

Verification of Pulsed Field Gel Electrophoresis (PFGE) Protocol for Molecular Typing of *Serratia marcescens*

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Abstract: *Serratia marcescens* has been described as an important cause of ocular infection with high incidence in contact lens-related keratitis. The only published method for molecular typing for *S. marcescens* is pulsed field gel electrophoresis. The aim of this study was to verify a protocol for PFGE for *S. marcescens*. Methods Fifty eight strains of multidrug-resistant *S. marcescens* were selected from a possible outbreak in a Hospital, Gainesville, Florida, USA. The PFGE protocol was performed according to Laboratory Manual of University of Health Network/Mount Sinai Hospital, Department of Microbiology, which included extra washing steps with preheated dH₂O and SWB with more incubation time at 55°C and using 5µl *Xba*I restriction enzyme for digestion for each strain. Results using 1µl *Xba*I enzyme and overnight incubation at 37°C gave unsuccessful results as lanes were smeared. Also using 5µl *Xba*I enzyme with overnight incubation gave the same unsuccessful results. While using 5µl *Xba*I enzyme with only 3hours incubation at 37°C gave good results as clear bands could be seen for all strains. Conclusion this protocol has proved its efficacy as the whole genomic DNA of all 58 selected strains of multidrug-resistant *Serratia marcescens* were seen as clear bands and no lanes were smeared.

Key words: *Serratia marcescens* • PFGE • Mount Sinai Hospital manual

INTRODUCTION

Serratia marcescens is a motile Gram negative bacillus shaped bacteria that belongs to the family Enterobacteriaceae. It was first thought to be non pathogenic and due to its ability to produce red pigmentation it was used as a marker in order to trace bacterial activity or transmission. However shortly after the famous Operation Sea Spray by the US Army, *S. marcescens* has been classified as an opportunistic human pathogen. Key characteristics of *S. marcescens* include the production of Dnase, lipase and gelatinase and it is oxidase negative. These bacteria grow well on standard media and produce a red to dark pink pigment that aids in identification [1]. *Serratia marcescens* frequently exhibits resistance to extended-spectrum β-lactams due to its ability to overproduce the chromosomal AmpC enzyme and to acquire plasmid-borne

extended-spectrum β-lactamases (ESBLs). Production of chromosomally encoded AmpC-type β-lactamase is the intrinsic mechanism of resistance to β-lactam antibiotics. *S. marcescens* generally has inducible expression of this enzyme and has been thought to be a natural chromosomal AmpC producer [2, 3].

S. marcescens are pathogenic and virulent because of their ability to adhere to host epithelial surfaces. Studies have shown that *S. marcescens* are capable of adhering to hydrocarbons and polystyrene allowing them to infect hosts by cell-surface hydrophobicity. Not only do these organisms adhere to biotic substrates but they are also found on abiotic substrates such as contact lenses. These organisms may possess pili that foster their adherence as well as an O-antigen that has a strong influence on the adhesion to abiotic and biotic surfaces. In particular, such interactions are mediated by large surface pili called type 1 fimbriae [4].

S. marcescens survives in environments and reservoirs such as drinking water pipes and hospital disinfectants as well as in medical instrumentation among other locations. There are many diseases that are associated with *S. marcescens*: sepsis, bacteremia, meningitis and cerebral abscesses, urinary tract infections, osteomyelitis, ocular infections and endocarditis. *S. marcescens* is also well known as a nosocomial pathogen and has been responsible for outbreaks particularly in critically ill neonates and patients in intensive care units. The biofilms produced are generally pathogenic in the body. Part of the problematic nature of this organism is its ability to colonize any surface. *S. marcescens* has been identified as the most common organism found in both the corneal scrapings and the contact lenses. *S. marcescens* has been described as an important cause of ocular infection with high incidence in contact lens-related keratitis [5, 6].

Endogenous endophthalmitis accounts for 10% of all endophthalmitis. Fungi are the most common causal pathogen followed by bacteria. *S. marcescens* is multiresistant Gram-negative bacillus that can produce a red pigment causing a pink hypopyon. As the incidence of endogenous endophthalmitis especially Gram-negative infections appears to be rising, then this aggressive organism may become a more common cause for this devastating condition [7].

