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DIFFERENTIATION BETWEEN HIGH AND LOW VIRULENCE *STAPHYLOCOCCUS AUREUS* STRAINS FROM RABBITS BY RANDOMLY AMPLIFIED POLYMORPHIC DNA (RAPD)-ANALYSIS.

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ABSTRACT

Randomly Amplified Polymorphic DNA (RAPD) typing was performed on 53 rabbit *Staphylococcus aureus* strains. Twenty-three strains isolated in thirteen different rabbitries with chronic problems of staphylococcosis, showed the same RAPD banding pattern. Twenty of these strains belonged to the "mixed CV-C" biotype and to the phage type 3A/3C/55/71, previously described to be highly virulent in rabbits, and three strains belonged to other biotypes or phage types. None of the strains isolated from rabbitries without chronic problems of staphylococcosis showed this specific RAPD pattern. RAPD-analysis can be used as a rapid and reliable test method to differentiate between the characteristic genotype corresponding to high virulence and other *S.aureus* strains from rabbits. This is useful for the diagnosis and prevention of introduction of these highly virulent strains in industrial rabbitries.

INTRODUCTION

In rabbits, problems of staphylococcosis arise when Staphylococcus aureus bacteria infect small dermal lesions and invade subcutaneous tissue (Okerman et al., 1984). All *S.aureus* infections of rabbits have a similar clinical appearance, with lesions of pododermatitis, subcutaneous abscesses and mastitis, and cases of septicaemia (Hagen, 1963; Okerman et al., 1984; Devriese et al., 1996). At the rabbit flock level, two types of *S.aureus* infections can be distinguished. In the first type, the infection remains limited to a small number of animals and has only a minor economic importance. In the second type of infection, *S.aureus* causes an epidemic spread of disease in the rabbitry. This leads to chronic problems that render infected rabbitries unremunerative. Strains causing an epidemic spread of disease in rabbitries usually belong to the biotype - phage type combination "mixed CV-C" - 3A/3C/55/71 (Okerman et al., 1984; Rossi et al., 1995; Hermans et al., 1999). On only one occasion, i.e. transiently from the end of 1994, another pathogenic type of *S.aureus* was described, notably the "mixed CV-C" biotype - phage type 29/79/42E/92/D11/HK2 (Devriese et al., 1996).

In order to avoid problems of staphylococcosis, the introduction of high virulence strains by carrier rabbits has to be prevented. Thus far, biotyping and phage typing were used to differentiate between high virulence and low virulence *S.aureus* strains in rabbits. These techniques are however laborious, time-consuming and may be difficult to standardise outside specialised laboratories (Parker, 1962). Randomly Amplified Polymorphic DNA (RAPD) typing (Williams et al., 1990), also called Arbitrarily Primed Polymerase Chain Reaction (AP-PCR) (Welsh and McClelland, 1990), is a technique suited for rapid detection of genomic polymorphisms. This detection method is based on a polymerase chain reaction with a single short oligonucleotide primer of arbitrary sequence. It was the aim of this study

to determine if RAPD typing can be used to differentiate highly virulent *S.aureus* strains causing an epidemic spread of disease in rabbitries from low virulence strains.

MATERIALS AND METHODS

Bacterial strains and growth conditions

In the present studies, 53 rabbit *S.aureus* strains were used. The strains belonged to five different biotypes, namely mixed CV-C, mixed CV-A, the human biotype, the poultry biotype and a type that could not be allotted (Devriese et al., 1981; Devriese, 1984). Twenty-two distinct phage type patterns (Parker, 1962) could be distinguished using the International Typing Set for human *S.aureus* strains (Parker, 1962). All strains were isolated in Belgium, except for one strain that was isolated in the United Kingdom, one strain from Spain and two strains from the Netherlands.

Twenty-eight rabbit strains were isolated from fourteen rabbitries with chronic problems of staphylococcosis. Of these 28 strains, twenty strains belonged to the biotype - phage type combination "mixed CV-C" - 3A/3C/55/71 and one strain to the biotype "mixed CV-C" and the phage type 29/79/42E/92/D11/HK2. Both these biotype - phage type combinations have been described as highly virulent in rabbitries (Okerman et al., 1984; Rossi et al., 1995; Devriese et al., 1996; Hermans et al., 1999). The remaining seven strains belonged to other biotype - phage type combinations.

