ITALIAN JOURNAL OF PUBLIC HEALTH

Rapid pulsed-field gel electrophoresis protocol for *Legionella pneumophila* typing

Massimiliano Orsini[1], Rosarita Amore[2], Bianca Maria Pietrangeli[3], Daniela Anastasi, Walter Ricciardi[2], Stefania Boccia[2]

[1]CRS4 Bioinformatica, Parco Tecnologico, Pixina Manna, Pula; [2]Institute of Hygiene, Università Cattolica del Sacro Cuore, Roma; [3]Department of Environmental Hygiene, ISPESL, Roma, Italy

Correspondence to: Stefania Boccia, Genetic Epidemiology and Molecular Biology Unit, Institute of Hygiene, Università Cattolica del Sacro Cuore, Largo Francesco Vito 1, 00168 Rome, Italy. E-mail: sboccia@rm.unicatt.it

Abstract

Background: Genomic DNA patterns generated by pulsed-field gel electrophoresis (PGFE) are highly specific for different strains of an organism and have significant value in epidemiologic investigations of infectious disease outbreaks. A disadvantage of PFGE is that the procedure requires up to 6 days to complete.
Methods: We developed a rapid PFGE protocol for subtyping *Legionella pneumophila* isolates based on the standardized protocol currently used. Various combinations of reaction conditions (e.g., lysis time and temperature, restriction enzyme concentration) and electrophoresis parameters were applied to devise a simple and rapid PFGE protocol that could also be used for frozen bacteria.
Results: PFGE analysis of *Legionella pneumophila* isolates can be completed in 26 hours using this protocol compared to 6 days for the conventional one.
Conclusions: We successfully applied a rapid PFGE protocol for *Legionella pneumophila* typing and comparison of the patterns obtained from the rapid compared with the conventional method showed that the rapid protocol gave identical and highly reproducible results.

Key words: legionella pneumophila, molecular typing, PFGE.

Introduction

Legionella pneumophila is widespread in aqueous environments and a common cause of nosocomial and community-acquired pneumonia. An accurate discrimination between

L. pneumophila isolates is important in epidemiological investigations for identifying common-sources cases, routes of diffusion, and bacteria distribution in water networks [1,2]. The most commonly used techniques that allow for bacteria typing are based on the detection of genomic DNA polymorphisms [3]. A number of methods have been validated, including monoclonal antibody typing, plasmid analysis and multilocus enzyme electrophoresis [1,4]. More accurate techniques based on genomic DNA polymorphisms have been developed in the last decades, such as pulsed-field gel electrophoresis (PFGE), ribotyping, arbitrarily primed polymerase chain reaction (AP-PCR), restriction fragment length polymorphism analysis (RFLP), and more recently amplified fragment length polymorphisms (AFLP) [5,6,7,8]. Currently AFLP is the reference method for *L. pneumophila* Serogroup (Sg) 1 genotyping [8], although PFGE of DNA macrorestriction digests remains the most used due to its high discriminatory power [9,10]. PFGE protocol involves DNA extraction from bacteria embedded in a gel, then the use of rare cutter restriction enzymes to generate a limited number (10 to 20) of high-molecular weights restriction fragments. These fragments are then separated by agarose gel electrophoresis using programmed variations in both the direction and the duration of the electric field (the 'pulsed field'). Nevertheless the time-consuming steps (6 days) of the current PFGE protocols for L. pneumophila [6] often preclude its use in monitoring the events during ongoing outbreaks.

In the last few years several authors have published rapid PFGE protocols for genotyping different bacteria species [11,12,13,14,15,16], however these protocols have never been applied to *L. pneumophila*. Here we describe the use of a new developed PFGE protocol for genotyping of *L. pneumophil*a isolates in less than 26 hours against 6 days of a traditional protocol [6]. In this paper we detail the workflow of the three main steps of

the rapid PFGE protocol by detailing: 1) DNA extraction procedure, 2) DNA restriction digestion, and 3) pulsed-field gel electrophoresis parameters. Each strain was processed in parallel with the rapid and the traditional protocol and results compared. The rapid protocol was also tested using frozen *L. pneumophila* as a starting sample.

Materials and methods

Six well-characterized isolates of *L. pneumophila* Sg1 were randomly selected among those collected during an hospital surveillance program in Rome [17] and processed with a traditional PFGE protocol [6] and the rapid one.

1-a) Rapid DNA extraction procedure.

