1)
3	AMP AMC	2	6	N38(1) OW03(1) OW04(1) OW05(1) OW06(1) OW09(1)
4	AMP AMC CTX CRO CAZ PIP AN GM NET CIP SXT	11	4	N08(2) N09(1) N16(1)
5	AMP AMC CTX CRO CAZ PIP PIP/TAZO GM CIP SXT	10	3	N11(3)
6	AMP AMC CTX CRO CAZ PIP GM NET CIP SXT	10	2	OW01(1) OW08(1)
7	AMP AMC CTX PIP GM CIP SXT	7	1	N03(1)
8	AMP AMC CTX CRO CAZ PIP PIP/TAZO AN GM NET CIP SXT	12	1	N11(1)
9	AMP AMC PIP GM SXT	5	1	N37(1)
10	AMP AMC CAZ PIP GM NET CIP SXT	8	1	OW10(1)

suscep. = susceptible agents, No. = number, NICU = neonatal intensive care unit,
N01-N38 = *Serratia marcescens* isolation from the neonates number 1-38 in the Neonatal Intensive Care Unit, C = *Serratia marcescens* isolation from sinks
OW01-OW10 = *Serratia marcescens* isolation from the other different ward number 1-10, D = *Serratia marcescens* isolation from drugs mixture for inhalation
N = *Serratia marcescens* isolation from normal saline for wiping eyes/mouth, AMP = ampicillin, AMC = amoxicillin/clavulanic acid, CTX = cefotaxime,
CRO = ceftriaxone, CEF/SUL = cefoperazone/sulbactam, CAZ = ceftazidime, FEP = cefepime, IPM = imipenem, PIP = piperacillin, PIP/TAZ = piperracillin/tazobactam,
AN = amikacin, GM = gentamicin, NET = netilmicin, CIP = ciprofloxacin, SXT = sulfamethoxazone/trimethoprim

Table 4.10. Percent susceptibility of the first isolates from each of the neonates and the environments in the NICU.

Antimicrobial agents	Specimens from NICU patients				Specimens From environments		
	T (36)	S (18)	P (1)	U (1)	N (1)	D (1)	C (3)
1. Ampicillin (AMP)	0	0	0	0	0	0	0
2. Amoxicillin/Clavulanic acid (AMC)	0	0	0	0	0	0	0
3. Cefotaxime (CTX)	0	0	0	0	0	0	0
4. Ceftriaxone (CRO)	0	0	0	0	0	0	0
5. Cefoperazone/Sulbactam (CEF/SUL)	100	100	100	100	100	100	100
6. Ceftazidime (CAZ)	0	0	0	0	0	0	0
7. Cefepime (FEP)	100	100	100	100	100	100	100
8. Imipenem (IPM)	100	100	100	100	100	100	100
9. Piperacillin (PIP)	0	0	0	0	0	0	0
10. Piperacillin/Tazobactam (PIP/TAZO)	100	100	100	100	100	100	100
11. Amikacin (AN)	97.22	100	100	100	100	100	100
12. Gentamicin (GM)	0	0	0	0	0	0	0
13. Netilmicin (NET)	97.22	100	100	100	100	100	100
14. Ciprofloxacin (CIP)	72.22	0	0	100	100	100	100
15. Sulfamethoxazone/Sulbactam (SXT)	0	0	0	0	0	0	0

T = throat swab, S = tracheal secretion, P = swab from inhalation set, U = urine, N = normal saline for wiping eyes/mouth, D = drugs mixture for inhalation, C = swab from sinks, Bl = blood, AMP = ampicillin, AMC = amoxicillin/clavulanic acid, CTX = cefotaxime, CRO = ceftriaxone,

CEF/SUL = cefoperazone/sulbactam, CAZ = ceftazidime, FEP = cefepime, IPM = imipenem, PIP = piperacillin, PIP/TAZ = piperracillin/tazobactam,

AN = amikacin, GM = gentamicin, NET = netilmicin, CIP = ciprofloxacin, SXT = sulfamethoxazone/trimethoprim

Table 4.11. The antimicrobial susceptibility of the isolates from the other different wards.

Antimicrobial agents	Number of susceptible isolates from other wards (10)
1. Ampicillin (AMP)	0
2. Amoxicillin/Clavulanic acid (AMC)	0
3. Cefotaxime (CTX)	6
4. Ceftriaxone (CRO)	6
5. Cefoperazone/Sulbactam (CEF/SUL)	7
6. Ceftazidime (CAZ)	5
7. Cefepime (FEP)	7
8. Imipenem (IPM)	7
9. Piperacillin (PIP)	5
10. Piperacillin/Tazobactam (PIP/TAZO)	7
11. Amikacin (AN)	7
12. Gentamicin (GM)	5
13. Netilmicin (NET)	7
14. Ciprofloxacin (CIP)	6
15. Sulfamethoxazone/Trimethoprim (SXT)	5

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Table 4.12. Pulsotypes of *S. marcescens* isolated from the neonates in the NICU.

Patient code	Throat swab (T)		Tracheal secretion (S)		Other specimens (O)	
	No. (total)	pulsotypes	No. (total)	Pulsotypes	No. (total)	pulsotypes
N01	12 (23)	A,A,A,AA,A	14 (22)	A,A,A,A,AA,A,	-	-
N02	4 (4)	A,A,A	2 (4)	A	-	-
N03	6 (19)	A,A,A	8 (17)	A,A,A,A	1^{p} (1)	A
N04	2 (2)	A	1 (2)	A	-	-
N05	1 (1)	A	-	-	-	-
N06	2 (2)	A	-	-	-	-
N07	1 (2)	A	1 (2)	A	-	-
N08	5 (8)	A,A,A	2 (8)	A	-	-
N09	6 (9)	A,A,A	-	-	-	-
N10	2 (4)	A,A	-	-	-	-
N11	6 (15)	A,A,A	8 (14)	A,A,A,A	-	-
N12	1 (2)	A	-	-	-	-
N13	3 (7)	A,A	1 (5)	A	-	-
N14	2 (4)	A,A	-	-	-	-
N15	1 (1)	A	-	-	-	-
N16	5 (7)	A,A,A	-	-	-	-
N17	11 (14)	A,A,A,A,A	3 (7)	A,A	-	-
N18	3 (7)	A,A	3 (6)	A,A	-	-
N19	1 (4)	A	2 (4)	A	-	-
N20	1 (4)	A	-	-	-	-
N21	1 (6)	A	-	-	-	-
N22	13 (16)	A,A,A,A,A	4 (6)	A,A	1^{u} (1)	A
N23	2 (6)	A	1 (2)	A	-	-
N24	3 (8)	A,A	-	-	-	-
N25	1 (4)	A	-	-	-	-
N26	5 (5)	A,A	1 (1)	A	-	-
N27	2 (6)	A,A	-	-	-	-
N28	4 (11)	A,A,A	-	-	-	-
N29	1 (1)	A	-	-	-	-
N30	1 (1)	A	-	-	-	-
N31	4 (5)	A,A	2 (4)	A	-	-
N32	4 (8)	A,A	2 (7)	A	-	-
N33	3 (5)	A,A	-	-	-	-
N34	1 (2)	A	-	-	-	-
N35	2 (3)	A	2 (3)	A	-	-
N36	2 (4)	A	2 (3)	A	-	-
N37*	-	-	1 (1)	A	-	-
N38*	-	-	1 (1)	B	-	-
Total	124 (208)	73	61 (119)	35	2 (2)	2

S. marcescens = *Serratia marcescens*, NICU = neonatal intensive care unit
p = swab from inhalation set, u = urine, No. = number
* = Isolates were collected from new cases 6 months after the specimen collection had finished.
- = specimen not collected
N01-N38 = *S. marcescens* isolated from neonates number 1-38

Table 4.13. Pulsotypes of *Serratia marcescens* isolated from the other fluid administered to the neonates and from the environments in the neonatal intensive care unit (NICU).

