OBSERVABLE REAL-TIME PULSED-FIELD GEL ELECTROPHORESIS

by

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B.Sc. Bioengineering, Istanbul Technical University,

Montana State University, 2013

Submitted to the Institute Institute of Biomedical Engineering

in partial fulfillment of

the requirements for the degree of

Master of Science

Biomedical Engineering

Boğaziçi University

2017

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ACKNOWLEDGEMENTS

I express my deep sense of gratitude to my respected and learned guides, Prof. Dr. Zühtü Tanıl Kocagöz, who has a very important role in my graduate education and Prof. Dr. Cengizhan Öztürk, who supported me from the beginning.

I also want express my deepest thanks to Prof. Dr. Banu Sancak and Assist. Prof. Dr. Onur Karatuna for their help.

I acknowledge with thanks for loving inspiration and timely guidance, Prof. Dr. Sesin Kocagöz and Phd. Sinem Öktem Okullu. I wish specially thank to my friends Alp Taştan, Görkem Gün, Selma Çat, Tuğçe Demir about their limitless encouragement.

The completion of this thesis could not have been possible without the support of my lovely parents, my mother Leyla Yılmaz, my father Kansu Kaya and Fatma Kaya.

This work was supported by TÜBİTAK Project No: 113M354, "Observable Pulsed Field Gel Electrophoresis".

ABSTRACT

OBSERVABLE REAL-TIME PULSED-FIELD GEL ELECTROPHORESIS

Pulsed-Field Gel Electrophoresis (PFGE) is a very important molecular research and diagnostic technique, used for the separation of very large DNA molecules. It enables DNA fingerprint analysis which can differentiate small number of sequence differences in genomes belonging to the same species. In this method the genome digested first by a restriction enzyme into large DNA fragments analyzed by PFGE. While DNA fingerprinting is used in forensic medicine for identifying the suspects or paternity, it enabled also the development of molecular epidemiology of infectious diseases. It is possible to identify the source and transmission routes of an epidemic by DNA fingerprinting of the strains of infectious agent isolated from a series of samples. It is very important to follow the transmission routes of the infectious agent in a hospital, country or even around the world, in order to take the precautions to stop the epidemic. Although DNA fingerprinting by PFGE is a very important and powerful technique, its application is very cumbersome, requires long time, experienced personnel and expensive equipment. These hinder the method from being widely used.

In a project that was completed four years ago, a method and an instrument called "Observable Real Time Electrophoresis (ORTE)" was developed and it won the Akın Çakmakçı Award, of Turkish Technology Development Foundation. In this thesis work the aim is to develope an Observable Real Time PFGE system. Its efficiency is evaluated by comparing the genomic fingerprints of bacterial and fungal strains that were previously isolated from hospital infections by Observable and classical PFGE. Observable PFGE has the potential to eliminate the need for hazardous ultraviolet light and carcinogen ethidium bromide. It provided pictures at every stage of electrophoresis enabling to choose the best picture for analysis of the results. Since Observable PFGE

enables to see the results early during electrophoresis we believe that compared to classical PFGE, it could save a lot of time and effort, in many diverse applications, once the system that we present the technological feasibility in this thesis work, is developed and tested further.

Keywords: Observable Real Time Electrophoresis, Pulsed Field Gel Electrophoresis, (PFGE), DNA fingerprinting, in vitro diagnostic biomedical device technologies.

ÖZET

İZLENEBİLİR DEĞİŞKEN ALANLI JEL ELEKTROFOREZİ

Değişken Alanlı Jel Elektroforezi (DAJE), veya (Pulsed Field Gel Electrophoresis PFGE) çok büyük DNA moleküllerini birbirinden ayırmak için kullanılan çok önemli bir moleküler araştırma ve tanı tekniğidir. Birbirine çok benzeyen genomlardaki az sayıda dizi farklılıklarını kolayca ayırt edebilen DNA parmak izi incelemesi yönteminin geliştirilmesini sağlamıştır. Bu yöntemde, bir restriksiyon enzimi ile büyük DNA parçalarına bölünen hücre genomu DAJE ile ayrıştırılarak incelenir. Parmak izi incelemesi adli tıpta babalık testi, suçlunun saptanması gibi amaçlarla kullanılırken, enfeksiyon hastalıklar alanında moleküler epidemiyolojinin gelişmesini sağlamıştır. Salgına yol açan enfeksiyon etkeninin çeşitli örneklerden elde edilen suşlarına uygulanan parmak izi incelemesi ile enfeksiyon kaynağı ve yayılma yolları saptanabilmektedir. Bir salgının hastanede, bir ülkede hatta dünyada yayılmasını bu yolla izlemek salgına engel olmak için gerekli önlemleri almak açısından büyük önem taşımaktadır. Bu derece önemli ve güçlü bir teknik olan DAJE maalesef uygulaması zor olan, uzun zaman alan, iyi eğitimli çalşan ve pahalı aygıtlar gerektiren bir teknik olduğundan yaygın olarak kullanılamamaktadır.

Dört yıl önce tamamlanan TÜBİTAK tarafından desteklenen bir proje çerçevesinde geliştirilen İzlenebilir Elektroforez adı verilen bir yöntem ve bu yöntemin uygulanmasını sağlayan bir aygıt, Türk Teknoloji Geliştirme Vakfı tarafından Akın Çakmakçı, sanayiye uygulanan tez çalışması ödülünü almıştı. Bu tez çalışmasında ise İzlenebilir Elektroforez adını verdiğimiz bir yöntem ve bu yöntemin uygulanmasını sağlayan bir aygıt geliştirilmiştir. Bu yöntemin etkinliğini saptamak için, hastane enfeksiyonuna sebep olan bakteri ve maya suşlarının sonuçları klasik DAJE ve İzlenebilir DAJE ile karşlaştırıldı. İzlenebilir DAJE'*nin*, klasik DAJE'*ye* göre araştırmacılara çok zaman

ve emek artırımı sağlayabileceği görüldü. İzlenebilir Elektroferezin kullanılabilmesi, klasik DAJE de kullanılması zorunlu olan zararlı ultraviyole ışığı ve kansorejen etidyum bromür kullanımını da ortadan kaldırabilecektir. Elektroforezin her basamağında fotoğraf çekilebilmesini sağlayan izlenebilir DAJE, sonucu analiz etmek için seçilebilecek en uygun fotoğrafın kullanıcı tarafından belirlenmesine olanak tanıyor, ve bu yolla eğer bu tez çalışmaları ile uygulanabilirliğini göstermeye başardığımız sistemin gelişimi ve geniş denemeleri tamamlanabilirse, klasik DAJE ile karşılaştırıldığında, çok fazla zaman ve efor kazanımı sağlayabilecektir.

Anahtar Kelimeler: İzlenebilir elektroforez, değişken alanlı jel elektroforez (DAJE), DNA parmakizi incelemesi, in vitro teşhis biyomedikal tıbbi cihazları.

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LIST OF SYMBOLS

F	Force
q	Charge of the Particle
E	Electrical Field
μ	Electrophoretic mobility
μg	Microgram
μl	Microliter

LIST OF ABBREVIATIONS

PFGE	Pulsed Field Gel Electrophoresis
ORT-PFGE	Observable Real Time Pulsed-Field Gel Electrophoresis
DNA	Deoxyribonucleic Acid
ORTE	Observable Real Time Electrohoresis
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
FIGE	Field-Inversion Gel Electrophoresis
TAFE	Transverse-Alternating Field Gel Electrophoresis
CHEF	Contour-Clamped Homogeneous Electric Fields
OFAGE	Orthogonal-Field Alternation Gel Electrophoresis
RGE	Rotating Gel Electrophoresis
PACE	Programmable Autonomously-Controlled Electrodes
PHOGE	Pulsed-Homogeneous Orthogonal Field Gel Electrophoresis
UV	Ultraviolet Light
LED	Light-Emitting Diode
CCD	Charged-couple Device
OD	Optical Density
NDM	New Delhi Metallobetalactamase
MRSA	Methicillin Resistant StaphyloccusAureus
TAE	Tris Acetat EDTA
TBE	Tris Boric Acid EDTA
TE	Tris EDTA

1. INTRODUCTION

Any scientist who is dealing with a material of a biological process often faces the problem of having to separate and examine the properties of high molecular weight substances such as proteins, nucleic acids, and complex lipids and carbohydrates. In all cases, it is necessary to change the properties considerably, giving as little damage to the molecules as possible. For this reason, existing methods of separation generally rely heavily on the physical and chemical properties of molecules to ensure that any biological activity of the molecule is kept at a maximum level. [1,2].

Electrophoretic methods are a group of related techniques for the analysis of proteins, nucleic acids and complex lipids and carbohydrates. These are among the most important aspects of biochemical analysis and play an important role in the clinical presentation of the disease in all aspects of forensic science, biotechnological research, food science, plant breeding, quality control and genetic engineering [1,2]. Electrophoresis is one of the methods used to separate biological molecules according to the effects on an electric field. It is known that the migration of charged solutes or particles may occur under the influence of an electric field in any liquid medium [3].

In 1937, the electrophoresis technique was introduced by the Swedish chemist Arne Tiselius for the separation of proteins. Nowadays it is used not only for separation of proteins but also for separation of various biomolecule classes including nucleic acids, carbohydrates and amino acids. The Tiselius$'s$ original equipment was a rectangular, sectional U-tube with fragile Schlieren optics (see Figure: 1.1). It has been observed that changes in the refractive index in different protein contents have passed. [6].

Standard agarose gel electrophoresis is the most popular technique to separate DNA molecules by size. Separation and purification of nucleic acids and proteins can be done by electrophoresis [1]. Electrophoresis of DNA is usually done by separating DNA molecules in an agarose gel. However, any other solid matrix like polyacrylamide can be used for this purpose. These molecules move through the gel under a static field

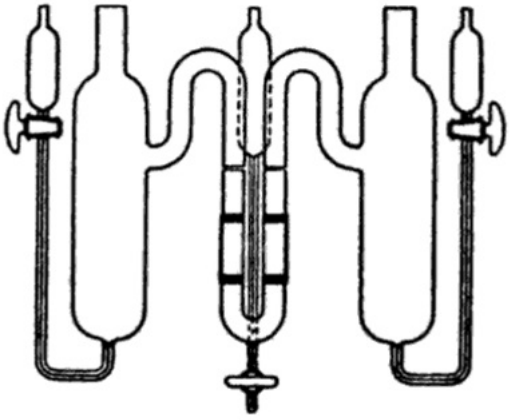

Figure 1.1: Electrophoresis apparatus for the moving boundary method, (Tiselius,1930) [6].

[1]. But the main limitation is the size of DNA molecules. DNA molecules between 100 base pairs (bp) to 50 kilo base pairs (kb) can be separated by conventional gel electrophoresis techniques [4]. The movement of molecules up to about 20 kb in size is permitted by normal agarose, but when the concentration of agarose is lowered, the resolution can be increased to up to 50 kb.

1.1. History of Pulsed Field Electrophoresis

Pulsed-Field Gel Electrophoresis (PFGE) was introduced by Schwartz, in 1982 [5]. PFGE is a very powerful diagnostic technique, which is used for the separation of large DNA molecules (see Figure 1.2). This device works with direct current electric field by changing its intensity and direction in a cyclic manner. The pulse time is called for the time interval that the field is any one direction. PFGE enabled scientists to analyze very wide range of DNA molecules [6] (i.e. from 50 kb microchromosomes of parasites to multimillion bp yeast chromosomes.) and with this technique scientists started to prepare physical maps of chromosomes [5].

PFGE has enabled to compare large genomic DNA from different sources, by a technique called DNA fingerprinting. In this method, the whole genomic DNA is digested by a restriction enzyme into large DNA fragments, usually bigger than 50 kb, which are then separated by PFGE. DNA fingerprinting has been used most commonly in forensic medicine for ownership identification of biological samples (see Figure

Figure 1.2: Pulsed Field Electrophoresis Systems (BIORED CHEF-DR II) and gel documentation system (BIORAD CHEMIDOC).

1.3), for paternity test and investigation of molecular epidemiology of infectious diseases, especially for identification of the source of infectious agents causing epidemics in hospitals.

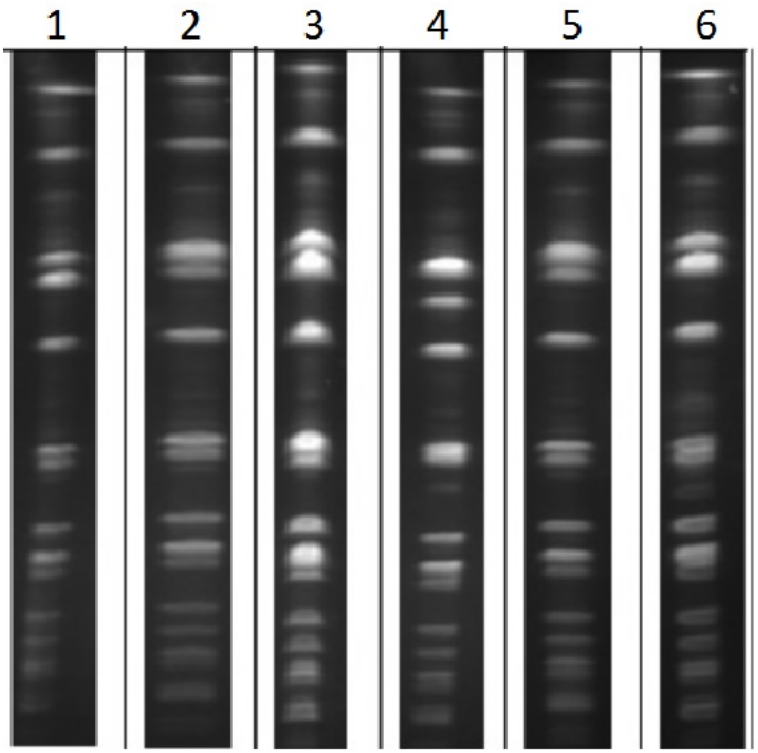

Figure 1.3: Ownership identification of Methicillin Resistant Staphyloccus Aureus (MRSA) samples. All strains sharing the same pulsotype.

1.2. Theory of Electrophoresis

Electrophoresis is the movement of charged particles in an electrical field. Positively charged particles move towards cathode during electrophoresis and thus are called the cations. Negatively charged particles move towards the anode and hence are called the anions [7].

A particle migration rate depends on the electrical field strength, the net charge of the particle and the density of the electrophoretic medium.

Electrophoretic separations are based upon the force (F) on a ion in an electrical field (E) [7].

$$F = q * E \tag{1.1}$$

F: The Force

E: Electrical Field

q: The charge of the Particle

The migration of the charged particle in the electric field, called the electrophoretic mobility (μ), is defined as,

$$\mu = v/E = q/f \tag{1.2}$$

v: The Velocity of the Charged Particle

f: Frictional Coefficient. (It relates to the size and the shape of the particle.)

Although modern electrophoresis equipment and systems do not vary greatly in shape and degree of automation, the basic components common to all systems are reservoirs containing buffer used in the process. The current is supplied via a platinum or carbon electrodes with a buffer from a power source and a separation medium connecting the two reservoirs. This entire device is enclosed to minimize evaporation and to protect both the system and the operator. The direct current power supply determines the polarity of the electrodes and delivers current to the medium as shown in figure 1.4 [8].

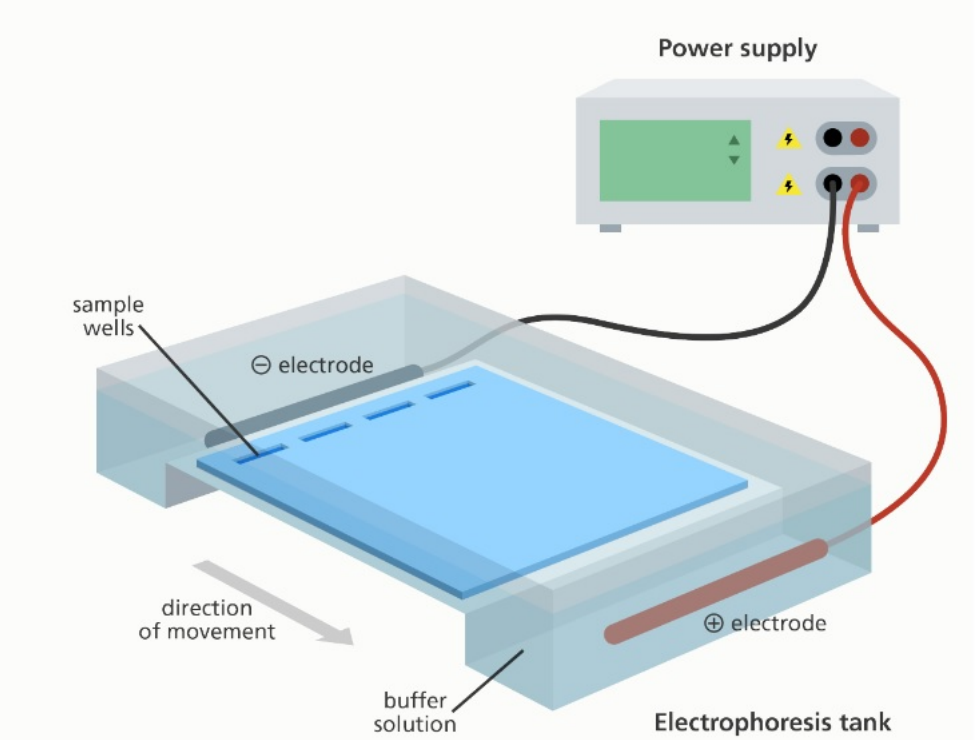

Figure 1.4: An illustration of a DNA electrophoresis device used to separate DNA fragments by size. A gel places in a buffer. The DNA samples are placed at the end of the gel than electrical current starts to pass. Negatively charged DNA moves towards the positive electrode [8].

