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Virulence type and tissue tropism of *Staphylococcus* strains originating from Hungarian rabbit farms

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ABSTRACT

Staphylococcosis has a major economic impact on rabbit farming worldwide. Previous studies described a highly virulent variant, which is disseminated across Europe. Such strains are reported to be capable of inducing uncontrollable outbreaks.

The authors describe a survey conducted on 374 *Staphylococcus* strains isolated from rabbit farms, mostly from Hungary, between 2009 and 2014, from a variety of pathological processes. The virulence type of the strains was determined using a multiplex PCR system.

84.2% of the strains belonged to a previously rarely isolated atypical highly virulent type. Only 6.1% belonged to the typical highly virulent genotype. Even low virulent strains were present at a higher percentage (6.4%). For a small group of strains (3.2%) the detection of the *femA* gene failed, indicating that these strains probably do not belong to the *Staphylococcus aureus* species.

The results reveal the possibility of the asymptomatic presence of highly virulent strains on rabbit farms. "Non-aureus" *Staphylococcus* sp. can also have a notable role in the etiology of rabbit staphylococcosis. An association with the lesions and the virulence type was demonstrated. Statistical analysis of data on organotropism showed a significant correlation between septicaemia and the highly virulent genotype.

1. Introduction

Staphylococcus aureus is one of the most important opportunistic pathogens, capable of infecting humans and a large variety of domesticated animals. Dermatitis and abscess formation, mastitis, respiratory diseases, urogenital infections and septicaemia can be caused by this organism in a variety of host species. Staphylococcal food poisoning (SFP) in humans is also an emerging problem. Other *Staphylococcus* species are described to be related with similar conditions of different species (Peton and Le Loir, 2014).

This disease in the domesticated European rabbit (*Oryctolagus cuniculus*) is usually presented as a subacute or chronic, purulent and necrotizing inflammation in different organ systems, manifested in an endemic pattern within the flock. Most commonly the infection of the skin, the subcutaneous tissue, the respiratory system and the mammary gland can be observed in practice. Neonatal septicaemia and mastitis of lactating does also frequently develop (Vetési et al., 1990). Staphylococcosis is a serious problem in rabbit farming. Kit mortality, premature elimination and replacement of diseased breeders, and slaughterhouse condemnations cause substantial losses to the industry (Hermans et al., 2003).

Characterization of the pathogens derived from diseased rabbits revealed the existence of highly virulent (HV) strains (Devriese et al., 1996). The evolution of specialized diagnostics resulted in a multiplex PCR method, which can be used to differentiate between strains of high virulence (HV) and low virulence (LV), and also to identify atypical highly virulent (aHV) strains (Vancraeynest et al., 2007). HV strains have the *bbp* (bone sialoprotein binding protein) gene (Vancraeynest et al., 2004) and *selm* gene, which is an allel within the *Staphylococcus* enterotoxin gene cluster (Vancraeynest et al., 2006a). Both HV and aHV strains

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have a nucleotide sequence named *flank*, which is identified to be specific for virulent strains (Hermans et al., 2000a; Hermans et al., 2001). HV, aHV and LV strains are all resulting an amplicon related to the *femA* gene in this multiplex PCR system. *FemA* is encoding a factor which is essential for methicillin resistance, and which is universally present in all *Staphylococcus aureus* isolates (Johnson et al., 1991; Vannuffel et al., 1995; Mehrotra et al., 2000).

HV strains are able to colonize domesticated rabbits permanently, they are proven to be particularly pathogenic for this species (Hermans et al., 2000b; Meulemans et al., 2007; Meulemans et al., 2011). The international dissemination of such strains has also been described (Vancraeynest et al., 2006b).

Staphylococcosis also has a major economic impact on Hungarian rabbit farming. Generally this disease is present on all farms, but epidemic spread can be prevented by management and medication. To the author's knowledge, genotypic characterization of *Staphylococcus* strains of rabbit origin has never been performed in Hungary. As modern rabbit farming in Hungary is based on French hybrid breeds, the presence of pathogens reported from Western European countries in Hungarian farms was predictable.

Our Department offers diagnostic services for rabbit producers. The bacterial pathogens isolated from diseased organs are routinely archived for scientific purposes. In the present paper we report the result of multiplex PCR performed on n = 374 *Staphylococcus* strains, originating from a total of 30 farm units, 28 from Hungary, representing approximately 85% of the Hungarian industrial rabbit population.