Pulsed field gel electrophoresis (PFGE) is used extensively throughout the world as it is a simple and straight forward method of subtyping within a species. PFGE can be used to find similarities between isolates from patients and in the case of an outbreak to track the source as a mean to prevent further infection. A standard method (PulseNet USA protocol) is commonly used within health departments as it can be applied to many genera (Centers for Disease Control and Prevention, Atlanta, Georgia, USA, 2004). Occasionally non-typeable strains are seen and problems with *Serratia marcescens* have arisen before [8].

The primary aim of this study was to verify a PFGE protocol for typing of *Serratia marcescens* isolates

MATERIALS AND METHODS

This study took place in the Department of Pathology, Immunology and Laboratory Medicine, Emerging Pathogens Institute, University of Florida, Gainesville, FL, USA. It involved 58 selected strains of multidrug-resistant *Serratia marcescens* referred by the

University Hospital Gainesville Florida. The selected strains were originated from quite wide variety of samples. We followed the Laboratory Manual of University of Health Network/Mount Sinai Hospital, Department of Microbiology, (2006) to perform the PFGE of *Serratia marcescens* with very few modifications.

Four buffers were prepared: The first buffer was *Serratia* cell suspension buffer (SCSB) prepared by adding 10ml of 1M Tris pH 8.0 to 20ml of 0.5M EDTA pH 8.0 and dilute to 100 ml using ddH₂O. The second buffer was *Serratia* cell lysis buffer (SCLB) prepared by adding 93.05gms of 0.5M EDTA to 5.0gms of 1% Sarkosyl (N-Laurylsarcosine, Sodium salt) and 20.0gms NaOH pellets all dissolved in 400mls of DH₂O then pH was adjusted to 9-9.5 and volume was brought to 500ml to be stored at 4°C. The third buffer was *Serratia* wash buffer (SWB) which was the Gram negative wash buffer prepared by adding 20 ml of 1M Tris HCl pH 8.0 to 100 ml of 0.5M EDTA pH 8.0 and was made up to one Liter with ddH₂O and autoclaved. The fourth buffer was TE buffer prepared by adding 2 ml of 0.5M EDTA pH 8.0 to 10 ml of 1M Tris HCl pH 7.6 and made up to one Liter with ddH₂O and autoclaved.

We worked on 13 isolates at a time. Three to five colonies of *S. marcescens* were inoculated into 3 ml LB broth and incubated at 37°C with shaking for 3-4 hours. 1% SeaKem Gold agarose (0.1 gm SKG in 10 ml SCSB) was prepared and kept in water bath 55-60°C until use.

Every bacterial growth was poured in a glass tube and measured in a spectrophotometer to adjust its optical density (OP) to 1.00 (range of 0.8-1.0) and that was done by diluting 1ml growth culture with 1.5-2ml LB broth, cultures were kept in ice during work. The adjusted cultures were transferred to the corresponding sterile labeled microcentrifuge tubes 1.5 ml and centrifuged at 13,000 rpm for 2 min to pellet. The pellet was dissolved into 200µl SCSB and vortex.

For casting the plugs, every isolate was to be processed alone and quickly by adding 10µl Proteinase K (20mg/ml) to the 200µl bacterial suspension and mixed by gently pipetting then 200µl SGA gel was added to the tube and gently pipetting the mixture up and down 2-3 times for mixing and immediately with the same pipette 200µl of the mixture was dispensed into the small disposable plug mold till it was completely filled with no bubbles, two plugs for each isolate could be made. Then the molds were placed in refrigerator at 4°C for 10-15 min to solidify the plugs.

