DEVELOPMENT OF A NEW ANTIBIOTIC COMPOSITION FOR A RABBIT SEMEN DILUTION MEDIUM (GALAP®)

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ABSTRACT

Given the alarming increasing of bacterial resistance, it was necessary to remove fluoroquinolone of semen dilution media, particularly GALAP® for rabbit semen. The purpose of this study was to search for new enrofloxacin-substitutable antibiotics and to study the effect of a new antibiotic composition of GALAP® based on semen motility and fecundity. Several bacterial strains were isolated from poor-quality ejaculates, and were tested for antibiotic resistance. Out of 15 antibiotics tested, gentamycin was the one that caught our attention by targeting 82.8 % of the colonies identified. In vivo tests were then carried out to analyze the effects of this antibiotic change on sperm parameters. Pools of good quality semen were diluted in original or gentamycin added GALAP®. Ejaculates were analyzed on the day of collection and up to 6 days storage. After 24h of storage, the motility in the new medium was reduced by 7.7 % compared to the original medium and this decrease was not amplified with storage time. At 6 days storage, no difference between the two media was detected. Females were then inseminated with semen pools diluted 1:10 in original or GALAP® with gentamycin. No difference in female fecundity was detected, between the 2 media. The decrease in motility does not impact the female reproductive performance when following the routine protocol of semen processing centers. To conclude, dilution of semen with the new antibiotic formulation of GALAP® is recommended for the insemination of rabbits.

Key words: Semen, Dilution media, Bacterial charges, Motility, Fecundity

INTRODUCTION

The success of AI in rabbits depends on the reproductive performance of the female, but other factors related to the quality of the semen are relevant and affect three essential parameters of female reproductive function: fertility, prolificacy and fecundity (fertility × prolificacy). Indeed, a correlation between sperm motility, semen concentration and female fecundity has been shown (Brun *et al.*, 2002; Lavara e*t al.*, 2005; Theau-Clément *et al.*, 2016). The bacterial load in the semen is another determinant of the quality of AI doses and thus of female productivity. High bacterial concentration, meaning contamination of the semen, has an adverse effect on the quality of the semen (Morrell and Wallgren, 2014). Bacteria produce toxic metabolites leading to DNA fragmentation and decrease sperm viability (Fraczek and Kurpisz, 2015). They can also lead to sperm agglutination, resulting in a decline of sperm motility (Althouse et al., 2008). In addition, doing AI with contaminated and poor quality doses impacts the health of the females, the fertilization rate and productivity (Maroto Martín *et al.*, 2010).

Therefore, the choice of semen medium is critical. In order to limit possible microbial contamination during semen processing, antibiotics are added to the semen medium (Morrell and Wallgren, 2014). GALAP® is one of the most frequently used dilution medium in Europe. Since it has been commercialized, it contains penicillin, streptomycin and enrofloxacin. The latter is part of the family of fluoroquinolones and has considerable importance in human medicine. Given the alarming increasing of bacterial resistance, the Pharmacovigilance Risk Assessment Committee (PRAC) recommends restricting the indications of these antibiotics (Francisco, 2018). The need to change the antibiotic composition of semen dilution media, particularly GALAP®, has emerged as an obvious

objective to be achieved while maintaining the quality of the diluent on sperm functions and fecundity at the same level.

In this context, our study was focused on the research and choice of a new substitute antibiotic to enrofloxacin in GALAP® medium, on its optimal dose concentration and its efficacy on the bacterial flora of the semen. Then, we studied the efficiency of a new GALAP® antibiotic formula on semen motility and female fecundity.

MATERIALS AND METHODS

Animals

Sexually mature rabbits originating from 2 different farms and 2 semen processing centers in France and Spain, were used as breeders. All rabbits were fed with a standard commercial diet and fresh water was provided ad libitum. The handling of the rabbits and experimental protocol were carried out in strict compliance with the sanitary approval of semen processing centers given by the National Federation of Rabbit Producer Groups approval.

Bacterial Charges Analyses

The analysis of the bacterial flora of collected ejaculates were carried out using 19 low quality semen obtained in Spain, and 10 better quality semen obtained in France. Semen were collected using a pre-heated (+45°C) artificial vagina. The poor quality raw ejaculates were put in laminoculture using the URITEST N kit (Liofilmchem, Roseto degli Abruzzi, Italy). The total number of colonies was counted after 24h at +36°C. Antibiograms were performed on isolated colonies. The Kirby-Bauer method was followed (Hudzicki, 2009). After bacteria inoculation, antibiotic discs were placed in the culture medium (Liofilmchem or Mast Group Ltd Mast House, Bootle L20 1EZ, UK). The inhibition spots were measured after 24h at +35°C, according to EUropean Committee on Antimicrobial Susceptibility Testing guidelines.

