VALIDATION OF A SAMPLING METHOD TO DETECT HEALTHY RABBIT *STAPHYLOCOCCUS AUREUS* CARRIERS

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ABSTRACT

Improvement of zootechnical performance and reduction of the use of antimicrobial agents could be achieved by adopting infective pathology control strategies based on selection of specific pathogen-free breeders. Chronic staphylococcosis, mostly caused by highly virulent *S. aureus* strains classified within the mixed CV-C biotype and the 3A/3C/55/71 phagetype, offers an ideal eradication policy target because it has a low diffusivity among animals, but may cause serious health impairment and it is difficult to control due to drug resistance and lack of vaccines. The main risk for staphylococcosis introduction is purchase of healthy carriers. Therefore, selection of high virulence *S. aureus* free breeders by screening them for carriage and the subsequent culling of infected subjects to prevent entry of infected animals on farm level is the basis of an eradication policy. The identification of healthy carriers of high virulence *S. aureus* is currently based on cultivation of the micro-organism and its characterization using traditional bacteriological methods. Biomolecular methods to distinguish high and low pathogenic *S. aureus* have been recently described, but they need proper in field validation and are not yet available for rabbit *S. aureus* high pathogenic screening. The standardization of sampling protocols that meet the needs of both increased analytic sensibility and technical and economic feasibility is of importance for a good screening and culling policy. The aim of the study is to evaluate a sampling method, especially looking for ideal body sites, for surveys based on rabbit as primary unit, and samples to be processed by traditional culture method prior to further characterisation of isolates. One hundred clinically healthy rabbits were enrolled. Each subject was sampled in four body sites (N: nose; E: outer ear; L: interdigital skin of hind legs; A: abdomen). Sterile cotton swabs scraped on skin surface were pre-incubated in Heart Infusion Broth (Oxoid) and than inoculated on a Baird-Parker Agar Base (Oxoid) which was RPF Supplement (Oxoid) enriched. Culture medium method with the biochemical identification of *S. aureus* by micromethod ID 32 STAPH (BioMérieux) was considered as the gold standard implying 100% specificity. Any rabbit with at least one positive sampling was considered a “true positive”, rabbits with all four samplings negative were considered ”true negatives”. 70% of analysed subjects hosted *S. aureus*. Sensibility was calculated for each body site and different combinations of two or more sample sites provided the following results: E=72.9%; A=52.9%; L=51.4%; N=64.3%; E+A=81.4%; E+L=81.4%; E+N=88.6%; E+A+L=88.6%; E+A+N=92.8%. The sampling and analysis procedure adopted resulted in a high percentage of *S. aureus* rabbit isolation. High sensibility of the E+A+L sample combination (88.6% sensibility; 95%CI: 81.1-96%) and the ease of sampling from the cutaneous areas while excluding the nose make this method of interest for detecting healthy rabbit *S. aureus* carriers.

Key words: *Staphylococcus aureus*, Rabbit, Carrier.

INTRODUCTION

The need to protect public health and the progressive extension of the concept of food quality to cover its productive phases as well (“farm to fork”) will have an increasing effect on the sanitary management of rabbit breeding where – as in other zootechnical segments – the current trend is characterized by the optimized use of pharmaceuticals in accordance with the principles of “prudent

use" (OIE, 2006) on one hand, and the creation of groups of breeders free from specific pathogens on the other. The latter aspect is directed at optimizing the zootechnical performance of highly-selected animals. Moreover it leads to the reduction of specific pathologies and the antimicrobial treatments commonly adopted for their control.

Staphylococcosis is probably the leading cause of death and discarding animals in rabbit reproduction units (Segura *et al.,* 2007). In particular, *Staphylococcus aureus* is responsible for mastitis, pododermatitis, pyometra and subcutaneous abscesses in breeders, and dermatitis, arthritis and enteritis in suckling rabbits contaminated by their mothers (Camarda *et al.,* 2007; Segura *et al.,* 2007).

Results of field studies and laboratory characterisation suggest that *S. aureus* strains can be spitted in two groups as regard to pathogenity (Devriese *et al.,* 1981; Hermans *et al.,* 2000); the high pathogenic strains display an increased ability to spread within the flock (Hermans *et al*., 1999) rather than an increasing of the injuries severity, so that compared with low pathogenic strains the within flock prevalence is higher for the previous strains and results in an endemic chronic staphylococcosis.

