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Molecular characterization (PCR-Based Methods) of *Staphylococcus aureus* isolated on dogs and cats

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ABSTRACT

A total of 36 isolates of *Staphylococcus aureus* from hospitalised and out patient dogs and cats were typed by RAPD-PCR, and 36 isolates were selected for further typing by ERIC-PCR and Coagulase gene-PCR and Coagulase gene RFLP, indicating a low degree of polymorphism in the coagulase genes. In this study, it is noticeable that RAPD-PCR displayed desirable typing quality by its ability to group the apparently related isolates from outpatient and hospitalised cats and dogs, whereas ERIC-PCR has the tendency to group the isolates into a single major cluster.

Key words: *Staphylococcus aureus*, RAPD-PCR, ERIC-PCR, Coagulase gene-

PCR and RFLP.

INTRODUCTION

A wide variety of diseases conditions associated with pathogenic staphylococci have been reported to contribute to major economic loss (Weigler *et al.*, 1990; Beck *et al.*, 1992; Altekruse *et al.*, 1997). Thus, preventive measures to control transmission of *S. aureus* in food or farms are necessary and it is now well accepted that an understanding of their epidemiological relationships is necessary to design effective control programme

(Aarestrup *et al.*, 1995; Annemuller *et al.*, 1999). Thus, microbiologist are required to determine the relatedness of staphylococcal isolates collected during the investigation of any outbreak or as a part of an on going surveillance system. While there are many different methods for typing *S. aureus*, not all methods divide the group of strains in a similar fashion. However, to our knowledge no study has compared techniques for typing *S. aureus* from dogs and cats in Malaysia.

In this study we used PCR-based techniques of enterobacterial repetitive intergenic consensus (ERIC-PCR), random amplified polymorphic DNA (RAPD-PCR) and the polymorphism of the coagulase gene to identify clusters of the isolates and determine the relatedness of the *S. aureus* strains isolated from dogs and cats.

MATERIALS AND METHODS

Bacterial Samples

Thirty-six *S. aureus* isolates from outpatient and hospitalised dogs and cats, of different sex, age and breeds were isolated from 408 samples collected from January to September 1997 at the University Putra Malaysia, Veterinary Hospital, Faculty of Veterinary Medicine,.

Genomic DNA isolation

Prior to amplification, genomic DNA of the *S. aureus* strains was extracted as described by Sambrook *et al.*, (1989).

Random amplified polymorphic DNA-PCR

Operon 10-mer Oligonucleotides set, designated as OPA-O1 to OPA-20, each with a (G+C) contents of 60% to 70% (Research Instruments, USA). The OPA-01: (5'-

CAGGCCCTTC 3'), OPA-07: (5'-GAAACGGGTG 3') and OPA-08: (5'-GTGACGTAGG 3') primers were chosen for the RAPD analysis. Twenty-five µl reaction mixtures were made consisting of 20 pmol of oligonucleotide primers. 1 µl (20-30 ng) of genomic DNA 3 mM $MgCl_2$, 0.2 mM of dNTPs mixture, 4 µl 10x concentrate of PCR buffer, and 1 unit of *Taq* DNA polymerase, made up to 25 µl with sterile distilled water. RAPD was carried out using a thermal cycler (Perkin Elmer 2400). The target sequences were amplified in 25 µl reaction volumes (after Pre-denaturation step at 94°C for 5 min) for 45 cycles 94°C for 1 min (denaturation), 36°C for 1 min (annealing) and 72 °C for 2 min (extension), followed by final extension 72 °C for 5 min.

