

EVALUATION OF THE CONJUNCTIVAL BACTERIAL FLORA IN 140 RABBITS (*Oryctolagus cuniculus*) FARMED IN SICILY ISLAND

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Summary: Normal conjunctival flora of animal eyes comprises of both bacterial and fungal organisms. Bacterial and fungal species isolated from healthy conjunctiva appear to vary with geographic location, as well as is influenced by age, sex, housing, and climate. The aim of this work was to evaluate the bacterial flora of the conjunctiva of rabbits from intensive livestock farming in Sicily Island, comparing differences in the isolation of bacteria, and evaluating the potential zoonosis risk for humans related to the bacterial species isolated. 140 rabbits of California and New Zealand breeds were examined, while 280 eyes swab were obtained. Isolation techniques, biochemical and enzymatic tests were performed. Statistical analysis performed shown that the zoonotic risk is statistically not significant. A 4 % of *Moraxella spp.*, 9 % of *Staphylococcus spp.*, 5 % of *Pasteurella multocida* and 9 % of *Staphylococcus aureus* were isolated on eye swabs. In the examined area may be excluded a human-health risk related to isolated pathogens, both because of low percentage of isolations and for small number of involved farms. Further investigations are necessary to continuous monitor the related epidemiological risk.

Key words: rabbit; microbiology; eye; Sicily Island

Introduction

A poorly defined microbial and mycological flora is present on domestic animals conjunctival mucous membranes. These microorganisms have an important role in defending ocular mucous membranes, by competing with pathogenic species and by limiting their capability to colonize ocular surface. Changes of the microbial equilibrium creates favourable conditions for the development of pathologic process (1). Under normal conditions,

microbial flora of the conjunctival sac is wide-ranging in relation to several factors, including geographical area and climate conditions, season, environmental hygiene, species, and specimen's immune system or performed medical treatments. Knowing the composition of the normal conjunctival flora during physiological conditions helps to recognize possible abnormalities. Gram-positive and Gram-negative bacteria are the most common microorganism isolated in most studies focused in ocular microbiology. Pathogenic bacteria frequently isolated by ocular surface of various domestic species are *Staphylococcus sp.*, *Streptococcus sp.*, *Corynebacterium sp.* and *Rhodococcus sp.*, *Listeria*

monocytogenes, and other Gram-negative bacteria such as *Pasteurella multocida*, *Pseudomonas sp.*, and *Moraxella sp* (2-7).

Bacterial flora show a discrepancy within geographic variables; therefore conclusions from others research performed in rabbits from different area, cannot be related to rabbits farmed in southern Europe, and particularly in an island. To authors' knowledge, no studies have been performed to evaluate physiological conjunctival flora of farmed rabbits in Sicily. The aim of this research is to evaluate the bacterial flora of the conjunctiva of rabbits from intensive livestock farming in Sicily Island, by comparing differences, the current literature, and therefore possible zoonotic risk.

Materials and methods

Animals

One hundred-forty (140) rabbits for meat production, of Californian and New Zealand breeds, have been included in this study (Table 1). Rabbits were sampled in eight (n=8) farms in Sicily Island. All of them were farmed in enclosed and climate-controlled barns. Rabbits were categorized into four groups according to production phases: fattened up rabbits (FR, ranging in age between 37 and 51 day, both male and female); bucks (ranging in age between 1 and 2 years); does (ranging in age from 7 months and 1.5 years); young does (ranging in age from 5 months to 1.5 years). Of the 140 examined rabbits, 59 were males (40 fattened; 19 bucks) and 81 females (24 does, 20 in fattening period; 37 young does). All examined rabbits were individually housed without litter except for the does; for does a litter with swarf was set a week before the expected date of parturition. Two days after parturition, litter was replaced with a new swarf, leaving just the fur used by the does to prepare the nest. Rabbits were exclusively

feed with cubed feedings tuff. The temperature inside the shed ranged from 20/25 °C overnight, 25/30 °C during the day, constantly for the whole year. Humidity ranged from 33 % to 75 %, often depending on the external weather.