A small loopful from three additional *L. pneumophila* isolates, grown on buffered charcoal-yeast extract agar (BCYE), was picked up using a sterile cotton swab from surface of culture plates and directly suspended in 500µL of PBS in 1.5mL conical bottom tub. In parallel, a small loopful of bacteria cells was picked-up from three frozen samples and transferred to a 1.5mL screw-capped tube containing 1mL of Phosphate Buffered Saline (PBS). In order to remove media traces, the cell suspension was inverted and centrifuged twice at 16,750X g for 3 min in a microcentrifuge.The pellet was then resuspended in 500µL of PBS (Figure 1) in 1.5mL conical bottom tube. The bacterial suspensions were all adjusted to 0.8 optical density (600nm) in PBS, using a spectrophotometer (Biophotometer, Eppendorf) and 500µL were then transferred into a new 1.5mL screw-capped tube. The lysis of bacteria cells was conducted testing different concentration of proteinase K and incubation times then detailed. Ten microliters of Proteinase K were directly added to the samples, before plug preparation, to a final concentration of 0.4 and 1.0 µg/µl, then gently mixed by inverting the tubes. Plugs were immediately prepared by adding to these samples one volume (nearly 500µL) of melted 1.8% pulsed-field agarose gel (BIO-RAD) in TE buffer 1X (10 mM Tris, 1 mM EDTA [pH 7.6]). Samples were then mixed and dispensed into a plug mold (BIO-RAD), which contains 100µL of suspension for each plug (in this way several plugs can be prepared for each sample). After solidification (4°C/5 min) plugs were transferred from the mould into a 2 mL conical bottom tube (two plugs for each tube) containing 1 mL of lysis buffer (10 mM Tris, 1 mM EDTA, 100mM NaCl, 1% Sarkosyl) and placed in a thermomixer (Eppendorf) at 55°C/600 rpm. Different incubation times were tested (15, 30, 60 min). Proteinase K was inactivated by thermal shock (80°C/15 min without shaking), thus avoiding use of hazardous material (phenylmethylsulfonyl fluoride, PMSF) (8). Lysis solution was then removed using a micropipette and 1mL of pre-warmed bidistilled water was added to the plugs. The tubes were incubated for 15min/50°C at 600 rpm in a thermomixer, then the water was removed and the washing step repeated twice using 1mL of TE 0.1X (50°C/10 min at 600rpm).At the end of these steps, the plugs were transferred in a clean 15 mL tube containing 5mL of fresh TE 0.1X for their storage at 4°C until restriction digestion.