Other samples	Number (total)	Pulsotypes
1. Normal saline for wiping the eyes/mouth of the patients	1 (32)	A
2. Drugs mixture for inhalation	1 (32)	A
3. Swab from sinks	3 (96)	A,A,A
Total	5 (160)	5

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Table 4.14. Pulsotypes of *S. marcescens* isolated from the patients in the other different wards.

Patient code	Throat swab (T)		Tracheal secretion (S)		Other (O)	
	No. (total)	Pulsotypes	No. (total)	pulsotypes	No. (total)	Pulsotypes
OW01	-	-	-	-	1^{bl} (1)	C
OW02	-	-	1 (1)	D	-	-
OW03	-	-	1 (1)	E	-	-
OW04	-	-	1 (1)	F	-	-
OW05	-	-	1 (1)	G	-	-
OW06	-	-	1 (1)	H	-	-
OW07	-	-	1 (1)	I	-	-
OW08	-	-	-	-	1^{u} (1)	J
OW09	-	-	1 (1)	K	-	-
OW10	-	-	-	-	1^{bl} (1)	L
Total	-	-	7 (7)	7	3 (3)	3

S. marcescens = *Serratia marcescens*, No. = number, bl = blood, u = urine,

OW01-OW10 = *S. marcescens* isolated from patients in the other different wards

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Table 4.15. Summary of the pulsotypes and the number of *S. marcescens* isolates in each type.

Type	Source										Total
	Number of specimens from NICU patients and environments (total)							Number of specimens from other ward (total)			
	T (124)	S (61)	P (1)	U (1)	N (32)	D (32)	C (96)	Bl (2)	S (7)	U (1)	
A	73	34	1	1	1	1	3	0	0	0	114
B	0	1	0	0	0	0	0	0	0	0	1
C	0	0	0	0	0	0	0	1	0	0	1
D	0	0	0	0	0	0	0	0	1	0	1
E	0	0	0	0	0	0	0	0	1	0	1
F	0	0	0	0	0	0	0	0	1	0	1
G	0	0	0	0	0	0	0	0	1	0	1
H	0	0	0	0	0	0	0	0	1	0	1
I	0	0	0	0	0	0	0	0	1	0	1
J	0	0	0	0	0	0	0	0	0	1	1
K	0	0	0	0	0	0	0	0	1	0	1
L	0	0	0	0	0	0	0	1	0	0	1
Total	73	35	1	1	1	1	3	2	7	1	125

S. marcescens = *Serratia marcescens*, NICU = neonatal intensive care unit, T = throat swab,
S = tracheal secretion, P = swab from inhalation set, U = urine,
N = normal saline for wiping eyes/mouth, D = drugs mixture for inhalation,
C = swab from sinks, Bl = blood

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Table 4.16. Pulsotypes and antibiograms of *S. marcescens* isolated from the neonates in the NICU.

No.	Date	Specimens	Types	Antibiograms
N01	27/4/00	T	A	2
	1/5/00	T	-	2
	12/5/00	T	A	2
		S	A	2
	23/5/00	S	A	2
	26/5/00	T	A	2
	30/5/00	T	-	2
		S	-	2
	2/6/00	S	A	1
	6/6/00	T	-	1
		S	-	1
	13/6/00	T	A	1
	16/6/00	T	-	1
		S	A	1
	20/6/00	T	A	1
		S	A	1
	23/6/00	T	-	1
		S	-	1
	30/6/00	S	-	1
	4/7/00	T	A	1
		S	-	1
	6/7/00	T	-	1
		S	A	1
	11/7/00	S	A	1
	13/7/00	S	-	1
	25/7/00	S	-	1
N02	27/4/00	T	A	2
	1/5/00	T	A	2
	12/5/00	T	-	2
		S	-	2
	16/5/00	T	A	2
		S	A	2
N03	27/4/00	T	A	2

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Table 4.16. (continue)

No.	Date	Specimens	Types	Antibiograms
N03	27/4/00	S	A	2
	28/4/00	P	A	2
	1/5/00	T	-	2
		S	-	2
	12/5/00	T	-	2
		S	-	2
	16/5/00	T	A	2
		S	A	2
	19/5/00	T	-	1
		S	-	2
	2/6/00	S	A	1
	6/6/00	T	A	1
		S	-	1
	13/6/00	S	A	1
N04	27/4/00	T	A	2
	1/5/00	T	A	2
		S	A	2
N05	28/4/00	T	A	2
N06	28/4/00	T	A	2
	1/5/00	T	-	2
N07	28/4/00	T	A	2
	1/5/00	S	A	2
N08	12/5/00	T	A	4
	16/5/00	T	-	1
	23/5/00	T	-	2
	26/5/00	T	A	2
		S	A	2
	30/5/00	T	A	1
		S	-	4
N09	16/5/00	T	A	2
	19/5/00	T	-	1
	23/5/00	T	A	4
	30/5/00	T	A	1
	2/6/00	T	-	1
	13/6/00	T	-	1

Table 4.16. (continue)

No.	Date	Specimens	Types	Antibiograms
N10	19/5/00	T	A	2
	23/5/00	T	A	2
N11	19/5/00	S	A	2
	23/5/00	T	A	5
		S	-	5
	26/5/00	T	-	5
		S	A	8
	30/5/00	T	-	1
		S	A	1
	13/6/00	T	A	1
		S	-	1
	20/6/00	T	-	1
		S	-	1
	23/6/00	S	A	1
	27/6/00	T	A	1
		S	A	1
N12	30/5/00	T	A	1
N13	13/6/00	T	A	1
		S	A	1
	20/6/00	T	A	1
	23/6/00	T	-	1
N14	13/6/00	T	A	1
	16/6/00	T	A	1
N15	30/5/00	T	A	1
N16	4/7/00	T	A	1
	6/7/00	T	-	1
	11/7/00	T	A	1
	13/7/00	T	-	1
	25/7/00	T	A	4
N17	11/7/00	T	A	1
		S	A	1
	13/7/00	T	A	1
		S	-	1
	20/7/00	T	-	1
		S	A	1
	25/7/00	T	A	1

Table 4.16. (continue)

No.	Date	Specimens	Types	Antibiograms
N17	27/7/00	T	-	1
	1/8/00	T	-	1
	3/8/00	T	A	1
	8/8/00	T	-	1
	10/8/00	T	A	1
	15/8/00	T	-	1
	17/8/00	T	-	1
N18	11/7/00	T	A	1
		S	A	1
	13/7/00	T	A	1
		S	-	1
	20/7/00	T	-	1
		S	A	1
N19	11/7/00	S	A	1
	13/7/00	T	A	1
		S	-	1
N20	11/7/00	T	A	1
N21	19/5/00	T	A	1
N22	20/7/00	T	A	1
	25/7/00	T	-	1
	27/7/00	T	-	1
	31/7/00	U	A	1
	1/8/00	T	A	1
		S	A	1
	3/8/00	T	-	1
		S	-	1
	8/8/00	T	-	1
		S	A	1
	10/8/00	T	-	1
		S	-	1
	15/8/00	T	A	1
	17/8/00	T	-	1
	22/8/00	T	-	1
	24/8/00	T	A	1
	28/8/00	T	-	1

Table 4.16. (continue)