ERIC-PCR

Oligodeoxribonucleotide primers with a G + C content of 50% were synthesised from Research Instruments, USA. The primers are: ERIC1R (3'-CACTTAGGGGTCCTCGAATGTA-5') and ERIC2 (5'-AAGTAAGTGACTGGGGT GAGCG-3'). The amplification protocol was performed as described by Van-Belkum *et al*. (1993). ERIC-PCR reactions were carried out in 25 µl containing 1 µl (20-30 ng) of genomic DNA, 1 mM $MgCl_2$, 1 µM of each primer, 0.2 mM of dNTPs mixture, 4 µl 10x concentrate of PCR buffer, and 1 unit of Taq DNA polymerase, made up to 25 µl with sterile distilled water. ERIC was carried out using a thermal cycler (Perkin Elmer 2400). The target sequences were amplified in 25 µl reaction volumes (after pre-denaturation step at 94°C for 10 min) for 35 cycles 94°C for 1 min (denaturation), 65°C for 8 min (annealing) and 72 °C for 2 min (extension), followed by final extension 72 °C for 16 min.

Coagulase gene PCR

Primers that used for coagulase gene PCR are: COAG2 (5'-CGAGACCAAGATTCAACAAG-3') and COAG3 (5'-AAAGAAAACCACTCACA

TCA-3'), which hybridised to sites 1632 to 1652 and 2589 to 2608 respectively were used (Research Instrument, USA). Each with a G + C content of 50%. The amplification protocol was in accordance to the method of Goh *et al*. (1992). PCR was carried out in 25 µl containing 1 µl (20-30 ng) of genomic DNA, 1 mM $MgCl_2$, 1 µM of each primer, 0.2 mM of dNTPs mixture, 4 µl 10x concentrate of PCR buffer, and 1 unit of *Taq* DNA polymerase, made up to 25 µl with sterile distilled water. Coagulase gene PCR was carried out using a thermal cycler (Perkin Elmer 2400). The target sequences were amplified in 25 µl reaction volumes (after Pre-denaturation step at 94°C for 3 min) for 40 cycles 94°C for 30 sec (denaturation), 56°C for 2 min (annealing) and 72 °C for 2 min (extension), followed by final extension 72 °C for 8 min.

The PCR amplification products were subjected to restriction endonuclease digestion of the fragments with *Alu*I restriction enzyme according to the manufacture's instructions.

The PCR amplification products were analysed by running 10 µl of the reaction on a 1% agarose gel, which was then stained with ethidium bromide and examined over UV illumination. A DNA ladder (Promega) was used as the DNA size marker.

STATISTICAL ANALYSIS

The fingerprints profiles of the *S. aureus* obtained using the combination of the three RAPD primers, ERIC primers and RFLP were scored visually. The data was recorded according to the presence and absence criterion (1= presence, 0= absence of band). The average similarity between two staphylococcus isolates represented by either RAPD-PCR, ERIC-PCR and RFLP was calculated according to the formula of Nei and Li (1979). The data was further analysed to generate the dendrogram with computer assisted RAPDistance software, using Neighbour-Joining Tree Program to produce the desired tree or dendrogram for cluster analysis.

RESULTS

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All the three primers (OPA-01, OPA-07 and OPA-08) successfully amplified polymorphic DNA from most of the 36 *S. aureus* strains. Eleven different fingerprinting profiles could be distinguished using primer OPA-01, however, the DNA of one isolate was not amplified. Although, the RAPD-PCR with primer OPA-07 generated 13 different fingerprinting profiles, but DNA of three isolates were not amplified. The level of differentiation was high when primer OPA-08 was used. RAPD-PCR with this primer generated 15 different fingerprinting profiles among all the 36 *S. aureus* isolates (Table 1). The dendrogram obtained from cluster analysis of the RAPD grouped the 36 *S. aureus* isolates into the clusters. The dendrogram showing estimates of the percentage similarities among these isolates on the basis of the combined results of the RAPD fingerprinting with three primers OPA-01, OPA-07 and OPA-08 was shown in (figure 1).

ERIC-PCR analysis of the 36 S. aureus *strains produced 19 different fingerprinting profiles (Table 1). The dendrogram obtained from cluster analysis of the ERIC-PCR grouped the 36 S. aureus strains into one major cluster and a single isolate (figure 2).*

The fingerprints obtained with COAG primers were distinctive and reproducible. The coagulase gene PCR gave only three fingerprinting for the 36 selected *S. aureus* as shown by the amplification of x, y and z bp fragments (Table 1). The positive control showed fragments of the amplified DNA with COAG2 and COAG3 primers and the negative control showed no fragments. *Alu*I restriction enzyme digestion of the coagulase gene PCR products for 36 *S. aureus* strains generated six fingerprinting profile as the PCR products of the same different sizes yielded different restriction profiles (Table 1). The dendrogram obtained from cluster analysis of the RFLP grouped the 36 *S. aureus* isolates into the main clusters (figure 3).