Before performing this the conjunctival swabs for each rabbit a medical data (race, gender, age, productive capacity, therapeutic treatments, type of litter used, kind of feeding, climate in the enclosures) were collected and a complete physical examination was performed. Upon complete physical examination, all sampled rabbits have good health conditions. Physical examination and sampling swabs were executed by an operator and an assistant, working in sterility. For each rabbits two eye-conjunctival swabs (1 right eye, 1 left eye, a total of 280 samples) were obtained. The swabs (Copan Transystem, Copan, Italy) were immediately placed in Amies transport medium (Dominique Dutscher, France) and then transported on ice to the Istituto Zooprofilattico Sperimentale "A. Mirri", Infectious Disease Section, Palermo (Italy).

Bacterial isolation and identification

The swabs were smeared directly on plates containing solid media, AS (Blood Agar) and MSA (Agar salt and mannitol), and incubated at the temperature of 37 °C in aerobic conditions and with enriched atmosphere of 5 % CO₂. After 24 h of incubation, bacterial colonies growth. Most noteworthy colonies were placed in pure culture. Further the identification of genus and species by using several biochemical and enzymatic assays were performed. Gram stain, catalase test, oxidase test, test of mobility, test of KIA (Kligler Iron Agar), growth in AGAR MacConkey, test of coagulase :, API 20 NE, API Staph were performed in order to identify the microorganism. On isolated strains antibiogram was performed (using Kirby Bauer method).

Table 1: Number of sampled rabbit

	Male		Female			Total in Farm
	Bucks	FR	Does	Young Does	FR	
Total	19	40	24	37	20	140

Statistical analysis

Statistical analysis was performed. Normal distribution of the studied parameters was verified (Kolmogorov-Sminrov). Statistical analysis of the data was performed by applying one-way analysis of variance for repeated measures (ANOVA) and by performing a Duncan's multiple comparison tests in order to evaluate the impact of causal agents on groups "farm", "phases", and "microorganism". For statistical analysis software Prism v. 5.00 (Graphpad Software Ltd., USA, 2003) was used. All data were expressed as mean $\pm$ standard deviation (SD).

Results

A blood agar showed colonies of *Pasteurella spp.*. A Pure culture and identification of the genus by using biochemical and enzymatic tests was performed. Tests showed: Gram stain: negative coccobacillus; Catalase test: positive; Oxidase test: negative; mobility test: negative; Test KIA (Kligler Iron Agar): fermentation of glucose without gas production; Growth in Agar-MacConckey: no colonies growth; API 20 NE: identified *Pasteurella multocida*. In agar-blood plates, several colonies with smooth margins and regular cream white had grown, attributable to the genus *Staphylococcus spp.*; a seeding phase on MSA was performed. Preparation of pure culture and identification of genus and species by using biochemical and enzymatic tests were performed; test results showed: Gram stain: positive cocci; Catalase test: positive; Oxidase test: negative; mobility test: negative; coagulase test: positive. Further

identification was performed in micro method by using API Staph that confirmed presence of *Staphylococcus aureus*. Again on Blood Agar, other colonies were smooth, with regular margins and creamy white, due to the genus *Staphylococcus spp.*. A pure culture and identification of the genus by using biochemical and enzymatic tests was performed. Results showed: Gram stain: positive cocci; Catalase test: positive; oxidase test: negative; mobility Test: negative; coagulase Test: negative. Considering the negativity in this last test, it was excluded presence of *Staphylococcus aureus* and *Staphylococcus pseudintermedius*, so the subspecies remained undefined (ie *Staphylococcus spp.*). The second type of colonies present on Blood Agar, were several minute colonies of 1-2 mm in diameter, smooth, flat, brown, attributable to the genus *Moraxella spp.*. A pure culture and identification of the genus by using biochemical and enzymatic tests was performed. Results showed: Gram stain: negative coccobacillus; catalase test: positive oxidase test: positive; Test mobility: negative; API 20 NE: *Moraxella spp.* was identified.

Results of 280 samples were the following (Table 2): 9 positive for *Pasteurella multocida*, 6 tampons were from females (3 does and 3 young does) and 3 males (2 in fattening period and 1 buck); 16 positive for *Staphylococcus aureus*; 14 tampons were from females (6 in fattening period, 5 does and 3 young does) and two males in fattening period; 4 positive for *Moraxella spp.*, 3 from to females (does only) and 1 to males (only during fattening period); 9 positive for *Staphylococcus spp.*, 6 from females (4 young does and 2 does) and 3 from males (fattening period). Totally, in farmed rabbit 38/180 samples were positive.