No.	Date	Specimens	Types	Antibiograms
N22	31/8/00	T	A	1
N23	1/8/00	T	A	1
	1/8/00	S	A	1
	3/8/00	T	-	1
N24	3/8/00	T	A	1
	8/8/00	T	-	1
	15/8/00	T	A	1
N25	8/8/00	T	A	1
N26	15/8/00	T	-	1
	17/8/00	T	A	1
	24/8/00	T	-	1
		S	A	1
N26	29/8/00	T	-	1
	31/8/00	T	A	1
N27	15/8/00	T	A	1
	17/8/00	T	A	1
N28	15/8/00	T	A	1
	17/8/00	T	-	1
	22/8/00	T	A	1
	24/8/00	T	A	1
N29	17/8/00	T	A	1
N30	22/8/00	T	A	1
N31	22/8/00	T	A	1
	24/8/00	T	-	1
	29/8/00	T	-	1
		S	-	1
	31/8/00	T	A	1
		S	A	1
N32	22/8/00	T	A	1
	24/8/00	T	-	1
	29/8/00	T	-	1
		S	A	1
	31/8/00	T	A	1
		S	-	1
N33	24/8/00	T	A	1

Table 4.16. (continue)

No.	Date	Specimens	Types	Antibiograms
N33	29/8/00	T	A	1
	31/8/00	T	-	1
N34	29/8/00	T	A	1
N35	29/8/00	T	A	1
		S	A	1
	31/8/00	T	-	1
		S	-	1
N36	29/8/00	T	A	1
		S	A	1
	31/8/00	T	-	1
		S	-	1
N37*	26/2/01	S	A	9
N38*	6/3/01	S	B	3

NICU = Neonatal Intensive Care Unit, *S. marcescens* = *Serratia marcescens*,
No. = neonates number,
N01-N38 = *S. marcescens* isolated from the neonates in the NICU,
T = throat swabs, S = tracheal secretion, U = urine
* = Isolates were collected from new cases 6 months after the specimen collection had finished.

Table 4.17. Pulsotypes and antibiograms of *S. marcescens* isolated from the environments in the NICU.

E	Date	Specimens	Types	Antibiograms
	30/6/00	N	A	1
	6/7/00	D	A	1
	13/7/00	C	A	1
	10/8/00	C	A	1
	17/8/00	C	A	1

S. marcescens = *Serratia marcescens*, NICU = Neonatal Intensive Care Unit,
E = environments, N = normal saline for wiping eyes/mouth,
D = drugs mixture for inhalation, C = swab from sinks

Table 4.18. Pulsotypes and antibiograms of *S. marcescens* isolated from the other different wards.

No.	Date	Specimens	Types	Antibiograms
OW01	20/7/00	BL	C	6
OW02	25/7/00	S	D	1
OW03	27/7/00	S	E	3
OW04	3/8/00	S	F	3
OW05	10/8/00	S	G	3
OW06	17/8/00	S	H	3
OW07	22/8/00	S	I	2
OW08	24/8/00	S	J	6
OW09	29/8/00	S	K	3
OW10	31/8/00	BL	L	10

S. marcescens = *Serratia marcescens*, No. = neonates number,

OW01-OW10 = *S. marcescens* isolated from the patients in the other different wards,

BL = blood, S = tracheal secretion

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1 2 3 4 5 6 7 8 9 10

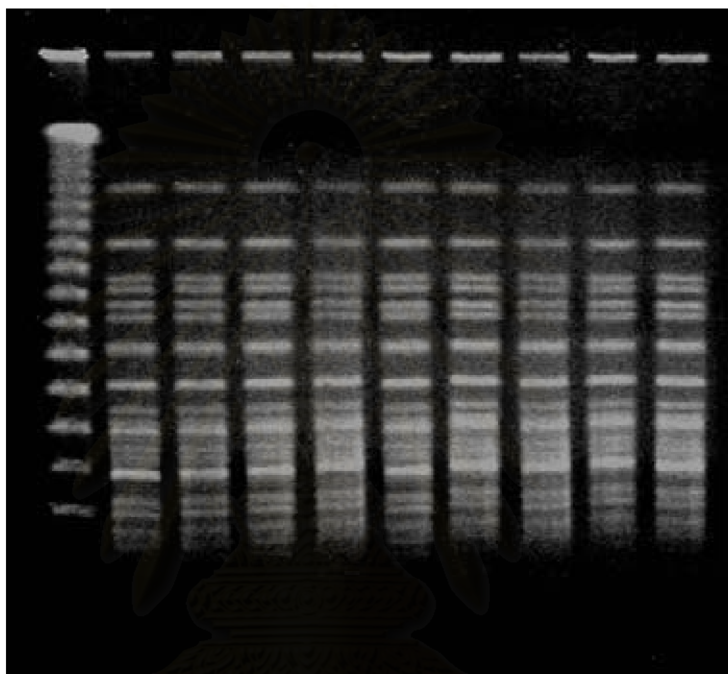

Figure 4.3. PFGE patterns of all *S. marcescens* isolates from the neonates and the environments in the NICU, digested with *Spe*I. The pulse time was 5 to 60 s at 6 v/cm and run time 22 h. Lane 1 show the lambda ladder (molecular marker) and lanes 2 to 10 show the PFGE pattern of isolates of *S. marcescens* from the 36 neonates and the 5 environments in NICU (pulsotype A).

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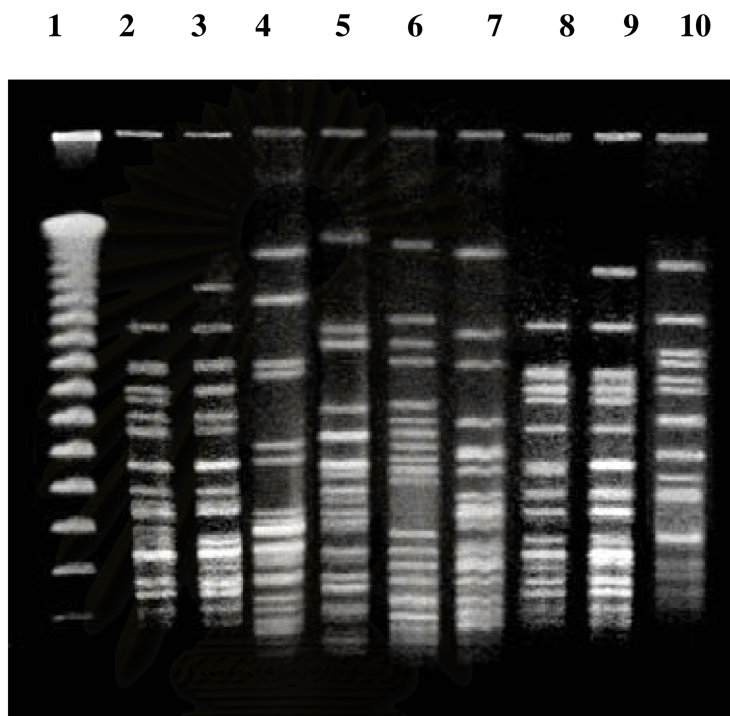

Figure 4.4(a). Comparison of PFGE patterns from *S. marcescens* isolates, digested with *Spe*I. The pulse time was 5 to 60 s at 6 v/cm and run time 22 h. Lane 1 show the lambda ladder (molecular marker). Lanes 2-9 show the PFGE patterns of isolates from the other different wards were pulsotypes C, D, E, F, G, H, I and J. Lane 10 show the PFGE pattern of isolates of *S. marcescens* from NICU was pulsotype A.

