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DETECTION OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* ISOLATED FROM MASTITIC BUFFALOES' MILK USING PULSED- FIELD GEL ELECTROPHORESIS (PFGE)

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SUMMARY

A total of 196 milk samples were examined bacteriologically for detection of methicillin resistant *Staphylococcus aureus* during the period from January to October 2004 and results obtained showed high incidence of isolation of *S. aureus* (38.46%) from a total 13 strains of staphylococci (29.54%). All obtained isolates were identified using a commercial kit system (API STAPH system consists of: Carrying out 19 tests using the API STAPH strip).

Antibiotic sensitivity test were done using five antibiotics, which are frequently used in mastitis therapy were chosen: penicillin-G, ampicillin, kanamycin, and cephalexine. The fifth compound was methicillin and results obtained showed no such strains could be found. *S. aureus* strains were susceptible to the antibiotics tested comprise 60 % (penicillin G), 80 % (kanamycin, cephalexine) and ampicillin (20 %).

Pathogenicity of the isolates were discussed including both haemolysin titre and pathogenicity in mice. The results obtained revealed that majority of the *S. aureus* (80%) isolates were haemolytic with the maximum haemolytic activity (1/1024) and concerning pathogenicity in mice mortality rate was 100%.

Genetic characterization of staphylococci by Pulsed Field Gel Electrophoresis (PFGE) analysis revealed no *S. aureus* resistance methicillin was recorded.

INTRODUCTION

Methicillin-resistant Staphylococcus aureus strains are a potential threat for food safety

because foodborne illness caused by methicillin-resistant *Staphylococcus aureus* has been reported even though these strains were only associated with nosocomial infections (Rilla et al., 2004).

Staphylococcus aureus is a major etiological agent of mastitis , which is the most economically important diseases to the dairy industry (yuji et al., 2001). Staphylococcal intoxication worldwide stands out as one of the main food borne diseases and considered to be the second or third most common food intoxication of microbiological origin (Atanassova et al., 2001).

Staphylococcus aureus is a gram-positive coccus that is responsible for a wide variety of community-acquired and hospital-acquired infections. *S. aureus* may cause relatively minor skin infections, but more severe infections, such as endocarditis, osteomyelitis, and toxic shock syndrome, they may result from the coordinated expression of virulence factors (Projan and Novick, 1997).

The increasing problems encountered and the intrinsic and acquired resistance to different antimicrobial compounds highlight the need for a rapid identification technique. as PCR and pulsed-field gel electrophoresis (PFGE) .In addition PFGE proved to be superior for interpretation of inter-strain relationships. Maria Miragaia et al. (2002) .

The penicillinase resistant penicillins (methicillin, nafcillin , oxcacillin , cloxacillin) have less potent antimicrobial activity against microorganisms that are sensitive to penicillin G, but they are effective against penicillinase producing *Staphylococcus aureus* (Chambers, 1997). Several global regulators of virulence in Staphylococcus aureus have been described. One of these systems, the accessory gene regulator system (agr), has been well described in vitro. agr consists of two divergently transcribed promoters; promoter 2 directs transcription of RNAII, which encodes the AgrBDCA quorum-sensing two-component system, while promoter 3 directs transcription of RNAIII, which is the effector molecule of the agr locus (Peng, et al., 1988). In vitro studies have revealed that RNAIII expression is highest during the postexponential and stationary phases of growth, during which RNAIII enhances expression of exotoxins and represses expression of surface-associated virulence factors. Although these in vitro studies demonstrated that agr gene plays a role in the global regulation of virulence factors (Alexa et al., 2004).

Methicillin is one of beta- lactam antibiotics, and resistant to hydrolysis by penicillinase produced from Staphylococci." Methicillin - resistant" is a generic term for beta- lactamase resistance by an acquired cell wall synthesizing enzyme (PBP2 or PBP2a, synonyms) coded by the mecA gene.

This work was aimed mainly to determine

a) The frequency of resistance of *Staphylococcus aureus* to various antimicrobial agents.

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a) The frequency of resistance of *Staphylococcus aureus* to various antimicrobial agents.

b) The relationship between antimicrobial resistance of the isolates and carriage of plasmids.

c) The correlation between some virulence mark and those of methicillin resistant strains.

MATERIALS AND METHODS

Bacteriological examination:

A total of 196 milk samples were examined bacteriologically for detection of methicillin resistant *Staphylococcus aureus* during the period from January to October 2004.

