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PULSED FIELD GEL ELECTROPHORESIS CHROMOSOMAL DNA RESTRICTION PATTERNS OF *STAPHYLOCOCCUS AUREUS* ISOLATED FROM CHICKENS

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ABSTRACT

A study on population and clonal variation analysis of DNA of *Staphylococcus aureus* isolated from chickens was carried out. All 50 strains of *Staphylococcus aureus* were processed for DNA clonal variation by pulsed field gel electrophoresis (PFGE). Pulsed field gel electrophoresis identified DNA clonal variation among the strains of *Staphylococcus aureus*. The strains showed difference in their numbers, size, patterns and arrangement of DNA bands. Three to four band differences were seen among the strains and therefore the strains were divided into 10 main groups and designated as A, B, C, D, E, F, G, H, I, J and also recognized as 3 biotypes.

Keywords: Chickens, colonial variation, DNA bands, PFGE, *Staphylococcus aureus*

INTRODUCTION

The infections caused by *Staphylococcus aureus* continue to be a problem for both, humans and animals (Eric and Miller, 2001 and Callija *et al.,* 2006)). Identification of strains causing infections is a major step in investigating the source of infection and in designing subsequent control measures. During the last decade, traditional methods of strains typing such as bacteriophage typing and serotyping have been superceded in many laboratories worldwide with newer molecular methods such as plasmid fingerprints, ribotyping, PCR based methods (Belkum, 1994) and analysis of chromosomal restriction patterns by pulsed field gel electrophoresis (PFGE) (Albeit *et al*., 1990; Finney, 1993; Maslow *et al*., 1993 Callija *et al*., 2006). However, bacteriophage typing is being still used in a number of large reference laboratories around the world for

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epidemiological studies of *Staphylococcus aureus* and serotyping as a tool for epidemiologic surveillance of *Salmonella* species (Olsen *et al*., 1994). Nowadays, PFGE comes closest to satisfying that need (Albeit *et al*., 1990).

Pulsed field gel electrophoresis involves embedding of organisms in agarose. lysing the organisms and digesting the chromosomal DNA with restriction enzymes that cleave infrequently (Finney, 1993; Maslow *et al.,* 1993. Callija *et al.,* 2006). The plugs of the agarose containing the chromosomal DNA fragments are loaded into the wells of an agarose gel and the restriction fragments are resolved in a pattern of discrete bands in the gel by an electrophoresis apparatus. The DNA restriction patterns of isolates are then compared with each other to recognise their relatedness. The present study was therefore designed to investigate and identify the clonal variation among the strains of *Staphylococcus aureus* isolated from chickens.

MATERIALS AND METHODS

The strains of *Staphylococcus aureus* obtained from differnt laboratories of various countries isolated from chickens (Table 1) were processed to record their DNA chromosomal restriction patterns. The protocol for preparation of chromosomal DNA designed by Goering and Winters (1992) was applied during present study. The chemical buffers such as TEN **(**TrisCl + ETDA +NaCl): 0.IMTrisCl 12.4 g; 0.15 M NaCl 8.5 g; 0.1 M ETDA37.4g), EC (6 mM TrisCl 0.725g; 0.1METDA 37.4g; 0.5% Brije (58) 5g; 0.2% sodium deoxycholate lg; 0.5% Sarkosyl 5g and 1M NaCI58.5g) and TE **(**TrisCl + ETDA: 10 mM TrisCl 1.2g and 5mM ETDA 1.7g) were prepared accordingly and pH was adjusted to 7.4 and then sterilized by steam sterilizer and kept in shelf till used. The sodium acetate solution (20mM) was also prepared for lysostaphin. The lysostaphin, (lmg/ml of 100µl concentration**)** l mg/ml of l00µlvolume concentration was diluted by adding 400µl of 20mM sodium acetate and brought up to 500u1 volume.