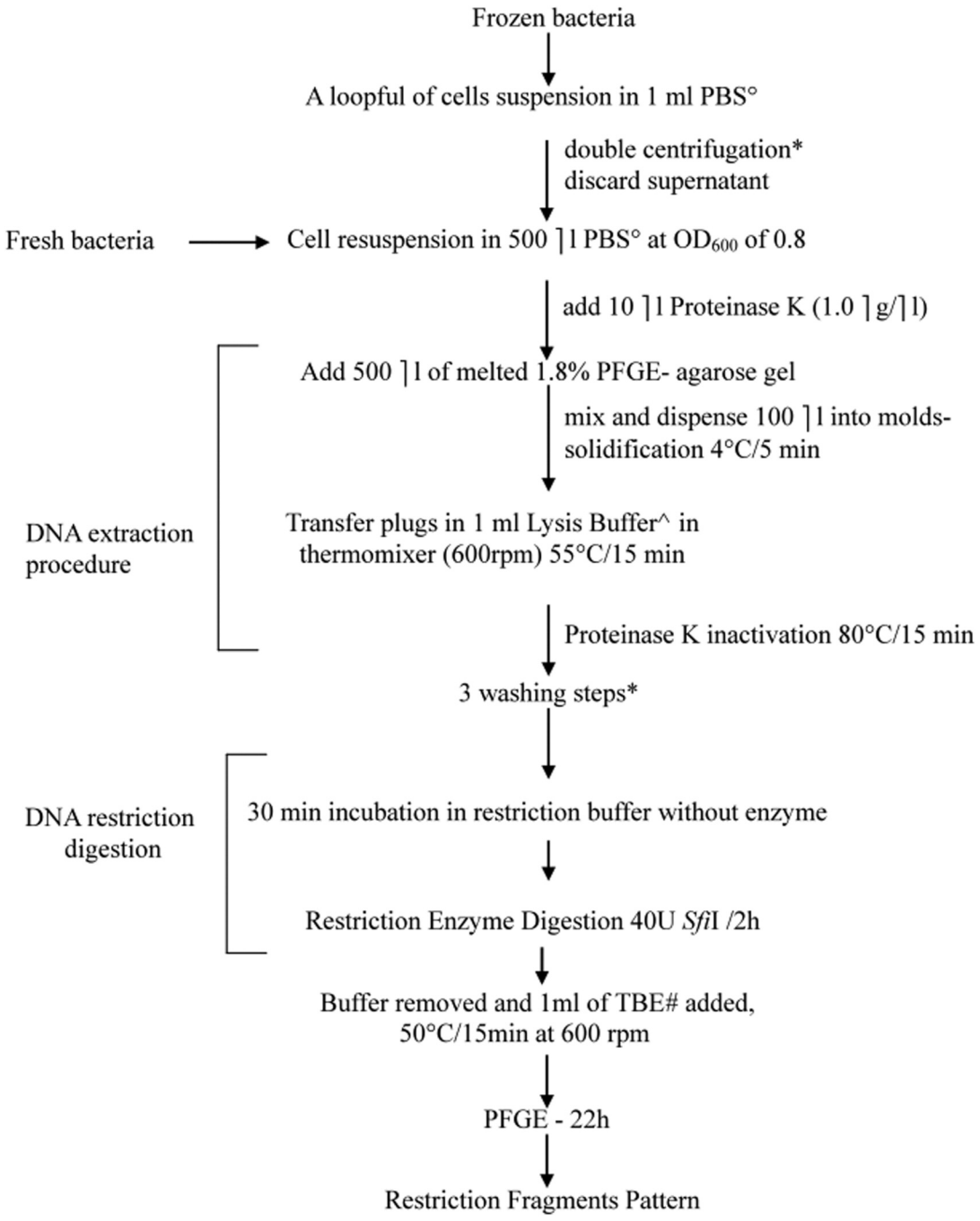
1-b) Traditional DNA extraction procedure.

A small loopful of bacteria cells was picked-up from three frozen samples and transferred to a 1.5mL screw-capped tube containing 1mL of Phosphate Buffered Saline (PBS). In order to remove media traces, the cell suspension was inverted and centrifuged twice at 16,750X g for 3 min in a microcentrifuge. The pellet was then resuspended in 1mL of Pett IV buffer (1.0 NaCl, 10mM Tris-HCl [pH 7.6]) in 2mL conical bottom tube. *L. pneumophila* cells of other three bacterial isolates grown on BCYE agar were picked up, harvested and resuspended in 1mL of Pett IV buffer in 2mL conical bottom tube. An equal volume of pulsed-field agarose gel was added to the suspension, mixed and pipetted into a plug mold and then allowed to solidify on ice. For lysis, the plugs were incubated at 37°C with gentle shaking in 2 volumes of EC lysis buffer (6mM Tris-HCl, 100 mM EDTA, 0.5% Brij 58, 0.2% deoxycholate, 0.5% N-lauroylsarcosine, 1mg of lysozyme per mL, 20 µg RNase per ml [pH 7.6]) per volume of plug. Following overnight incubation, the plugs were transferred to ESP (0.5M EDTA [pH 8.0], 1.0% N-lauroylsarcosine, 1mg PK per mL) and were incubated for 48 h at 50°C with one change of ESP at 24 h.To inactivate the PK, plugs were incubated in 10mM Tris-0.1 mM EDTA-1.0 mM PMSF (pH 7.5) overnight at 37°C. The plugs were then washed twice for 2 h each with TE 0.1X and stored at 4°C.

2-a) Rapid DNA restriction digestion.

For restriction analysis a plug slice of 2 to 4 mm wide was equilibrated in a 2mL conical bottom tube with 300 µL of 1X restriction buffer supplied by the enzyme manufacturer at 37(C/30 min. Buffer was removed and DNA digested using SfiI enzyme (Promega). Different concentrations of SfiI enzyme were tested (10U, 20U and 40U) in a final volume of 100µL. Tubes were placed in a

Figure 1. Schematic representation of the main steps for rapid DNA preparation of *L. pneumophila* for Pulsed Field Gel Electrophoresis (PFGE).



thermomixer at 50(C/2 h at 300 rpm (according to manufacture instruction). Buffer solution was removed using a micropipette and 1mL of 0.5X Tris-Borate-EDTA buffer (89mM Tris-HCl, 89 mM boric acid, 25 mM EDTA pH8.0) was added to the plug. Tubes were incubated 15 min/50°C at 600 rpm in a thermomixer. Plugs were ready to be sealed into 0.8% pulsed-field agarose gel.