Semen collection for motility analysis, dose packaging and artificial insemination

The semen of 105 males was harvested in the facilities of the semen processing center in France. Semen must have aqueous white appearance. The ejaculates were pooled in groups of 3 to 5. The pools were diluted (1:10) in different pre-warmed (+37°C) GALAP®: antibiotic-free (AB free), original and new antibiotic composition (new). After one hour of equilibration at + 23°C, the quality of the semen pools was assessed after dilution in the appropriate media (1:4), incubation for 10 min at + 37°C, and motility analysis using a computer-assisted semen analyzer (IVOS II, Hamilton Thorne, Beverly, USA). After quality assessment, one part of the semen pools was stored at +17°C in order to follow semen quality during storage time. The other part of the semen pools was used to carry out the insemination doses in 0.5 mL straws (around 24.10^8 total sperm cells/dose). Only the pools with a total motility above 70 % were kept. The doses were stored 24h at +17°C before AI. Multiparous rabbits, distributed in 2 breeding farms, were inseminated: 180 with the semen diluted in the original GALAP®; 180 with the new antibiotic composition of GALAP®. AI was performed using a curved plastic sheath. Immediately after AI, each does received an intramuscular injection of 5 µg of lecirelin.

Statistical analysis

A Chi-square test was used to interpret the presence of bacterial colonies. A GLMM procedure was used to analyze the relationship between the different GALAP® and the semen quality and fecundity (GALAP® media and/or day of storage as the fixed factors; semen pool, breeding farm, bands, parity, and age as random factors). Statistical analysis were performed on R software, version 1.1.463 (R Core Team, 2014). Significant difference in the results was consider when p-value < 0.05.

RESULTS AND DISCUSSION

Determination of the sensitivity to various antibiotics of bacterial colonies of poor-quality semen.
The inoculation of 19 poor-quality ejaculates allowed us to identify up to 48 bacterial colonies. The isolation of some of these colonies allowed us to perform 15 antibiograms. Only 36.6 % of the bacterial colonies tested were sensitive to enrofloxacin, initially present in GALAP® (Table 1). Of the

Table 1: Antibiograms made from poor quality ejaculate samples. Percentage (%) of bacterial colonies isolated, which are sensitive, moderately sensitive (intermediate) and resistant to the action of different antibiotics (ns = not significant). Not all antibiograms were presented in this table.

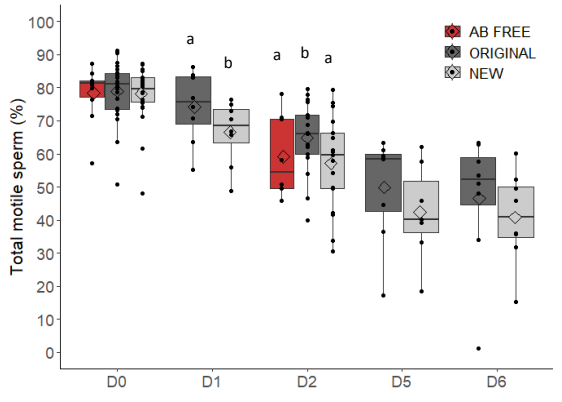
ANTIBIOTICS (µg)	Resistant %	Intermediate %	Sensible %	TOTAL no. of colony analysed	*p. value*
ENROFLOXACIN 5	44,7	19,1	36,2	47	
AMIKACIN 30	20,8	2,1	77,1	48	ns
GENTAMICYN 10	17,0	0,0	83,0	47	ns
GENTAMYCIN 30	10,3	6,9	82,8	29	*0,048
LINCOMICIN 15	75,9	0,0	24,1	29	*0,048
PENICILIN G 1 IU	82,8	0,0	17,2	29	*0,013
TYLOSIN 30	77,3	2,3	20,5	44	*0,048

15 antibiotics tested, we showed that 2 could replace the original antibiotic mixture with a wider bacterial spectrum than enrofloxacin and penicillin combined. Although amikacin 30 µg showed interesting efficacy against the bacterial strains identified (77.1 % of sensitivity), gentamycin 30 µg had the broadest bacterial spectrum by targeting nearly 83 % of the bacterial strains identified. Gentamycin completely inhibited the growth of 90.9 % of the streptococci spp. and 100 % of the staphylococci and protei spp. identified in this study (data not shown). Gentamycin was shown to prevent the bacterial development on its own, at the same level than the original antibiotic mixture. In addition to the fact that this antibiotic has the advantage of being stable in aqueous solution for more than 12 months (Berendsen *et al.*, 2011), we sought to validate a GALAP® medium with a new antibiotic composition, with the addition of 0.3 g/L of gentamycin.