1.3. ORTE (Observable Real Time Electrophoresis)

In an earlier research project at Acıbadem University (funded by TUBITAK project no:104M292), Tanıl Kocagöz, Sinem Öktem Okullu et. al. have developed a method and instrument called Observable Real Time Electrophoresis (ORTE). The aim of that project was to develop an electrophoresis device in which molecules separated by electrophoresis can be directly visualized and assessed. They aimed to eliminate the use of toxic dyes like ETBr and ultraviolet light that is hazardous to both DNA and to naked eye, which are used to visualize DNA molecules in classical electrophoresis methods. For these purposes, an electrophoresis device in which light emitting diodes with wavelengths at visual range were used as the light source and DNA binding fluorescent dyes with excitation wavelength corresponding to the light source which enabled to visualize DNA molecules separated by gel electrophoresis was utilized. The whole system was placed in a pyramid shaped black box with a camera placed on top and the camera was connected to a computer for real time visualization, recording and taking pictures of electrophoresis [2].

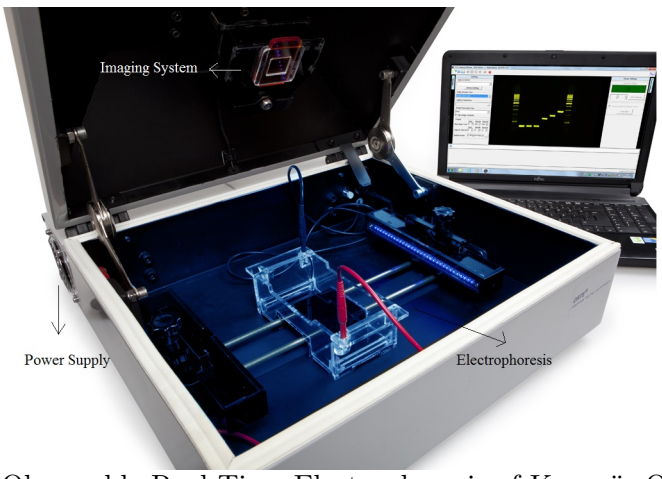

Figure 1.5: Observable Real Time Electrophoresis of Kocagöz,Okullu et al.

1.4. Theory of Pulsed Field Gel Electrophoresis

The principle of pulsed field gel electrophoresis depends on the two different electric field. After the first electric field is activated, DNA molecules are started to move. Than, first electric field is gone and second electric field is applied to the gel at same angle with the first one. DNA molecule is changed conformation and

reoriented. The reorientation time is related with the length of the molecule. More time is needed for the larger DNA molecules, due to the physical properties of the agarose gel. Therefore large molecules must redirect a larger portion of each switching cycle before DNA molecules begins to pass through gel. Because the all fields are equal in terms of length and voltage, the DNA molecules reflect the sum of the longer path actually taken, traveling along a straight path beneath the gel [11].

The components of a pulsed field gel system are a gel box, a cooler, a power supply,and a switching unit (see Figure:1.6).

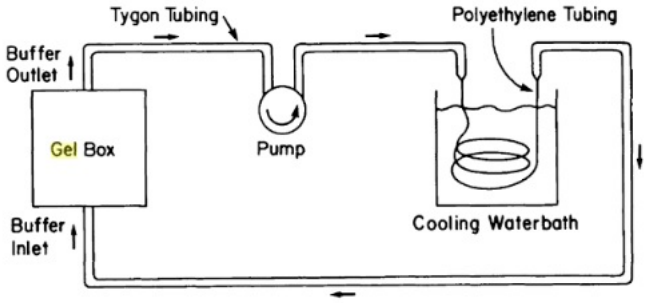

Figure 1.6: Components of a PFGE system [11]

1.4.1. Gel Box

The fundamental configuration of PFGE boxes consists of an immobilized gel inside of a variety of electrodes and a method for circulating the electrophoresis buffer. 10 volts/cm is the main voltage gradient in PFGE, and 15 volts/cm is the main voltage gradient in Field-Inversion Gel Electrophoresis (FIGE). Heat-exchange mechanism is also important for the control of buffer [9].

1.4.2. High Voltage Power Supply

Pulsed-field gel electrophoresis electrodes usually 25-50 cm apart and the range of voltage gradients are 1.5-15 volts/cm. Maximum voltage can be increased up to 750 volts. The current is 0.5 amperes. Tris Boric acid EDTA (TBE) is used for the running buffer at 14°C [12].

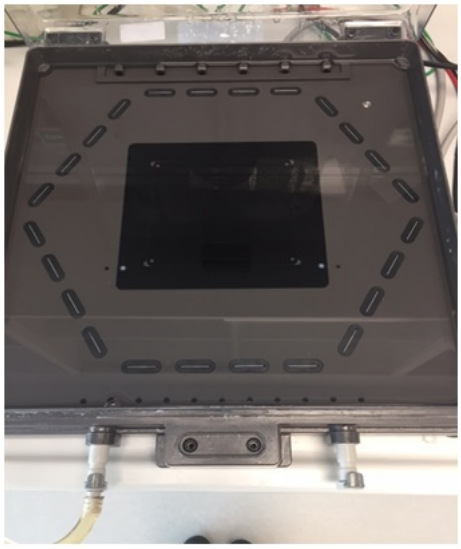

Figure 1.7: Gel box

1.4.3. Switch Unit

Switch interval that is controlled by a computer, is the most important part for the separation. Fast switching is required for the separation of DNA fragments between 2 to 50 kb. Electrode and switching voltage control circuits and also metal oxide semiconductor field effect transistors are the main parts of the switching units. In PFGE systems, high-voltage solid-state electronics is used to eliminate the drawbacks of electromechanical relays. The capacity of rapid switching, ample voltage and currents (0.1 ms, 750 V, 0.5 ampers) is the advantage of these relays. These mechanical assemblies can control the reorientation points between electric fields [9].

1.4.4. Computer Program

High quality algorithm is important for the switch times, since it should be sufficiently quick enough for the short switch times (1 ms) and also good resolution for the switch interval increments. The most generally utilized methodology is the linear switch interval ramping in view of its basic implementation. The highest run time ought to be could extend up to around two weeks to separation of huge DNA particles [10].

Figure 1.8: High Voltage Power Supply and Switch Unit

1.4.5. Cooler

Recirculation of the buffer is an essential element, as it sifts temperature varieties inside of the gel. DNA particle movement is effected by temperature, and consistent temperature over the gel is expected to guarantee even migration in each of the paths. Buffer is recycled at a rate of 450 ml/min by a reciprocating solenoid pump. The buffer is cooled in its store tank by icy water around 5°C coursed. In a normal run, buffer temperature maintained at 14°C [10, 11].

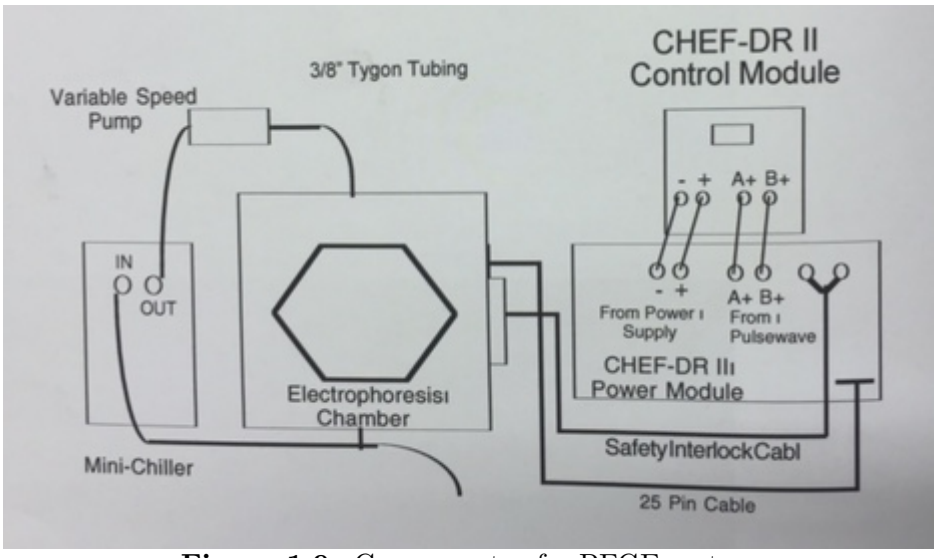

Figure 1.9: Components of a PFGE system

1.5. Staining

After PFGE, molecules need to be stained with appropriate dyes. There are numerous sorts of nucleic acid dyes. Some of them are put into the electrophoresis gel and few dyes are utilized to stain the gels after electrophoresis [13].

1.5.1. Ethidium Bromide (EtBr)

Ethidium bromide (EtBr) ties by embeddings itself between the stacked bases in double-stranded DNA. The ring structure of ethidium is hydrophobic and takes its place among the rings of thebases in DNA. When illuminated by ultraviolet light, it will fluoresce with an orange color [14].Ethidium Bromides excitation peak is at 300 nm (UV) and it can be excited also 518 nm, if there is suitable LED power. Figure 1.10 shows excitation and emission spectral for Ethidium Bromide.

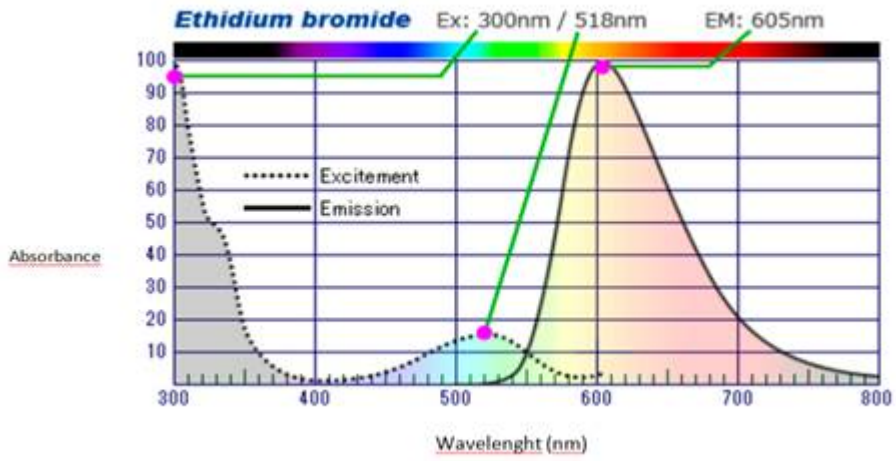

Figure 1.10: Excitation and emission spectral for Ethidium Bromide [12].

Ethidium Bromide enables visualization of DNA in a very sensitive manner, but it is famously hazardous. It an extremely solid mutagen, and might be a cancer-causing agent or teratogenic. Its MSDS reports express that it is hurtful if gulped and extremely lethal by inhaling. It is also hazardous to the eyes, respiratory framework and skin. Furthermore it conveys the danger of irreversible damages. Ethidium bromide can consequently represent a noteworthy wellbeing issue for the scientist and it is an ecological hazard during disposal [13].

1.5.2. SYBR Green I (SG)

SYBR Green I is an asymmetrical cyanine dye utilized as a nucleic acid stain in moleculer science and it ties to double stranded DNA. The subsequent dye complex ingests blue light (max= 488 nm) and radiates green light (max= 522 nm) [16]. SYBR green is utilized as a dye for the evaluation of double stranded DNA in real time Polymerase Chain Reaction (PCR). It is likewise used to imagine DNA in gel electrophoresis. SYBR Green I is promoted as an safer alternative dye to mutagen ethidium bromide, being both more secure to work with and free from the intricate waste transfer issues of ethidium [16].

1.5.3. SYBR Gold

The most noteworthy widely used nucleic stains created to date are ideally energized utilizing short-wavelength ultraviolet or visible light. This is a hindrance for labs equipped just with 306-or 312-nm UV transilluminators. SYBR Gold is an unsymmetrical cyanine dye that overcomes this problem. This dye, SYBR Gold, has two fluorescence excitation maxima when bound to DNA, one focused at roughly 300 nm and one at around 495 nm. When utilized with 300-nm transillumination and Polaroid highly contrasting photography, SYBR Gold stain is more sensitive than other dyes. It is also more sensitive than other dyes for identifying double stranded DNA, single-stranded DNA, and RNA [17].

SYBR Gold is also the most sensitive fluorescent stain for distinguishing double or single-stranded DNA and RNA in electrophoretic gels, utilizing standard ultraviolet transilluminators, outperforming even the affectability of SYBR Green gel stains. SYBR Gold stain is an exclusive unsymmetrical cyanine color that exhibits 1000-fold fluorescence increase after bind to nucleic acids and has a high quantum yield (0.6). 495 nm is the excitation maxima for nucleic acid complexes in the visible and 300 nm in the ultraviolet light (see Figure 1.11). 537 nm is the emission maximum. According to researchs [18] SYBR Gold stain is more sensitive than ethidium bromide for detecting DNA and RNA in denaturing urea, glyoxal, and formaldehyde gels, even with 300

nm transillumination. SYBR Gold stain has additionally more sensitive than SYBR Green II dye for detecting single strand conformation polymorphism (SSCP) products. SYBR Gold stain enters thick and high concentration agarose gels quickly, and even formaldehyde agarose gels do not require destaining, because of the low background fluorescence of the unbound dye [18].

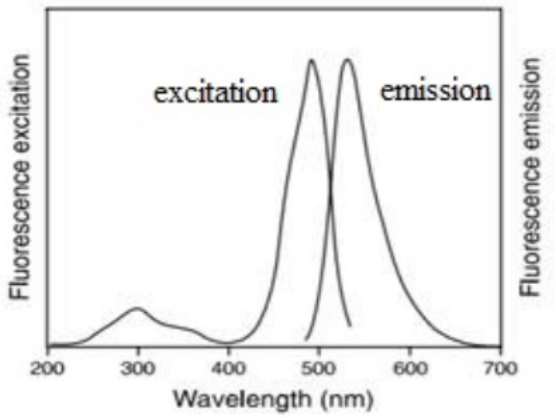

Figure 1.11: Excitation and emission spectra of SYBR Gold nucleic acid gel stain bound to double-stranded DNA [18].

1.5.4. Gel Green

Gel Green is a sensitive, stable and naturally safe green fluorescent nucleic acid dye particularly intended for gel staining. Gel Green has UV excitation between 250 nm and 300 nm and an absorption peak on 500 nm (see Figure 1.12). Along these lines, Gel Green is perfect with either a 254 nm or 300 nm UV transilluminator. Gel Green is much more sensitive than SYBR Safe. Dissimilar to SYBR dyes, which are known to be unsteady, Gel Green is exceptionally steady, both hydrolytically and thermally. Gel Green is noncytotoxic and nonmutagenic at concentrations well over the working concentrations for gel staining, as a result of the dye's inability to cross cell membranes [19].

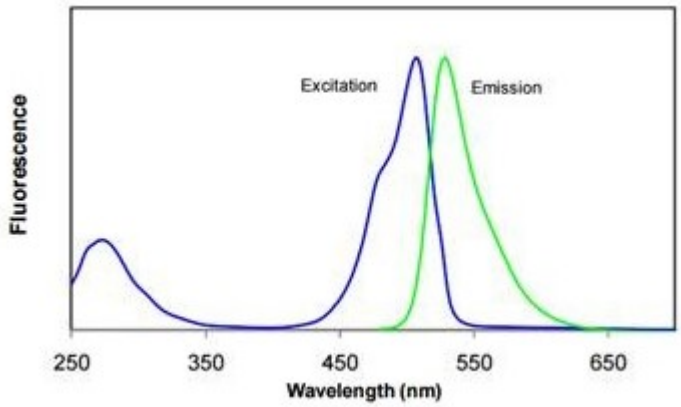

Figure 1.12: Excitation and emission spectra of Gel Green bound to dsDNA in TBE buffer [19].