Due to endemic disease Hermans (2003) concluded that the only solution for farmers facing problems with high virulence *S. aureus* strains is to slaughter the entire flock. Field evidences seem to confirm the hypothesis and actually in the mid-term farms infected with high-virulence strains would undergo a progressive reduction in productivity associated with the greater incidence of pathologies and the higher costs incurred for the therapy and prophylaxis necessary.

S. aureus is a widely distributed micro-organism that lives on human skin and animal fur. This makes the complete eradication of *S. aureus* an improbable hypothesis, whereas the possibility of eliminating highly virulent strains appears feasible even in the field. These strains seem to be clonal in origin and their spread might be linked to the sale of breeders produced by a limited number of selection centers that subsequently infected farms all over Europe (Vancraeynest *et al.,* 2006). For the same reason, it is plausible to assume that an eradication policy of high pathogenic *S. aureus* of selected breeders will lead to the establishment of farms free from the pathogens, provided that adequate bio-security measures are adopted. The lack of serological tests capable to distinguish rabbits infected with highly virulent strains from those infected with low virulence strains obliges eradication programs performed with traditional bacteriological test or bio-molecular test whenever available for field screenings.

The main risk of introducing high virulence *S. aureus* in a previous free herd is through healthy carriers. In order to develop a pre-introduction test, whether it will be based on traditional or biomolecular methods, it is of importance to standardize and validate the sampling protocol at the individual stage. Previous papers report that within herd prevalence can be affected by the overall sensibility of the test used to identify *S. aureus* carriers (Hermans *et al*., 1999). Aim of the study is to evaluate a sampling method for surveys based on rabbit as primary unit and samples to be processed by traditional culture method prior to further characterisation of isolates. The ability of the purposed protocol to correctly identify the healthy carriers of *S. aureus* in a field situation, where several variables determine the final result, was estimated.

MATERIALS AND METHODS

One hundred of clinically healthy rabbits older than 40 days and of 43 farms origin were enrolled. Sampling was performed using sterile cotton swabs previously moistened with HIB (Heart Infusion Broth) rubbed on the skin of the outer ear (E), the skin of abdomen (A), the interdigital skin of the hind leg (L) and on the mucosa of the nasal cavity (N). For the animal welfare the latter sampling was performed with cotton swabs for paediatric use introduced in the nasal cavity to a mean one centimetre depth. Once sampled the cotton end of the swab was inserted in a test tube filled with HIB (5 ml) incubated at 37°C+/-1°C for 24 hours; than 30 µl of broth medium was inoculated on a Baird-Parker Agar Base (Oxoid) plate added with RPF Supplement (Oxoid) and incubated at 37°C+/-1°C for 24-48 hours. Colonies of *S. aureus* was identified according to morphology: white, grey, or black colonies

surrounded by an opaque ring of fibrin precipitate. Doubtful colonies were re-incubated in Columbia Agar Base medium (Oxoid) added with sheep red blood cells (5% v/v) and identified using normal biochemical procedures (ID 32 STAPH, BioMérieux).

The degree of purity of the isolates and the number of *S. aureus* colonies observed were also recorded in order to assess a reduction of sensibility associable to bacterial contamination. Contamination affects the ability of the medium to support *S. aureus* growth and the operator's ability to morphologically identify colonies.

The following interpretation scheme was used to quantify the *S. aureus* colonies: negative sample = 0; 1-10 colonies = 1; 11-50 colonies = 2; 51-300 colonies = 3; confluent growth = 4. As regards the assessment of the degree of isolation purity, the following arbitrary classification was adopted: pure growth; limited contamination (1-30 colonies); elevated contamination (30-300 colonies); heavy contamination with non-interpretable sample (>300 contaminating colonies).

For the purpose of data analysis, rabbits with at least one positive sampling were encountered as "true positive" regardless of the number of colonies identified, whereas rabbits that tested negative at all 4 sampling points were considered "true negatives". Sensibility of each sampling site and any possible combination of them was then estimated. The culture medium method and the biochemical identification of *S. aureus* using micromethod ID 32 STAPH (BioMérieux) was considered the gold standard and thus a 100% specificity was assumed. The relationship between *S. aureus* isolation and plate contamination was analysed using the Chi^2 test.