The stock culture of all 50 strains of *Staphylococcus aureus* was primarily streaked out on TSA (Tryptone Soya Agar) medium and incubated at 37°C overnight. A pure colony from each strain was isolated, inoculated into 5ml TSB medium and incubated overnight in shaker incubator with 200rpm at 37°C. From overnight culture, 1ml of each strain was transferred in cryonic tubes and 300µl of glycerol were added, and then stored in deep freezer at -70°C.

Pulsed field gel electrophoresis

All 50 strains of *Staphylococcus aureus* (Table 1) were grown in 5ml TSB medium in universal tubes for overnight in shaker incubator with 200rpm at 37°C.Only 0.7ml (700 µl) of overnight culture was added in eppendorf tubes of each strain and then harvested by centrifugation in micro-centrifuge machine at 7000rpm for 2 minutes. The supernatant was removed and left the pellet in the eppendorf tubes. TEN buffer, 1ml was added to each eppendorf (containing bacterial cells) and was vortexed properly to mix the pellet accordingly and then

centrifuged in micro-centrifuge machine at 7000rpm for 2 minutes to wash the bacterial cells and then TEN buffer was removed from each eppendorf tube.

The washed bacterial cells were resuspended in 0.3ml (300µ1) autoclaved in EC buffer and vortexed. A 2u1 of a 1mg/ml of l00µl volume of lysostaphin (Ambicin 1, Applied Microbiology, England) was prepared in 20 mM sodium acetate and added to the bacterial suspension tubes and each tube was subsequently vortexed. However, a 2% (2g) sea plaque agarose (Bio-Rad Laboratories, England, UK) was dissolved in 100ml EC buffer, sterilized and then 300µ1 of agarose were added to lysostaphin cell suspension.

The suspension was briefly vortexed and quickly pipetted through the help of micro-pipette into the plug molds. Before adding to plug molds, all molds were cleaned and marked with the strain numbers. The plugs were allowed to solidify at room temperature for about 15 minutes. After solidification, the plugs were removed from the plug molds and placed in tubes (16x100mm) containing. 3ml EC buffer. The plugs were then kept in incubator at 37°C for 1 hour. After complete lysing of cells, the EC buffer was removed by micro-pipette to avoid any damage to the plugs and replaced with 3ml of sterilized fresh TE buffer and tubes were again kept in incubator/water-bath at 55°C for 1 hour. After that, the TE buffer was replaced with fresh TE buffer (3ml) and stored at 4°C in refrigerator till further analysis.

Restriction enzyme

The restriction enzyme SmaI (20U) of 20,000 units (New England Bio Lab. Beverly Mass) with 10X (20units =l µl) was prepared in buffer (12.5 µ1) and 111.5µl double sterilized distilled water were added and brought up to total volume of 125 µ1 for a single plug sample (normal size).

Plug digestion

The plugs prepared from different strains were taken out from TE buffer (placed in the tube along with TE buffer) and placed on a glass slide/Petri dish. The half of the plug was incised with scalpel/forceps and placed in eppendorf containing 125µl total restriction enzyme and half of the plug was placed back in the tube and kept in the refrigerator at 4°C, as done before. The plugs in the restriction enzyme were placed in the shaker incubator at 140rpm for 2 hours at 26°C and then transferred to refrigerator till analyzed.

Preparation of running gel

Before preparation of running gel, 0.5X TBE buffer was made from 10 X TBE concentrations (5ml of 10X TBE buffer in 100ml of distilled water). For better performance of 0.5x TBE, 4mg of thio-urea was added in 2 litre of distilled water. After that, 1% (lg in 100ml distilled water) Seakem agarose (Bio-Rad Richmond Calif) was weighed and added to 100ml distilled water, dissolved by steam

sterilizer, allowed to cool and then 100µ1 of thio-urea (4mg in 2ml distilled water) were added into the running gel.