1.6. Types of Pulsed-Field Gel Electrophoresis

There are very different types of PFGE to increase the size resolution and they use different pulsed-field electric fields (see Figure 1.13). These are Field-Inversion Gel Electrophoresis (FIGE), Transverse-Alternating Field Gel Electrophoresis (TAFE), Contour-Clamped Homogeneous Electric Fields (CHEF), Orthogonal-Field Alternation Gel Electrophoresis (OFAGE), Rotating Gel Electrophoresis (RGE), Programmable Autonomously-Controlled Electrodes (PACE), and Pulsed-Homogeneous Orthogonal Field Gel Electrophoresis (PHOGE).

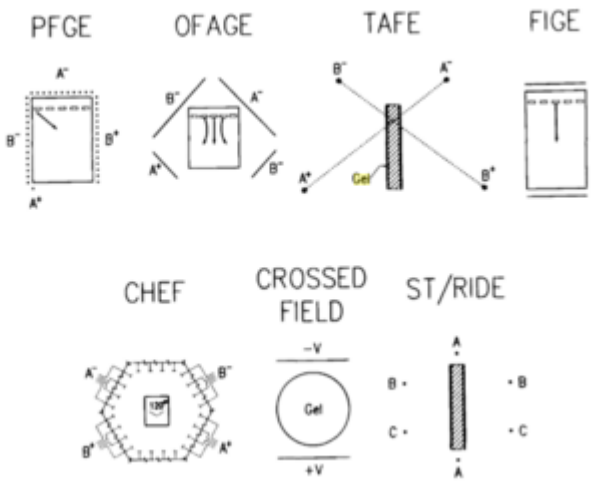

Figure 1.13: PFGE systems with their schematic diagrams [11]

1.6.1. Field-Inversion Gel Electrophoresis (FIGE)

FIGE was developed by Carle, Frank and Olsen, in 1986 [6]. It is based on the periodic inversion of an electric field in one dimension. There are two fields, which are apart from each other by 180 [6]. The main thing about FIGE is the way of direction. Backward pulse is always smaller than the forward direction (see Figure 1.13) [7].

1.6.2. Transverse-Alternating Field Gel Electrophoresis (TAFE)

TAFE has been used to separate 2000 bp to 7 million bp. The difference of TAFE from other electrophoresis method is the electrode arrangements that are across lanes of the gel. Gel is always placed at vertical position in electrophoresis tank and the DNA molecules go through zigzag paths in gel (see Figure 1.13) [8].

1.6.3. Contour-Clamped Homogenous Electric Fields (CHEF)

CHEF is the most popularly used apparatus. Electric fields can be controlled by a technique in which multiple electrodes are placed along a hexagonal shape contour and clamped to predefined electric possibilities. 24 non-passive electrodes are placed around the contour. Between the positive to the negative poles, CHEF uses an angle of 120 and DNA over 50 kilobases can be separated. Alternate switching positions are

the main difference of CHEF from the other electrophoresis types (see Figure 1.13) [20].

1.6.4. Orthogonal-Field Alternation Gel Electrophoresis (OFAGE)

In 1984, Carle and Olson reported the device that used two different electric fields [22]. This two nonhomogeneous electric fields and the varied angle between them were the main difference of the OFAGE than other electrophoresis types. The angle between the electric fields changes from 90 to 180. Yeast chromosomes can be separated in OFAGE (see Figure 1.13) [23].

1.6.5. Rotating Gel Electrophoresis (RGE)

Orientation of the electric fields should change for the separation of large DNA molecules in pulsed-field systems. However, Southern described another way to separate fragments in 1987, which is a periodically rotating a gel within a constant electric field [23] or by rotating the electric field mechanically around the gel. When the gel rotates between the two parallel electrodes, field becomes homogeneous and can be easily switched. DNA fragments between 50 kb to 5000 kb can be separated with the RGE (see Figure 1.13) [23].

1.6.6. Programmable Autonomously-Controlled Electrodes (PACE)

In 1988, Clark described a system, that can control all parameters in electric field by different regulation of the voltages on 24 electrodes arranged in a closed contour [24]. The main difference in this system is the voltage of each electrode. They can be controlled by a computer, not like CHEF, which is controlled by a different circuit. However, complex electronic hardware increases the cost of PFGE system (see Figure 1.13) [24].

1.7. Light Source Used For Image Analysing In Electrophoresis

1.7.1. Ultraviolet Light (UV)

UV is a segment of the electromagnetic range simply past violet which is at the short wavelength end of visible light as observed by the human eye. UV cannot be seen by the human eye, but rather when it falls on specific materials it might make them get to be distinctly fluorescent and reflect obvious light back to our eyes. There are three distinctive types of UV light. These are UVA (315-400 nm) which is ordinarily known as dark light , UVB (280-315 nm) is perilous with overdose and will bring about the skin to smolder and rinkle, and UVC (100-280 nm) that is extremely unsafe and for the most part hindered from the sun by the world's climate [28].

1.7.2. Light Emitting Diode (LED)

Recently, tendancy towards less expensive, steadier light for fluorescence has stretched out to light-emitting diodes (LEDs). The use of LEDs are preferred because of their long lifetime, little size, minimal usage of electricity, stable power, high efficiency and numerous radiating wavelengths extending from red to blue therefore they are now turned into an appealing option of light source for fluorescence identification. Another preferred standpoint of LEDs is that they are cost effective at wavelengths crossing the whole standard range, including close UV and close IR. In respect to lasers, industrially accessible LEDs radiate expansive band emissions from 350 to 1550 nm, with 291 nm LED as being developed lately [29].

1.8. Imaging and Detection Technologies For Image Analysis In Pulsed Field Gel Electrophoresis

For image analysis, the image capture is the first step. In the field of electrophoresis, two main devices are used for image capture. Scanner is the first one and charged-couple device (CCD) camera is the other one. In recent years important advancements have been achieved in the area of image capture, particularly in the field of

fluorescence and chemiluminescent recognition. The success of image analysis depends on high resolution and sensitive light detection of the CCD camera framework [2].

1.9. Interpreting PFGE Patterns

The patterns of restricted DNA isolates are collated with each other to identify their relations. At present, there are no institutionalized criteria for examination the patterns. Subsequently, different researchers seeing the same PFGE results may come at very different conclusions [34]. According to researchers Fred C. Tenover, Robert D. Arbeit et. al. four different categories of genetic and epidemiologic relatedness were defined (see Table 1.1):

- **Indistinguishable**; Bands of isolates should be same numbers and the same apparent size.
- **Closely relate**; Isolates should be closely related, in example, PFGE pattern may differentiate with a single genetic event.
- **Possibly related**; Two independent genetic events can be an example of this catagorization, it can be explained also with the 4-6 band differences between bands.
- **Unrelated**; PFGE patterns differs from the outbreak pattern by changes consistent with three or more independent genetic events [35, 36, 37].

1.10. Aim of the Thesis

In spite of the fact that DNA fingerprinting by PFGE is a critical and effective procedure, its application is exceptionally cumbersome, requires long time, experienced staff and costly hardware. These hinder the technique from being widely utilized. The aims of this thesis were to explore the feasibility of an observable PFGE devices i.e. to be able to see and record the separation of large DNA fragments during the whole run of electrophoresis; without utilizing U.V. light, (which is hazardous both to human eye, skin and DNA molecules) and ethidium bromide, a carcinogenic dye commonly used for DNA visualization.

Category	**No. of genetic differences compared with out-break strain**	**Typical no. of fragment differences compared with outbreak pattern**	**Epidemiologic Interpretation**
Indistinguishable	0	0	isolate is the part of the out-break
Closely related	1	2-3	Isolate is the probably part of the out-break
Possibly related	2	4-6	Isolate is the possibly part of the out-break
Different	3	7	Isolate is not part of the outbreak

Table 1.1: Criteria for interpreting PFGE patterns modified from [34].

2. METHODS

The detailed protocol for pulsed field gel electrophoresis is adapted from Chung et al.[37] and this protocol consists of six main parts after the cultured cells are harvested; wash cell suspension, determine cell concentration resuspend at predetermined concentration, mix cells with liquid low melting agarose, digest with proteinase K with EDTA and wash samples, restriction of DNA discs and preperation of DNA discs. All contents of the solutions can be reached in Appendix C.

Several variables are very important for the DNA separation: a pulse time, the electrical field strength and shape, gel, sample concentration and temperature.

2.1. Pulsed-Field Gel Electrophoresis Detailed Protocol

2.1.1. Harvest Cultured Cells and Wash Cell Suspension

A loopfull of the bacterial culture stock was plated onto a sheep blood agar plate by an inoculating loop and incubated overnight at 37°C. Next day, a colony was transferred into tryptic soy broth (TSB) and incubated at 37°C overnight in shaking incubator. For isolation of genomic DNA, 500 μl of the overnight cultures were transferred into 1.5 ml microcentrifuge tubes. The bacteria were sedimented by centrifugation at 13000 rpm for 2 minutes and supernatant was removed from the pellet. Pellets of bacteria were resuspended by adding 0.5 ml of PIV, and sedimented again by centrifuging. The supernatant was discarded and 200 μl of PIV was added to each tube.

2.1.2. Determine Cell Concentration

Cell concentration was calculated by using spectrophotometer (HITACHI U-1900). 5 μl of cell suspensions was pipetted into cuvettes, which were containing 1 ml of PIV. Optical density (OD) was measured for each sample at 620 nm. PIV was

added to each tube to obtain a final OD of 5.0 according to the formula in equation 2.1.

$$\frac{OD}{5.0}200 = \frac{Vadd}{210}210* \tag{2.1}$$

- **OD**; Optical density measured in cuvette.
- **200**; Dilution in cuvette (5 μl of cell suspension into 1 ml of PIV).
- **5.0**; Desired final OD of the bacterial suspension in tubes.
- **Vadd**; Volume calculate to add in order to have a final OD.
- **210**; Original volume of the bacterial suspension
- **210***; Total final volume.

2.1.3. Mix Cells with Liquid Low Melting Agarose

For the preparation of low melting agarose, 10 ml of PIV was added to 0.15 g of low melting agarose and heated to dissolve the agarose completely. Dissolved agarose was kept at 42°C.

A glass plate was coated with parafilm. Parafilm surface was rinsed with 70% ethanol. Microscope slides were evenly placed along the sides of the plate. 100 μl of the cell suspensions was mixed with 100 μl of the low melting agarose. 20 μl droplets were deposited onto the glass plate, which was coated with parafilm.

The droplets were covered with glass slides, by placing these on the slides previously placed on the sides of the glass plate as shown in figure 2.1. Thus agarose disks about 1 mm thickness containing bacteria were prepared. These steps were repeated for each cell suspensions.

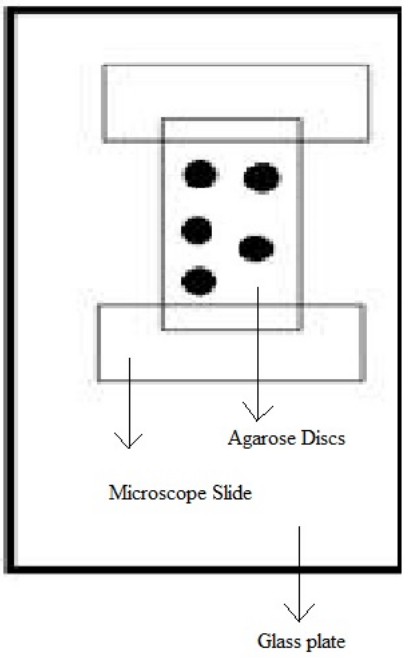

Figure 2.1: Schematic representation of tray assembly for DNA disc preparation.

2.1.4. Digest Cells with Proteinase K and Wash Samples

After all disks were solidified, microscope slides were lifted off and disks were transferred to 15 ml centrifuge tubes, which were filled with 0.5 ml of EC lysis solution (Appendix C). They were incubated at 37°C for at least 3 hours until all disks became transparent. Than 0.5 ml of ESP solution (Appendix C) was added to tubes and disks were incubated overnight with proteinase K at 50°C. For Gram negative bacteria, lysozyme was added, while lysostaphine was added to Gram positive bacteria. Next day, disks were washed with Tris-EDTA solution at least 5 times.

2.1.5. Restriction of DNA Discs

Reaction mix for restriction enzyme digestion was prepared. Appendix A contains the information about manufacturers of utilized chemicals.

Enzyme Type	SmaI (EURx)	XbaI (EURx)
Site	3-G G G C C C-5 5-C C C G G G-3	3-A G A T C T-5 5-T C T A G A-3
Source	*S*erratia marcescens	*X*anthomonas badrii
Standard Protocol	1-2 μg pure DNA 5 μl 10x Buffer Acet 0.5 μl BSA 1-2 U SmaI	1-2 μg pure DNA 5 μl 10x Buffer Acet 0.5 μl BSA 1-2 U XbaI

Table 2.1: Features and standard protocol of restriction enzymes [38,39].

2.1.6. Preperation of the Gel and PFGE Apparatus

Digested genomic DNA inside the disks with Xba-I for Gram negative bacteria and Sma-I for gram positive bacteria, was loaded into 1 percent agarose gel in 0.5 x TBE solutions and was run on CHEF-DR II instrument. Molecular standards, CHEF DNA Size Marker-S.cerevisia and CHEF DNA Size Standard- Lambda Ladder, were used in all gels to compare the results from several gels. PFGE device (Chef Drive II,

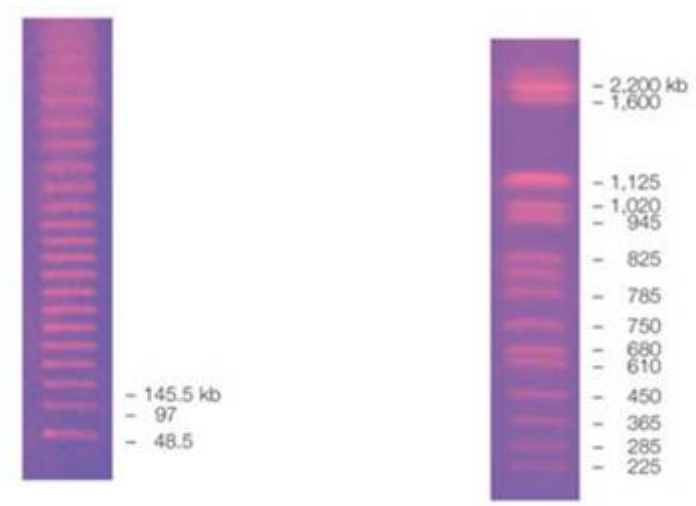

Figure 2.2: CHEF DNA Size Marker-S.cerevisia and CHEF DNA Size Standard- Lambda Ladder [41]

Biorad) was used for standard PFGE application. This device has hexagonal gel box with 24 electrodes, direct current electric source, a device that can change the electric field, cooler and reciprocating solenoid pump, which is used for the recycling the buffer.

For PFGE, 5xTris Borate EDTA (TBE) buffer solution was prepared and was filled into the electrophoresis tank after diluting 10 times with deionized water to make

it 0.5x. Using the same solution, a 1 percent high strength agarose gel was prepared. For this purpose, 2 grams of agarose were placed in 200 ml 0.5x TBE and boiled in a microwave oven. After cooling the gel solution for a while, it was placed into a gel-forming plate and the comb was placed to form wells. The comb was removed and the agarose discs prepared for DNA fingerprinting were loaded into the wells.

The gel was placed in the electrophoresis tank. Pump and tubing were connected between the buffer solution cooling system and the electrophoresis tank. The cooling (Bio-Rad Laboratories, USA) system was adjusted to maintain the buffer solution at 14C throughout the entire process. The electrodes were connected to the power supply which provided variable field. The voltage was set to 5-6 volts per gel centimeter, 35 seconds in diagonal directions, and 5 seconds in the vertical direction in interstices. The electrophoresis was run for 16 to 24 hours. After the PFGE was completed, the gel was placed into ethidium bromide solution for 30 minutes. DNA fragments were visualized and photographed in ChemiDoc XRS gel documentation system (Bio-Rad Laboratories, USA). These disks were used in both PFGE and Observable PFGE [16].

2.2. Design of Observable PFGE

Observable PFGE was built as a 50x50x15cm square box (see Figure 2.3) that can do electrophoresis and visualize gel at the same time. This box was covered with pyramidal cap. The system was placed into dark to eliminate the outside light that could cause any background, to detect the fluorescence of dyes. A rectangular window (see Figure 2.4) was placed in front of the pyramidal cap to visualize gel with naked eye during the run and an orange color filter was placed to this window to filter out the blue light.

A camera and an orange filter were inside the cap (see Figure 2.5), in the middle, to provide continuous imaging of the gel.Camera was mounted on a mechanical device that enabled the movement of camera, which can zoom in and focus the gel. A silicone gasket was placed between the lid and the box to prevent light leakage. CCD camera was used to produce sharper images.