The cream and supernatant fluid were discarded. A loopfull from the sediment was streaked onto the surface of blood agar, Oxoid No. 110 . Baird parker and mannitol salt agar plates. All inoculated plates were incubated at 37°C for 24-48 hrs. Suspected growing colonies were studied morphologically, culturally and biochemically according to Quinn et al. (2002).

Identification of the isolates:

The identification of the isolates were carried out with the API STAPH system consists of : Carrying out 19 tests using the API STAPH strip and the results were interpreted using the API STAPH data base.

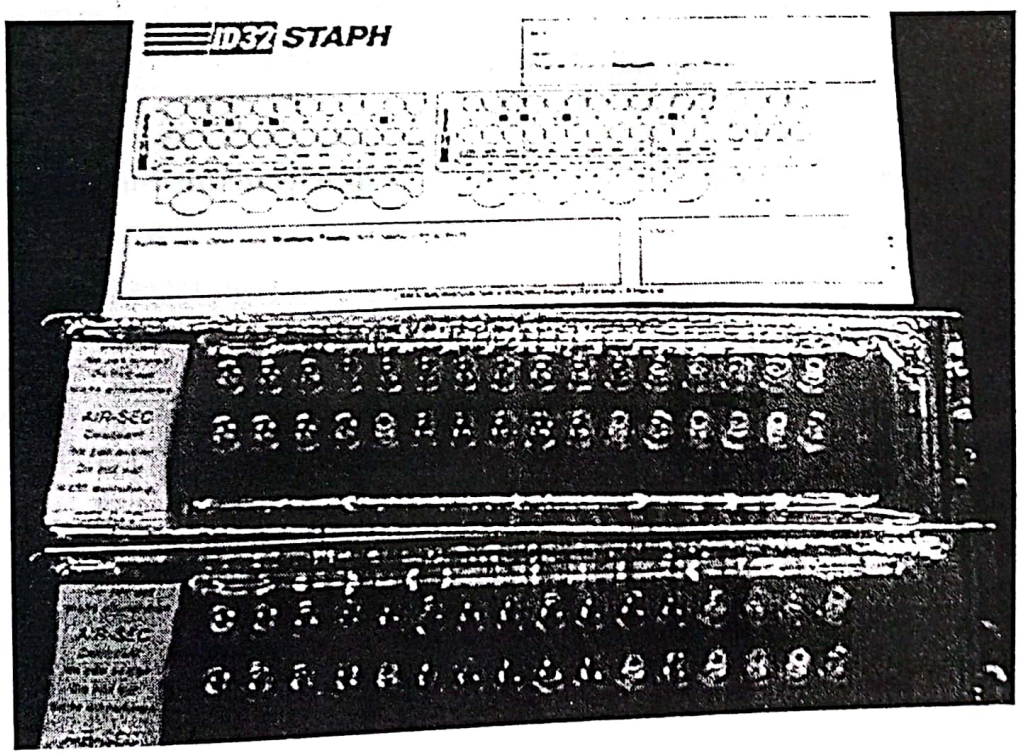


Photo (1): shows the API STAPH strip
This test was applied on the laboratory of Microbiology (Faculty of Agriculture Kobe University. Japan)

Antibiotic sensitivity test:

Five antibiotics, which are frequently used in mastitis therapy, were chosen: penicillin-G, ampicillin, kanamycin, and cephalexine. The fifth compound was methicillin using agar disk diffusion methods.

Detection of haemolysin titre

a- Preparation of haemolysin (Feder et al. , 1994)

Single-colony grown on tryptic soy agar (TSA) plate containing 5% sheep blood (24 h. at 37°C in 6% CO_2) was sub cultured onto TSA plate and incubated at 6% CO_2 for 16 h at 37°C . Colonies were harvested from TSA plates with cold RPMI 1640 medium and were transferred to an ice chilled flask. The RPMI suspension was incubated aerobically for 1 h at 40°C , then centrifuged at 12.000 Xg for 10 min. (4°C) and the supernatant was filtered through a 0.45 µm pore size filter. All filtrates were chilled on ice and haemolysin assays were conducted immediately.

b- Haemolysin assay (Marchlewicz and Duncan, 1980)

The test was conducted into 96 U shape haemagglutinating plates. 25 µl of PBS (pH 7.2) were added to each well. 25 µl of culture supernatant were add to the first well and then subjected to double fold serial dilution.

25 µl of washed sheep RBCs (1%) were added to all wells.

3 wells containing 25 µl of washed sheep RBCs were included in the test as a negative culture.