Processing of PFGE

The gel cast was cleaned and reassembled properly to avoid any leakage of gel and then comb was placed. The gel was poured off (100ml) in the cast and allowed to solidify at least for 20 minutes. The comb was removed and the plugs were cut into small pieces (containing DMA), and the wells were loaded with the small pieces of plugs, finally the wells of both ends were also loaded with Bacteriophage Lambda DNA Concatamers (Bio-Rad) as size standards and thus served as a control for running parameters of the CHEF-DR units. The wells were sealed with 0.8 % Sea plaque agarose (prepared in EC buffer). Before placing the gel into the electrophoresis cell, the cell was cooled by running 0.5X TBE buffer (2 liters) and brought the temperature for cooling the unit up to 12-14°C. The gel was placed into the electrophoresis tank and the running parameters were allowed, initial pulse 5 seconds, final pulse 40 seconds, voltage 6V and at 12-14°C for 20 hours.

Table 1. The isolates of *Staphylococcus aureus* recovered from different sources and used in the present investigation.

Culture or Code No. of Isolates	Date Isolation	Location	Biotypes	Protein A
AV-24	1999	Ireland	Poultry	Negative
AV-34	1999	Ireland	NHS-a	Negative
AV-35	1999	Ireland	Poultry	Negative
AV-36	1999	Ireland	Poultry	Negative
AV-82	1999	Ireland	Poultry	Negative
AV-86	1999	Ireland	Poultry	Negative
AV-301	1999	Ireland	Slaughterhouse	Positive
AV-303	1999	Ireland	Slaughterhouse	Positive
AV-496	1999	Ireland	NHS-b	Positive
DS56	1999	N Ireland	Poultry, oesteomeylitis	-
DS67	1999	N. Ireland	Poultry, oesteomeylitis	-
DS68	1999	N. Ireland	Poultry, oesteomeylitis	-
DS72	1976	N. Ireland	Poultry, oesteomeylitis	-
00-8661	8/1999	NE.Georeia	Poultry	-
00-9705	11/1999	NE. Georgia	Poultry	-
00-9686	11/1999	NE. Georgia	Poultry	-

Contd on next page

10217	01/2000	NE. Georgia	Poultry	-
10009	12/1999	Iowa	Poultry	-
00-7439	05/1999	NE. Georgia	Poultry	-
00-9679	11/1999	NE. Georgia	Poultry	-
99-7184	04/1999	NE. Georgia	Poultry	-
10105	01/2000	NE. Georgia	Poultry	-
00-8566	08/1999	NE. Georgia	Poultry	-
00-9779	11/1999	NE. Georgia	Poultry	-
00-8078	06/1999	NE. Georgia	Poultry,	-
00-9448	10/1999	NE. Georgia	Poultry	-
99-5281	01/19999	NE. Georgia	Poultry	-
99-5613	02/1999	NE. Georgia	Poultry	-
99-5612	02/1999	NE. Georgia	Poultry	-
00-7608	05/1999	NE. Georgia	Poultry	-
00-8954	08/1999	NE. Georgia	Poultry	-
C1X2	1976	Belgian	Nose of young hen	-
C1X8	1976	Belgian	Nose of broiler	-
C!X18	1976	Belgian	Nose of adult lay. Hen	-
C1X30	1976	Belgian	Poultry	-
C1X38	1976	Belgian	Poultry	-
C1X51	1976	Belgian	Poultry	-
C1X77	1976	Belgian	Poultry	-
253	04/2004	Edin.UK	Canteen staff member	-
254	04/2004	Edinburgh UK	Canteen staff member	-
335	06/2004	Cumbria. UK	A bird from a farm	-
489	11/2004	Glasgow. UK	Hock joint of a bird	-
492	11/2004	Glasgow. UK	Hock joint of a bird	-
494	18/2004	Glasgow. UK	Hock joint of a bird	-
495	11/2004	Glasgow. UK	Metatarsus of a bird	-
512	12/2004	Edinburgh UK.	Canteen staff member	-
513	12/2004	Edinburgh UK	Canteen staff member	-
514	12/2004	Edinburgh UK	Canteen staff member	-
634	05/2005	Dunfermlinc UK	Liver of young bird	-

After 20 hours, the electrophoresis cell was switched off and then gel was taken out from the tank and stained in ethidium bromide (l0mg /ml), for 30 minutes then washed with distilled water for again 30 minutes and visualized under photo camera microscope, pictures were taken and analysed for size, pattern, number and arrangement of bands yielded by PFGE. The pattern, size, number of bands etc were analysed by visual observation and formed different groups. The relatedness of the strains were analysed by computer software.