DISCUSSION

All isolates were typeable using the combination of three primers in RAPD analysis. However, we observed that certain isolates were untypeable with a particular primer but

were typeable when using another primer. None typeable isolates could be interpreted as the loss or absence of specific sites for primer binding in the in the chromosomal DNA in these isolates The combination of the three primers increased the efficiency of RAPD and gave 33 different profiles (Saulnier *et al*., 1993 and Myllys *et al.*, 1997). However, by the primers OPA-01, OPA-07 and OPA-08 isolates from the same animal gave the same profile despite of the site of isolation (skin, nose and ear) and some profiles were shared between hospitalised and out patient animals. In RAPD-PCR, majority of the isolates of *S. aureus* from hospitalised cats were separated into two distinct groups. However, the *S. aureus* isolates from both hospitalised and outpatient dogs were clustered as a single group (Figure 1).

All the 36 isolates are typeable using ERIC1R and ERIC2 primers and gave 19 different profiles. No specific fingerprinting profiles can be correlated according to the site (skin, nose and ear). In addition the results of the present study allowed us to recognise ERIC-PCR type identity across different strains as 23 of 36 of isolates selected from out patient dogs and hospitalised cats clustered together in one group and with a single isolate from out patient dog (Figure 2).

The limited polymorphism of the coagulase gene PCR rendered it as the least discriminatory typing method used only few profiles were distinguished. The coagulase gene has been described to contain a series of repetitive 81 bp DNA sequences which differ, both in the number of tandem repeats and location of *Alu*I restriction sites among different isolates, and has been used for molecular subtyping of *S. aureus* (Goh *et al.*, 1992; Schwarzkopf and Karch, 1994; Omega *et al*. 1999; Annemuller *et al.*, 1999). The *Alu*I restriction analysis of the amplified coagulase gene sequences allowed us to confirm six patterns among the *S. aureus* strains. The low number of profiles seen within the study is an advantage as it provided a convenient method, which was easy to interpret and compare the isolates examined, due to use of only one restriction enzyme. The number of bands could be expanded by the use of multiple restriction enzymes (Tenover *et al.*, 1994; Schwarzkopf and Helge, 1994; Hookey *et al*. 1998; Omega *et al*. 1999). The RFLP cluster analysis

showed that there are two main clusters, consisting of strains from hospitalised and outpatient dogs and cats from the various body sites examined.

It is of interest that individual hospitalised or outpatient dogs or cats are infected with strains of *S. aureus* with six different RFLP-PCR profiles of the coagulase genes. This implies that virulence of a *S. aureus* strain varies for the individual animals examined as defined by Raimundo *et al.* (1999). This will have implications in attempts to determine the virulence of *S. aureus* strains by both *in vitro* and *in vivo* investigations as it may lead to a complex results. Based on the methodology used, one could determine the relatedness of organism involved in seemingly common sources as each method offers a different level of discrimination in the ability to distinguish apparently related organisms. In the case of *S. aureus* in this study, coagulase gene typing appeared to be the most macroscopic of the typing methods used. This method had a tendency of placing the isolates into only two major groups. RAPD and ERIC methods showed higher levels of resolution but RAPD had the desirable quality of being able to cluster the apparently related *S. aureus* isolates such as outpatient and hospitalised cats and all isolates from dogs. In the study using molecular-based typing methods, RAPD-PCR and ERIC-PCR typing methods are reported to be superior in term of its ability to discriminate between strains and almost similar in the differentiation level and were found to be useful for preminiary epidemiological investigation (Lipman *et al.*, 1996; Schmitz *et al.*, 1998).