Twenty-five strains were obtained from twelve rabbitries without problems of staphylococcosis. None of these strains belonged to the "high virulence" biotype - phage type combinations.

The strains were grown on Columbia agar (Gibco, Paisley, United Kingdom) supplemented with 5 % ovine blood and incubated overnight at 37°C in a 5 % CO_2-enriched environment.

Extraction of genomic DNA and RAPD-PCR

One colony of each isolate was suspended into 20 µl of lysisbuffer (0.25 % sodium dodecyl sulphate and 0.05 N NaOH) according to Niemann et al. (1997). Samples were incubated for 5 min at 95°C and centrifuged briefly at 16000 x g at room temperature to collect the contents to the bottom of the tube. After adding 180 µl of distilled water to these contents and centrifugation for 5 min at 16000 x g, these sample preparations were stored at - 20°C and the supernatant was used as the DNA-extract. DNA amplification was done using Ready-To-Go RAPD Analysis Beads (Amersham Pharmacia Biotech, Uppsala, Sweden), consisting of buffer, dATP, dCTP, dGTP, dTTP and Taq polymerase, to which 1 µM of primer and 1/15 volume of sample preparation were added in a total volume of 25 µl. Each reaction mixture was centrifuged briefly at 16000 x g at room temperature and overlaid with 50 µl of mineral oil. The cycling programme was performed in a Gene E PCR cycler (Techne, Cambridge, United Kingdom) and consisted of one cycle of [94°C, 5 min; 35°C, 5 min; 72°C, 5 min], 30 cycles of [94°C, 30 sec; 35°C, 1 min; 72°C, 1 min] and 5 min at 72°C. The primer was a decamer, designated RAPD 4 M, synthesized by BioSource, Fleurus, Belgium and had the following sequence: 5'-AAGACGCCGT-3'. Reliability of the technique was determined by amplifying eighteen strains twice and five strains at five separate occasions.

Gel electrophoresis and photography

Two microlitres of sample buffer (50 % glycerol, 1 mM cresol red) were mixed with seven microlitres of PCR mixture. Electrophoresis was done in a gel containing 2 % Metaphor Agarose (FMC BioProducts, Rockland, Maine), 0.5 % Multi Purpose Agarose (Boehringer Mannheim, Brussels, Belgium) and 50 µg/l ethidium bromide (Sigma-Aldrich, Bornem, Belgium) in 1 X TBE buffer (Gibco, Paisley, United Kingdom). Gels were run for two hours

at 175 V in a Sub-cell tank (Bio-Rad, Nazareth, Belgium) containing 0.5 X TBE buffer with 50 µg/l ethidium bromide. After electrophoresis, gels were visualised under U.V. light and photographed. The Gene Ruler™ 100 bp DNA Ladder Plus (MBI Fermentas, St. Leon-Rot, Germany) was used as a DNA size marker. Gels were compared both visually and by means of GelCompar software (Applied Maths, Kortrijk, Belgium).
For the eighteen strains that were amplified twice, the two separate PCR products were run on different gels. The results were compared visually. For the five strains that were amplified five times, a comparison of the PCR products of identical strains was performed both on the same gel and on different gels. Variability caused by gel electrophoresis was evaluated by means of GelCompar software (Applied Maths, Kortrijk, Belgium). The similarity was calculated with Pearson correlation, using Ward's clustering algorithm with fine optimization.

RESULTS

The 53 *S.aureus* strains from rabbits produced thirteen RAPD fingerprints (a - m) as shown in Figure 1a and 1b. Results of RAPD typing of the different strains are given thereafter.