RESULTS AND DISCUSSION

An epidemiological study on chromosomal DNA clonal variation among different strains of *Staphylococcus aureus* isolated from chickens was carried-out by Pulsed Field Gel Electrophoresis (PFGE) and results are presented in Tables 1 and 2 and Figures 1 and 2. A total of 88% strains produced fragments of DNA successfully while remaining did not produce any DNA bands (12%) this might be due to DNA was not properly obtained. All 50 strains of *Staphylococcus aureus* isolated from poultry birds and only 44 strains were processed properly and grouped in 10 major groups (A-J) representing their size, pattern, number, and arrangement of DNA fragments. A very narrow difference among the strains was observed. The characteristics of the DNA fragments of the different strains demonstrated in the present epidemiological survey are summarized in Table 2.

Table 2. Characteristics of DNA clones of *Staphylococcus aureus* strains isolated from chickens generated by PFGE and grouped in different groups.

Groups	Number of strains	Number of DNA bands Bands	% of strains	Characteristics of DNA clones
A	2	10	4	Pairs and singles
B	6	14	12	Four, pairs but thicker
C	8	11	7	Pairs but thinner
D	7	9	14	Singles, triple but some thicker
E	4	10	8	Pairs, triple, singles
F	4	9	8	Singles, triple and pairs
G	1 6	11	2	Singles, and triple
H	6	10	12	Pairs, triple and pairs
I	2	13	4	Singles, pairs but somewhat thicker
J	4	12	9	Singles

The strains of *Staphylococcus aureus* isolated from poultry birds and typed by PFGE were analysed by computer software that displayed the relatedness of the strains through a dendogram (Fig. 1).