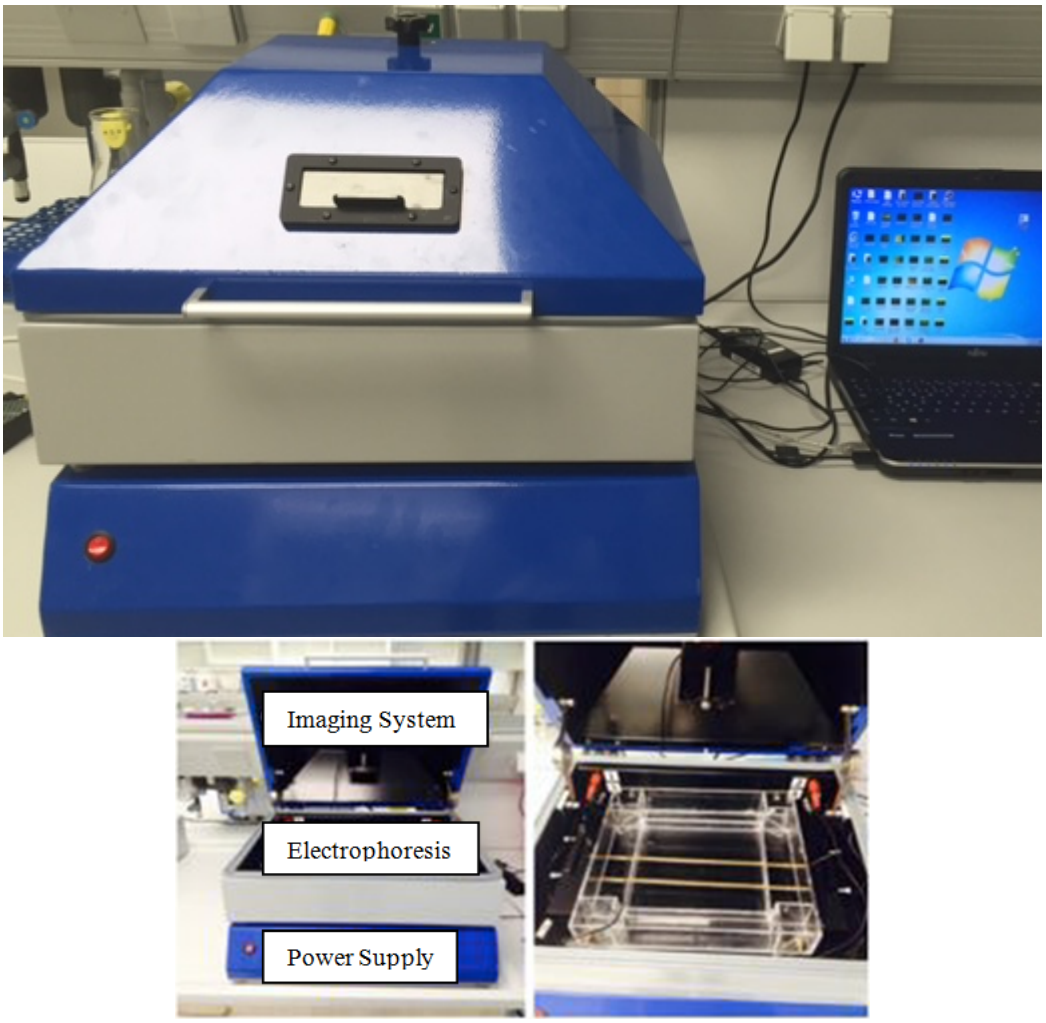

Figure 2.3: Observable Real-Time PFGE. Imaging System, electrophoresis and power supply combined in one device.

A fan was placed on the back face of the cap and vent holes were punched at the bottom of the box (see Figure 2.6). This enabled to keep the buffer solution and agarose gel at room temperature during electrophoresis and eliminated the need for a buffer cooling system present in standard PFGE instrument.

Under the main electrophoresis box, an electrical system that provides pulsed-field electric current and power to the blue LED light was placed. Both boxes were painted with appropriate enamel paint to make them corrosion resistant. The LEDs were either 3 or 5mm in diameter. The lighting system consisted of a series of LED lights. These produced 470 nm wavelength blue light, which is close to excitation peaks of SYBR Green, SYBR Gold and Gel Green dyes.Ten LEDs were attached to each other in a row and one set was placed on each side of the electrophoresis tank.

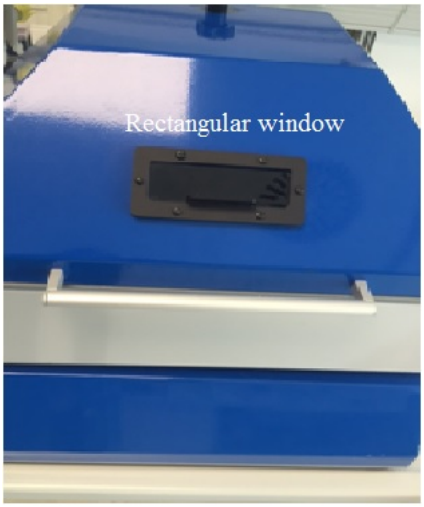

Figure 2.4: A rectangular window was placed in front of the pyramidal cap to visualize gel with naked eye during the run.

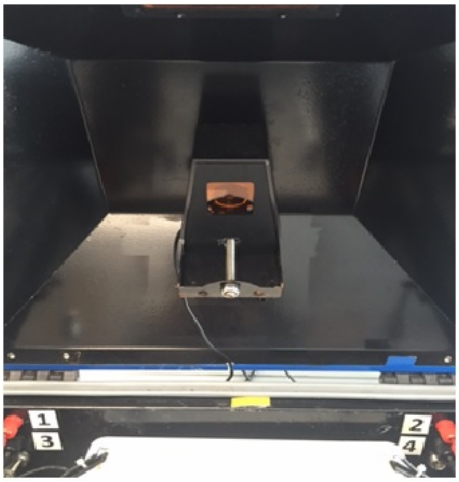

Figure 2.5: A camera and an orange filter.

At the beginning of the study, LED lamps working with 3 Volts were used. Later, they were replaced with more powerful LEDs that required 12 Volts, to increase sensitivity. An electronic card that supplies the electric current to four electrodes to create the desired positive or negative polarity period was prepared. It was possible to create electric field in any direction by using different combinations of positive and negative charges on the electrodes (see Figure 2.7).

The electronic part consists of two separate main parts, which are located inside the ORT-PFGE device.

1; The digital part that reads the parameters from the computer section by reading from the "USB" port and converts it to the signal output and hosts software on it.

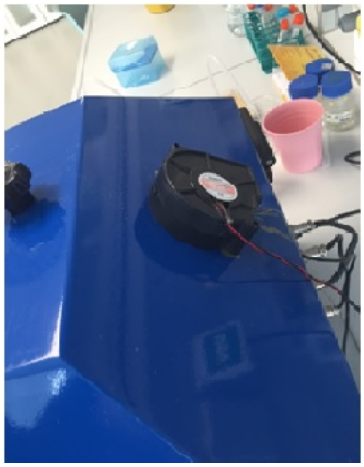

Figure 2.6: A fan was placed on the back face of the cap and vent holes were punched at the bottom of the box.

2; Power card that detects the signals coming from the digital part and provides the desired voltage and direction outputs.

When you set the desired direction and voltage from the computer software and start the system, this information is sent to the ORT-PFGE device via the "USB" port. The digital card in the ORTE device, which is connected to the "USB" port of the computer, converts this information into a simple electrical signal that we know by reading it. In short, we convert the information called "USB" into a digital signal.

Subsequently, the information translated into a digital signal is transmitted to the main power board. The main power card opens and closes the contacts corresponding to the desired voltage and direction according to the signal received. All information about electronic material brands can find in Appendix B.

The necessary timing settings are made by the software on the computer at the end of each time information is sent back to the digital card and the next job is being done.

Arduino, open-source electronic prototyping platform, was used for developing a computer program. A computer program was developed to control the amount of current, strength and direction of the electric field and change the total and switch time. The program also enabled to make these adjustments in cycles. The original

imaging program of ORTE was used to make camera adjustments for zooming, focusing, brightness and contrast of the pictures and for obtaining pictures periodically as desired. The program also enabled recording and playback of the pictures as a film at the end of electrophoresis, with the aim of selecting and reviewing the desired photo.

In figure 2.8, as shown on the computer screen, the device has been made to be able to apply voltage cyclically in 8 different directions for the desired time. Circular programs can be easily written by preparing the direction table.

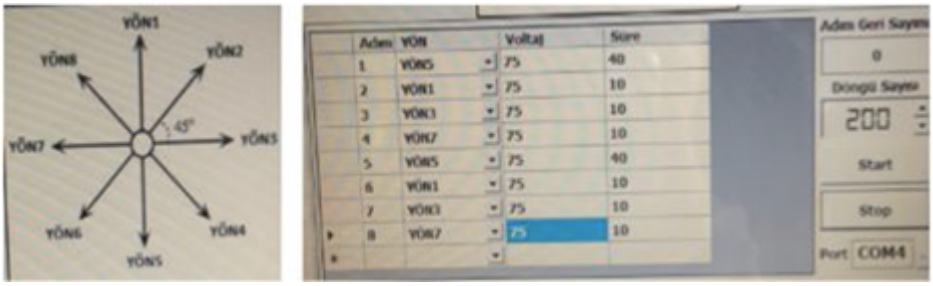

Figure 2.7: ORT-PFGE software interfere displaying showing the electric field directions and the programing screen for the cyclic direction table.

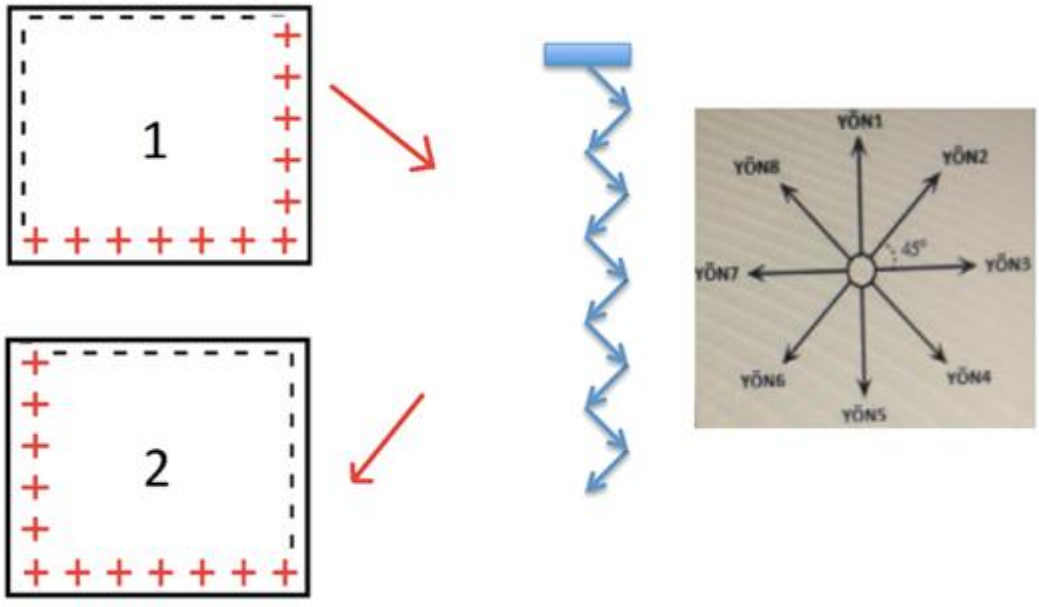

Figure 2.8: Adjustment of charges on the electrodes of Ort-PFGE for adjusting the direction of electric field.

Four types of electrophoresis tanks were prepared during the thesis. Each new electrophoresis tank design was made with the aim of eliminating the problems arising during the previous studies and allowing the experiments to be carried out as desired.

For the development of the ORT-PFGE, firstly a square electrophoresis gel box was built. Platin electrodes were placed on the four corners of the gel box. The depth of the box was deep enough to get enough solution to cover the gel. By applying current to the upper and lower electrodes, an electric field was created by in the vertical direction. With the top and one side being negative, bottom and other side being positive it was possible to create an oblique electric field. By the experiments made with this container, DNA fragments in the middle of the gel were linearly separated in the vertical axis. However, the samples on the edges moved sideways and get out of the gel after a while.

Then, the electrodes were shortened and mounted in the middle of the edges to produce a second electrophoresis tank. It was observed that the samples loaded on the side of the gel scan be run without deviating from the vertical axis in this new tank. However, it was not possible to separate large DNA fragments by this tank.

Thinking that oblique electric fields cannot be created properly when the electrodes are placed in the middle of the sides, we have built a third thank by putting the electrodes on the corners. This tank provided 45 oblique electrical fields to the horizontal axis. Although it was possible to separate large DNA fragments by this tank the resolution was not satisfactory. We have thought that oblique electric fields other than 45 may increase the resolution.

In order to test the efficiencies of using oblique electric fields with different angles we have built a fourth electrophoresis thank in which the position of electrodes can be moved to any position as desired. In this fourth instrument, with the aim creating more linear electric fields we increased the dimensions of the tank. The blue light sources were mounted on the edges of the electrophoresis chamber to illuminate the gel more evenly (see Figure 2.9).

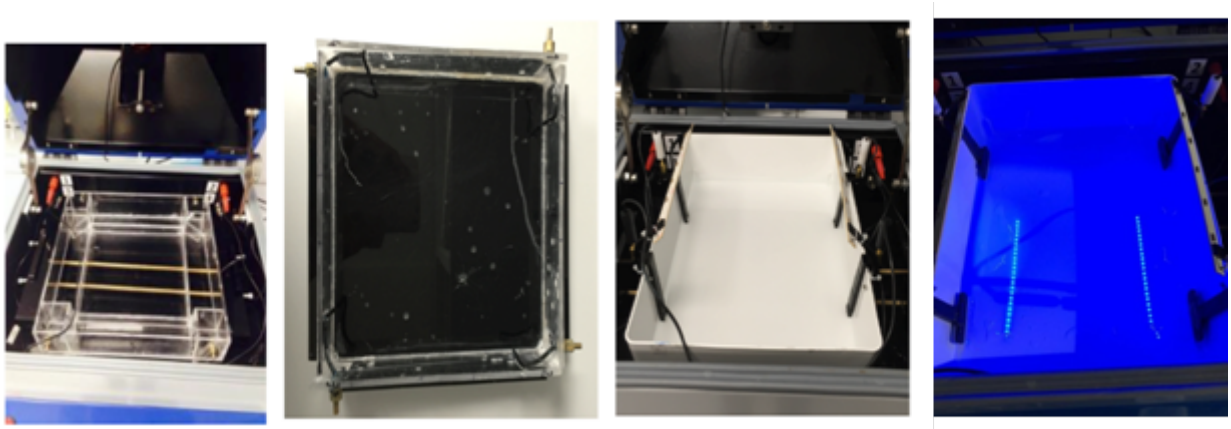

Figure 2.9: Electrophoresis vessels produced during project: Left: The electrophoresis tank in which the electrodes are extended along the edges in a square shape. (The electrodes were shortened in a modification of this tank). Middle: the model where the electrodes are placed on the corners. Right (two pictures): A rectangular-shaped electrophoresis tank (with and without blue light illumination)with mobiles electrodes that can be placed at any desired position.

In studies conducted with the fourth electrophoresis tank, it is desirable to isolate DNA molecules were moved slowly than we observed. This is due to the shortness of the platinum wires in the electrodes. In addition, during electrophoresis, gas bubbles arising from electrolysis cause current transmission from the wires. Changing location of electrodes eliminated this problem. The lower portions of the electrodes are expanded to place the platinum wires in the electrophoresis buffer area parts increased to about 3 times (see Figure 2.10).

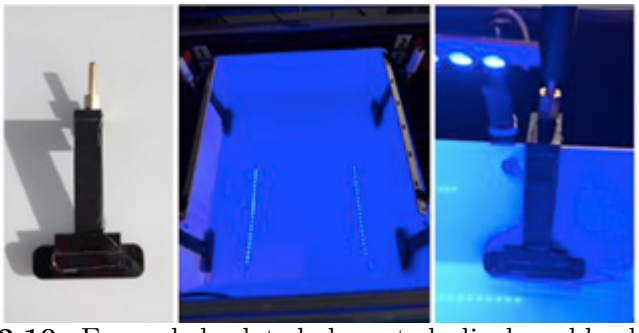

Figure 2.10: Expanded, plated elongated, displaceable electrodes.

For the staining of DNA, three different nucleic acid binding fluorescent dyes were used. These were SYBR Green I, SYBR Gold and Gel Green. Under blue light, all three fluorescent dyes produce green colored light. The dyes were obtained as 10.000x concentrated stock solutions. 1μl of 100 times diluted stock solution was enough to

stain a disk in 10 μl buffer.