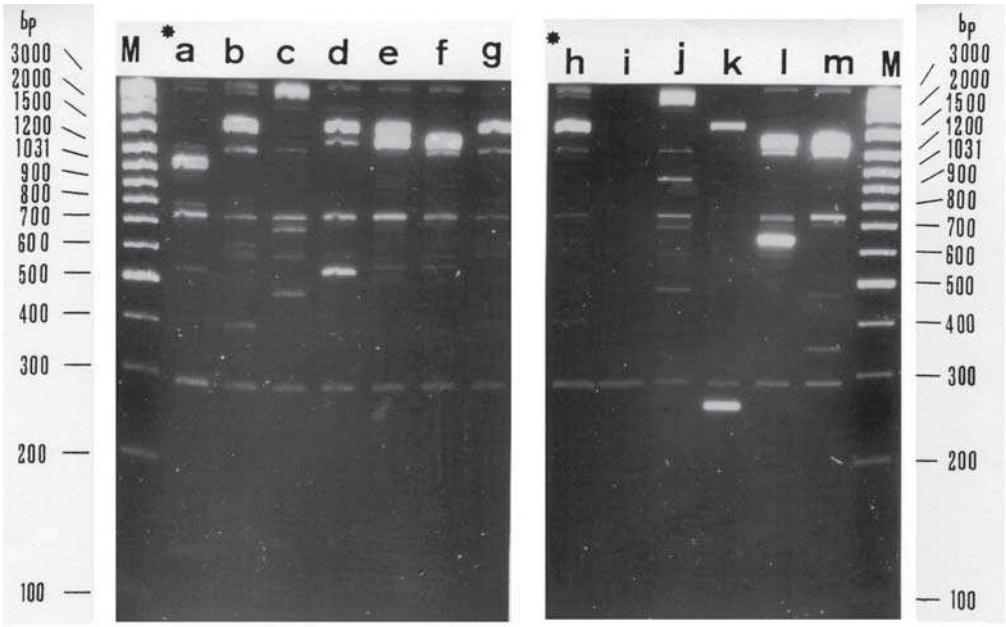

Figure 1a and 1b: Agarose gel electrophoresis after RAPD typing of rabbit *S.aureus* strains, using primer RAPD 4 M.
M: DNA size marker used was Gene Ruler™ 100 bp DNA Ladder Plus (MBI Fermentas, St. Leon-Rot, Germany). *RAPD profiles (**a-m**) are indicated above.

Twenty-three rabbit strains examined showed the RAPD type a, which consisted of 9 distinct DNA fragments, of 3300, 2950, 1280, 1080, 740, 700, 530, 290 and 120 base pairs (bp). Twenty of these strains belonged to the biotype - phage type combination "mixed CV-C" - 3A/3C/55/71. The three other strains belonged to the biotype - phage type combinations "mixed CV-A" - 3A/3C/55/71, "mixed CV-C" - 29/52/52A/80/3A/3C/55/71/6/ 42E/47/53/54/75/84 and "mixed CV-C" - 29/3A/3C/55/71/6/42E/47/54/75/84/94, respectively. All these strains were isolated in rabbitries experiencing chronic problems of staphylococcosis. Five strains isolated in rabbitries with severe problems, but not belonging to the biotype - phage type combination "mixed CV-C" - 3A/3C/55/71, showed a profile that

differed from RAPD type a. Furthermore, none of the strains isolated in rabbitries without chronic problems of staphylococcosis showed the RAPD type a profile.

Thirteen rabbit *S.aureus* strains belonged to RAPD type b and two strains each to RAPD types d, e, f, i and j. RAPD types c, g, h, k, l and m included one strain. One strain produced no banding pattern at all on gel electrophoresis, even after repeated testing.
Similarity levels between strains determined visually as having the same pattern, varied between 95.5 ± 0.9 % and 99.5 ± 0.0 % for strains within the same gels. For the eighteen strains that were tested twice and run on different gels, results were comparable, although some variability in the separation and intensity of the amplimers was found. For the five strains that were tested five times, the similarity level found varied from 93.6 ± 3.4 % to 96.6 ± 1.5 % for comparison between different gels, and from 95.5 ± 0.9 % to 97.7 ± 0.8 % for comparison within the same gel. RAPD type a was consistently clearly distinguishable from other RAPD profiles.