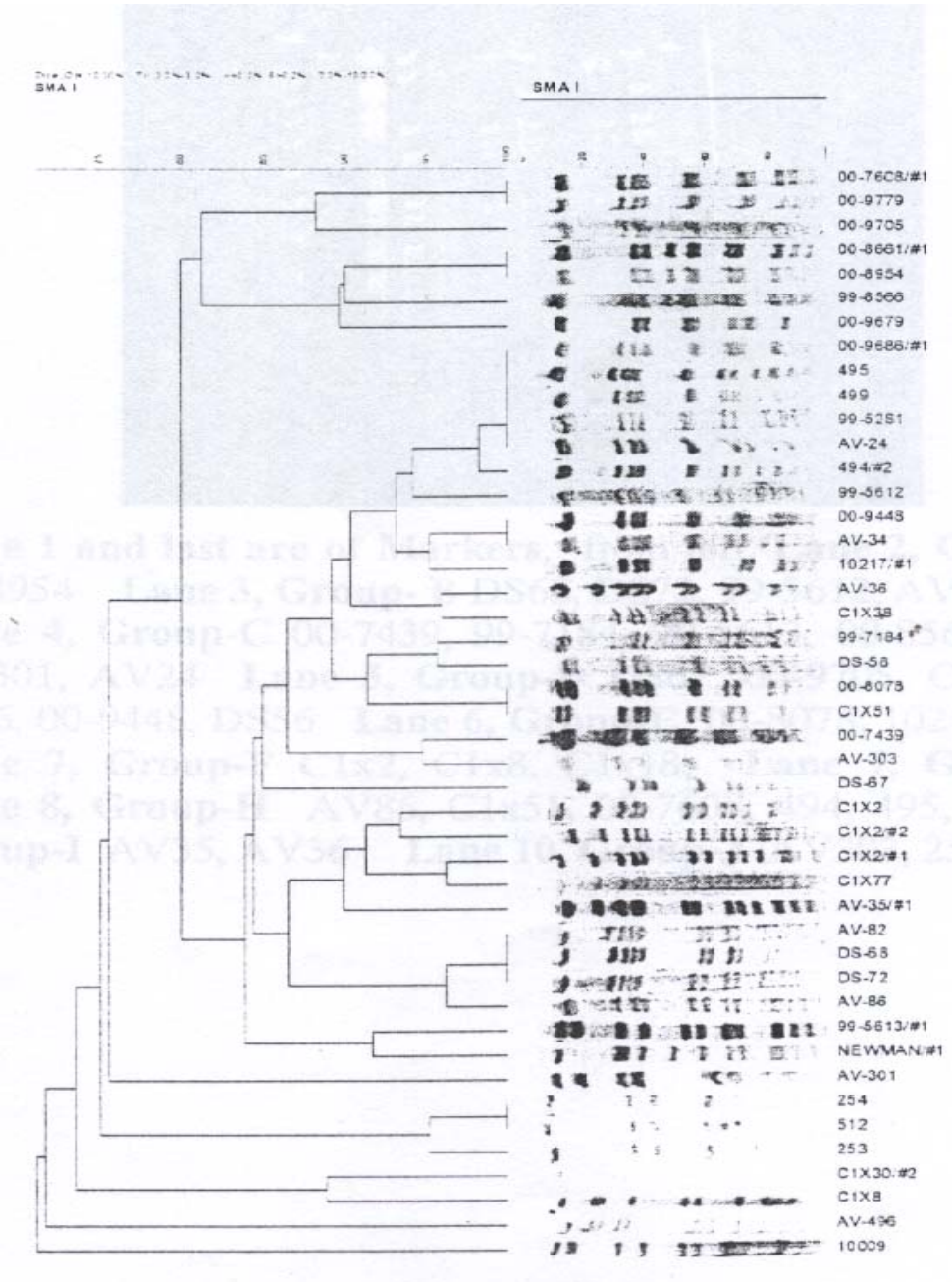

Figure 1. Dendogrammatically presentation of relatedness and un-relatedness of strains of *Staphylococcus aureus* based on their DNA clones analyzed by software generated by PFGE

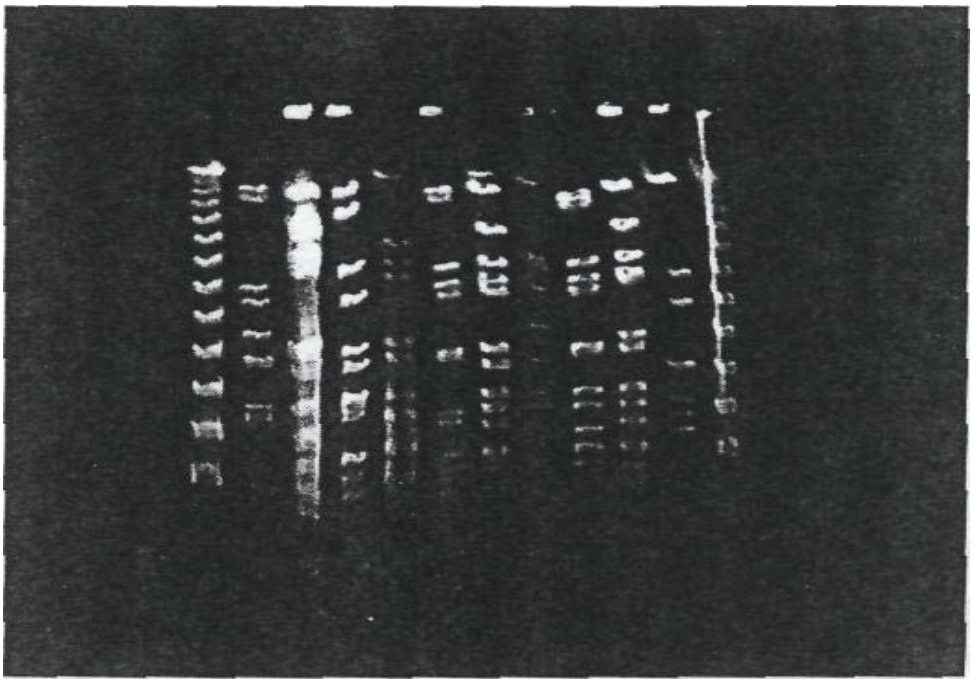

Figure 2. DNA fingerprints of strains of *Staphylococcus aureus* isolated from chickens generated by PFGE and grouped in 10 main groups and each group contains following strains.