Table 2: Positivity to bacteria in sampled rabbits distinguished by sex and breeding/fattening period

Bacteria	Male		Female			Total
	Bucks	FR	Does	Young Does	FR	
<i>Pasteurella multocida</i>	1	2	3	3	0	9
<i>Saphylococcus aureus</i>	0	2	5	3	6	16
<i>Moraxella spp.</i>	0	1	3	0	0	4
<i>Spahylococcus spp.</i>	0	3	2	4	0	9

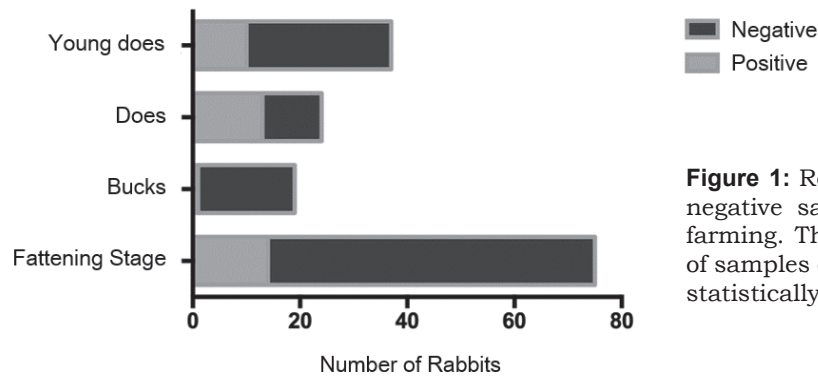


Figure 1: Relationship between positive and negative samples according to the type of farming. This chart shows that the number of samples obtained in rabbits fattening, was statistically lower compared to the negative

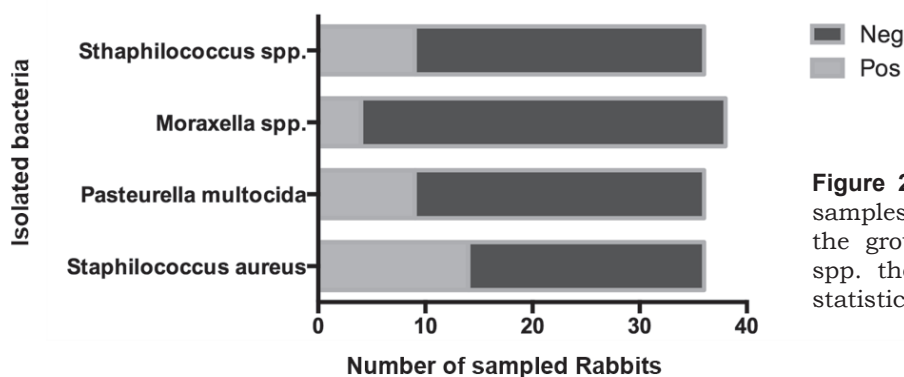


Figure 2: Number of positive and negative samples sorted by isolated bacteria. In the groups *Moraxella* and *Staphylococcus* spp. the number of positive samples was statistically lower than the negative

After grouping the obtained results, statistical analyses were performed and they showed a significant impact of the etiological agent (i.e. any of pathogens obtained in our investigation) on: Farm = $P < 0.05$; phases = $P < 0.05$; Single microorganism = $P < 0.05$. Duncan's multiple comparison test, revealed that, positive samples was statistically lower than negative. In addition number of positive samples was statistically lower than negative in the group of fattening rabbit (Fig.1). Finally, we could admit that the *Moraxella* spp. and *Staphylococcus* spp. positive samples were statistically lower than the negative (Fig.2).

Discussion

Gram positive organisms predominated in current study. The most frequently isolated Gram-positive bacteria were *Staphylococcus* spp., *Pasteurella* spp. and *Moraxella* spp.. *Staphylococcus* spp. has the highest isolation rate in our study, and as reported in literature is commonly found on health conjunctiva of most