2-b) Traditional DNA restriction digestion.

A 2-mm square of the plug was placed in a 2mL conical bottom tube with of 100µL of 1X restriction buffer supplied by the enzyme manufacturer.After 1 h equilibration at 37°C, 5U of SfiI was added and the tubes incubated at 50°C overnight. Buffer solution was removed using a micropipette and 1mL of 0.5X Tris-Borate-EDTA (TBE) buffer (89mM Tris-HCl, 89 mM boric acid, 25 mM EDTA pH8.0) was added to the plug. Tubes were incubated at 50°C/15 min at 600 rpm in a

thermomixer. The plugs were then loaded into 0.8% pulsed-field agarose gel.

3-a) Rapid PFGE of DNA. Electrophoresis parameters were optimized using the autoalgorithm on CHEF MAPPER III apparatus (BIO-RAD) setting a range of expected fragments of 40-600 kb. Fragments were separated in a 0.8% agarose gel (0.5X TBE buffer), with running conditions of 200V for 22 h at 14°C with switch times of 1s (initial) and 54 s (final). Gels were stained in ethidium bromide (0.5 µg/mL for 15 min), destained in water and photographed by Polaroid 667 film under UV illumination.

3-b) Standard PFGE of DNA. Fragments were separated in a 1% agarose gel (0.5X TBE buffer), with running conditions of 200V for 24 h at 14°C with switch times of 7s (initial) and 74 s (final).

Results

Comparisons of PFGE patterns produced by the conventional and rapid method provided indistinguishable results for the six strains tested, as shown in Figure 2. The optimization of the three steps revealed that:

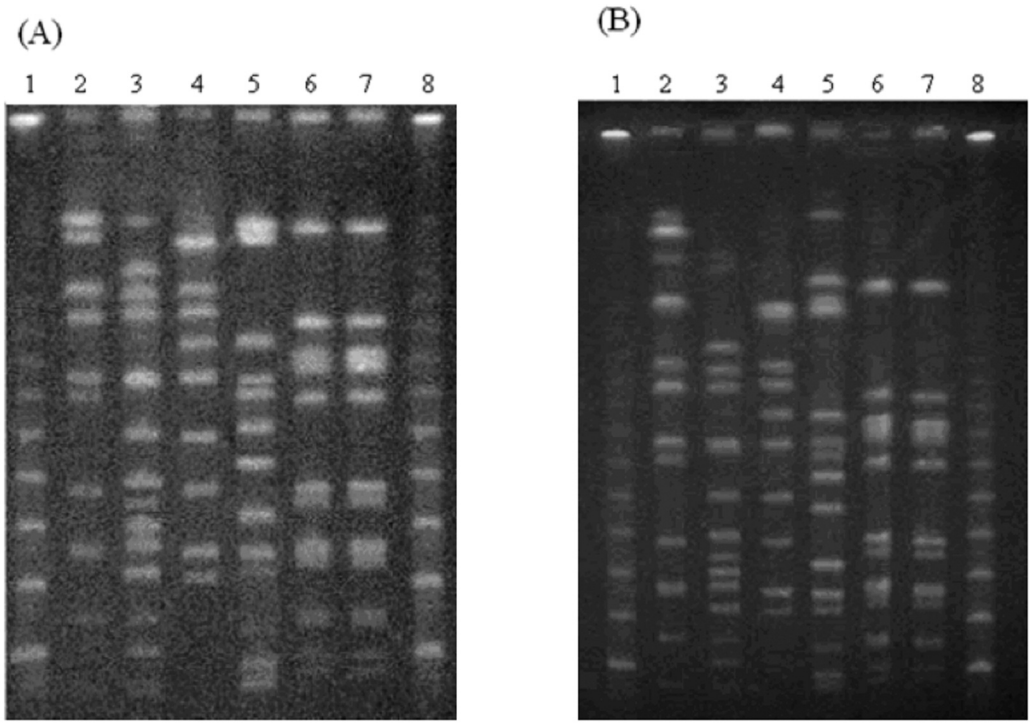
Rapid DNA extraction procedure: no major differences were observed in a different range of PK concentration and incubation time spanning from 15 to 60 minutes, thus we suggest to incubate the plugs 15 minutes in lysis buffer using a PK concentration of 1.0 µg/µl.A brief incubation time seems to have a better perform, probably due to a lower quantity of DNA available for the digestion process. In fact, when the incubation time was protracted for 60 min, exceeding DNA remains indigested at the up side of the lane and partially digested fragments appear (data not shown).