The uneven distribution of *S. aureus* observed at various body sites sampled partially reflects marked genetic differences among the *S. aureus* population from the animals examined as revealed by genetic polymorphism analysis. The present work sheds more lights on the diversity of a single bacterial species on dogs and cats and it poses a very relevant experimental question. As different body sites host populations with very different degree of genetic polymorphism, it is questionable whether a particular body site can be considered as the fundamental sample unit in studies concerning body colonisation by micoorganisms. As a consequence, random sampling without taking into account the patterns of bacterial populations colonization sites provide information of a little use for

epidemiology study. In this study, we observed that isolation rates of *S. aureus* from the various body sites were in the order of ear>skin>nose and nose>skin >ear for cats (Table 1). This type of information is essential and is of basic importance for effective bacteriological sampling in an epidemiological study or as a preventive diagnostic test.

ACKNOWLEDGEMENT

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Table 1. Distribution of *S. aureus* isolates according to the RAPD[a]-PCR, ERIC[b]-PCR, COAG[c]-PCR and RFLP[d] fingerprinting profiles.

No	Source	OPA-01	OPA-07	OPA-08	ERIC	COAG	RFLP
1	13HDS	1	1	1			
2	13HDE*	1	2	1	1	1	1
3	2ODN*	1	3	2	2	2	2
4	3ODE*	1	4	2	3	2	2
5	10ODS*	1	5	3	-	1	3
6	10ODE	2	6	4			
7	16ODE*	3	7	2	3	1	1
8	18ODS*	3	8	3	3	1	4
9	20ODN*	3	4	4	4	1	4
10	24ODS*	3	-	5	5	1	3
11	25ODS*	3	9	5	6	1	4
12	25ODE	3	9	5			
13	27ODE*	4	10	6	7	1	4
14	29ODE*	4	11	7	8	1	4
15	5HCS*	5	12	8	9	1	3
16	5HCN	5	12	8			
17	5HCE1	5	12	9			
18	5HCE2	5	12	8			
19	29HCN	6	12	10			
20	29HCE*	6	12	6	10	2	4
21	31HCE*	6	6	11	11	1	4
22	33HCN*	7	12	11	12	3	3
23	35HCE*	7	6	10	13	1	2
24	50HCN*	7	6	6	14	2	1
25	61HCN*	8	-	12	15	1	1

26	1OCS	-	2	13			
27	1OCN*	9	6	13	16	1	3
28	1OCE	9	12	13			
29	8OCS*	9	13	13	17	1	3
30	9OCS	8	-	14			
31	9OCE*	8	13	14	18	1	5
32	10OCS	10	14	8			
33	10OCN*	10	14	8	2	1	5
34	10OCE	10	12	13			
35	14OCS*	9	12	13	19	2	6
36	17OCE*	11	13	15	15	1	3

[a] Random Amplification Polymorphyc DNA primers, OPA-01, OPA-07 and OPA-08.

[b] Enterobacterial repetitive intergenic concensus.

[c] Coagulase gene PCR.

[d] Restriction Fragment Length Polmorphyism

D= dog, C= cat, H= hospitalised, O= out patient. S= skin, N= nose, E= ear.

الملخص باللغة العربية

التوصيف الجزيئي بواسطة تفاعل مضاعفة جينات من سلسلة الحامض النووي المنقوص الأكسجين للبكتريا العنقودية الذهبية المعزولة من القطط و الكلاب

عند تصنيف 36 عينة من البكتريا العنقودية الذهبية بواسطة تفاعل مضاعفة جينات من سلسلة الحامض النووي المنقوص الأكسجين. وجد أن هنالك 36/33 نمط من طريقة مضاعفة جينات عشوائية (RAPD-PCR) و أن هنالك 36/19 نمط بواسطة مضاعفة جينات مكررة متراصة من البكتريا المعوية (ERIC-PCR) و أن هناك 36/3 عند استخدام طريقة ألجين المسئول من التجلط عند هذه البكتريا (Coagulase gene) و أن هنالك 36/6 نمط عند تقطيع هذا ألجين.