2. Materials and methods

2.1. Bacterial strains

Staphylococcus sp. strains used in this study were collected from 2009 to 2014, from a total of 30 industrial rabbit farms (data table provided as Appendix A in Supplementary material). Twenty-eight of the farms are located in Hungary, one in Austria (XXVI) and the other in Slovakia (XXIV). The doe population of the farms has an average size of 2943 (±2219 SD; median: 1800, range 300–22,000). Thus this strain collection represents approximately 88300 does and their progeny. In all cases, diseased rabbits were submitted for diagnostic examination to our laboratory by the respective farmers.

Strains were isolated from infected organs using standard methods (Quinn et al., 1994). The isolates that were identified as *Staphylococcus* sp. had the following characteristics: Gram-positive, catalase-positive, oxidase-negative cocci with clustered aggregations, forming medium-sized, haemolysing, yellow or grayish pigment producing colonies. Strains were archived at −80 °C using standard methods (Murray et al., 2003).

2.2. Multiplex PCR

Extraction of total DNA was performed using the Qiagen DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. Multiplex polymerase chain reaction (PCR) for amplification of virulence determinants was performed as described by Vancraeynest et al. (2007) using an Eppendorf MasterCycler PCR instrument (Eppendorf, Hamburg, Germany).

We selected a typical HV strain as positive control from the group of specimens used in previous studies, labelled "Sp17". It is originating from a Spanish rabbit farm with severe staphylococcal mastitis problems. This strain belongs to the typical highly virulent rabbit *S. aureus* clone, as it shows a mixed CV-C biotype (Devriese, 1984), it is sensitive to phages of phage group II (3A, 3C and 71), it shows the multiplex PCR pattern specific for highly virulent *S. aureus* strains (Vancraeynest et al., 2007), and has pulsed field type N2 and *spa* type t645 (Vancraeynest et al., 2006b).

2.3. Statistical analysis

To facilitate statistical analysis, we have grouped the examined strains into six categories, according to the organ system they originated from. Dermatitis, sore hocks, subcutaneous abscesses and conjunctivitis were grouped into a "cutaneous" (CUT) group. We had fourteen (3.74%) isolates originating from the small intestine of suckling rabbits with diarrhea, and lacking any other cultivatable bacteria (e.g., *E. coli*) in their samples. We decided to separate this group for the sake of analysis as "intestinal" (INT). However, such infection is not described in other sources, the intestinal pathogenicity of these strains were not proven with experiments, and the intestinal presence of the bacteria could be explained by the mastitis of the mother of the examined kits. Mastitis (MAST) and metritis (MET) cases were enrolled into separate groups. The respiratory disease (RESP) group was made of pneumonia and pleuropneumonia cases. Cases of splenitis, hepatitis, polyserositis without pneumonia, peritonitis, arthritis and meningitis were all grouped into "septicaemia and bacteremia" (SEPT).

Correspondence analysis and association plots were utilized to illustrate associations between virulence type and tissue tropism.

A joint plot figure contains both row and column variables. The scalar multiplication of these vectors is the data value of the corresponding cell of the table. The ellipsoid outlines indicate the 95% confidence interval (Greenacre, 2007). An association plot can be built on a two-dimensional contingency table containing frequency data. The figures are based on the Pearson's chi-square test, which examines the differences between the observed and predicted frequencies. The Cohen-Friendly association figure represents all cells of the table as rectangles. The area of the rectangle is proportional to the difference between the observed and the expected frequency values. The rectangles in each row are placed above or under a baseline, showing whether the data in the cell indicate a greater or smaller observed frequency compared to the expected value, respectively. Significant differences are marked by color as well (blue for greater and red for smaller). The number of cases is shown in the boxes. The associations suggested by the illustrative plots were confirmed by Fisher's exact test.

All statistical analyses were conducted using the R 2.12.0 statistical software (Ihaka and Gentleman, 1996). The correspondence analysis was conducted using the package 'anacor', while for the association plot the package 'vcd' was used. For significance level $p < 0.05$ was used.

3. Results

3.1. Summary of multiplex PCR results

The majority of the strains (84.2%, n = 315) belonged to a previously rarely isolated aHV type. We detected 23 (6.1%) HV strains. In cases when more than one strain was isolated from one outbreak, HV and aHV strains were regularly detected simultaneously from different animals with similar lesions. HV isolates were found on only six farms (20% of all examined farms), 15 (65%) of them originating from a single farm. Only 30% of all strains (n = 50) isolated from this farm were of the HV genotype. The isolates from 2009 and 2010 had only one HV strains each year (1,0 and 1,9% respectively). The strains from 2011 and 2012 had 4 (7,1%) and 3 (5,1%) HV genotypes, respectively. In 2013 we isolated 12 HV strains (16,9%). In 2014, due to the depopulation of some