Pathogenicity in mice (Yoshida and Takuchi, 1970)

Cell suspensions were prepared from cultures grown on mod. staphylococcus 110 agar. The organisms were gently washed with mod staphylococcus 110 broth and suspended in the same medium. These cell suspensions were added to colony forming unit / ml of each cell suspension contain $1x10^9$ (cfu/ml). 0.5 ml was injected I/P into each group of 6 mice. The number of mice that died during first 2 weeks after challenge was recorded. Those isolates caused death of 60% or more of the animals were regarded as virulent and isolates which caused death of 40% or less were considered avirulent.

Genetic characterization of staphylococci by PFGE analysis

The protocol of Pulsed- Field Gel Electrophoresis (PFGE) for Staphylococcus was carried out according to Chung et al. (2000) as follows:

Culturing the isolates

The isolates were cultured into 5 ml of brain heart infusion broth and incubated overnight with shaking (130 rpm) at 37°C

0.7 ml of the culture was centrifuged at 8.000 xg for 10 minutes and harvested by removing the supernatant. Cells were washed once in 1 ml of

TEN buffer (0.1 M Tris - HCl, 0.15 M NaCl, 0.1 M EDTA, pH 7.5) and centrifuged again at 8.000 xgm for 10 minutes.

The washed cells were resuspended in 0.3 ml of autoclaved EC buffer (6 mM Tris-HCl, 1 M NaCl, 0.1 M EDTA, 0, 5% Brij 58, 0.2% deoxycholate, o.5% Sarkosyl, pH 7.5).

10 µl of a lysostaphin solution (1mg/ml in 20 mM sodium acetate, pH 4.5) was added to the cell suspension, which was immediately vortexed and placed into 300µl of warm (55°C) 2% low-melting temperature agarose prepared with EC buffer. The agarose cell suspension was immediately vortexed and transferred into a well of a 10-well sample plug mold. After the plug solidified, the plug was placed in a tube containing 3 ml of EC buffer and the cells in the plug was lysed for 1 to 2 hours at 37 °C, until the plug was cleared (complete lysis).

After lysis, the EC buffer is decanted and replaced with 3 ml of TE buffer (10 mM Tris-HCL, 1 mM EDTA, pH 7.6), then incubated at 55°C for 1 hour.

The plug was then transferred to 3 ml of fresh TE buffer for storage at 4 °C until further analysis.

Digestion with Sma I and Electrophoresis. (Tenover et al., 1995).

125µl of restriction enzyme buffer solution containing 20 U of Sma I (110.5µl of sterile distilled water, 12,5 µl of 10 x NE buffer 4, 2.0µl of Sma I (20.000µ /ml) is compounded.

For DNA digestion, the agarose plug was cut into small slices (2x4x1.5 mm) and placed in the restriction enzyme solution, and then incubated with shaking at 130 rpm for 2 hours at 25°C.

During digestion, 15-well of 1% GTG agarose gel slab (12,5 x 14 x 0,9 cm) is prepared in 100 ml of 0,5 x Tris borate EDTA buffer (89 mM Tris- HCl, 89 mM boric acid, 2 mM EDTA, pH8,0), and air was removed from 2.500 ml of 0.5 x TBE buffer for electrophoresis.

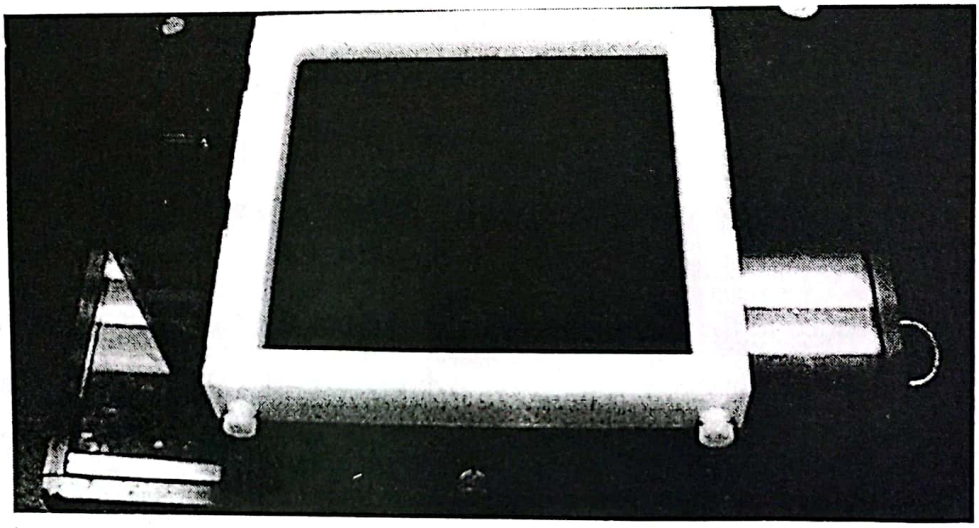
After digestion, the agarose plug was very carefully inserted into the well of gel slab and sealed with 0.8% low- melting temperature agarose prepared with 0.5 x TBE buffer.