RESULTS AND DISCUSSION

The proposed protocol was based on sampling of four body sites and allowed to isolate *S. aureus* in 70% of analysed rabbits (Table 1); Hermans (1999) reported an isolation rate of *S. aureus* in 60% of subjects by sampling 10 different body sites but with a culture protocol that did not consider the pre-incubation in liquid medium. The HIB incubation technique may increases the sensibility of the culture medium and allow the multiplication of low numbers of bacterial cells that otherwise might not have been survived and displayed with direct inoculation on a selective agar plate medium. This step was included as our aim was to increase the sensibility of the whole procedure in order to detect as much healthy carriers as possible.

The high percentage of positive rabbits can be explained by the fact that *S. aureus* is one of the most common pathogens found in breeders, which immediately after birth at lactation can contaminate their newborn through the close physical contact that ensues. The possibility that the high prevalence of *S. aureus* carriers we registered might be related to the pathogenic ability of isolates was investigated but the association was not statistically significative (Agnoletti *et al.*, 2008). It is also possible that our results are related to the pathogenic ability of strains isolated even if we did not observe any difference (Agnoletti *et al.*, 2008).

The farmer can also be a source of contamination of both animal and human strains himself through his frequent handling of the rabbits; actually approximately 30% of human population is permanently colonized by *S. aureus* on the skin (Tenover and Gorwitz, 2006).

Rabbits were found to be 70 true positive and 30 true negative. Seven rabbits tested positive only to sample E, three rabbits tested positive to sample A, 5 tested positive to sample L, and 8 tested positive to sample N.

Table 1 shows the sensibility ranging from 51.4 to 100% as regard to any possible combination of body sampling sites we used. The nasal sampling site was obviously the time consuming due to the animal's natural tendency to close its nostrils when inserting the swab; moreover it is a sampling that affects the animal welfare. In this regard, the choice of the E+A+L combination without nasal

sampling allowed an acceptable (88.6%) sensibility and once defined an appropriate number of samples to collect in the herd it offers a good drawback for *S. aureus* control program in the field. Table 2 shows the number of *S. aureus* colonies observed and the degree of contamination of isolates for each sampling site. A statistically significant correlation between the sample contamination and *S. aureus* isolation was observed for all sampling sites($P<0.05$ for each sampling site), it is possible that sample contamination might reduce the method sensibility and produce false negatives.

Table 1: Sensibility of standardized protocol to detect healthy rabbit *S. aureus* carriers

Sample	No. of true +	No. of false -	Sensibility	Sensibility: 95% CI: limit	
				Low	High
E	51	19	72.9	62.4	83.2
A	37	33	52.9	411	64.5
L	36	34	51.4	39.7	63.1
N	45	25	64.3	53.1	75.5
E+A	57	13	81.4	72.3	90.5
E+L	57	13	81.4	72.3	90.5
E+N	62	8	88.6	81.1	96.0
E+A+L	62	8	88.6	81.1	96.0
E+A+N	65	5	92.8	86.8	98.9
E+A+L+N	70	0	100.0	100	100

E: outer ear; A: abdomen; L: interdigital skin of hind leg; N: nose

Table 2: Colony numbers of *S. aureus* and bacterial contaminants in different sampling sites

Sampling site	Colony number of *S. aureus*					Colony number of bacterial contaminants			
	0	1-10	11-50	51-300	confluent growth	absent	limited	elevated	contaminated sample
E	49	3	13	30	5	31	18	49	2
A	63	4	5	22	6	22	11	59	8
L	64	5	7	22	2	27	17	53	3
N	55	2	8	26	2	39	21	37	3

E: outer ear; A: abdomen; L: interdigital skin of hind leg; N: nose

CONCLUSIONS

Eradication programs of highly virulent *S. aureus* strains based on "test and cull" are at the present based on traditional bacteriological methods for the identification of healthy carriers. In field screening and rapid methods are hoped but not jet available.

For the purpose, groups of selected breeders are individually controlled in order to detect high virulence strains of *S. aureus*, and if at least one rabbit in the group tests positive the entire group of animals is culled. Screening at the individual stage are not economically feasible for farms and a drawback must be accepted between the sampling accuracy and its feasibility in field conditions. We propose a protocol that samples 4 anatomical sites in each animal (nose, abdomen, the interdigital skin of hind leg, and the inside of the outer ear) and allowed the isolation of *S. aureus* in 70% of the group examined; the sampling method can be simplified by excluding the nasal swab, which is time consuming and welfare affecting, and meanwhile increasing the number of samples collected in the herd to compensate the loss of sensibility.

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