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عليه يمكن استخدام طريقة مضاعفة جينات عشوائية و طريقة مضاعفة جينات مكررة متراصة من البكتريا المعوية لتوصيف هذه البكتريا. أما طريقة تقطيع ألجين فيمكن زيادة فعاليتها بواسطة زيادة عدد الإنزيمات التي تعمل علي تقطيع ألجين .

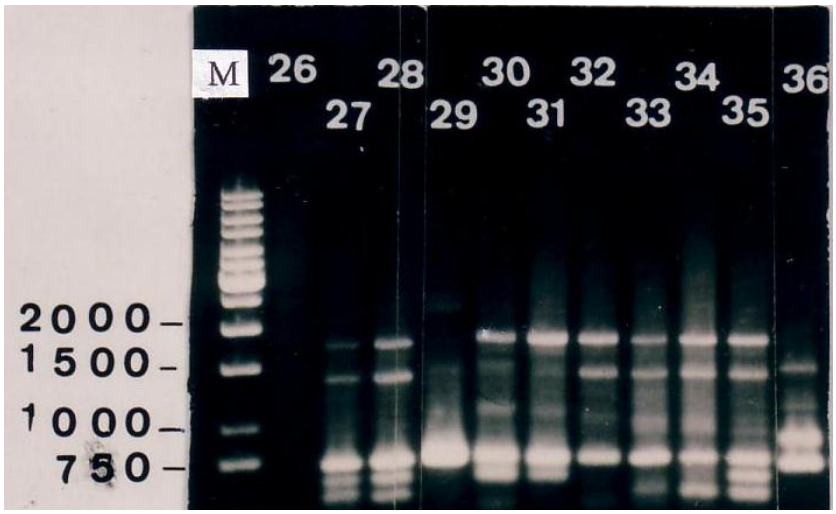

Figure 1: RAPD profiles of *S. aureus* obtained with primer OPA-01 electrophoresed on 1% agarose gel. Isolate number refer to the order in which they were isolated. Lane M, 1Kb molecular weight size marker in base pair.

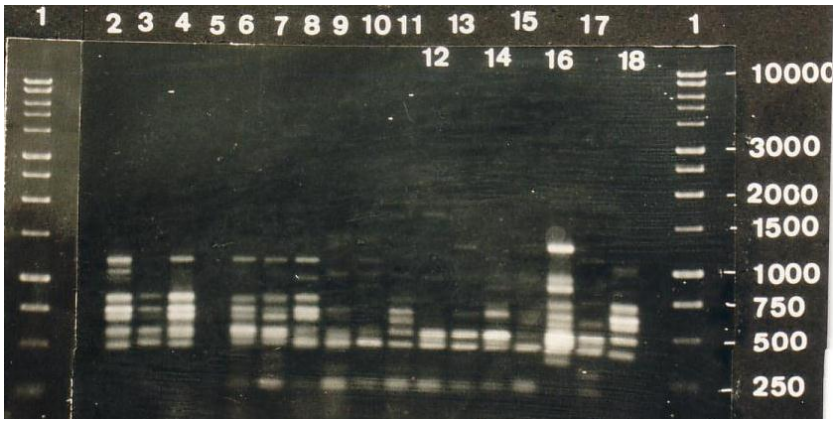

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Figure 2: ERIC profiles of *S. aureus* obtained with ERIC 1R and ERIC2 primers electrophoresed on 1% agarose gel. Isolate number refer to the order in which they were isolated. Lane 20, 1Kb molecular weight size marker in base pair.