50 ml Falcon tubes were labeled with the isolate number, each filled with 2ml lysis sol. (SCLB) and 40µl PK into which the corresponding 2 plugs would be immersed and that was done by placing the plug mold on top of the tube after removing the tape from its bottom and the plug was pushed out by inserting the plunger into the well to dispense the plug. Then tubes were placed in a rack and incubated in H₂O bath at 55°C overnight with the level of H₂O was above the level of lysis buffer in the tubes. Washing steps was taking place on the second day; the Falcon 50 ml tubes were removed from the water bath and the blue caps were replaced by appropriately labeled green screened screw caps. The lysis solution was decanted completely and the edge of the green cap was touched with absorbent paper towel to ensure complete removal of liquid in this step and subsequent wash steps. First wash was with 25-35ml pre-heated (55°C)(dH₂O added to each tube and invert sideways to rinse through then the rack was placed in a shaker water bath at 55°C for 15 min then water was decanted from all tubes. Again the wash step was repeated exactly. The third wash was with 15ml pre-heated SWB and also incubated in the shaker water bath at 55°C for 15 min. Then SWB was decanted from all tubes and another 15ml pre-heated SWB was added for the fourth and final wash step which lasted for 2 hours in the shaker water bath at 55°C. (every time when tubes were in the shaker they were covered with both the blue caps above the green ones and when decant the wash the blue caps were removed). After decant of SWB a 5ml TE buffer was put into each tube where plugs were completely immersed then the green caps were replaced by the blue ones to be kept in refrigerator at 4°C till next day when the step of restriction digestion of DNA in agarose plugs with *Xba*I was proceeded, pre restriction incubation step was done; restriction buffer 10X was prepared by adding 180µl sterile dH₂O to 20µl R buffer thus 200µl was put into each labeled 1.5ml microcentrifuge tube. The plug was taken out of the TE buffer with spatula placed in a sterile Petri dish and were cut into 2 halves with razor blade and transferred into the corresponding labeled microcentrifuge tubes containing diluted RB to be incubated in a 37°C water bath for 15 min. The restriction enzyme master mix (Enzyme/10Xbuffer mix) was prepared for all isolates in one tube, calculations were done as 5µl *Xba*I restriction enzyme (New England BioLabs, USA) for each isolate added to 175µl H₂O and 20µl RB. For 13 samples; 65 µl *Xba*I enzyme was added to 2275µl H₂O and 260µl restriction buffer. Total volume was 2600µl in one 3ml tube. The microcentrifuge tubes were taken out from

incubation, the buffer was aspirated using 200µl pipette and decanted. 200µl restriction enzyme master mix was added to each tube with gentle tapping to be sure that plug halves were under enzyme mixture then incubated in 37°C water bath for 3 hours. During that incubation period we prepared 0.5X Tris-Borate EDTA buffer (TBE) needed for stopping reaction and for preparing SKG agarose gel also 10X TBE needed for electrophoresis running buffer. 1% Seakem Gold (SKG) agarose in 0.5X TBE was prepared for 14-cm-wide gel form (15 wells) by dissolving 1gm SKG agarose into 100 ml 0.5X TBE which was prepared by adding 5 ml of 10X TBE to 95ml dH₂O. Also small volume of melted 1% SKG agarose was needed for sealing the wells and prepared by dissolving 0.1gm SKG agarose into 10 ml 0.5X TBE which was prepared by adding 0.5 ml of 10X TBE to 9.5ml dH₂O to be kept in 55°C-60°C water bath until used. The gel form (casting stand) was perfectly leveled on leveling table and the front of comb holder and teeth was facing the bottom of gel frame with the comb teeth were touching the gel platform. The agarose was poured carefully and left for 30 min to solidify. During that time the electrophoresis chamber was prepared ; the black gel frame was placed into it and 2200ml buffer was freshly prepared and added (110ml 10X TBE was completed with dH₂O to 2200ml), 3 buttons of power supply, pump and cooling module (14°C) were turned on and the program was entered by pushing two state button to select gradient 6 and entered, run time 18 hours were selected and entered, included angle, 120, initial switch time 2.16 sec, final switch time 54.17 sec, entered ramping factor showed linear and machine was ready for start run. After 3 hours incubation, enzyme/buffer mixture was removed and 200µl 0.5X TBE was added to each tube and incubated at room temperature for 5 min to stop reaction. Comb was removed. plug halves of the first sample was transferred into sterile Petri dish, excess buffer was removed with tissue, selected half was put on the tapered end of a spatula to be applied inside the well and with the help of another spatula the plug was loaded to fit inside the well and was gently pushed to the bottom and front of the well with the wide end of the spatula with no bubbles. Loading of samples started from the second to the 14th well, while the first well and the last one (No 15) were loaded with Lambda Ladder PFG Marker (New England BioLabs, USA). After loading all the 15 wells they were sealed with the prepared melted 1% SKG agarose using pipette and allowed to harden 2-3 min then excess agarose was removed from front and back of wells using a blade and spatula. Unscrew to remove end gates from gel form