3. RESULTS

3.1. Optimization of Sample Preparation

Discs containing restriction DNA fragments prepared from different bacterial isolates were first evaluated with standard PFGE method to understand whether the DNA fragments, were prepared properly, before using them in ORT-PFGE. For this purpose growing of microorganisms, preparation of agarose discs, obtaining genomic DNA, and cutting methods with restriction enzymes were optimized. Carbapenamase producing gram negative bacilli, which also have epidemiological importance, were selected for the first group of organisms that were studied. Escherichia coli and Klebsiella pneumoniae strains, that were grown and collected at the clinical microbiology laboratory from patient samples, were included in the study. A classical variable field electrophoresis system was employed and evaluation of microorganism genomes started. After the DNA molecules were separated by electrophoresis, they were stained with ethidium bromide and made visible in ultraviolet light. Genomic DNA restriction products were not cleaved in the first trial due to device-induced problems (see Figure 3.1).

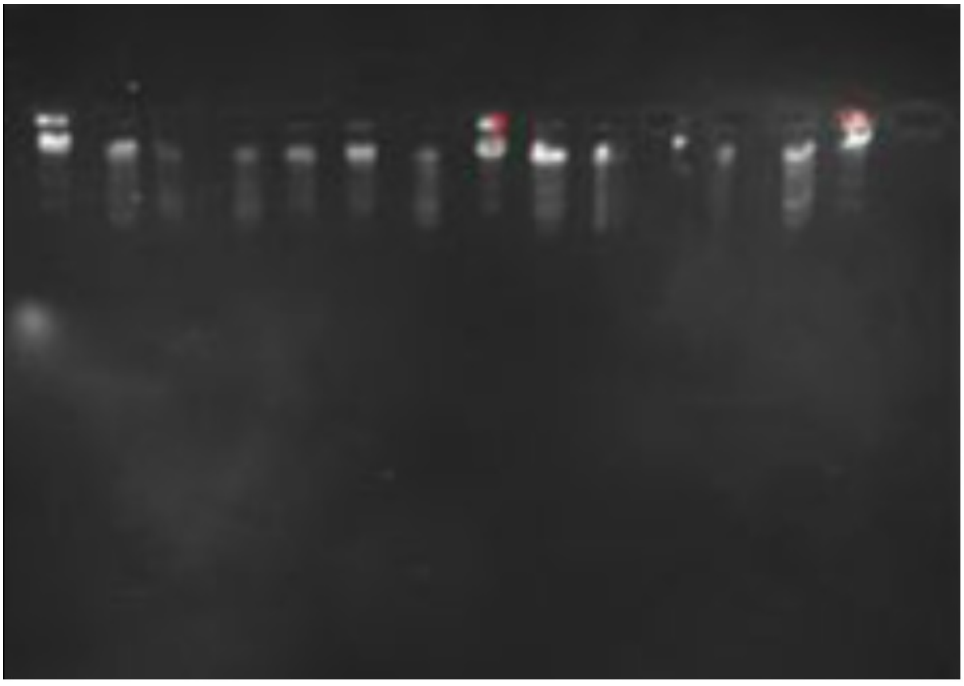

Figure 3.1: Genomic DNA cutting products that can not be degraded by PFGE. It is observed that the DNA samples were cut off but could not be separated by electrophoresis.

Results suggested that separation inside 1 % high stability agarose gel, in 0.5x Tris Borat EDTA buffer with 0.5 Volt/cm gel voltage for 35 seconds cross-sided, 5 seconds vertical at transitions and total running time for 22 hours, were the best result giving parameters in standard PFGE. With this revised protocol we have evaluated the phylogenetic relationships of New Delhi Metallobetalactamase (-NDM-) strains resistant to carbapenems, which are rapidly spreading in the world in recent years, by PFGE conducted using these parameters (Figure 3.2).

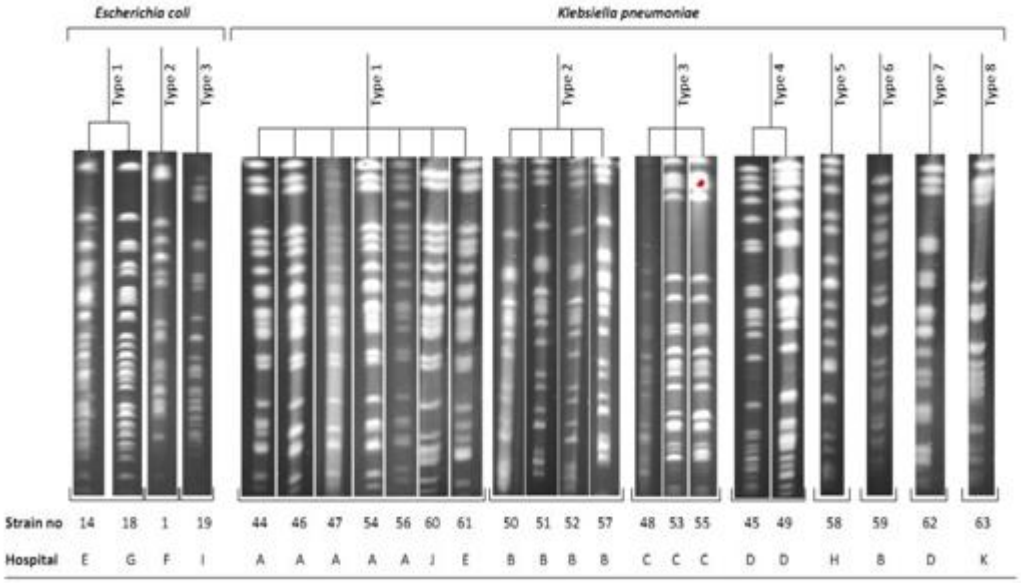

Figure 3.2: Pulsed Field Gel Electrophoresis patterns of blaNDM-1 positive Escherichia coli and Klebsiella pneumoniae isolates

The epidemiological spread of NDM-type 1 carbapenemase producing Klebsiella pneumoniae strains has been studied in detail Gel images were analyzed with Totallab image analysis software (Total Lab Ltd., United Kingdom). We have prepared a dendrogram using the same software. Thirty-five different patterns were identified for 55 strains examined (see Figure 3.3).

The dendrogram describes the combination of clusters at each step of the analysis along with a numerical measurement of similarity [42]. A graphical procedure that represents the emergence of a hierarchical clustering method. A dendrogram is defined as a binary tree that contains an elongated root with all the data items on its leaves. Traditionally, all leaves are shown at the same level of the drawing [43].

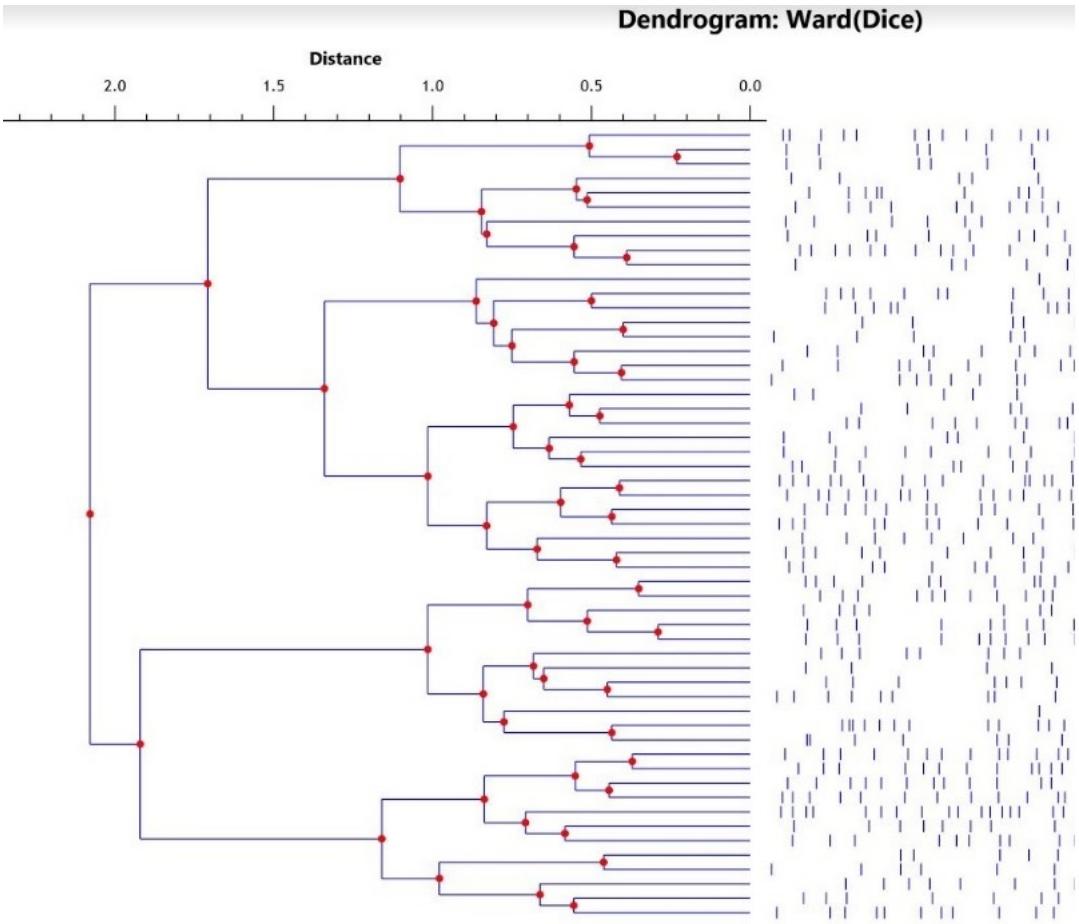

Figure 3.3: Dendograms showing the clonal proximity of NDM1-positive Klebsiella pneumoniae strains.

Second PFGE study was done with Methicillin Resistant Staphyloccus Aureus-MRSA isolates, which is one of the most problematic agents of hospital infections. MRSA strains were isolated by our research partner, Hacettepe University, Clinical Microbiology department. MRSA strains represented gram-positive organisms in our study and also they were very important for epidemiological studies. MRSA disks were prepared by using a similar optimized protocol of section 3.1, which was used in gram-negative bacterial isolates. For lysing the cell walls, lysostaphine, which is more effective on Gram positive bacterial cell wall, was used in addition to lysozyme. DNA molecules released from the cells were cut with the SmaI restriction enzyme. Restriction fragment of DNA were seperated by using of standard PFGE.

A good seperation was obtained by testing various variables. The best result was obtained with 5 seconds of current direction change; 35 seconds of cross current time,

23 hours of run time and voltage of 6.0 V / cm gel. After electrophoresis, the gel was stained with ethidium bromide and photographed using an ultraviolet transilluminator in a conventional gel imaging system (see Figure 3.4).

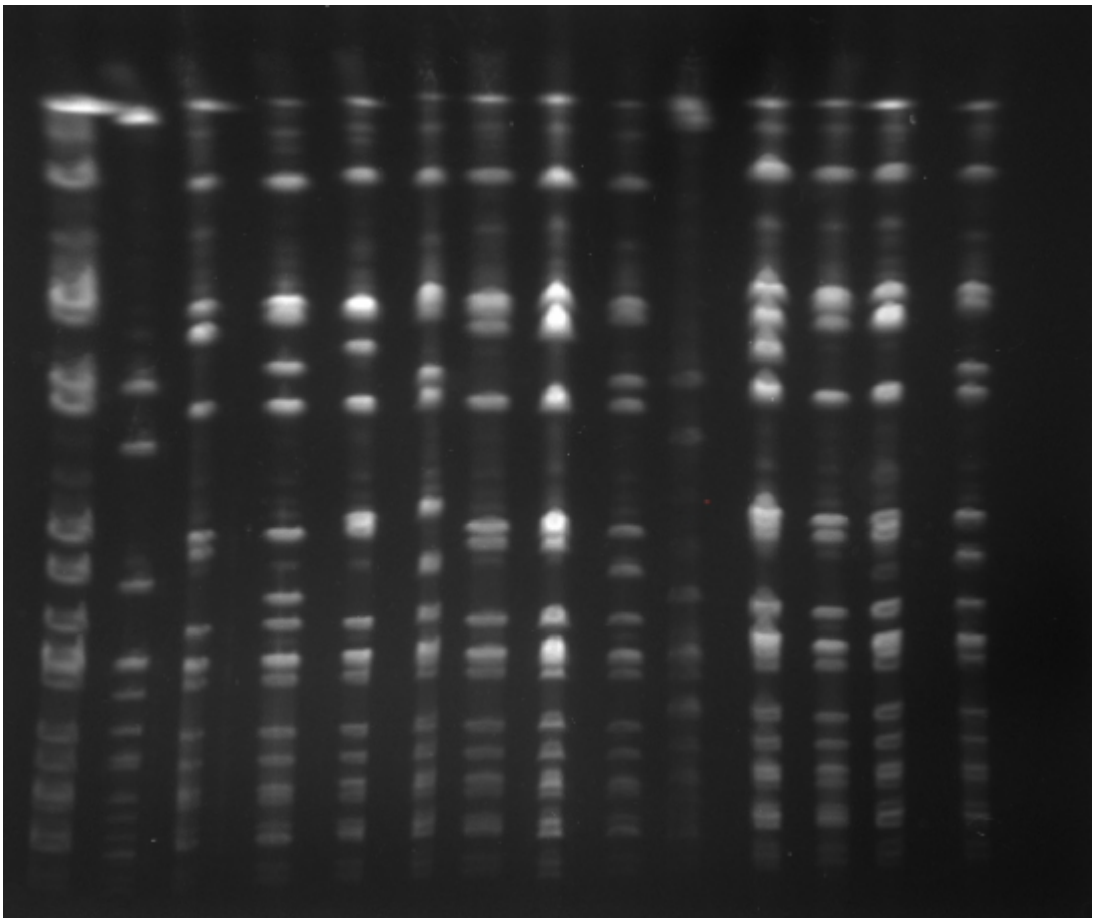

Figure 3.4: DNA fingerprints of MRSA strains with classical PFGE. Most of the patterns being identical in most of the strains indicate that these isolates come from the same source.

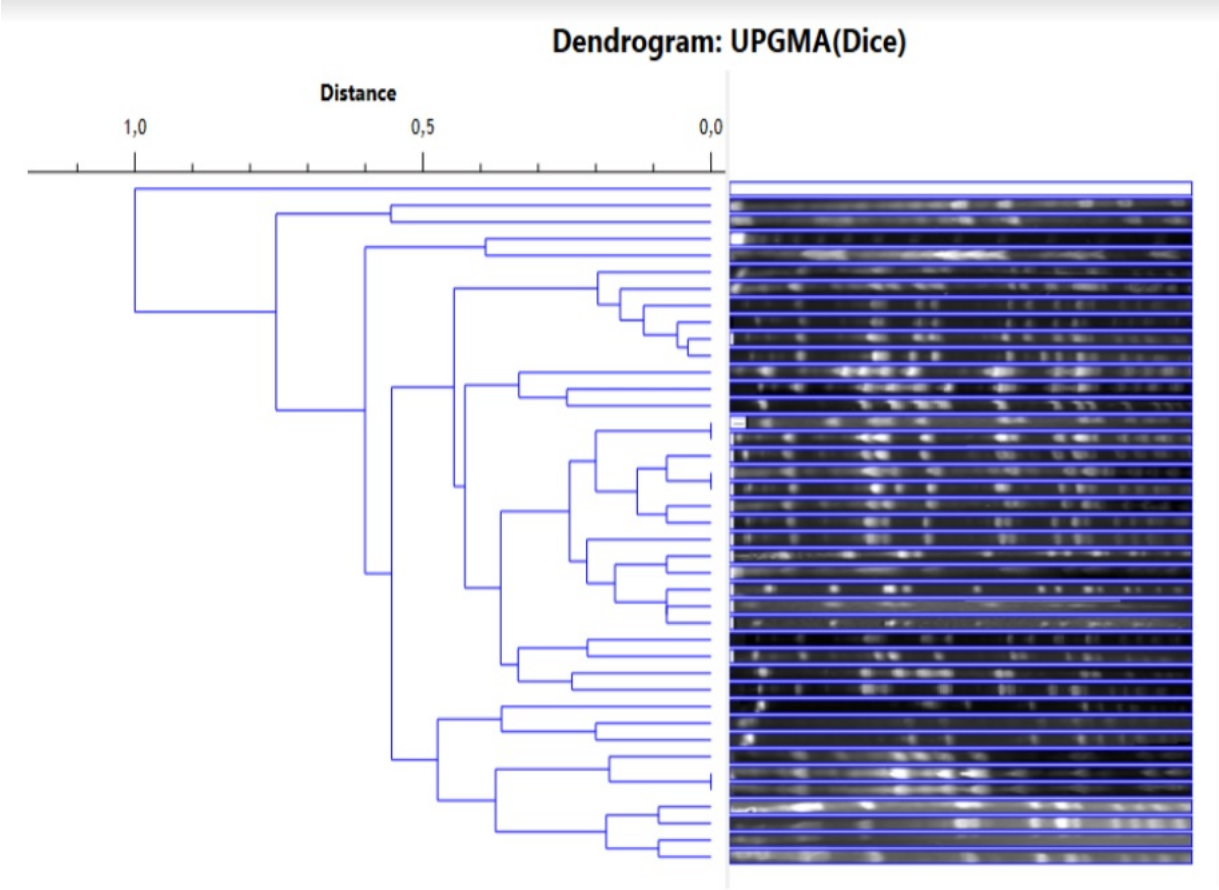

Figure 3.5: Diagram showing the phylogenetic proximity of MRSA isolates isolated from Hacettepe University Hospital.