Restriction DNA digestion: different enzyme units do not appear to affect restriction performance, however partially digested fragments at lower concentrations (10 or 20 U) were appreciated, so that we suggest to use 40U enzyme concentration.

Rapid PFGE of DNA: a typical electrophoresis profile for an optimal separation of fragment ranging from 40 up to 500 kb require 26 h of running time (0.8% agarose gel, 24-27 hours are required using a 1.0% agarose gel), however when rapid results are required these parameters allow the electrophoresis to be already stopped after 22 hours (Fig.2-A). In the Fig. 2 differences in the

Figure 2. Pulsed Field Gel Electrophoresis of Sfi I digested chromosomal DNA of six *L. pneumophila* environmental isolates extracted and processed by (A) rapid and (B) standard method.

Lanes (A and B panels): 1, 8, Lambda ladder marker (New England, Biolabs); 2-3, *L. pneumophila* isolates processed directly from frozen samples; 4-7, fresh cultured *L. pneumophila* isolates.



duration of the electrophoresis run may be appreciated: in the panel B the DNA fragments were separated in a 0.8% gel for 24 hours while in the panel A identical samples were resolved in 22 hours. It is evident that in the panel B the fragments can be more clearly detected and analyzed, while in the panel A the pattern result more packed (especially for high molecular size bands), nevertheless genetic relationship among isolates can be well established and this is the main goal of a PFGE protocol.

Discussion

Legionellosis accounts for 2-15% and 10-25% of nosocomial and community acquired pneumonia with a mortality rate of 20% and 40%, respectively [18]. The availability of a rapid and reliable method for genotyping *L. pneumophila* isolates is often required for determining the relatedness of the isolates collected during an outbreak investigation, or as a part of a nosocomial surveillance program. PFGE has a high reproducibility, discriminatory power and ease of interpretation, however it requires several days to complete [6]. Although rapid PFGE protocols have been developed for several bacteria species [11,12,13,14,15,16], the procedure here reported is the first we know to be optimized especially for *L.pneumophila* bacteria, also working directly from frozen samples.

We shortened the standard PFGE assay primarily by 1) utilizing directly frozen bacteria, 2) reducing the time allowed for all enzymatic reactions, a modifications employed by others to shorten similar procedures [16,12], 3) inactivating the PK using a thermal shock instead of incubation with PMSF overnight, and 4) shortening the run time to 22 hours (from 24 hours). From our point of view one of the key parameters appear to be the rate between the DNA available for the restriction process and the quantity of enzyme, with best results obtained by incubating samples for 15 min in lysis buffer (PK 1.0 µg/µl) and then digesting them with 40U of restriction enzyme when shaking. One more important modification with respect to the standard protocol was to change the switch times and gel concentration, so that the electrophoresis run can allow a clear separation of the DNA fragments in 22 hours. Other rapid PFGE protocols have been shortened by eliminating PK digestion, but we were unable to obtain satisfactory results when applying this modification. Some authors [16] also shortened PFGE procedure by electrophorese DNA in agarose gel with ethidium bromide, thus eliminating the need to stain and destain the gel. We did not prefer to shorten the protocol at this point just for keeping the PFGE apparatus not contaminated.

In conclusion, this report describes a rapid PFGE protocol for *L. pneumophila* typing directly from frozen samples in less than 26 hours. Some critical steps in the rapid protocols are described and discussed, with the best parameters clearly indicated. This approach has broad indications for both epidemiologic investigation and during ongoing outbreaks for the rapid comparison of stored bacteria with fresh ones implicated in the epidemic event.

References

1) Luck PC, Helbig JH, Gunter U, Assmann M, Blau R, Koch H, Klepp M. Epidemiological investigation by macrorestriction analysis and by using monoclonal antibodies of nosocomial pneumonia caused by Legionella pneumophila serogroup 10. J Clin Microbiol 1994;32:2692-7.

2) Perola O, Kauppinen J, Kusnetsov J, Heikkinen J, Jokinen C, Katila ML. Nosocomial Legionella pneumophila serogroup 5 outbreak associated with persistent colonization of a hospital water system. Apmis 2002;110:863-8.