was done and excess agarose was removed from sides and bottom of casting platform with tissue. The loaded casted gel was kept on casting platform and carefully placed inside black gel frame in electrophoresis chamber, cover was closed and button start run was pushed. The fourth day when electrophoresis run was over, the equipment was turned off and the gel with the black base was taken out for staining by sliding the gel into a Pyrex tray with 400 ml distilled H₂O to which 40µl ethidium bromide was added. The tray was covered with aluminum foil and put onto the shaker for 30 min at room temperature. Then the gel was washed by sliding it into another tray filled with dH₂O twice and then the gel left into dH₂O, covered and put onto shaker for 15 min. Washing was repeated again and gel was left covered with dH₂O for another 15 min. Finally the gel was photographed under ultraviolet light using Gel Doc XR+(BIO-RAD, USA).

RESULTS AND DISCUSSION

In this study two trials with modifications regarding restriction digestion step was done the first trial included using 1µl *XbaI* enzyme and overnight incubation at 37°C and that gave unsuccessful result as lanes were smeared. In the second trial we used 5µl *XbaI* enzyme with overnight incubation at 37°C and also we got the same unsuccessful result as shown in (Fig. 1). Finally we used 5µl *XbaI* enzyme with only 3hours incubation at 37°C and results were satisfying as clear bands could be seen for all 58 *Serratia marcescens* strains (Figs. 1-5).

Although the discriminatory power of conventional pulsed field gel electrophoresis (C-PFGE) profiling is high some drawbacks were reported and encountered such as its laborious and time-consuming steps, as well as the extended time needed to complete the procedure to overcome these drawbacks Alaidan *et al.* [10] applied modifications to the typical protocol previously reported by Alfizah *et al.* [11] their modifications included reducing the incubation period of lysis, washing and digestion by restriction enzymes resulting in a rapid procedure in two-day. PFGE protocols with *S. marcescens* generally use 18 to 24 h for cell lysis and to shorten the lysis time, 40µl of freshly prepared lysozyme (10mg/ml) were mixed with the 40µl of proteinase K (10mg/ml) and added to the bacterial cell suspensions prepared directly from the colonies and immediately incubated at 37°C for 15 min before mixing with LMP agarose. Additionally using a time of 150 min with higher incubation temperature 55°C

to facilitate cell lysis resulted in reduction of lysis time described in the C- PFGE protocol. They also modified the washing step by using preheated sterile water and TE buffer, the washing time was reduced to 75 min. Restriction enzyme digestion was also shortened from a conventional overnight incubation to 4 h or less without increasing the amount of enzyme used which was 50 U of *SpeI*.

However this rapid protocol described by Alaidan *et al.* [10] was performed earlier by our team and results were not satisfying (data not shown).

O'Reilly [8] tried some modifications with PFGE standard method (PulseNet USA protocol Centers for Disease Control and Prevention, Atlanta, Georgia, USA, 2004) He tried different methods to counter the autodegradation with *S. marcescens* isolates as doubling the amount of thiourea in the running buffer which stopped the DNA degradation of one third of the isolates then better improvement was obtained when the cell suspensions were heated before processing. The cell lysis buffer was made with no Tris and were either 50 mM or 100 mM EDTA. The final successful method with all isolates was used 16 h cultures and suspended the bacteria in ice cold 100 mM EDTA before heating them to 75°C for 10 min. Then the run with 20 ml of 50mM thiourea in 2.5 l of TBE. The restriction enzyme used for digestion was *XbaI*. He concluded that this modification is easy to perform in laboratory that routinely uses PulseNet protocol because it only uses one extra solution and one extra step.

PFGE typing was performed by Miranda *et al.* [12] to analyze an outbreak in a neonatal intensive care unit (NICU), PFGE of whole-cell DNA digested by *SpeI* was used as a marker of strain identity.

Also Shi *et al.* [13] reported that minimal cutting of *S. marcescens* DNA by *XbaI* and overactive cutting of *S. marcescens* DNA by *DraI*, *SspI*, *ApaI*, *SmaI* and *SfiI* were found (data not shown). *SpeI* was found to be the most suitable restriction endonuclease for digestion of *S. marcescens* DNA in PFGE typing in their study.