Bacteriophage lambda DNA concatemers (lambda ladder) was used as size standards and served as a control for the running parameters.

Electrophoresis was performed at 14°C in 0.5 x TBE buffer from which air is removed. The running parameters were as follows: initial pulse, 5s; final pulse, 40s; voltage, 6/cm; and time, 22 hours. Pomp setting is adjusted at 65 to 70.

PFGE analysis.

After electrophoresis, the gel was stained with ethidium bromide (2ul/ml) for 25 minutes. After staining, the gel was washed with distilled water for 2 hours and the gel is photographed under UV light.



RESULTS AND DISCUSSION

Bovine mastitis is a major disease that affects the dairy industry and *S. aureus* is one of the most frequently isolated pathogens from both clinical and chronic infections (Bramley , 1992). Mastitis is the most significant cause of economic losses of dairy industry. Although several bacterial pathogens can cause mastitis, *Staphylococcus aureus* has emerged as one of the most prevalent ones, and once it is established in the mammary gland, it is difficult to eradicate (Stringfellow et al., 1991). To establish an infectious process *S.aureus* must overcome the different host defense mechanisms (Phagocytosis, elimination by milking etc) in order to reach and colonize the ductular and alveolar mammary gland region (Aguilar et al., 2001).

Table (1) Incidence of *Staphylococuus aureus* isolated from milk samples

Number of examined milk samples	Number of bacteriologically positive samples		Total number of *Staphylococcus* species		Number. of *Staphylococcus aureus* species	
	No.	%*	No.	%	No.	%**
196	44	22.4	13	29.5	5	38.5

* % = percentage was calculated according to total number of examined samples (196)

** % = percentage was calculated according to total number of staphylococcus speiess (13).

Table (1) showed that out of 196 milk samples from buffaloes showing signs of mastitis 44 samples were found to be positive to bacteriological examination with an incidence of 22.4%. Detection of samples revealed the isolation of Staphylococcus species from 13 samples with the percentage of 6.6. On the other hand *Staphylococcus aureus* were isolated from 5 samples with an incidence of 38.46 %.and this high incidence of isolation are in concurrence with those of Verma (1988), Capurro et al. (1999), Seddek et al. (1999) and Vitayalakshmi et al. (2001) who found that *S.aureus* isolated with a high incidence than the other types of Staphylococcus species.

One of the virulence factors was haemolysin activity and the importance of this factor was also demonstrated by Feder et al. (1994). Results obtained revealed that majority of *Staphylococcus aureus* isolates (80%) were haemolytic with the maximum haemolytic activity was 1/1024 (Table 2). Cifrizn et al. (1996) reported that lyses of bovine erythrocytes was due to primarily to β toxin but the presence of α toxin in culture supernatant from *Staphylococcus aureus* didnít increase the lyses of bovine erythrocytes.

Table (2): Haemolysin titre of *Staphylococcus aureus* isolated from mastitic milk samples.

Source of isolates	Haemolysin titer						
	1 /32	1/64	1/128	1/ 256	1/512	3 / 1024	Total positive
Clinical mastitis	0 / 5	0 / 5	0 / 5	1 /5	1 /5	2/5	4/5
	0 %	0 %	0 %	20 %	20 %	40 %	80 %

Titre > 128 considered positive

Pathogenicity of *S. aureus* in mice were discussed in the present study to bring into being the risk of Staphylococcus aureus isolated from mastitic milk . As shown in Table (3), mice were infected experimentally with *Staphylococcus aureus* isolates by I/P rout. The mortality rate was highest in the period 3-6 days and 1-3 days and decrease gradually within the following days but with an end incidence reach 100% and this mortality was explained by Joyce et al. (1984) who proved that, death after challenged through endotoxic shock by enterotoxin "C" of *Staphylococcus aureus* induced blockade of reticuloendothelial system.