1 2 3 4 5 6 7 8 9 10

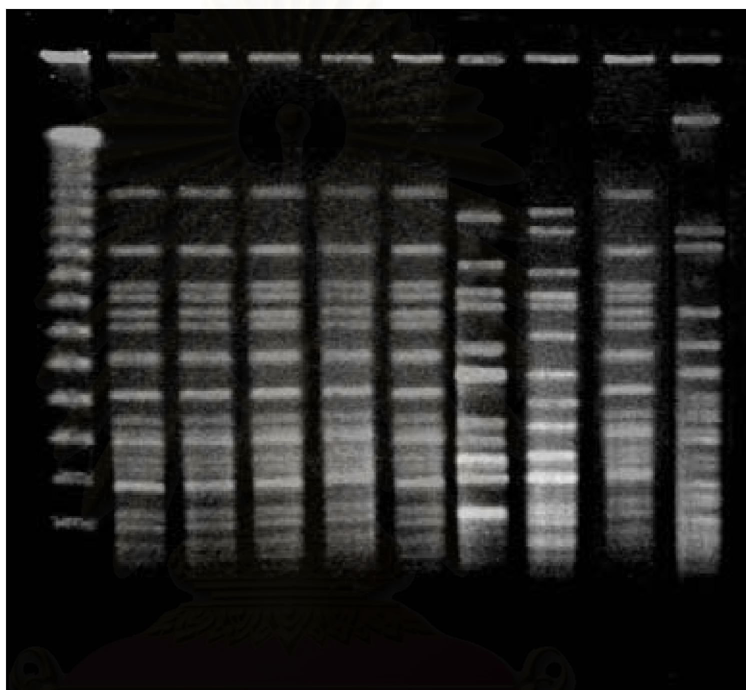

Figure 4.4(b). Comparison of PFGE patterns from *S. marcescens* isolates, digested with *Spe*I. The pulse time was 5 to 60 s at 6 v/cm and run time 22 h. Lane 1 show the lambda ladder (molecular marker). Lanes 2-6 show the PFGE pattern of isolates of *S. marcescens* from NICU was pulsotype A. Lanes 7-8 show the patterns of isolates from the other different wards were pulsotypes K and L. Lane 9-10 show the patterns of isolates of *S. marcescens* from two neonates in NICU whom the specimen collected at 6 months after the previous 4-month study period were pulsotype A and B.

CHAPTER V

DISCUSSION

S. marcescens is a well-recognized hospital-acquired pathogen causing many type of infections especially in neonates (34) and immunocompromised patients (36). Results from the studies during the 1970s indicated that neonatal colonization and infections by *S. marcescens* were rare events; however, from 1981 to 1997 the number of publications documenting epidemics of *S. marcescens* infections in a number of NICUs had increased considerably (40).

An epidemic or disease outbreak is the occurrence of disease at an unusual (unexpected) frequency. Because the word "epidemic" tends to create fear in a population, that term is usually reserved for a problem of wider than local implications, and the term "outbreak" is usually used for a localized epidemic. Nevertheless, the two terms are often used interchangeably (55). The emergence of a disease outbreak requires immediately action to determine the origin of the problem, and ultimately, to prevent other persons from becoming affected (56).

This study is one of the very first studies on *S. marcescens* outbreaks in Thailand. There have been several cases of infections due to *S. marcescens* in neonates admitted in the NICU at Siriraj hospital shortly before and during the time of this study. After reviewing the report of isolation rate of *S. marcescens* from the Department of Microbiology, Siriraj hospital during 1994 to 1999, it was found that the spreading of colonization was increased drastically 34.62% during the 4 months of study (May to August, 2000).

This prevalence was high, similar to that obtained in the study done by Van O, et al. which showed that an outbreak of colonization and infection with *S. marcescens* occurred in a neonatal intensive care unit (NICU) (Leiden University Hospital, The Netherland). The outbreak was recognized when two infants on the NICU developed septicemia and the third infant developed purulent conjunctivitis with *S. marcescens.* After recognition of the outbreak, rectal and pharyngeal swabs were taken from all other infants on the NICU and *S. marcescens* was isolated from five preterm infants (gastational age 25-30 weeks) (32).

Berthelot P, et al. investigated an outbreak of *S. marcescens* in a marternity hospital (Clinique Michelet, Saint-Etienne; France) (November 1994 to May 1995). They showed that 13 of the 104 (12.50%) patients in the Neonatal Unit (NU) were colonized with *S. marcescens*, mainly in stool; another baby developed bacteremia during the first 4 month of the study. The prevalence of *S. marcescens* colonization was increased to 20.91% in the following 2 months, but no infection occurred. The delivery rooms were suspected to be the initial place of contamination. Culture of the environmental samples led to the isolation of *S. marcescens* in the tocography transducers. The focus of infection control measures to the delivery rooms led to the end of the outbreak (34).

In this study, *S. marcescens* were isolated from both neonates and contaminated environments in NICU. It was clearly shown that environments in this study were not the important sources because the prevalence of *S. marcescens* isolated from environment was very low in spite of the fact that there have been many reports suggesting that *S. marcescens* has the property to survive in many sources, hospital environments and equipments. In addition, most of *S. marcescens* isolates were obtained only from throat swabs of 18 patients which indicated that colonized patients

should be the important sources of these infecting organisms. Supporting evidence on the endogenous source was clearly shown that *S. marcescens* were isolated from the throat swab of 8 patients before their tracheal secretions were positive. In contrast, the organisms were also isolated from tracheal secretion before they were isolated from throat swabs in 3 patients; thus also indicating the external sources.

In addition, the organisms sometimes disappeared from the throat swabs and tracheal secretions of the same neonates. It could be explained by the fact that these neonates might receive the antimicrobial agents causing the disappearance of the organism. Thus, organisms might reappear again afterward.

All *S. marcescens* isolates were tested for antimicrobial susceptibility agents. The antimicrobial agents tested were selected by the clinicians at Siriraj hospital, for the treatment of gram negative bacterial infection. Even though most of the isolates (144/202) (71.29%) were still susceptible to the carbapenem, piperacillin+β-lactamase inhibitor, aminoglycosides and quinolones but they were resistant to almost all the β-lactam antibiotics included the third generation cephalosporins. The results obtained from this part of the study were concordance with the report by Yu WL, et al. (57) and many other previous studies (10, 38) which showed that most of the *S. marcescens* in their studies were susceptible to the carbapenem and aminoglycoside but were resistant to penicillins and first to third generation cephalosporins.

Cephalosporins were the most frequently administered to the *S. marcescens* infected patients, but the *S. marcescens* isolates in this study were very resistant to these agents possibly because the isolates could produced β-lactamase which destroy β-lactam antibiotics.

The comparison of percentage susceptibility of the first isolates from various specimens showed that the isolates from NICU were less susceptible to the

antimicrobial agents tested than the isolates from the other different wards. It could be suggested that the use of various kinds of antimicrobial agents for a long time might be the cause of the development of resistant organisms. Therefore, the results obtained from the susceptibility test might be used as the guideline in the selection of antimicrobial agents in the treatment of *S. marcescens* and as the precautions of the long-term use of antimicrobial agents.

The study on *S. marcescens* prevalence alone was not enough for the epidemiologic study of *S. marcescens* in NICU. Hence, the molecular typing by pulsed-field gel electrophoresis (PFGE) was done to complete this part of the study. The antimicrobial susceptibility patterns of *S. marcescens* isolates (antibiograms) was also included in combination with PFGE in order to obtain the epidemiological data.

Even though, the use of antimicrobial susceptibility patterns, is simple and frequently used by clinicians for the typing of the outbreak strains, it was clearly shown in this study that antibiograms may not accurately predict molecular relatedness. The clonally distinct pathogens may share similar antibiograms and conversely, antibiotic resistance factor can be acquired by clonally related organisms during the course of an outbreak. This indicated that the use of antibiograms to trace the epidemiology of *S. marcescens* is not appropriate because of the variety of antibiograms due to the instability in this phenotype characteristic.