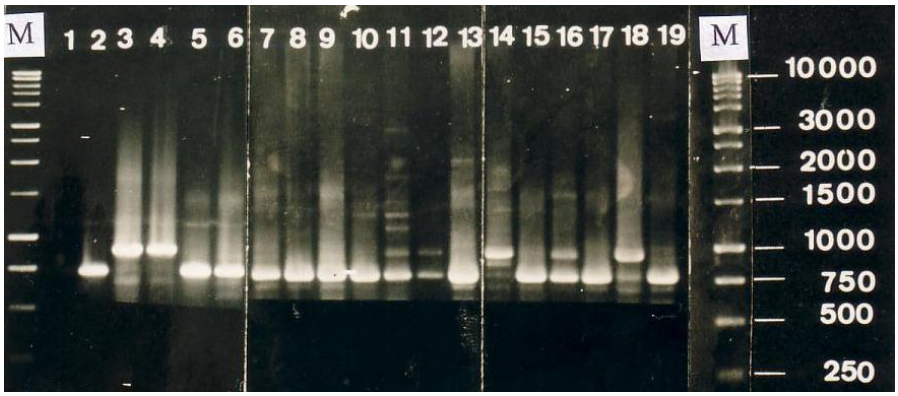

Figure 3: Coagulase gene profiles of *S. aureus* obtained with COAG2 and COAG3 primers electrophoresed on 1% agarose gel. Isolate number refer to the order in which they were isolated. Lane M, 1Kb molecular weight size marker in base pair.

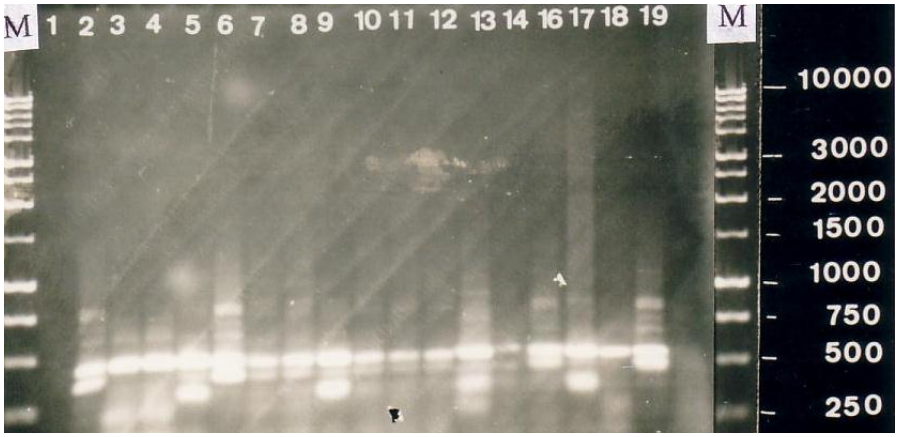

Figure 4: RFLP generated by restriction endonuclease of coagulase gene profiles of *S. aureus* using *Alu*1 restriction enzyme electrophoresed on 1% agarose gel. Isolate number refer to the order in which they were isolated. Lane 19, 1Kb molecular weight size marker in base pair.