Table (3): Pathogenicity of *Staphylococcus aureus* isolated from mastitic milk samples in mice

Source of isolates	Total	No. of inoculated mice	No. of dead mice per day					No. of dead mice	Mortality rate
			1-3	3-6	6-9	9-12	12-14		
Clinical mastitis	5	30	8	13	6	3	0	30	100

The emergence and worldwide spread of methicillin-resistant *Staphylococcus aureus* (MRSA) between the early 1960s and the late 1990s have begun to pose serious threats to the chemotherapy of staphylococcal diseases worldwide. The genetic determinant of methicillin resistance in Methicillin resistance Staphylocccus aureus is the acquired gene mecA, which encodes the low-affinity penicillin-binding protein 2A (PBP2A), which, according to current theory, can function as a surrogate transpeptidase in the presence of high concentrations of -lactam antibiotics that inactivate the four high-affinity PBPs native to S. aureus (Goni et al., 2004). In addition, the mecA gene and the associated large (40-to 60-kb) mec element are not native to *S. aureus* but were acquired from an extraspecies source by an unknown mechanism. The nature of the extraspecies source, i.e., the evolutionary origin of mecA and the formation of the mec element, has remained largely a matter of speculation (Savolainen et al., 2001).

In methicillin-resistant strains of *S. aureus*, the *mec*A gene provides a unique and broad range of resistance to all -lactam antibiotics. Surprisingly, strains of *S.sciuri* carrying the structurally similar *mec*A homologue were found to be uniformly susceptible to -lactam antibiotics, including even penicillin. The contrast between the striking structural similarity of the *S.sciuri* mecA homologue to the mecA gene of *S. aureus* and the complete lack of associated antibiotic resistance in the case of *S.sciuri* prompted us to explore possible structural changes in the *S.sciuri* mecA homologue and its transcription in -lactam-resistant mutants. The observations described in this communication suggest that the antibiotic pressure selects for a unique structural change in the regulatory sequence of the mecA homologue, converting it to an antibiotic resistance determinant capable of expressing the resistant phenotype even in the genetic background of *S. aureus*. Shangwei et al. (1998).

Staphylococcus aureus is a pathogen for numerous animal species and humans. Human isolates of *S. aureus*, unlike animal isolates, are frequently resistant to the penicillinase-resistant penicillins. Organisms exhibiting this type of resistance are referred to as methicillin (oxacillin)-resistant *S. aureus* (MRSA). In the 1980s, MRSA emerged as a major clinical and epidemiological pathogen in human hospitals (Kloos and Bannerman, 1995). The seriousness of this problem has been compounded by the fact that these organisms are frequently resistant to most of the commonly used antimicrobial agents, including the aminoglycosides, macrolides, chloramphenicol, and tetracycline. Although initially susceptible to the fluoroquinolones, MRSA strains have rapidly developed widespread resistance to this class of antimicrobial agent (Mandell, et al., 1995).

The activity of selected antimicrobial agents against *Staphylococcus aureus* was determined with the agar disk diffusion test to determine the diameter of the zone of inhibition.. Four antibiotics, which are frequently used in mastitis therapy, were chosen: penicillin-G, ampicillin, kanamycin, and cephalexine. The fifth compound was used to detect methicillin-resistant *S. aureus*, but no such strains could be found. According to the evaluation criteria, 60.0 (penicillin) to 80.0 % (kanamycin, cephalexine) *S. aureus* isolates were susceptible to the antibiotics tested.

Table (4) Results of sensitivity test of *S. aureus* to different antibiotics

Antimicrobial	*S. aureus*	
	Sensitive	Resistant
	%	%
Cephalexine	80.0	20.0
Ampicillin	20.0	80.0
Kanamycin	80.0	20.0
Penicillin	60.0	40.0
Methicillin	100.0	0.0

Results obtained in this study showed that all isolates were sensitive to methicillin with an incidence of 100% and this observation were in accordance with that mentioned by Maria Miragaia et al. (2002) who recorded that the frequency of methicillin-resistant *Staphylococcus aureus* is very low and attributed this to the strict infection control and restrictive antibiotic use policies. In addition, Hartmann et al. (1997) reported that concerning the isolation of MRSA in a veterinary environment, it was concluded that the isolates were not of animal origin and were most likely from humans. Results of sensitivity test of *S. aureus* to the rest of antibiotics used concerning in particular ampicillin and penicillin were focused due to the relative high resistance observed and this explained by (National Committee for Clinical Laboratory Standards. 1997) that MRSA strains should be considered to be resistant to all cephalosporins, cephems, and other β-lactams, such as ampicillin-sulbactam, amoxicillin-clavulanic acid, tiarcillin-clavulanic acid, piperacillin-tazobactam, and the carbapenems, regardless of the in vitro test results obtained with those agents. The justification for this is the poor clinical response to those antimicrobial agents by MRSA.