Recently PFGE has been proved to be more accurate in discriminating between unrelated organisms than biotyping, serotyping, phage typing and plasmid analysis (35). PFGE technique must be performed on specialized, expensive equipment. Additionally the technique requires that the restriction enzyme used cuts the genomic DNA into 10-20 easily distinguishable fragments, to allow accurate identification of polymorphism. The appropriate restriction enzymes have been

determined for most clinically relevant pathogens. The procedure is labor-intensive and requires experience and technical expertise, but the analysis can be completed within approximately 10 days with standard protocols to yield a very accurate assessment of genetic relatedness.

There were 2 pulsotypes (A and B) from *S. marcescens* isolated from the neonates, the environments in the NICU but at least 10 pulsotypes from the other different wards; A, B, C, D, E, F, G, H, I, J, K and L. The predominant pulsotype in this study was pulsotype A. All except one isolate from NICU were pulsotype A. The pulsotype B isolate was isolated from the specimen collected from one of the patients at 6 months after the 4 month study period was ended. The pulsotype A was still persisted in NICU because it could be isolated from one neonate whom the specimens were collected after 6 months. In addition, the parallel study was performed during the same time the isolates from the other different wards were shown to be other 10 different pulsotypes including C, D, E, F, G, H, I, J, K and L. This suggested that the pulsotype A might be the outbreak strain of the NICU at Siriraj hospital and had not been transmitted to the other different wards. The result from this part of the study was similar to the results obtained by the various previous investigations (10, 15, 40) who successfully showed the epidemic strains which were responsible for the outbreak. However, these investigations also suggested the cross-transmission among patients in the NICU.

In this study, PFGE analysis provided more reliable results than the antibiograms. This typing method could facilitate the reliable evaluation of the clonal relationship of *S. marcescens* isolates and the identification of the common sources of an outbreak in NICU.

In summary, the results in this study indicated that there was an outbreak of *S. marcescens* colonization in the NICU because all the isolates from this patient unit were all in the same pulsotype A while the isolates from the other different wards where the specimens were collected at the same period of time were in many different types. The PFGE patterns were not correlated with the results of antibiograms because resistant determinants might be plasmid encoded which had low stability while the patterns of total DNA digestion by PFGE were not affected by low molecular weight plasmid DNA.

The results from this study provided a useful epidemiologic informations of hospital-acquired *S. marcescens* isolates from the NICU. The investigation of nosocomial outbreak is very important in the control and the spread prevention of this pathogen. Similar to many other reports, the environmental source for the outbreak still could not be identified, even though, the occurrence of the environmental isolates seemed to be due to the contamination suggesting the possibility of personnel transmission via environments.

In addition, the antimicrobial susceptibility patterns are useful for medical personal for the selection of the antimicrobial agents in the treatment of *S. marcescens* infected patients. Carbapenem, piperacillin+β-lactamase inhibitor, aminoglycoside and quinolones were still effective against the isolates from the NICU, which were resistant to the beta lactams antibiotics including the third generation cephalosporins.

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CHAPTER VI

CONCLUSION

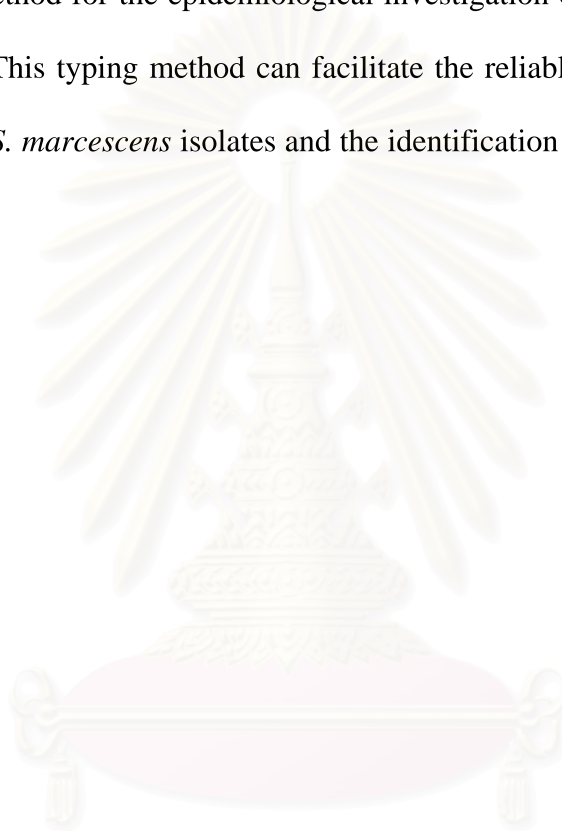
There was an outbreak of *S. marcescens* in the NICU at Siriraj hospital. The prevalence of *S. marcescens* in this unit was 34.62% which was much higher than the recovery rate from the other wards at the same time of study. However, there was a low prevalence of *S. marcescens* in environments in the unit, *S. marcescens* might be transmitted from one colonized patient to others who might develop the respiratory tract infection later on. The route of transmission of *S. marcescens* between patients and environments could not be completely concluded.

It was shown that cefoperazone/sulbactam, cefepime, imipenem, piperacillin/tazobactam were still the most effective agents against *S. marcescens* infection. In addition, amikacin, netilmicin and ciprofloxacin were also shown to be useful in treatment of *S. marcescens* infection, because of the high percentage of the susceptible strains (≥ 70%). However, the *S. marcescens* isolates were resisted to multiple antimicrobial agents including ampicillin, amoxicillin/clavulanic acid, cefotaxime, ceftriaxone, ceftazidime, piperacillin, gentamicin and sulfamethoxazone/trimethoprim.

The PFGE typing could distinguished all the 125 *S. marcescens* isolates into 12 pulsotypes; A, B, C, D, E, F, G, H, I, J, K and L. Most of the *S. marcescens* isolated from the NICU were pulsotype A except one isolate from the specimen which was collected at 6 months after the previous 4-month study period, was pulsotype B. In the parallel study, the pulsotypes of *S. marcescens* isolates from the other different wards were totally different (C, D, E, F, G, H, I, J, K and L). It could be suggested

that *S. marcescens* pulsotype A was the epidemic (outbreak strain) in the NICU at Siriraj hospital and were not transmitted to the other different wards because the pulsotype of *S. marcescens* isolated from the neonates and the environments in the NICU were different from the other wards.

In conclusion, PFGE typing was found to be a highly discriminatory and reproducible method for the epidemiological investigation of *S. marcescens* infection in the NICU. This typing method can facilitate the reliable evaluation of the clonal relationship of *S. marcescens* isolates and the identification of the common sources of outbreaks.



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APPENDICES

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APPENDIX I

Media, Chemical Agents, Materials, Instruments and Identification Procedures.

A. Media

A1. Mueller Hinton agar medium (Becton Dickinson, USA)

Beef, Infusion form	300.0 g
Bacto casamino acids, Technical	17.5 g
Starch	1.5 g
Bacto agar	17.0 g
Distilled water	1000.0 ml

pH: 7.3 +/- 0.1 at 25°C

A2. Tryptic soy agar (Mearck, Germany)

Peptone from casein	15.0 g
Peptone from soymeal	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Distilled water	1000.0 ml

pH: 7.3 +/- 0.2 at 25°C

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A3. Tryptic soy broth (Mearck, Germany)

Peptone from casein	17.0 g
Peptone from soymeal	3.0 g
D(+)Glucose	2.5 g
Sodium chloride	5.0 g
di-Potassium Hydrogen Phosphate	2.5 g

Distilled water 1000.0 ml

pH: 7.3 +/- 0.2 at 25°C

Media preparation:

All of ingradients were dissolved in distilled water, heat to boiling and then steriled by autoclaving at 121°C, 15 pounds/inch^2 pressure, for 15 minutes. The sterile medium was cooled to 45°C to 50°C, and dispensed into sterile plates. For sterile Tryptic soy broth, 5 ml were dispensed into each tube before autoclaving.