Analysis of semen motility parameters over the post-semen collection time.

At collection day (D0), ejaculates showed a percentage of total motility of 77.7 ± 10.2 % (Figure 1). After 24h of storage at +17°C (day 1, D1), the percentage of total motile sperm stored in the new GALAP® was 7.7 % significantly lower than sperm stored in the original medium (p = 0.0001), without affecting the proportion of progressive spermatozoa nor the velocity of the motile spermatozoa (data not shown). At D2, the difference (7.8 %) was still significant between the 2 antibiotics formula (p = 0.0028). The new formula was nevertheless not significantly different from the antibiotic free medium. After D5, the gap of 7.6 % in the percentage of motile spermatozoa between the 2 media was maintained. However, the difference was not anymore significant at D6 because of the high variability observed between pools of semen diluted in the original medium. These decreases in semen motility corroborate with the results obtained in a study carried out on stallion semen from Aurich and Spergser (2007). Gentamycin in GALAP®, although preventing bacterial development, negatively affects sperm motility only in the first 24h of storage and is not worsened by storage time. Gloria *et al.* (2014) suggests that the toxicity of gentamycin is related to the cooling phase of the semen since no reduction in sperm motility was found after dilution and incubation at room temperature of the semen. However, no further explanation has been given in the literature.

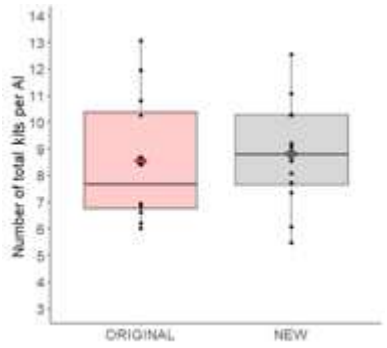
Figure 1: Percentage of total motile spermatozoa after semen dilution in different GALAP®: antibiotic-free (AB FREE), original (ORIGINAL) and with gentamycin (NEW). Semen pools was analyzed at different day after collection (D0, D1, D2, D5, D6). (n = 8 to 26). Different lower case letters indicates significant difference.



Effect of the new GALAP® antibiotic composition on fertility and prolificacy.

Fecundity (number of total kits per AI) has been recovered after AI of does with semen diluted in the different GALAP®. Although motility has been shown to be a determining factor in female fecundity, AI results showed no negative impact of the loss of motility on the does fecundity (p = 0.517), and would even allow to give 0.3 extra kits per AI (Original : 8.5 ± 2.4 kits/AI ; New : 8.8 ± 2.0 kits/AI).

Figure 2: Female fecundity after AI with semen diluted in the original or new GALAP® media (n = 22 pools, 15 AI / pools)



However, a larger study would be necessary to show the significance of this result. Since the concentration and total number of motile sperm cells are critical to the success of AI, Theau-Clément *et al.* (2016) demonstrated that the use of doses at 30.10^8 cells/ml, containing a total minimum of 17.10^8 motile sperm cells, results in an optimized fertility rate. Thus, packaging doses concentrated at around 25.10^8 sperm cells allowed us to obtain a total sperm count in excess of the minimum 17.10^8. Our doses contained 74 and 66 % motile sperm cells in the original and gentamycin GALAP® respectively, giving a motile cell count of between 37 and 33.10^8.

CONCLUSIONS

Gentamycin, due to its wide bacterial spectrum and its stability in aqueous solution, has shown its effectiveness on semen from French farms. However, dilution of the semen in GALAP® gentamycin causes a drop in sperm motility within 24h post-collection, probably related to the known toxicity of gentamycin. In addition to perform ejaculate pools and heterospermic dosing to limit the individual effect of males, conditioning the semen to a concentration of 50.10^8 spermatozoa/ml, may provide an appropriate balance degree between motility and antibiotic toxicity to maintain best fertility performance. Under these conditions, the new medium with gentamycin shows similar efficacy to the original GALAP®. The substitution of the antibiotic cocktail of the original GALAP®, with gentamicin does not induce any financial loss for the breeders.

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