And Fleisch *et al.* [14] in their study to investigate three consecutive outbreaks of *S. marcescens* in neonatal intensive care unit, where lysis of bacteria embedded in agarose by lysozyme and proteinase K followed by digestion of chromosomal DNA with infrequently cleaving restriction endonucleases *SpeI*.

PFGE plugs containing whole genomic DNA of meropenem resistance *S. marcescens* isolates were digested with *SpeI* and *S1* nuclease, respectively by Suh *et al.* [2].

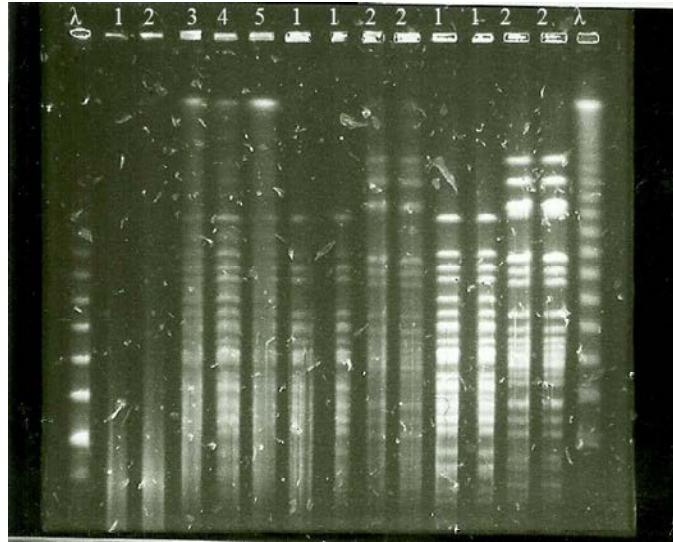


Fig. 1: Sm PFGE 2014-06-13 13hr 53min

Lanes from 1-5 were *Serratia marcescens* strains from No. 1 to No. 5 included in the first trial where restriction digestion was done using 1µl *Xba*I enzyme for overnight incubation at 37°C.

The next four lanes were for repeated strains No 1 and No 2 using 5µl *Xba*I enzyme for overnight incubation at 37°C.

The last four lanes were for repeated strains No 1 and No 2 using 5µl *Xba*I restriction enzyme with only 3hours incubation at 37°C.

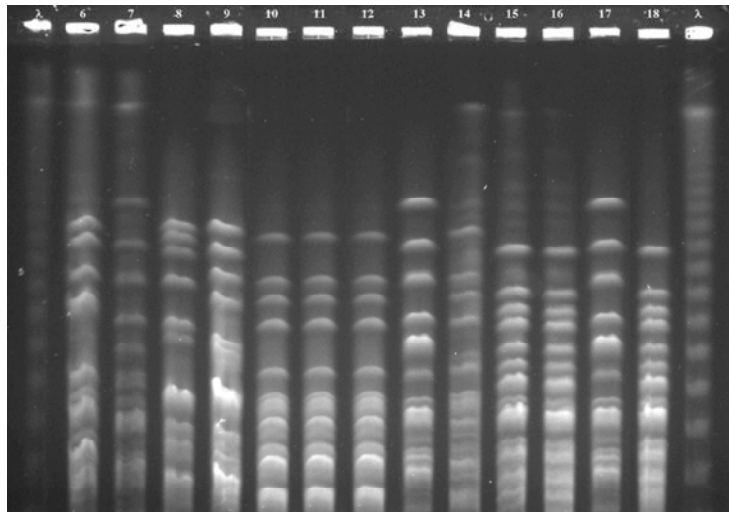


Fig. 2: Sm PFGE 2014-06-26 13hr 59min. From strain No. 6 to No 18

Isolates were considered to be the same strain if all bands matched, to be subtypes of the same strain if one to three bands differed and to be different strains if more than three bands differed [9]

Naumiuk *et al.* [15] reported *Serratia marcescens* to be a significant agent of hospital infections that mainly affects patients in neonatal and surgical units and

patients in intensive care units (ICUs) however PFGE typing was performed using *Xba*I restriction patterns of the isolates' genomic DNAs.