B. Chemical agents

Low melting point agarose (Promega, USA)

Ultrapure agarose (Gibco BRL, Spain)

Brij-58 (Sigma, USA)

Sodium deoxycholate (Sigma, USA)

Sodium lauroyl sarcosine (Sigma, USA)

Proteinase K (Amresco, USA)

Tris (Hydroxymethyl) (Amresco, USA)

Sodium chloride (Merck, USA)

EDTA (Amresco, USA)

Boric acid (Bio-Rad, USA)

C. Materials

15-ml snap-top tubes (Fisher, USA)

5-ml snap-top tubes (Fisher, USA)

15-ml round bottom tube, screw cap (Pyrex, USA)

Insert mold (Bio-Rad, USA)

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D. Instruments

Incubator 37°C (Memmert, Germany)

Shaking waterbath (United Instrument, USA)

Turbidity meter

Vortex Mixer (Scientifix, USA)

Digital sliding vernier caliper

Roller (Life Science, USA)

Refrigerator centrifuge (4°C) (Sigma, USA)

Refrigerator (-20°C) (Listed Household Freezer, USA)

Automatic pipette, p20/p200/p1000 (Gilson Medical Electronic, France)

pH meter (Beckman, USA)

Millipore filter

Pulsed-Field Gel Box (Bio-Rad, USA)

Pump, Gel Molds (Bio-Rad, USA)

Cooling system (Bio-Rad, USA)

Power supply, Pulse wave switcher (Bio-Rad, USA)

E. Enzyme and Molecular Marker

RNase (Amresco, USA)

Lysozyme (Amresco, USA)

Proteinase K (Amresco, USA)

*Spe*I (Boehringer, Germany)

λ ladder marker (Bio-Rad, USA)

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F. Identification procedures

F1. Gram staining procedure

Gram crystal violet solution

Gram iodine solution

Gram safranin solution

95% ethanol

Staining procedure: The organisms were smeared on a clean slide and allowed to dry. The slide was heated with a flame to fix the smear. Gram crystal violet was dropped on the smear. After minute, the slide was then washed with water and drained. Next, gram iodine solution was dropped on the smear, and washed with the water after 1 minute. The smear was decolorized with 95% ethanol and then washed with water. Gram safranin solution was next dropped on the smear in order to use as counterstain for 30 seconds. The smear was allowed to dry and then examined by microscope under 100x objective lens over the entire smear.

F2. Biochemical tests

F2.1. Arabinose fermentation

Virtually all the members of the Enterobacteriaceae had the ability to ferment the carbohydrate L-arabinose. An important exception was *S. marcescens*, which could not. Therefore this test was very helpful in the separation of *S. marcescens* from other members of its genus, as well as from other organisms in the family *Enterobacteriaceae*. A suitable carbohydrate fermentation base medium such as cystine trypticase agar (CTA), was inoculated with a loopful of the colony to be tested. Fermentation of the arabinose was accompanied by a lowering of the pH of the medium, resulting in the indicator changing to a yellow color after overnight incubation.

F2.2. Citrate agar, Simmons

Purpose: Simmons citrate agar is used to distinguish gram-negative bacteria based on their ability to utilize as a sole source of carbon.

Principle and interpretation: Several theories have been proposed to explain the mechanism of citrate agar. Only one is presented here. Organisms that metabolize citrate as a sole source of carbon cleave citrate to oxaloacetate and acetate via the citritase enzyme. Another enzyme, oxaloacetate decarboxylase, then converts oxaloacetate to pyruvate and CO_2. CO_2 combines with sodium and water to form Na_2CO_3 , an alkaline compound. As a result, the pH of the medium rises and the indicator (bromthymol blue) changes from green to Prussian blue. Presence of the blue color constitutes a positive finding for citrate utilization.

Ingredients and Preparation: Mix the following ingredients, heat to boiling, dispense into test tubes, and sterilize at 121°C for 15 minutes. Cool each tube of medium in a slanted position.

Sodium citrate	2 g
NaCl	5 g
$MgSO_4$	0.2 g
Ammonium dihydrogen phosphate	1 g
Dipotassium phosphate	1 g
Bromthymol blue	80 mg
Agar	15 g
Distilled water	1 L

Final pH 6.9

Procedure: Lightly inoculate the test organism to the surface of citrate medium, incubate at 35°C for 24 to 48 hours, and observe for a Prussian blue color change.

F2.3. Deoxyribonuclease test

Purpose: DNase test agar is used to detect DNase activity in species of aerobic bacteria.

Principle and interpretation: Several methods have been developed to determine DNase activity: hydrochloric acid precipitation and the toluidine blue and methyl green methods. DNase agar contained DNA in complex with a green dye that maked the plate a light green color. The *S. marcescens* was inoculated on the plate as a single streak and incubated for 24 hours at room temperature (DNase enzymes were more active at this temperature). If the organism produced the enzyme DNase, the agar around the streak will appeared clear because the DNA complex had been degraded and the dye released. A negative reaction would show no clearing around the streak. Positive and negative controls should be run on the same plate to make interpretation easier.

The hydrochloric acid (HCl) method is based on the fact that undegraded DNA is precipitated by acid, whereas oligonucleotides liberated by DNase activity are soluble. The organism is streaked on DNase test agar, incubated, and flooded with 1N HCl. If DNA has not been degraded, it precipitates when the acid is added and opaque areas of precipitated DNA appear. However, if DNA has been degraded, the oligonucleotides are dissolved by the acid and a zone of clearing is evident around the bacterial growth.

Toluidine blue and methyl green are metachromatic dyes. When toluidine blue is bound to DNA, the dye appears blue; however, when DNase activity

occurs and DNA is degraded to oligonucleotides, the dye appears rose pink in the area of the medium in which degradation occurred. Similarly, methyl green is green when bound with DNA, but when DNA is hydrolyzed, the methyl green is released and becomes colorless. Toluidine blue is toxic to many species of gram-positive bacteria. Thus, the HCl hydrolysis or methyl green methods should be used to test these bacteria for ability to produce DNase.

Ingredients and preparation: Mix the ingredients, heat to boiling, sterilize at 121°C for 15 minutes, and dispense into sterile Petri plates.

DNA	2 g
Papaic digest of soybean meal, *USP*	5 g
Papaic digest of casein, *USP*	15 g
NaCl	5 g
Agar	15 g
Distilled water	1 L

Final pH 7.3

Optional ingredient:

Toluidine blue	100 mg

DNase reagent, 1N HCl:

HCl, concentrated	2.8 ml
Distilled water	97.2 ml

Procedure: Inoculate DNase test agar with the organism, incubate at 35°C for 18 to 24 hours, and flood the plate with 1N HCl if a metachromatic dye is not included in the medium.

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F2.4. Indole test

Purpose: Indole broth is used for distinguishing between bacteria based on ability to produce indole form tryptophan.

Principle and interpretation: Indole broth contains tryptophan-rich peptone and NaCl. The tryptophan present in peptone is oxidized by certain bacteria to indole, skatole, and indoleacetic acid. The intracellular enzymes that are responsible for metabolizing tryptophan to these compounds are collectively termed tryptophanase. Indole is detected in broth cultures of bacteria with an alcoholic *p*-dimethylaminobenzaldehyde reagent. Indole reacts with the aldehyde to give a red product in the alcoholic layer of the broth-reagent mixture.

Two reagents were used to detect indole: Kovac's and Ehrlich. Ehrlich reagent is believed to be more sensitive than Kovac's and is recommended for detection of indole production by anaerobic bacteria and nonfermentative gram-negative organisms. Kovac's reagent was used initially to classify members of the family *Enterobacteriaceae* and should be used with these organisms.