3.2. Dye Optimization of ORT-PFGE Device

In order to make DNA molecules visible during ORT-PFGE, different fluorescent dyes, that binds to DNA and give fluorescence when illuminated by blue light were evaluated. For this purpose SYBR Green, SYBR Gold and Gel Green, which have these features, were included in the study. We have observed that SYBR Gold and Gel Green dyes dissolved agarose discs at high concentration, making difficult to load the discs into gel wells. Putting SYBR Green in the electrophoresis gel enabled better separation, than staining the discs.

In order to be able to monitor PFGE with observable real-time electrophoresis with blue LED light, we have started to experiment with dyes that can be used instead of etidium bromide. For this, firstly SYBR Gold, which is also used for observable

real-time electrophoresis, was used.

For staining, 5 μl loading buffer solution for stock and stock solution 1/100 5μl of SYBR Gold was used. It was observed that samples that we worked with SYBR Gold could not be separated by electrophoresis. Considering that the concentration of dye was too high, cause of that the added volume of 5 μl dye was dropped to 3 μl and experiment was tried again. In figure 3.6 it was decided to try SYBR Green and Gel Green as an alternative to SYBR Gold. The samples were painted with 3 different dye types and ethidium bromide.

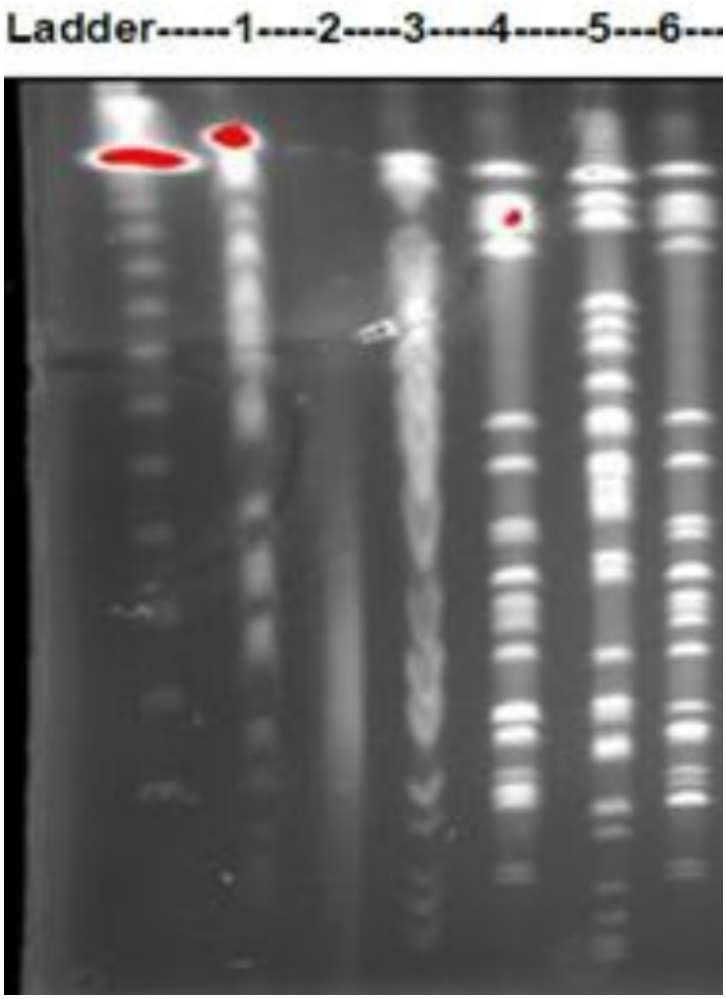

Figure 3.6: Genomic DNA staining products with various fluorescent dyes. Columns: 1-SYBR Gold; 2: SYBR Green, 3: Gel Green, 4-6: ethidium bromide stained samples.

To evaluate the difference in the separation in the DNA fragments using prestained and unstained discs, the gels were stained by ethidium bromide after completion of the

run in ORT-PFGE. Prestained fragments were observed and photographed by ORT-PFGE and unstained fragments were visualized by classical gel documentation system after staining by ethidium bromide. The electrophoresis of the agarose discs containing genomic DNA was loaded into the gel, followed by a staining experiment with Gel Green as shown in figure 3.7.

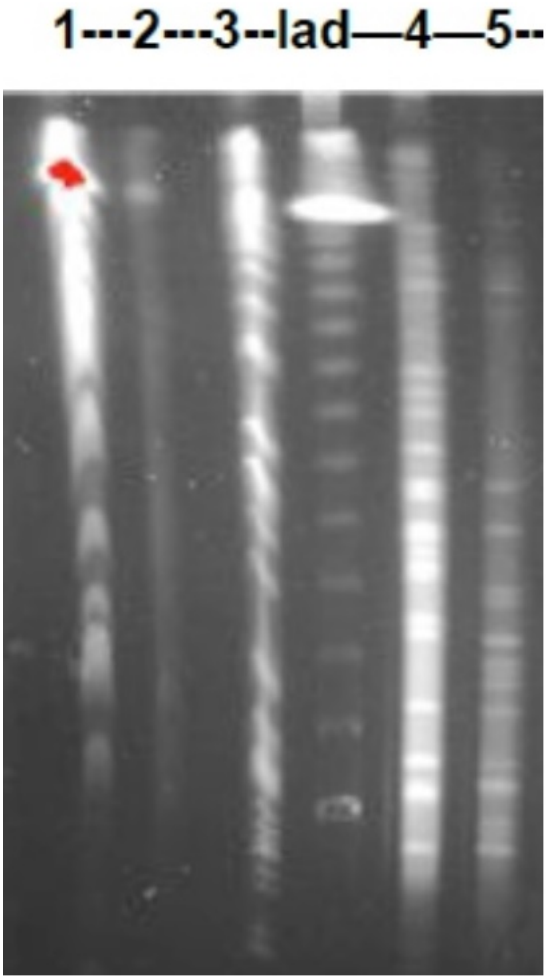

Figure 3.7: 1 and 2: gel green added after gel is added; 2-5: samples stained with ethidium bromide.

We have observed that some dyes inhibited the separation of DNA fragments. Lowering the concentration of these dyes has been tested to overcome this problem.

Lowering the concentration of both SYBR Gold and SYBR Green enabled better separation of DNA fragments. Lower concentrations of SYBR Gold and SYBR Green stains were sufficient to visualize DNA fragments; however it was not possible to see the

DNA fragments at low concentrations of Gel Green. Discs painted with stock Gel Green with 1/100, 1/200, 1/400, 1/800 dilution (see Figure 3.8). As the dye concentration decreases, the decomposition rate of the molecules increases.

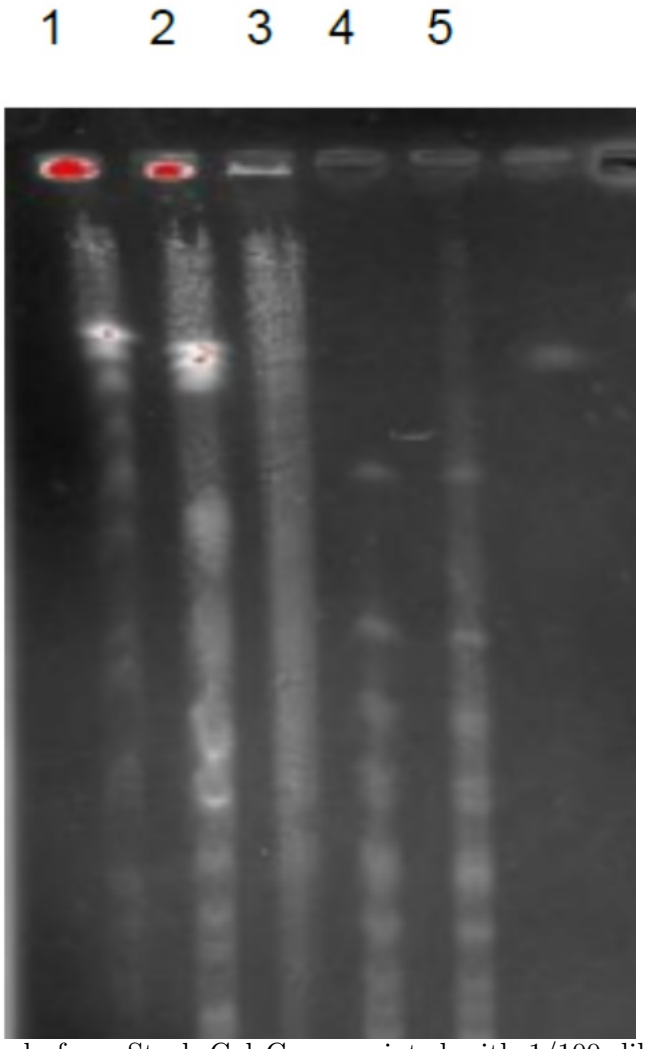

Figure 3.8: 3: Sample from Stock Gel Green painted with 1/100 dilution. 2: 1/2 times diluted dye 1: 1/4 times diluted dye 4 and 5: same sample stained at 1/8 dilution.

As the dilution rate increased with the experiment with Gel Green stain at different dilutions, it was observed that the molecules became better and the DNA bands became better visible. This result could only be achieved after gel separation, after dyeing with ethidium bromide and after observing DNA bands in the ultraviolet transilluminator. When observed at the blue LED lamps, diluted Gel Green dyed stained samples that could not be distinguished.

In conclusion, it was decided to continue the work with SYBR Gold and SYBR GREEN, considering that Gel Green dye could not be used in the observable PFGE, because of its lower sensitivity. SYBR Gold provided best results in terms of electrophoretic separation and visualization in blue light (see Figure 3.9). Under blue light without ethidium bromide staining, SYBR Green and Gel Green samples were not seen in the photo since they were not prestained.

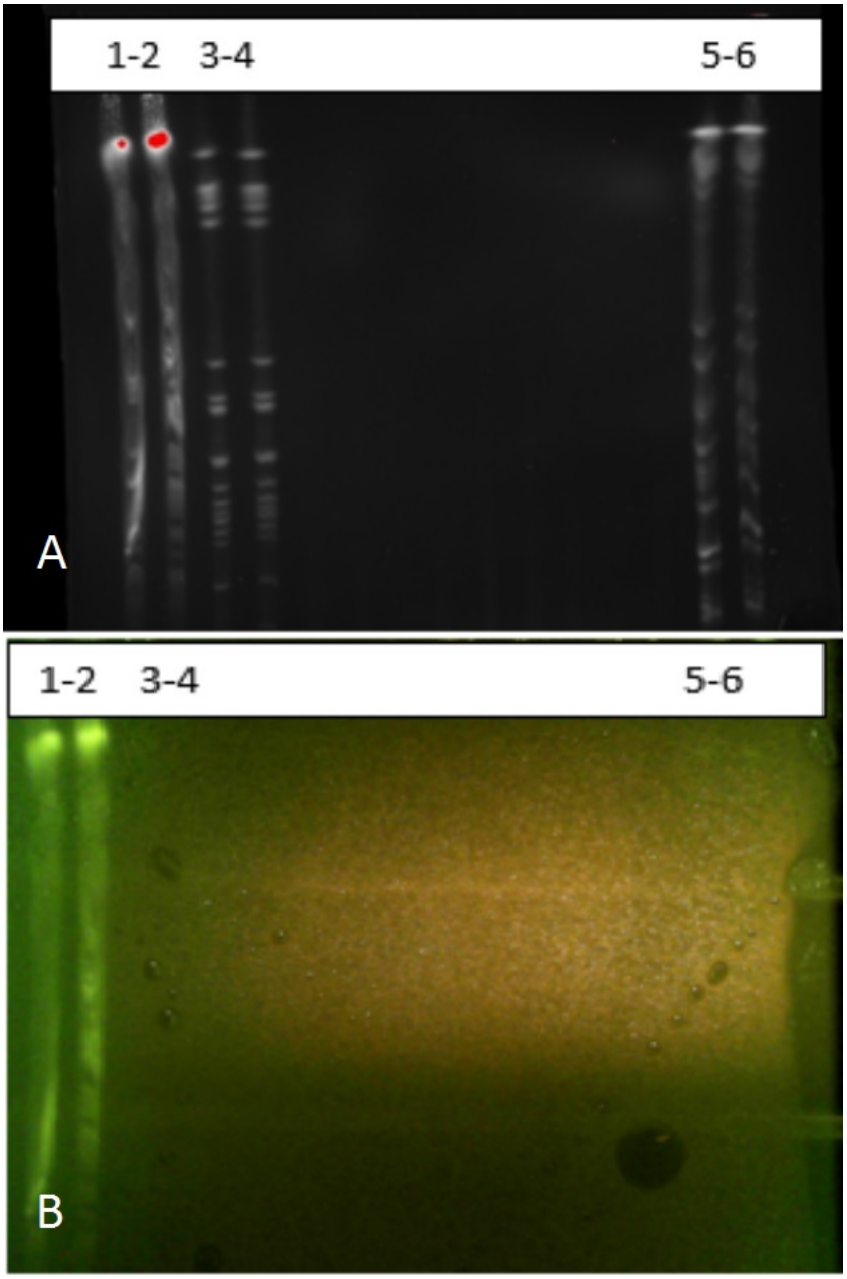

Figure 3.9: Comparison of three different dyes in terms of electrophoretic separation and visualization under UV and blue light: 1 and 2 SYBR Gold, 3 and 4 SYBR Green, 5 and 6 Gel Green stained examples. A: The visualization of the gel over ultraviolet transilimunator after staining with ethidium bromide. B: Under blue light without ethidium bromide staining.

It was observed that the discs loaded with the fluorescent dye started to deteriorate physical structures while waiting in the paint. It was also observed that not only physically, but also molecules were not properly separated in PFGE. In working with 3 different dyes, this problem was found at least with SYBR Green (see Figure 3.10).

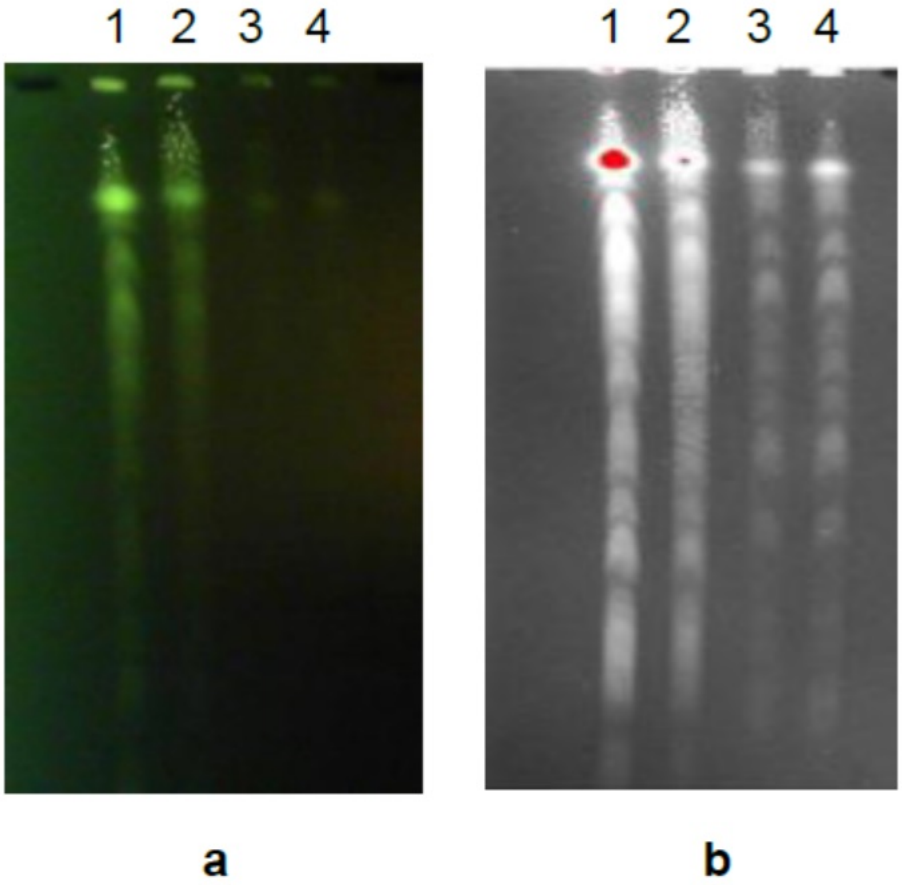

Figure 3.10: 4 samples painted with SYBR Green dye (a) under blue light (a) and with ethidium bromide (b) after ultraviolet light images 1 and 2, 1/100 diluted dye from stock dye; 3 and 4, samples stained with 1/200 diluted dye.