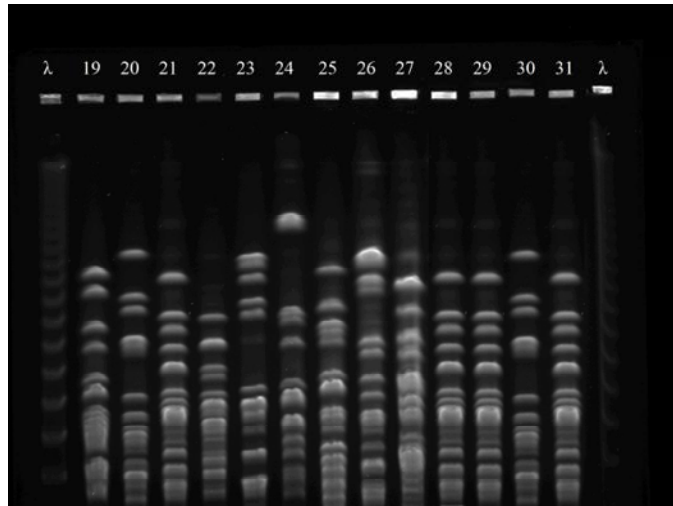


Fig. 3: Sm PFGE 2014-07-17 13hr 38min. From strain No.19 to No 31

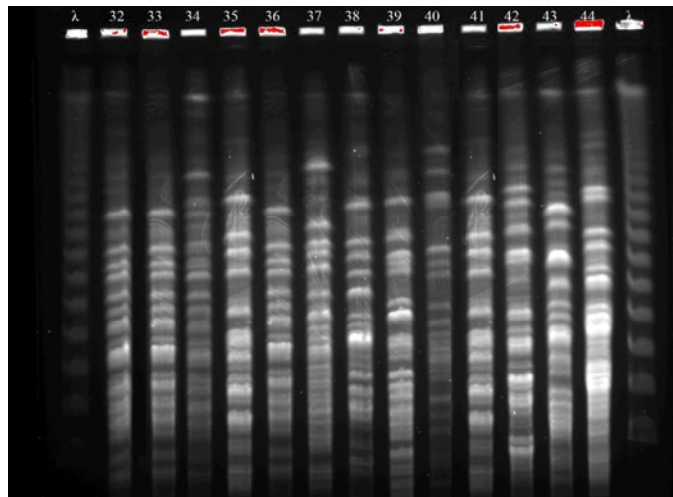


Fig. 4: Sm PFGE 2014-07-24 13hr 20min. From strain No. 32 to No. 44

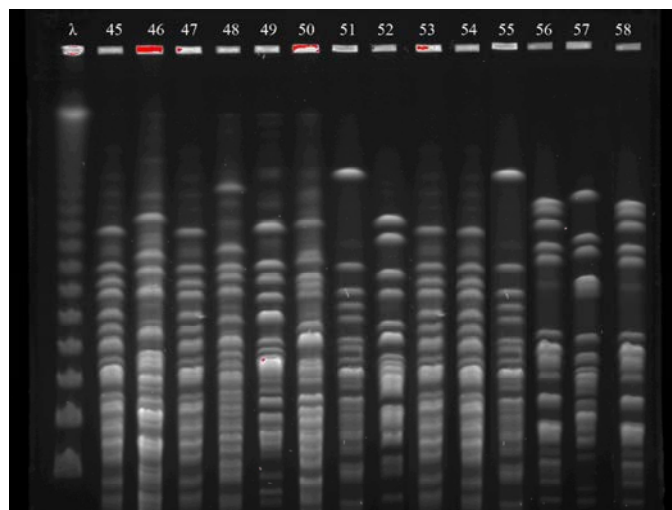


Fig. 5: Sm PFGE 2014-08-02 15hr 58min. From strain No 45to No. 58

CONCLUSION

PFGE protocol in this study included four washing steps and digestion of the genomic DNA by the restriction enzyme *Xba*I. This protocol proved its efficacy as the whole genomic DNA of all 58 selected strains of multidrug-resistant *Serratia marcescens* were seen as clear bands and no lanes were smeared.

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