Lane 1 and last are of Markers, from left, Lane 2, Group A- 00-8661,00-8954 Lane 3, Group- B DS68, DS72, 99-5612, AV496, AV82,Clx38 Lane 4, Group-C 00-7439, 99-7184, 99-5613, 00-8566,00-9679,10009,AV301, AV24 Lane 5, Group-D DS67, 00-9705,Clx30,00-9779,00-9686, 00-9448, DS56 Lane 6, Group-E 00-8078,10217,99-5281,AV34. Lane 7, Group-F Clx2, Clx8, Clxl8, Lane7, Group-GNewman, Lane 8, Group-H AV86, Clx51, 00-7608, 494, 495, 499 Lane 9, Group-I AV35, AV36 Lane 10, Group-J AV303, 253, 254 and 512

A total 50 strains of poultry were typed by PFGE and categorized in 10 different groups on the basis of their chromosomal DNA restriction size, patterns, numbers, and arrangements of bands. Calleja *et al.(*2006) carried out an investigation on molecular typing of *Staphylococcus aureus* isolates recovered from rabbit meat to determine the diversity and possible origin of the organism through PFGE, observed the highest discrimination index (D=0.966) and identified 19 patterns (more than one band difference and in 10 biotypes more than three band differences). Similar kind of investigation on typing of. *Staphylococcus aureus* isolated from different sources, a two methods comparison was made by Bannerman *et al.* (1995). The typing of the *Staphylococcus aureus* isolates/strains was done on the banding patterns. However, the isolates were divided in to different groups. They described

epidemiologically related isolates that appeared identical in size and number of bands were considered to represent the same strains which was designated strain A. Isolate banding patterns differed from main pattern because of one, two or more differences due to mutation were considered of a subtype within main group recorded as A1, A2 and A3 and the isolate banding patterns differed from the main pattern by four or more bands which can not be described by at most two genetic events were identified of a different strains and designated the strains as B. C, and D groups.

Many workers worldwide explained that if the isolates are designated indistinguishable if their restriction patterns have the same numbers of bands and the corresponding bands have the same apparent size, the epidemiologic interpretation of the results is that the isolates are all considered to represent the same strain (Tenover *et al.,* 1995). An isolate is considered to be closely related to the outbreak strain if its PFGE pattern differs from the outbreak pattern by changes consistent with a single genetic event; such changes typically result in two to three band differences. Where new chromosomal restriction site could divide in two small fragments, should approximate the size of one large fragment. The loss of original large fragment is one band difference and the appearance of two smaller fragments represent two additional band differences, thus there is three band difference between the out break pattern and that of the test isolate, such an isolate is considered to be closely related. Variations of two to three bands have been observed in strains of some species when they are cultured repeatedly over time or isolated multiple times from the same animal species (Tenover *et al.,* 1995). Further, that if PFGE pattern differs from outbreak-pattern by changes consistent with three or more independent genetic events, generally more than seven band differences, this implies that <50% of the well-resolved fragments present in the patterns from such an isolate should be present in the outbreak pattern (Tenover, *et al.,* 1995).

It is clear from the present investigation that the results obtained and strains typed and grouped in main 10 groups are very similar in all respect to the descriptions given by the workers for the typing and grouping of *Staphylococcus aureus* strains recovered from chickens or other animals

CONCLUSION

Pulsed field gel electrophoresis identified DNA clonal variation among the strains of *Staphylococcus aureus*. The strains showed difference in their numbers, size, patterns and arrangement of DNA bands. Three to four band differences were seen among the strains and divided into 10 main groups and also in 3 biotypes.

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