Ingredients and preparation: Mix the ingredients, heat to boiling, dispense into tubes, and sterilize at 121°C for 15 minutes.

Indole broth:

Pancreatic digest of casein, *USP*	20 g
NaCl	5 g
Distilled water	1 L

Final pH 7.2

Reagents:

Kovac's indole reagent. Dissolve the aldehyde in the alcohol and slowly add acid to the mixture.

Alcohol, amyl or isoamyl	150 ml
p-Dimethylaminobenzaldehyde	10 g
Hydrocholic acid, concentrated	50 ml

Procedure: Inoculate the test organism into indole broth, incubate at 35°C for 18 to 24 hours, and test as follows.

Indole test: Add five drops of Kovac's reagent directly to the broth culture, shake gently, and observe for development of a red color in the upper alcohol layer.

F2.5. Malonate broth

Purpose: Malonate broth is used for differentiation of members of the family *Enterobacteriaceae*, espectially *Salmonella* spp.

Principle and interpretation: Malonate broth tests for utilization of sodium malonate as a sole source of carbon. The medium contains buffer, pH indicator, sodium malonate, required salts, and a small amount of yeast extract and glucose. The pH indicator, bromthymol blue, is a deep Prussian blue at its alkaline end point (pH 7.6), yellow at its acidic end point (pH 6.0), and green when uninoculated (pH 6.7). Bacteria that are capable of using malonate as a source of energy and carbon produce alkaline byproducts that change the color of the medium to blue. Bacteria that are unable to use malonate as a carbon source usually do not grow and the pH of the medium does not change; the indicator remains green. Some malonate-negative strains may produce a yellow color owing to fermentation of glucose.

Ingredients and preparation: Mix the ingredients, heat to boiling, dispense into tubes, and sterilize at 121°C for 15 minutes.

Yeast extract	1 g

Ammonium sulfate	2 g
Dipotassium phosphate	0.6 g
Monopotassium phosphate	0.4 g
NaCl	2 g
Sodium malonate	3 g
D-Glucose	0.25 g
Bromthymol blue	0.025 g
Distilled water	1 L

Final pH 6.7

Procedure: Inoculate the test organism into malonate broth and incubate at 35°C for 18 to 24 hours.

F2.6. Methyl red-Voges-Proskauer broth

Purpose: Methyl red-Voges-Proskauer (MR-VP) broth is useful for distinguishing between members of the family *Enterobacteriaceae* based on their ability to produce acetylmethylcarbinol (acetoin) and strong acids from fermentation of glucose. The broth, which contains protein, glucose, and phosphate buffer, is used for the MR test and the VP test.

Principle and interpretation: Members of the family *Enterobacteriaceae* may be divided metabolically into two groups: the mixed acid producers and the butylene glycol producers. The mixed acid producers such as *Escherichia coli* produce large amounts of organic acids including lactic, acetic, formic, and succinic. Butylene glycol producers such as *Klebsiella* and *Enterobacter* spp. produce smaller amounts of organic acids and large amounts of neutral products, especially 2,3-butanediol.

The MR test is used to distinguish the mixed acid producers. In this test a methyl red indicator is added to the MR-VP test broth after incubation. At a pH of 4.4 the indicator remains red, and at the pH of 6.0 it become yellow. The MR-positive organisms are those that produce large amounts of acid and a red color, whereas the MR-negative organisms produce a yellow color.

The VP test detects the presence of acetoin, or acetylmethylcarbinol, an intermediate in the production of butylene glycol. In this test two reagents, α-naphthol and 40% KOH, are added to the test broth after appropriate incubation. The broth-reagent mixture is then mixed thoroughly to expose the medium atmospheric oxygen. If acetoin is present, it is oxidized in the presence of air and KOH to diacetyl. Diacetyl then reacts with the guanidine components of peptone, in the presence of α-naphthol, to form a red color (α-naphthol serves as a catalyst and acts as a color intensifier). Development of a red color is a positive VP test result.

Ingredients and preparation: Mix the ingredients, heat to boiling, dispense into tubes (1 ml per tube), and sterilize at 121°C for 15 minutes.

Pancreatic digest of casein and peptic Digest of animal tissue, USP	7 g
D-Glucose	5 g
Dipotassium phosphate	5 g
Distilled water	1 L

Final pH 6.9

Reagents:

Methyl red reagent: Dissolve the methyl red in alcohol and add the distilled water. Store at room or refrigerator temperature.

Methyl red	50 mg

Ethyl alcohol, 95%	150 ml
Distilled water	100 ml

Voges-Proskauer reagents:

VP-1:

α-naphthol	5 g
Ethyl alcohol, absolute	100 ml

VP-2:

Potassium hydroxide	40 g
Distilled water, q.s. to	100 ml

Procedure: Inoculate the test organism to two tubes of MR-VP broth, each containing 1 ml, and incubate for 1 to 3 days at 35°C.

Methyl red test: Add five drops of methyl red reagent to one broth culture and observe for development of a red color. This is a positive MR test, which is indicative of mixed acid fermentation.

Voges-Proskauer test: Add 0.6 ml of VP-1 reagent to another broth culture, shake the tube, and add 0.2 ml of VP-2 reagent. The reagents must be added in the preceding sequence. Shake the tube gently. Allow the tube to stand for at least 15 minutes and observe for formation of a red color. This is a positive VP test and indicates butylene glycol fermentation. Hold tubes in which results are negative for an additional 45 minutes, since maximum color development occurs within 1 hour after the reagent is added. Ignore a copper color of the medium, which occurs after 1 hour's incubation. This color is due to reaction between α-naphthol and KOH.

F2.7. Triple sugar iron agar

Purpose: Triple sugar iron (TSI) agar is a screening medium used to identify gram-negative bacilli based on ability to ferment the carbohydrates glucose, sucrose, and lactose to produce H_2S gas.

Principle and interpretation: TSI agar contain protein, NaCl, lactose, sucrose, dextrose, a sulfur source, an H_2S indicator, a pH indicator, and agar. The medium includes ten times as much lactose and sucrose as glucose. Bacteria that ferment glucose produce a variety of acids, turning the color of the medium from red to yellow. Larger amounts of acid are produced in the butt of the tube (fermentation) than in the slant of the tube (respiration). Organisms growing on TSI also from alkaline products from the oxidative decarboxylation of peptone. These alkaline products neutralize the small amounts of acids present in the slant but are unable to neutralize the large amounts of acid present in the butt. Thus, the appearance of an alkaline (red) slant and an acid (yellow) butt after 24 hours incubation indicates that the organism is a glucose fermenter but is unable to ferment lactose and sucrose.

Bacteria that ferment lactose or sucrose (or broth), in addition to glucose, reduce such large amonts of acid that the oxidative deamination of protein that may occur in the slant does not yield enough alkaline products to cause a reversion of pH in that region. Thus, these bacteria produce an acid slant and acid butt. It is impossible to determine from the TSI reaction whether both lactose and sucrose are being fermented or only one of these carbohydrates is being fermented; individual carbohydrate fermentation tests are required to make this assessment.

Gas production (CO_2 and hydrogen) is detected by the presence of cracks or bubbles in the medium. These are formed when the accumulated gas escapes.

H_2S gas is produced as a results of the reduction of thiosulfate. H_2S is a colorless gas and can be detected only in the presence of an indicator, in this case ferric ammonium sulfate. H_2S combines with the ferric ions of ferric ammonium sulfate to produce the insoluble black precipitate ferrous sulfide. Reduction of thiosulfate proceeds only in an acid environment, and blackening usually occurs in the butt of the tube. Although the black precipitate may frequently obscure the color of the butt, it can be assumed that the organism is a glucose fermenter because of the requirement for an acid environment. The reactions can be summarized as follow:

Alkaline slant/acid butt: glucose only fermented

Acid slant/acid butt: glucose and sucrose fermented or glucose and lactose fermented or glucose, lactose, and sucrose fermented

Bubbles or cracks present: gas produced

Black precipitate present: H_2S gas produced

Ingredients and preparation: Mix the ingredients, heat to boiling, dispense into tubes, and sterilize at 121°C for 15 minutes, and allow tubes of medium to cool in a slanted position.