DISCUSSION

Several methods are available for typing *S.aureus* strains. Until now, only biotyping and phage typing were useful to distinguish between high virulence and low virulence *S.aureus* strains in rabbits. Strains belonging to the biotype "mixed CV-C" and to the phage type 3A/3C/55/71 are known to be highly virulent in commercial rabbitries (Okerman et al., 1984; Rossi et al., 1995; Hermans et al, 1999). In the present study, RAPD fingerprinting from strains with these properties was performed. All twenty strains belonging to this biotype-phage type combination, isolated in thirteen different rabbitries suffering from severe problems of staphylococcosis, showed the same profile on gel electrophoresis. These strains included seventeen *S.aureus* strains isolated during 1997-1998 in ten Belgian rabbitries, one strain that was isolated in Belgium in 1983, one strain isolated in Spain, and one strain isolated in the United Kingdom. The fact that these strains were isolated in Belgium with a fifteen year - interval and were isolated in different countries, may indicate the occurrence of a clonal type of *S.aureus*, which causes severe problems of staphylococcosis in rabbits. This situation is analogous to that in dairy cows, where it is supposed that mastitis is caused by only a few high virulent clones of *S.aureus*, which have a broad geographic dissemination (Fitzgerald et al., 1997).

Since phage typing is difficult to standardise in the field and can only be applied in specialised laboratories (Parker, 1962), RAPD typing may provide a more rapid and a reliable method to distinguish between high and low virulence *S.aureus* strains. Moreover, RAPD typing is simpler to execute and interpret compared to other genotyping methods, such as genomic restriction endonuclease fingerprinting and DNA hybridization (Matthews et al., 1994). The rapidity of the method and its cost-effectiveness (Van Belkum et al., 1995a) make it suitable for use as a preventive diagnostic test.

Since antibiotics and eradication of diseased animals are not able to clear high virulence strains from affected rabbitries, introduction of such strains in rabbitries by carrier rabbits has to be prevented in order to avoid problems of staphylococcosis. Before new animals are introduced in a rabbit flock, it could be possible to sample them for the presence of *S.aureus*, and when found positive, to type the strains by RAPD-analysis. If these rabbits appear to be carriers of the high virulence strains, they should not be introduced in the rabbitry.
In the present study, typing five strains five times showed that banding patterns of RAPD typing performed at different occasions have a high level of similarity. The level of similarity

was a little higher within the same gel than between different gels. This confirms earlier findings that gel electrophoresis is still a cause of a certain experimental variability (Van Belkum et al., 1995a), especially regarding the separation and intensity of the amplimers. When RAPD-analysis is used to detect high virulence *S.aureus* strains, it is therefore useful to include a known high virulence strain in the test as a positive control.

RAPD typing as a preventive diagnostic test may be useful, but there is a restriction, however. Strain PI 41/95, which was tested in this study, was isolated from a rabbitry with severe staphylococcosis in 1995. This strain does not belong to the common rabbit-pathogenic biotype - phage type combination mixed CV-C – 3A/3C/55/71, but has nevertheless been described as highly virulent in rabbitries (Devriese et al., 1996). Still the RAPD pattern of this strain did not belong to type a. No strains with the biotype - phagetype combination of strain PI 41/95 were isolated during a recent screening in Belgian rabbitries (Hermans et al., 1999). However it is probable that in the future new pathogenic *S.aureus* types will appear in certain flocks. Characterisation of *S.aureus* strains isolated from severe outbreaks and not belonging to RAPD type a, will therefore be necessary.

Three strains showing the "virulent" RAPD type a did not belong to the common rabbit-pathogenic biotype - phage type combination "mixed CV-C" - 3A/3C/55/71. They were all isolated in rabbitries suffering from chronic problems of staphylococcosis. In these herds, strains belonging to the "high virulence" biotype - phage type combination were also present. Strain KH 383, belonged to the phage type 3A/3C/55/71 but produced staphylokinase and showed growth type A on crystal violet agar instead of growth type C. This might indicate that growth characteristics on crystal violet agar and production of staphylokinase can alter in the field. Indeed, lysogenic conversion of staphylokinase production has been described (Kondo et al., 1976). Two "mixed CV-C" strains (KH 119 and KH 522) showing the "virulent" RAPD type a, not only were susceptible to phages 3A, 3C, 55 and 71, but also to several other phages. This might indicate that phage-sensitivity can alter in the field as well, or that phage typing may be able to differentiate between strains that show the same RAPD-pattern. Further studies are required to determine the virulence of these three strains.
In two rabbitries with chronic problems of staphylococcosis, not only "mixed CV-C" - 3A/3C/55/71 strains were isolated, but also four strains belonging to biotype - phage type combinations which are considered to be of low virulence. These strains did not show RAPD type a. These results confirm that high and low virulence strains can be present in the same rabbitries and even on the same rabbit (Hermans et al., 1999).

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