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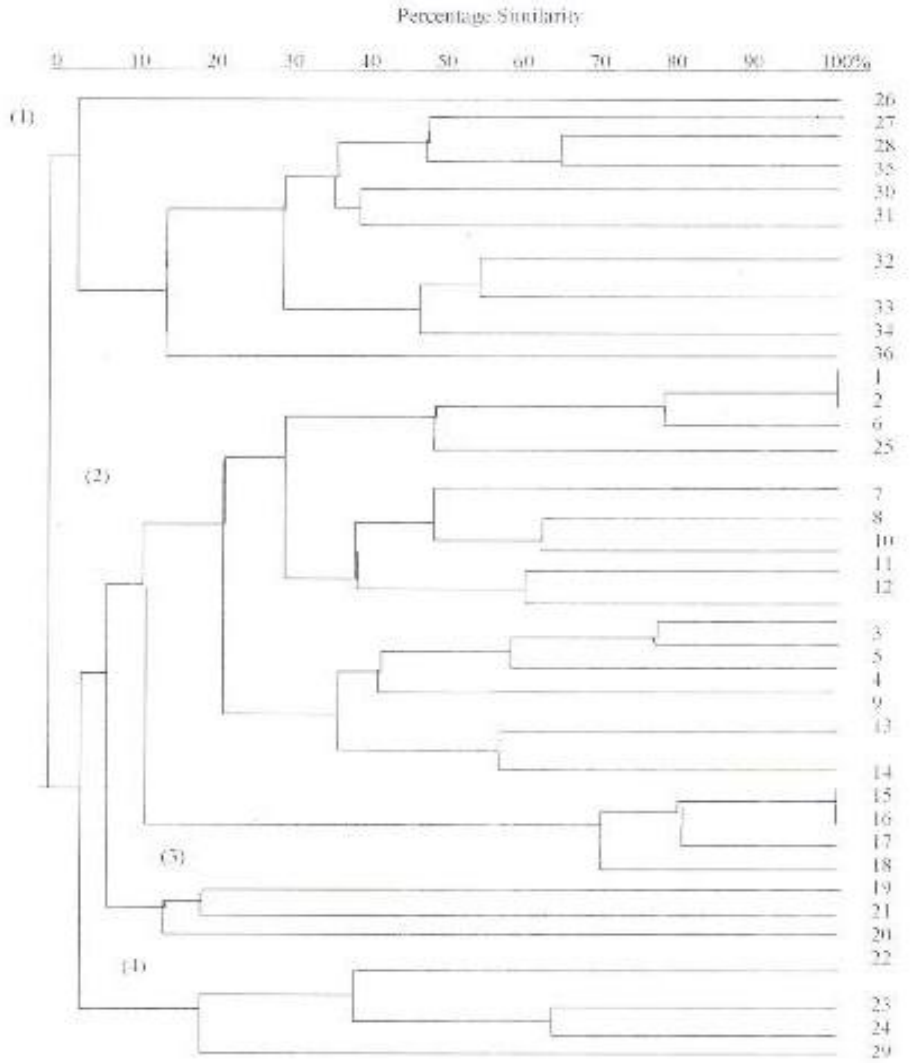

Figure 5: The dendrogram generated from matrix data obtained from RAPD-PCR. The patristic distance showed the genetic dissimilarity among the *N. gonorrhoeae* isolates. This is the combined result of the three primers: OPA-01, OPA-07 and OPA-08. Lane numbers correspond to isolate numbers given in Table 6.1.

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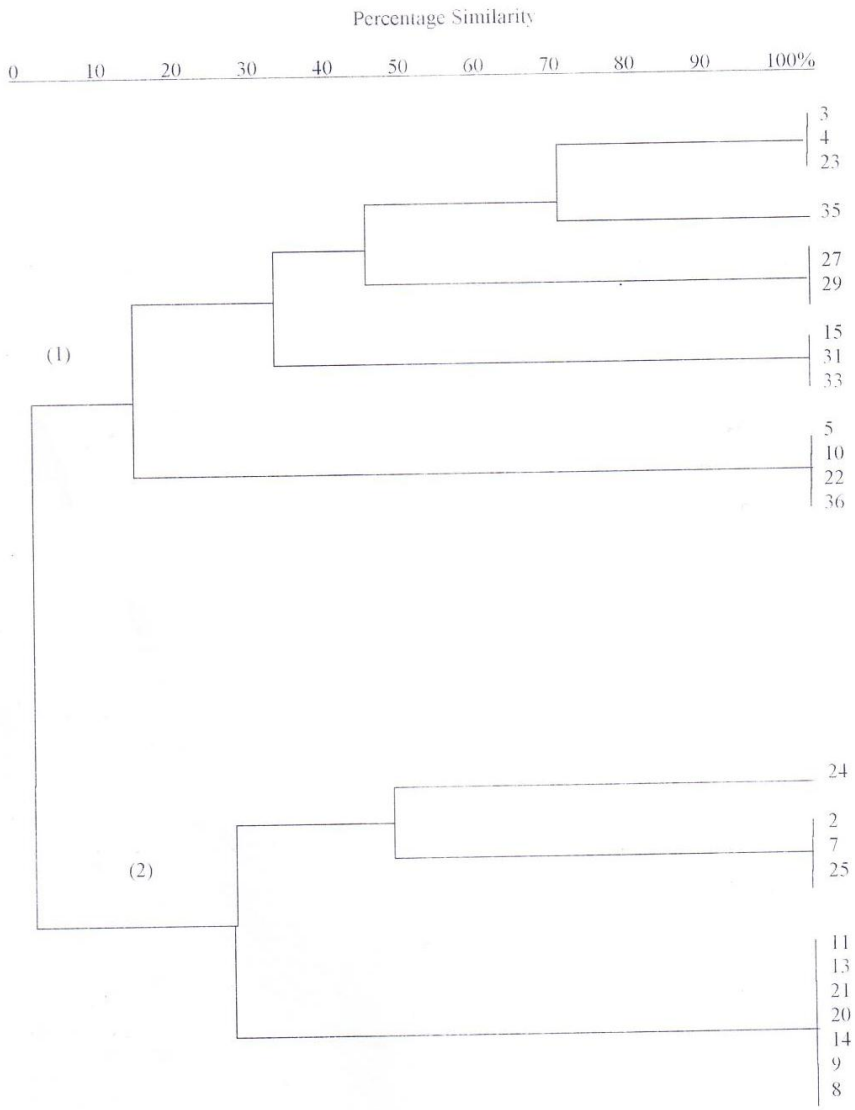