Considering that increasing the dye concentration could bind DNA molecules to each other before electrophoresis and that the problem of decomposition could be caused by this, it was tried to put the dye within the electrophoresis gel instead of putting the dye on discs containing genomic DNA before electrophoresis. SYBR Green dye was prepared by adding 25 μl stock SYBR Green dye in to 250 ml prepared gel, adhering to the gel dyeing method described in the user guide of SYBR Green [104].

By this application it was possible to separate restriction fragments of genomic DNA by PFGE and simultaneously observing them in blue light (see Figure 3.11).

Figure 3.11: Image taken with blue light of the samples stained with SYBR Green. (None of the two samples stained before electrophoresis.)

3.3. Optimization of Electrical Field Directions

According to the software program we have prepared, we can run our electrodes in 8 different directions (see Figure 2.7 and Figure 2.8). We tried three different options because there are so many possibilities that we can work. It was decided to try the three cyclic programs shown in figure 3.12 in the first step because there are so many different possibilities that we can work with in the direction and time cycle.

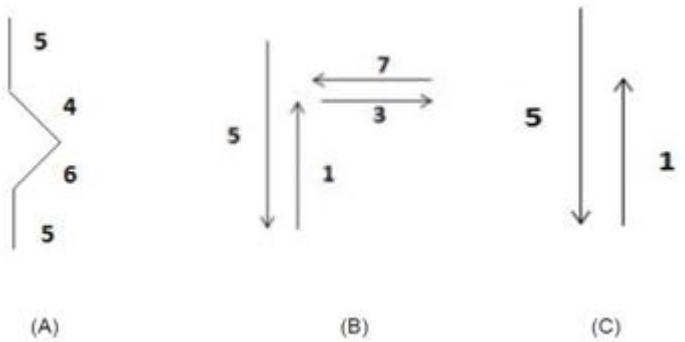

Figure 3.12: Three programs prepared using different aspects of ORT-PFGE. A: 5-4-6 orientations; B: 5-1-3-7 directions, C: 5-1 directions.

In ORT-PFGE, in the first experiment using circles 5-4-6, with 100 V for each direction and 30 seconds, the DNA molecules in the samples remained in the pit were not seperated.

Secondly, the same samples were tried to be separated by applying 75 V in 5-1-3-7 directions and 40, 10, 10 and 10 seconds in cycles with the same sequence and dyes. It was not possible for the DNA molecules to be observed in the gel after this application.

The use of 5-1 directions cyclically in ORT-PFGE, with application of 70 V in these directions for 40 and 10 seconds, allowed DNA molecules to be isolated, even at the desired separation force (see Figure 3.13).

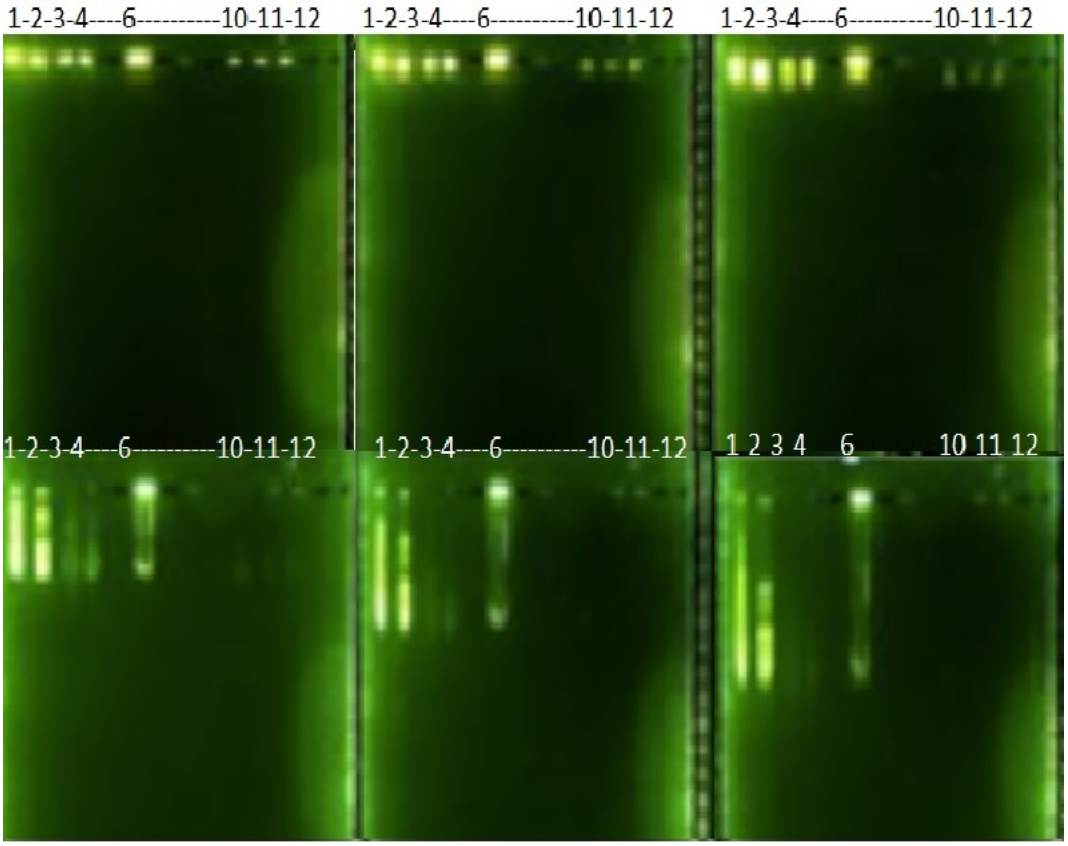

Figure 3.13: Photographs that are attempted to be separated by ORT-PFGE using the 5-1 direction and automatically saved at regular intervals with the ORT-PFGE program. (Examples 1 and 2 are SYBR Gold, samples 3 and 4 and 6 are SYBR Green 10, 11 and 12 are Gel Green dye.)

The ability of electrophoresis to be traced for the first time in this experiment allowed us to follow the movement of DNA molecules until the end of electrophoresis. With the first two examples, SYBR Gold, we observed that bands were gradually formed. It has been detected that Gel Green has not been able to see the molecules from the beginning of the separation. It was observed that after 3 rd hours of electrophoresis, the band of DNA molecules began to separate, but at the same time their visibility decreased. Although the molecules in SYBR Green-stained samples could be seen at first, it was observed that as the electrophoresis progressed, it became disappeared.

During the studies, It was determined that the samples could not be linearly processed with the first square electrophoresis tank, which had elongated electrodes extending along its edges.

In the first studies of ORT-PFGE, an electrophoresis tank extending from a 4-sided rectangular wire electrode designed in a square shape was used. The first observation with this tank was that it could not carry forward the forward samples linearly from the pits. While the samples loaded on the center, they were moving linearly from the middle, the samples loaded on the sides were moving on a circular curve outwardly, and the samples on the side were going out (see Figure 3.14).

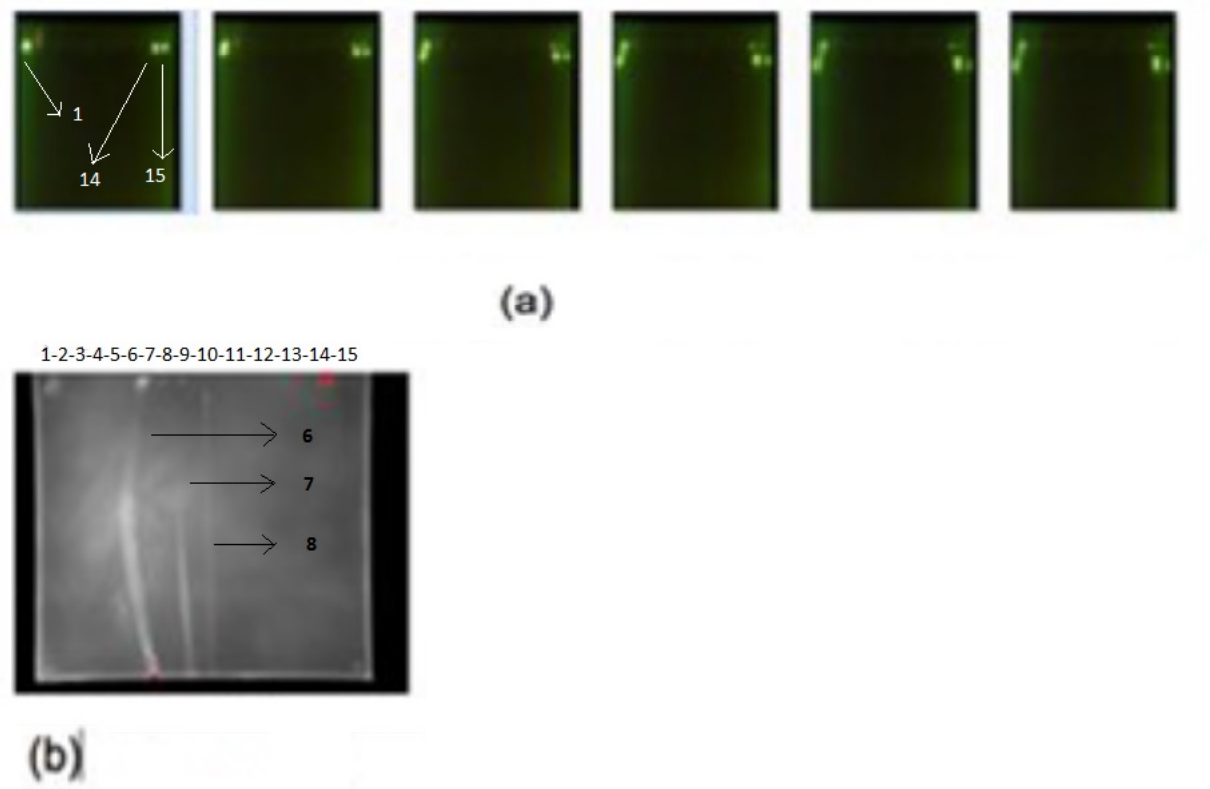

Figure 3.14: A: Image of the samples taken with ORT-PFGE every 5 minutes and with the dye in blue light. ("Chef DNA Size Standard Lambda ladder" was installed on the 1st and 5th wells.) The sample was stained with Gel Green, 6, 7 and 8 with ethidium bromide. The sample at the 14th well was stained with SYBR Gold and the sample at the 15th place with SYBR Green.) B: A photograph of the sample taken with ethidium bromide.

The problem was solved by shortening the electrodes to length of the electrodes to 1/3 of their original size. By this change, linear separation of the samples was achieved. However, the separation of genomic DNA was still not satisfactory. To improve the seperation many experiments on the parameters of the direction of the current (different angles to the vertical axis), voltage and duration applied in each direction were done.

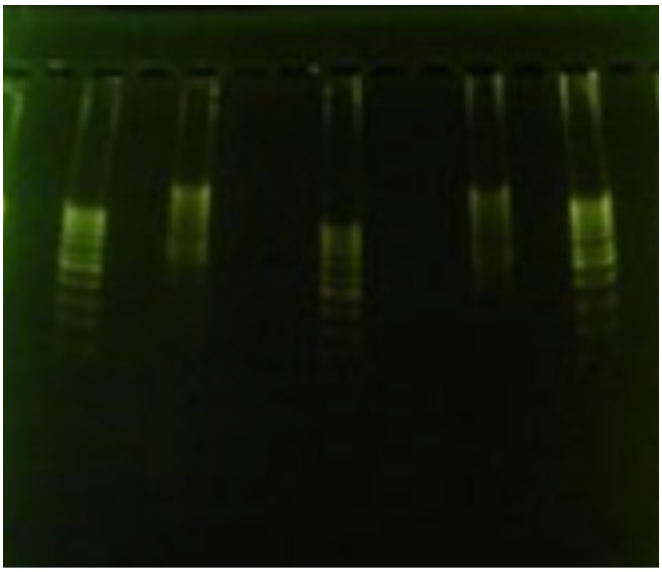

Figure 3.15: In ORT-PFGE the samples can be linearly separate after the electrodes were shortened and taken to the center of the edges. The example was 100 bases of ladder.

A new electrophoresis tank was designed and manufactured and the electrodes were placed to the corners in order to try various angle of the current. Numerous trials have been carried out involving numerous parameter changes, such as gel, electric field directions, and durations at different concentrations. When this parameter was changed, ORT-PFGE experiments were repeated once more by various combinations of the other parameters, current direction, duration and voltage. As a result of these experiments, it was observed that separation was best by 45° angle, 100 volts and 35 seconds in each direction (see Figure 3.16).

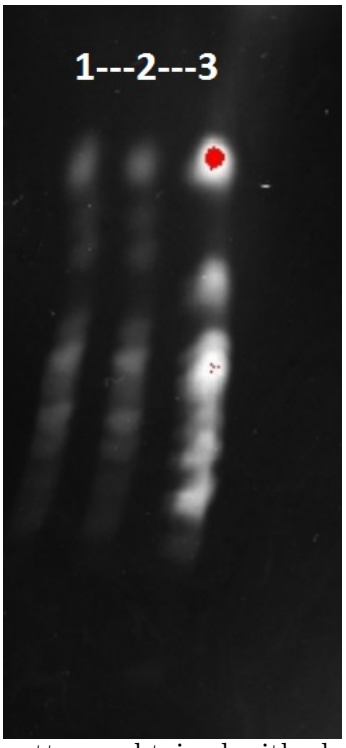

Figure 3.16: DNA fingerprint patterns obtained with electrodes placed in corners by 45° angle, 100 volts and 35 seconds in 4-6 direction.1 and 2 samples were stained with ethidium bromide, 3 was stained with SYBR Gold

3.4. Optimization of Chemical Gel Compositions for ORT-PFGE

In order to increase the separation power, gels containing agarose at different concentrations were assayed. 0.6 percent agarose gel was enabled best separation of DNA fragments (see Figure 3.17). When this parameter was changed, ORT-PFGE experiments were repeated by various combinations of the other parameters, current direction, duration and voltage described as in the previous section 3.3.

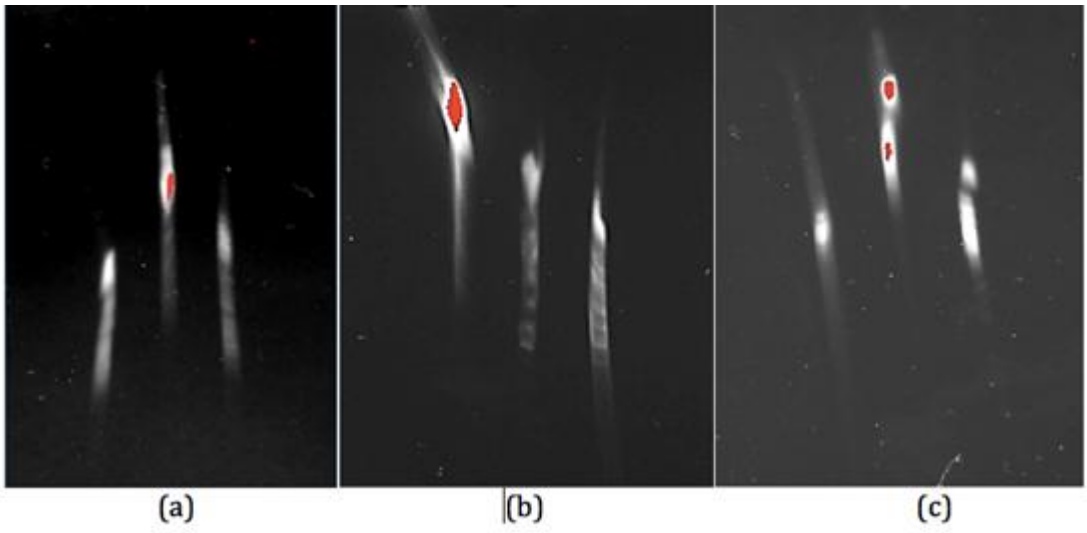

Figure 3.17: Separation studies using agarose at different concentrations: A: 0.5 percent agarose; B: 0.6 percent agarose; C: 0.7 percent agarose.

In an effort to improve the separation power we have tried changing the composition of the electrophoresis gel. Firstly, we tried to add ethylpropilecellulose, which is used to prepare soft gels in industry. We have tried gels with different proportions of agarose and ethylpropylcellulose. The effect of cellulosic was negative in the studies. A large number of trials were performed by adding agarose with high strength, melt agarose at low temperature and polyethylene glycol in the same way at different concentrations. The best results were obtained with gel containing 0.5 percent agarose, 1 percent polyethylene glycol (see Figure 3.18).