Using of the pulsed gel electrophoresis was significant in this research to accomplish our results and this observation were in accordance with that mentioned by Maria Miragaia et al. (2002) that PFGE met the requirements for the molecular characterization of MRSE strains, identifying the major clones, as well as the diversity among them.

The existence of common PFGE types among MRSA isolates from distant origins can result either from geographic dissemination of these MRSA strains among the various countries or from independent and convergent evolution of distinct strains within these different locations. The first hypothesis seems to be more plausible than the second, based on the following observations. *S. aureus* is endogenous to the human skin flora and is therefore easily transmissible. In fact, the dissemination of the resistant counterparts (MRSA) was already described in the hospital environment (Dominguez et al., 1996 and Villari et al., 2000),

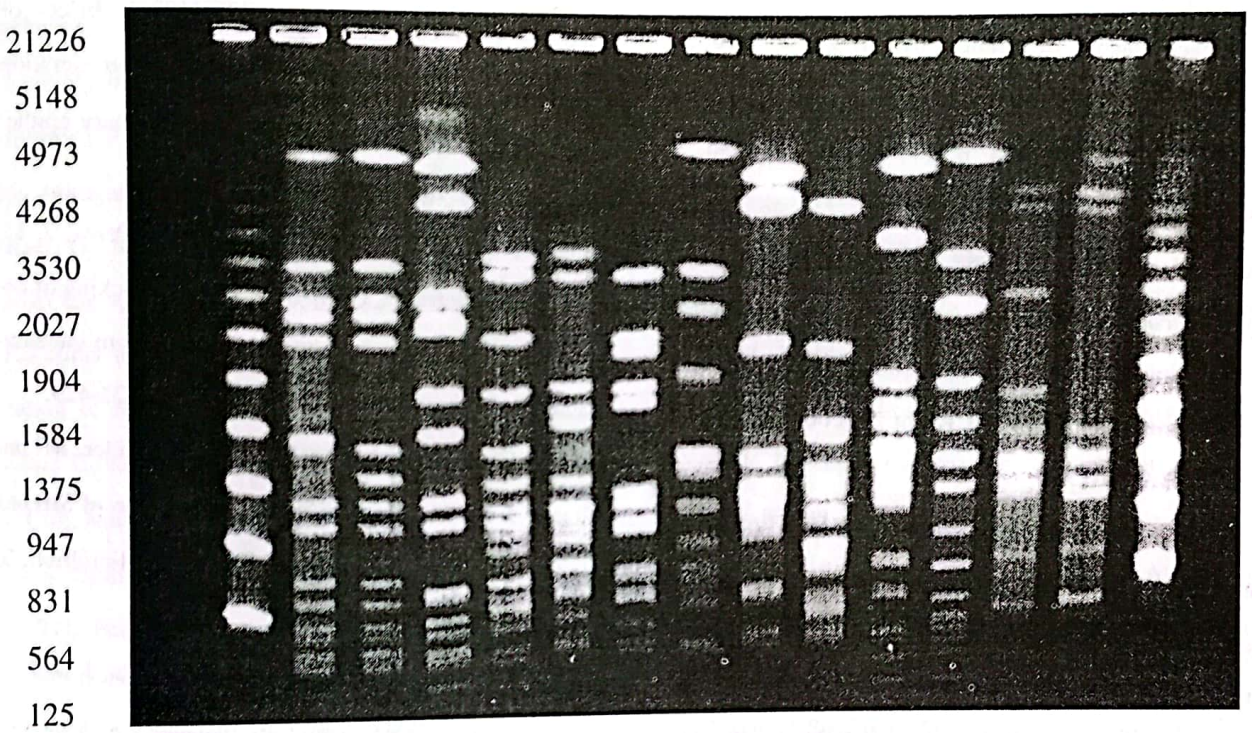


Photo (3) : Pulsed field gel electrophoresis product

Marker : conventional lambada DNA marker: suitable for sizing linear double stranded DNA fragments in agarose gel.

Lambada DNA is digested to completion with appropriate restriction endonuclease, purified and dissolved in a storage buffer.

Premixed with a loading dye solution for direct loading onto agarose gel.

The lambada DNA marker can be easily visualized by ethidium bromide.

Range: 13 fragments (in bp) 21226-5148-4973-4268-3530-2027-1904-1584-1375-947-831-564-125).

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