3) Fry NK, Alexiou-Daniel S, Bangsborg J M, Bernander S, Castellani-Pastoris M Etienne J, Forsblom B, Gaia V, Helbig J, Lindsay D, Luck P C, Pelaz C, Uldum S A, Harrison T G. A multicenter evaluation of genotypic methods for the epidemiologic typing of Legionella pneumophila serogroup 1: results of a pan-European study. Clin Microbiol Infect 1999;5: 462-77.

4) Mamolen M, Breiman RF, Barbaree J, Gunn A, Stone KM, Spika JS, Tennis DT, Mao SH , Vogt RL. Use of multiple molecular subtyping techniques to investigate a Legionnaires' disease outbreak due to identical strains at two tourist lodges. J Clin Microbiol 1993;31:2584-8.

5) Price CS, Huynh H, Paule S, Hollis RJ, Noskin GA, Pfaller M, Peterson LR. Comparison of an automated ribotyping system to restriction endonuclease analysis and pulsed-field gel electrophoresis for differentiating vancomycin-resistant Enterococcus faecium isolates. J Clin Microbiol 2002;40:1858-61.

6) Schoonmaker D, Heimberger T Birkhead G. Comparison of ribotyping and restriction enzyme analysis using pulsed-field gel electrophoresis for distinguishing Legionella pneumophila isolates obtained during a nosocomial outbreak. J Clin Microbiol 1992;30:1491-8.

7) van Belkum, A, Struelens Q , Quint W. Typing of Legionella pneumophila strains by polymerase- chain reaction mediated DNA fingerprinting. J Clin Microbiol 1992;30:2198-200.

8) Fry NK, Bangsborg JM, Bergmans A, Bernander S, Etienne J, Franzin L, et al. Designation of European Working Group on Legionella infection amplified fragment length polymorphism types of Legionella pneumophila serogroup 1 and results of inter-centre proficiency testing using a standard protocol. Eur J Clin Micro Inf Dis 2002;21:722-8.

9) Aurell H, Etienne J, Forey F, Reyrolle M, Pascale G, Farge P, Decludt B, Campese C, Vandenesch F, Jarraud S. Legionella pneumophila serogroup 1 strain Paris: endemic distribution throughout France. J Clin Microbiol 2003;41:3320-2.

10) Boccia S, Amore R, Stenico A, Moroder L, Orsini M, Romano Spica V, Ricciardi G. Molecular epidemiology by automated ribotyping and pulsed field gel electrophoresis of Legionella pneumophila environmental isolates representing nine different serogroups. Epidemiol Infect 2005;133:1097-105.

11) Ribot EM, Fitzgerald C, Kubota K, Swaminathan B, Barrett

TJ. Rapid pulsed-field gel electrophoresis protocol for subtyping of Campylobacter jejuni. J Clin Microbiol 2001;39:1889-94.
12) Chang N, L Chui. A standardized protocol for the rapid preparation of bacterial DNA for pulsed-field gel electrophoresis. Diagn Microbiol Infect Dis 1998;31:275-9.
13) Benson JA , Ferrieri P. Rapid Pulsed-Field Gel Electrophoresis Method for Group B Streptococcus Isolates. J Clin Microbiol 2001;39:3006-8.
14) Katsuda K, Iguchi M,Tuboi T, Nishimori K,Tanaka K, Uccida I, Eguchi M. Rapid molecular typing of Listeria monocytogenes by pulsed-field gel electrophoresis. Res Vet Sci 2000;69:99-100.
15) Gautom RK. Rapid pulsed-field gel electrophoresis protocol for typing of Escherichia coli O157:H7 and other gram-negative organisms in 1 day. J Clin Microbiol 1997;35:2977-80.
16) Matushek MG, Bonten MJ, Hayden MK. Rapid preparation of bacterial DNA for pulsed-field gel electrophoresis. J Clin Microbiol 1996;34:2598-600.
17) Boccia S, Laurenti P, Borella P, Moscato U, Capalbo G, Cambieri A, et al. Prospective three year surveillance for nosocomial and environmental legionella: implications for infection control. Inf Contr Hosp Epidemiol 2006;27:459-65.
18) Fields BS, Benson RF, Besser RE. Legionella and Legionnaires' disease: 25 years of investigation. Clinical Microbiology Reviews 2002;15:506-26.