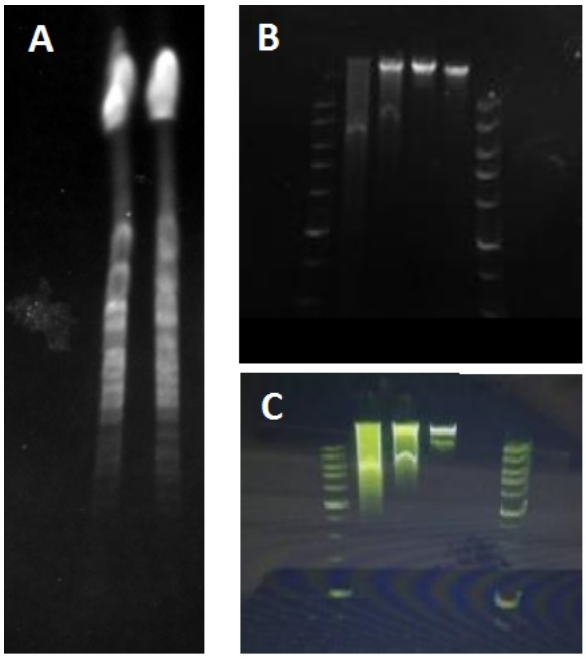

Figure 3.18: Separation studies using agarose, ethylpropilecellulose and polyethyleneglycole at different concentrations: Image A shows gel with 0.5 percent agarose and 1 percent polyethylene glycol, mage B and C shows 0.2 percent cellulose and 0.5 percent agarose. B: A photograph of the samples taken with ethidium bromide. c: A photograph of the samples taken under blue light.

The gel and the buffer solution were cooled and the electrophoresis was carried out in the cold room, but different results were not obtained from the experiments performed at room temperature, considering that the temperature might be an important parameter for increasing the separation power. In order to reach the classical PFGE level of separation power, it was possible to create electric fields in different directions, and the electrodes were moved to work by producing moving electrophoresis tank.

3.5. Effects of Melting of Discs on ORT-PFGE

The oval shape of the discs was caused sometimes the deformed shape of the image on the end of the experiment. For this reason, it is tried to load the samples liquid form. Until now, all studies used discs in its solid state. 20 μl of distilled water, 10 μl of loading dye and 1 μl of fluorescent dye were combined with a disk and heated at 70°C for 5 minutes. (see Figure 3.19)

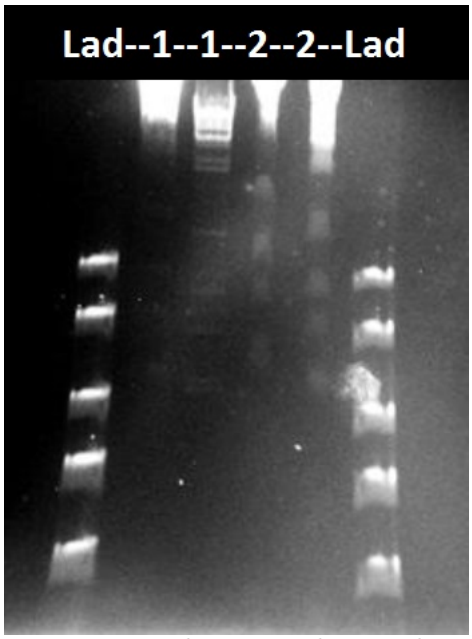

Figure 3.19: The 0.6 percent agarose gel contained same discs, 1:melting method and two other same discs, 2: that prepared with normal protocol.

3.6. Final Conditions

Standard PFGE method tried and 1% high stability agarose gel, in 0.5x TBE buffer with 6.0 volt/cm gel voltage for 35 seconds cross-sided, 5 seconds vertical at transitions and total running time for 23 hours were the best results giving parameters in standard PFGE. Totallab Image Analysis Software was choosen for dendrogram preperation.

According to dye optimization experiments, SYBR Gold was choosen for the fluorescent dye that is used in ORT-PFGE. According to optimization studies with ORT-PFGE, final design was carried out in 0.6% high stability agarose gel at 45° angle for 35 seconds and 100 volt in 4-6 directions (see Figure 2.7 and Figure 2.8) with melting discs.

4. DISCUSSION

PFGE is a very important molecular research and diagnostic technique used to separate very large DNA molecules from each other. However, it is not widely used because it is a cumbersome technique, which is difficult to implement, time consuming, requires well-trained personnel and expensive devices. In this study we have developed an observable PFGE device which has potentially several advantages over classical PFGE.

In order to develop observable PFGE we first set up standard PFGE for different groups of organisms, as the reference method. Carbapenamase producing gram negative bacilli, which have epidemiological importance in terms of causing hospital infections, were selected as the first group of organisms to be studied. Escherichia coli and Klebsiella pneumoniae collection strains that were previously isolated from patient samples, were included in the study. Many trials suggested that separation inside 1 percent high stability agarose gel, in 0.5x Tris Borat EDTA buffer with 0.5 Volt/cm gel voltage for 35 seconds cross-sided, 5 seconds vertical at transitions and totally for 16 to 22 hours, were the best result giving conditions in standard PFGE.

Next a series of new PFGE systems are designed and constnell in which DNA fragments can be visualized and recorded during the whole electrophoresis run. We have named this new method Observable real-time PFGE (ORT-PFGE). In this system the cancer-causing ethidium bromide and ultraviolet light (UV), which is hazardous both for eyes and DNA molecules, were replaced by blue light and fluorescent dyes.

4.1. Final Design

At the final design a new gel electrophoresis system that can work with 4 electrodes rather than 24. Since this system can be kept running at room temperature by air cooling and it enabled us to eliminate the requirement for a liquid cooling system, which is required in traditional PFGE.

In ORT-PFGE, photographs of the running electrophoresis gel can be taken with desired intervals; another gel documentation system for taking a picture of the gel is not needed. ORT-PFGE takes pictures at every stages of the run making DNA fingerprinting analysis easy by enabling to choose the best picture, which saves time and brings down the expenses.

ORT-PFGE eliminates the need for ultraviolet light that damages the DNA molecules separated during electrophoresis, and for carcinogenic etidium bromide by using DNA dyes that give fluorescence with blue light.

The development of the ORT-PFGE device and its methodology required testing of a large number of variables in combination, in a systematic approach. Variables included different gel compositions and substance concentrations, fluorescent dyes that make the DNA traceable during electrophoresis, different concentrations of these dyes, staining the discs or electrophoresis gels by these dyes, cycles of electrophoresis directions, angles of electric field directions, durations of steps in the cycle, the power of the electric field in each step.

A new electrophoresis system with 4 electrodes instead of 24 has been developed, a new gel that allows large DNAs to be separated at room temperature, and it has been understood that the cooling system required for classic PFGE is not required.

At ORT-PFGE, pictures of the gel are taken at desired intervals during the entire electrophoresis process. Photos showing the best resolution can be selected and used for the examination, and there is no need to analyze the gel with a separate gel documentation system and transfer of gel from one instrument to the other at the end of electrophoresis.

OPFGE is a system that reduces the cost and simplifies the DNA fingerprint examination. Melting of the disks before loading to the gel has been shown to be better than normal standard loading the discs without melting. It has been found that the results of the separation of DNA fragment, melted discs were much more successful

than solid-loaded discs.

4.1.1. Different Gel Compositions and Substance Concentrations

In order to increase the separation power, gels containing agarose at different concentrations were assayed. 0.6 percent agarose gel was enabled best separation of DNA fragments.

In an effort to improve the separation power we have tried changing the composition of the electrophoresis gel. Firstly, ethylpropilecellulose was tried, which is used to prepare soft gels in industry. The effect of cellulosic was negative in the studies. Later on experiments were performed by adding agarose with high strength, low melting and polyethylene glycol in the same way at different concentrations. The best results were obtained with gel containing 0.5 percent agarose, 1 percent polyethylene glycol. However, 0.6 percent agarose gel was better than the other composite gels.

4.1.2. Fluorescent Dyes that Make the DNA Traceable During Electrophoresis, Different Concentrations of These Dyes

In ORTE DNA molecules were stained with fluorescent dyes before loading into gel. This did not pose any problem in the separation of DNA molecules. Whereas in ORT-PFGE, fluorescent DNA stains inhibited the separation of molecules by electrophoresis when the samples were loaded into DNA molecules before gel loading. This was thought to be due to the large groupings of very large DNAs attached to the fluorescent dyes, so that the molecules cannot pass through the electrophoresis gel pores.

In standard electrophoresis, DNA molecules, usually up to 10 kilobases (kb) in size, are separated. However, the size of DNA molecules separated by PFGE is usually over 100kb. Since molecules as large as these cannot pass through the gel pores, molecules are separated by creating cyclic variable electric fields. Fluorescent dyes used to image DNA bind to DNA by electrostatic forces. The high concentration of dye may be the reason for aggregation of very large DNA by forming bridges between

the molecules. This can inhibit their passage through the gel pores. In our experiments it was seen that reducing the fluorescence dye concentrations facilitated the separation of DNA molecules by electrophoresis. However, reducing the amount of dyes excessively prevented the molecules from being observed during ORT-PFGE.

Numerous trials were conducted using different concentrations of dyes. When SYBR Gold and SYBR Green were studied, it was observed that the results obtained were more successful as the concentration decreased. Considering that increasing the dye concentration may bind DNA molecules to each other before electrophoresis and that the problem of blocking of separation may be caused by this, instead of putting the dyes onto discs, which were containing genomic DNA before electrophoresis, it was tried to put the dyes into the gel. Result was successful and good separation was achieved. Normally when we worked with ORTE device, very small amount of dye (1 l of 1/100 stock dye) was used when dyeing the sample. However, high amount of dye (25 l stock SYBR Green dye) was used when the high strength agarose gel was stained, which is also very expensive. That means it is possible to stain 2500 samples instead of 13 samples when the samples are stained instead of agarose gel.

It was decided to continue the work with SYBR Gold and SYBR GREEN, considering that Gel Green dye could not be used in the observable PFGE, because of its lower sensitivity.

4.1.3. Cycles of Electrophoresis Directions

In order to be able to reach the classical PFGE level of separation power, we have tried applying different directions of electric fields. To make this easy electrodes were made mobile on the electrophoresis tank.

First it was not possible to keep the current constant during electrophoresis. It was thought that the reason for this was the gas bubbles forming by the electrolysis, on the short platinum wires. In order to remedy this situation, the parts of the moving electrodes which extend into the electrophoresis buffer solution were extended to

increase their length by 3 times.

4.1.4. Limitations

For further development of the system, a variety of variable combinations may be tried, such as different gel compositions, dye concentrations, voltage used for separation, electric field direction. During the preparation of the disc, some errors may be occurring during preparation of buffer, enzyme, lysis or proteinase K solution. Some DNA dyes (SYBR Gold, SYBR Green) can disrupt the structure of the agarose disc. Because of the square-shape box, the way of electric or switch unit maynot be enough for the separation of agarose disc. Platin wire inside the box may not be long enough for electric current. High voltage may cause the increase in the temperature of buffer solution. Because of that a cooler may be needed to add to system. High-strength agarose may melt because of high temperature of the buffer in the box. Type of agarose can be changed during the experiments. The device need to be optimized further the technical feasibility of this technique is shown in this thesis. If the separation may be improved by these trials, we believe that ORT-PFGE, which has important advantages compared to the classical PFGE, can find widespread use.

5. CONCLUSION

With this thesis, a working prototype of an advanced PFGE (Pulsed Field Gel Electrophoresis) device was developed and called ORT-PFGE (Observable Real Time-PFGE). PFGE has an important role in the prevention of epidemics. However researchers do not prefer to use this device because of the difficulties. The ORT-PFGE system is important tool and is expected to find usages in areas such as DNA fingerprinting, determination of criminals, paternity testing. Urgenly needed progress in the prevention of epidemics requires more cost effective devices like the ones explored in this thesis work. ORT-PFGE has an original design, combining the electrophoresis and visualizing of gel together in the same device, which could save significant time and effort. New types of gels may still need to be developed further for ORT-PFGE, where the use of a carcinogenic dye, ethidium bromide could be eliminated. With the further development of the separation power of this ORT-PFGE system, it could be converted into a more practical device for several important applications.

APPENDIX A: Manufacturers of Utilized Chemicals

Molecular Weight Marker	Roche
Tris Base Ultra Pure	BioShopCanada
SmaI	EurX
XbaI	EurX
AGAROSE, High Gel Strength (PulseField)	BioShopCanada
EDTA Biotechnology Grade	BioShopCanada
Certified PCR LowMeltAgarose	BioShopCanada
CHEF Dna Size Standard, lambda ladder	Biorad
CHEF DNA Size Marker, S.cerevisiae	Biorad
Lyticase	SigmaAldrich
RnaseA	Thermofisher
Lysosyme	SigmaAldrich
Lysostaphine	SigmaAldrich
N-Lauroyl Sarcosine Sodium Salt	SigmaAldrich
SYBR Gold Flourescense Dye	Thermofisher
SYBR Green Flourescense Dye	Thermofisher
Gel Green Flourescense Dye	Biotium

APPENDIX B: Other Material Brands

5 mm diameter and 10000mcd power blue LED (LCB 12 WD)	SunLED
5 mm diameter and 10000mcd power blue LED (LCB 11 WD)	SunLED
Metal Box	Gl Pano
Camera	Orite
CCD camera	IRIS Digital Color
Camera Lens	IRIS Camera Lens
TV Card	AverMedia
Computer	Aidata
Electrophoresis Tank	Obitek EDU101, METU
Power Supply	Obitek Mini-Power100, METU
UV Transilluminator	BioRad

APPENDIX C: Solutions

1 M Tris Ph 8.0 (1 Liter)

Weight out 121.1 grams of Trizma base and add 800 ml of distilled H20. Adjust to pH 8.0 by adding approximately 42 ml of concentrated HCL. Adjust volume to 1 liter with distelled water.

0.5 M EDTA Ph 8.0 (1 Liter)

Weight out 186.1 grams of EDTA (disodium salt, m.w. = 372.2) and add 800 ml of distilled water. Stir and adjust to pH 8.0 with NaOH. Then add the rest of the distilled water until the volume reaches 1 liter.

PIV (500 ml): 10 Mm Tris Ph 8.0, 1.0 M NaCl

1 M Tris Ph 8.0	5 ml
Sodium chloride	29.2 grams
Add distilled water up to 500 ml	

EC solution (500 ml)

Final concentration	Stock solution to prepare 500 ml
6 Mm Tris Ph 8.0	1M Tris Ph 8.0 3ml
1 M NaCl	NaCL 29.2 grams
100Mm-EDTA Ph 8.0	0.5 M EDTA Ph 8.0 100 ml
0.2 percent Na-deoxycholate	Na-deoxycholate 1 gram
0.5 percent Na-laurylsarcosine	Na-laurylsarcosine 2.5 grams
Add distilled water up to 500 ml	

RNase A

Dissolve 30 mg of pancreatic RNase (Sigma) in 3 ml of water and boil at 100C for 15 minutes. Aliquot and store at -20C.

EC lysis solution (15 ml of EC)

Final concentration	to prepare 15 ml
50 μg/ml RNase A	10 mg/ml RNase A 75μl
100 μg/ml Lysozyme	20 mg/ml Lysozyme 75μl
50 μg/ml Lysostaphin	10 mg/ml Lysostaphin 75μl
Add 12.75 ml EC solution	

ES (500 ml)

Weight out 93.1 grams of EDTA (disodium salt) and dissolve in 400 ml distilled water. Then adjust the Ph to 9.0 by adding NaOH pellets (approximately 10 grams). Add 5 grams of sarcosyl. Adjust the volume to 500 mls with distilled water.

ESP (15 ml)

Final concentration	to prepare 15 ml ESP
1 mg/ml Proteinase K	15 mg Proteinase K
	15 ml ES

1 M Tris Ph 7.5 (1 liter)

Dissolve 121.1 grams of Trizma base in 800 ml distilled water. Add concentrated HCl until the Ph reaches 7.5. Adjust the volume to 1 liter with dH2O.

Tris-EDTA (TE) (1 liter)

Final concentration	Stock solution to prepare 1000 ml
10 Mm EDTA Ph 8.0	0.5 M EDTA Ph 8.0 2ml
Add distilled water up to 988 ml	

50x TAE Stock Buffer Solution (1 liter)

50x TAE Stock Buffer Solution (1 liter)

Final concentration	Stock solution to prepare 1000 ml
Tris base (2-amino-2-hydroxymethyl-propane-1,3-diol) (2 mole)	242 g
Glacial acetic acid (57.19 ml = 1 mole)	57.1 ml
0.5 M Na2 EDTA (ph 8.0)	100 ml
Add distilled water up to 1000 ml	

Final concentration	Stock solution to prepare 1000 ml
Tris base (2-amino-2-hydroxymethyl-propane-1,3-diol) (2 mole)	54 g
Boric acid	27.5 g
0.5 M Na2 EDTA (ph 8.0)	20 ml
Add distilled water up to 1000 ml	

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