domestic animals (8-10). Our findings are not similar to the study carried out by Okuda and Campbell (11) on 54 New Zealand white rabbits where *Bacillus* species have been found as the most common isolated organism. However, they are just opportunistic pathogens and the most frequently isolated organisms in cases of canine bacterial conjunctivitis (12). The evolution of farming techniques and the emergence of antibiotic resistance over time have increased the spread of staphylococcal infections, especially in backyard flocks intensive cattle, sheep and goats, rabbits, equids and pigs (13-14). In recent years Foti et al. (15) have isolated methicillin-resistant *Staphylococcus aureus* from the healthy conjunctival sac of donkeys. *Staphylococcus aureus* MRSA, is an important cause of nosocomial and emerging infections. The widespread use of cephalosporin and fluoroquinolones on medicated feed in farmed rabbits may be promote in the coming years further spread of MRSA, with dangerous consequences for rabbits farming. Luckily in our study, this strain was

not encountered. In addition, our findings were compatible to a study performed on rabbits by Cooper et al. (16) in terms of species of bacteria. In Cooper et al. study *Staphylococcus* species were the most commonly recovered organisms, and other organisms isolated with less frequency were *Pasteurella* species, and *Moraxella* species. In contrast with this report, in present study *Micrococcus* spp., *Bacillus* spp., and other bacteria were not founded. Frequency of isolation was similar for *Pasteurella* spp. (6 %) and *Moraxella* spp. (4 %). If compared with the finding in other farmed animals like pigs, results from our study is similar to the existent literature (17), which shown a high prevalence (78 %) of *Staphylococcus* spp. Unlike, a recent report in horses, shown a high presence of *Acinetobacter* spp. (18), but not isolated in our report. Regarding *Moraxella* spp., they have been isolated from the healthy conjunctival sac of dogs, horses, cattle, goats, sheep (19-24). In Human *Moraxellaceae* are normally was isolated at the level of the oropharynx, mucous membranes, skin and genital tract, and although the pathogenic and zoonotic potential of this bacterium has not been clearly documented, this possibility may occur (25). *Pasteurella* spp. are frequently encountered in rabbit farms, causing enormous economic damage direct and indirect (26); pasteurellosis is one of the most important diseases of the rabbit and this species may act as a carrier of the pathogen. Although *Pasteurella* infections in humans are still very rare, especially for children or for farms technician could be an significant issue (27).

From obtained data on our study, we can admit that since the limited number of isolations, the zoonotic risk is negligible despite isolated bacteria are potentially pathogenic to humans. Particularly in studied farms *Moraxella* spp. was found in 4 % and *Staphylococcus* spp. was isolated in 9 % of eye swabs, and *Pasteurella multocida* was revealed in 5% and *Staphylococcus aureus* was isolated in 9 % of eye swabs. However, must be admitted that in the examined area, sanitary risk related to the presence of these pathogens may be excluded. Further investigations are needed to increase a constant monitoring of the Island. Moreover, because of zoonosis are transmitted also through ingestion of infected meat, obtained data are encouraging, as we have recorded a prevalence of negativity in rabbits during fattening period in both farms.

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OCENA BAKTERIJSKE FLORE OČESNIH VEZNIC 140 KUNCEV (*Oryctolagus cuniculus*) GOJENIH NA SICILIJU

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Povzetek: V normalni flori veznice živalskih oči so bakterijski in glivični mikroorganizmi. Bakterijske in glivične vrste, izolirane iz zdravih veznic, se spreminjajo z geografsko lokacijo, nanje pa vplivajo tudi starost, spol, način reje živali in podnebje. Namen dela je bil oceniti bakterijsko floro veznic kuncev intenzivne reje na Siciliji ter primerjati razlike v izolaciji bakterij in ocenjevanju potencialnih tveganj za obolenje ljudi, povezanih z najdenimi bakterijskimi vrstami. Pregledanih je bilo 140 kuncev kalifornijske in novozelandske pasme in tako je bilo pridobljenih 280 očesnih brisov. Opravljeni so bili izolacija mikroorganizmov ter biokemijski in encimski testi. Statistična analiza je pokazala, da je tveganje za pojav zoonoz zelo majhno. Iz brisov oči so bili izolirani v 4 % *Moraxella spp.*, v 9 % *Staphylococcus spp.*, v 5 % *Pasteurella multocida* in v 9 % *Staphylococcus aureus*. V proučevanem območju se lahko izključi nevarnost za zdravje ljudi, povezano z izoliranimi patogeni, tako zaradi nizkega odstotka izolacij kot majhnega števila vključenih rej. Nadaljnje raziskave so potrebne za nadaljevanje spremljanja, povezanega z epidemiološkim tveganjem.

Ključne besede: kunec; mikrobiologija; oko; Sicilija