Figure 7: The dendrogram generated from matrix data obtained from RFLP. The patristic distance showed the genetic dissimilarity among the *S. aureus* isolates. Lane numbers correspond to isolate numbers given in Table 6.1.

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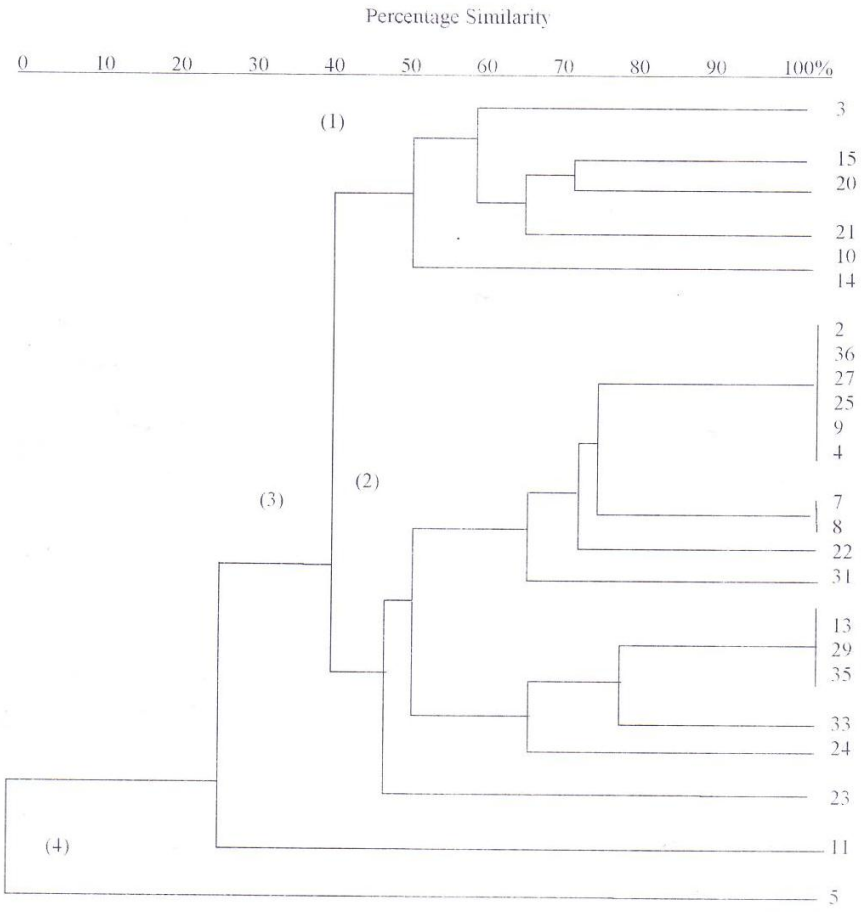

Figure 6: The dendrogram generated from matrix data obtained from ERIC-PCR. The patristic distance showed the genetic dissimilarity among the *S. aureus* isolates. Lane numbers correspond to isolate numbers given in Table 6.1.