Pancreatic digest of casein, *USP*	10 g
Peptic digest of animal tissue, USP	10 g
NaCl	1 g
Lactose	10 g
Sucrose	10 g
D-Glucose	1 g
Ferric ammonium sulfate	0.2 g
Sodium thiosulfate	0.2 g
Phenol red	25 mg

Agar	13 g
Distilled water	1 L

Final pH 7.3-7.4

Procedure: Inoculate test cultures to TSI agar by first touching a sterile bacteriologic needle to a colony and then stabbing the needle into the deep agar region of the medium. Hence withdrawing the needle, move it from side to side over the surface of the medium. Incubate cultures at 35°C for 18 to 24 hours. Examine cultures for color of the slant, butt, gas cracks, and blackening caused by H_2S.

F2.8. Urea agar

Purpose: Urea agar are used for distinguishing between species of aerobic bacteria based on ability to hydrolyze urea.

Principle and interpretation: A variety of media are used to test for ability to hydrolyze urea. The hydrolysis of urea by urease to ammonia is accompanied by a rise in pH of the medium and a concomitant change in the color of the indicator from yellow to red.

Ingredients and preparation: Mix urea basal ingredients, sterilize by filtration, and add sterile agar solution (50°C). Mix and dispense into tubes, and allow tubes of medium to cool in a slanted position.

Urea base:

Pancreatic digest of gelatin, USP	1 g
NaCl	5 g
Monopotassium phosphate	2 g
D-Glucose	1 g
Urea	20 g
Phenol red	12 mg

Distilled water 100 ml

Final pH 6.8

Agar solution:

Agar 15 g

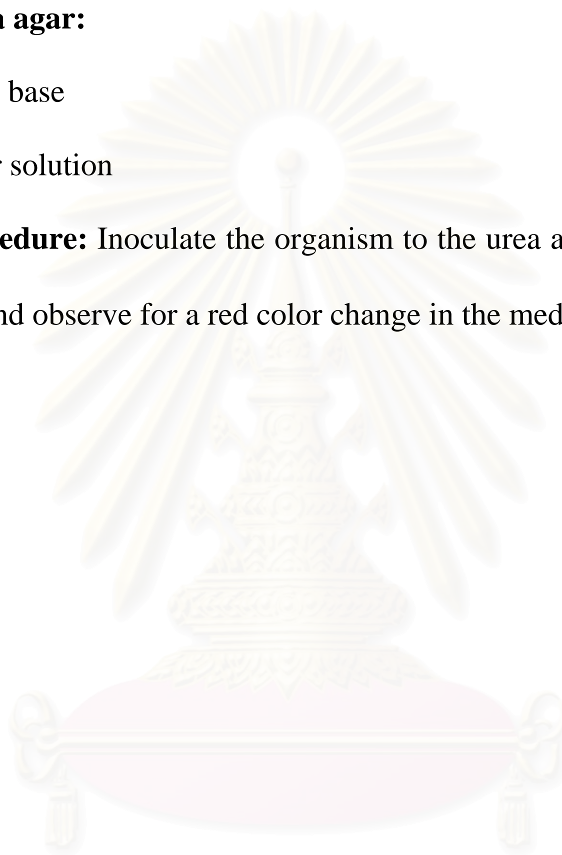
Distilled water 900 ml

Urea agar:

Urea base 100 ml

Agar solution 900 ml

Procedure: Inoculate the organism to the urea agar, incubate for 24 to 48 hours at 35°C, and observe for a red color change in the medium.



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APPENDIX II

Reagents

1. PIV buffer:

- 10 mM Tris (pH 7.6)	0.6057 g
- 1M NaCl	29.22 g
- Ultrapure water	500.00 ml

All ingradients were dissolved in 500 ml of ultrapure water. The buffer adjusted the pH to 7.6, and then sterile at 121°C, 15 pounds/inches^2 pressure. The PIV buffer was stroed at 4°C.

2. Lysis buffer:

- 6 mM Tris (pH 7.6)	0.0726 g
- 1M NaCl	5.884 g
- 100 mM EDTA (pH 7.6)	3.7224 g
- 0.5% Brij-58	0.5 g
- 0.2% Sodium deoxycholate	0.2 g
- 0.5% Sodium lauroyl sarcosine	0.5 g
- Ultrapure water	500.00 ml

All ingredients were dissolved in 500 ml of ultrapure water, and then adjusted the pH to 7.6. The buffer was steriled by millipore filter, and then stroed at 4°C.

RNase stock solution

The 10 mg of RNase was dissolved in 1 ml of sterile water, and then heated in boiling water for 20 to 30 min to destroy DNase. The 100 µl of RNase

was dispensed in each microcentrifuge tube, and freezed at -20°C. (These aliquots could be refrozen up to two times)

Lysozyme stock solution

The 0.5 g of lysozyme was dissolved in 10 ml of sterile water (final concentration, 50 mg/ml or 10 mg/ml). The 200 µl of lysozyme was dispensed in each microcentrifuge tube, and freezed at -20°C. (These aliquots could be refrozen once)

Lysis solution

- 20 µg of RNase per ml
- 1 mg of lysozyme per ml
- Lysis buffer

The lysis solution was prepared by mixing the 80 µl of RNase stock solution, 800 µl of lysozyme stock solution, and 40 ml of lysis buffer together.

3. ES buffer

- 0.5M EDTA (pH 8.0)	93.06 g
- 10% Sodium lauryl sarcosine	50.00 g
- Ultrapure water	500.00 ml

All ingradients were dissolved in 500 ml of ultrapure water, and then adjusted the pH to 8.0. The buffer was sterile by millipore filter and stored at 4°C.

20x Proteinase K stock solution

One hundred grams of proteinase K was dissolved in 50 ml of ES buffer and then incubated at 50°C for 1 h. The solution was stored at 4°C.

ESP solution

- 100 µg of Proteinase K per ml of ES buffer
- ES buffer

Ten milliliters of 20x Proteinase K was added to 190 ml of ES buffer, and then mixed thoroughly. The solution was stored at 4°C.

4. 1xTE buffer

- 10 mM Tris (pH 7.6)	0.6057 g
- 0.1 M EDTA (pH 7.6)	0.0186 g
- Ultrapure water	500.00 ml

All ingradients were dissolved in 500 ml of ultrapure water, and then adjusted the pH to 7.6. The buffer was steriled at 121°C, 15 pounds/inches^2 pressure and stroed at 4°C.

5. 10x TBE buffer

- 0.1 M Tris (pH 8.5)	108.00 g
- 0.1 M Boric acid	55.00 g
- 4 mM EDTA	9.30 g
- Sterile ultrapure water	1000.00 ml

All ingradients were dissolved in 500 ml of sterile ultrapure water and then adjusted the pH to 8.2-8.4. 500 ml of sterile ultrapure water was added and then mixed thoroughly. The solution was stored at 4°C.

6. Ethidium bromide solution

One pellet (11 mg) of ethidium bromide was dissolved in 11 ml of ultrapure water.

Working solution: The 40 µl ethidium bromide stock solution was mixed with 300 ml ultrapure water before used.

BIOGRAPHY

Miss Thidarat Buachuen was born on November 17, 1976 in Bangkok, Thailand. She geraduated with the Bachelor degree of Science in Biology from Faculty of Science, Chulalongkorn University in 1997.

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