EVALUATION OF THE PERSISTENCE OF ANTIBIOTICS RESIDUES IN DRINKING WATER DISTRIBUTION SYSTEM AFTER STATIC AND DYNAMIC WASHING

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ABSTRACT

Some critical problems in modern rabbit intensive farming could be related to the use of antimicrobials administered through drinking water. The industrial breeding of rabbits is a short production cycle and this method of therapeutic administration has an increasing importance in quantitative terms.
In this study the presence of different classes of antimicrobials simultaneously in the same sample was analysed, and the persistence of active antimicrobial ingredients at different points of the water distribution system was verified. In particular, after having carried out either "static or dynamic washing" of the lines and before introducing another cycle of animals, in the production logic "all in, all out". Multiresidue analysis for analytes belonging to ten different group of antimicrobials was done by LC-HRMS and a comparison of the results obtained by using the two different washing system was done. At the end of the cycle, many molecules were present at different concentration, some resulting from the last cycle treatments, others from previous cycles. At the same time, the comparison between different cleaning systems used at the end of the cycle highlighted evident differences. This study highlighted significant elements on the use of antimicrobials and gave indications on how to to reduce the potential risk of multiple contamination.

Key words: antimicrobials, residues, drinking water, washing

INTRODUCTION

The methods of using antimicrobials in the various livestock chains is argued by the operators involved at different levels in production, even if there are several national and European indication for their correct and rational use (www.salute.gov.it; https://eur-lex.europa.eu). Even the use, and sometimes misuse, of antimicrobials in rabbit breeding has been object of discussion for long time, and studies carried out over time have shown that it could be critical in quantitative terms (Agnoletti et al., 2018; Grilli et al., 2017). Nowadays, the downward trend is evident, but it is not still enough to fully satisfy the demanding requests coming from the consumer world. With time, the methods of administration have also changed in the rabbit production management: the administration through medicated feed is decreasing, also due to the growing attention towards the problem of carry-over, and contemporarily the use of drinking water as a mean of administration is increasing. Some aspects of the methods of administration are focused on how to optimize this intervention, as well as to minimize possible concomitant risks, i.e. those related to incorrect distribution and to the presence of residues beyond the duration of prescribed therapy in the case of medicated feed. Moreover, antimicrobials' residues significantly contribute to increase the risk of selection of resistant microbial strains. In the intensive rabbit production, high attention is given to biosafety measures, both external and internal to the breeding units. Among the different production techniques, the most used today i.e. Dual Band

provides, given the two reproduction and growth phases coexisting in the same site, the so-called "closed cycle breeding". Thus, two connected but structurally divided environments with the same type of cage, firstly equipped for the maternal phase (birth and breastfeeding) and, then, after the removal of the nest and transfer of the mother, also for group fattening. Therefore, the production cycle for growing rabbits occurs in the same environment and for most of them in the same cage. At the end of the cycle, when 10-12 weeks old, rabbits are moved to slaughterhouse, the full site (cages, equipment, fans, sheds, etc.) is washed and disinfected, and left empty for a variable period before the reintroduction of pregnant females for a new production cycle.
In this study, we planned to carry out in two similar farms managed by the same operator, at the end of the cycle when rabbits were removed, a series of water withdrawals from different points of the drinking lines. The scope was to determine the degree of persistence of antibiotic residues in the watering lines by comparing the results of the above-mentioned water withdrawals with a corresponding set of samples taken, after full environmental washing and disinfections, seven days post introduction of breeding animals.

MATERIALS AND METHODS

Animals and experimental design

Two "closed loop sites" were used for the study. The first site consisted of conventional type cages, i.e. dual-purpose cage (W38-L102-H39), where 5-6 rabbits are housed during the growing phase, while the upper row has comeback cages (W38-L43.5-H28). At weaning (35 dd), the breeding animals are moved to a next room of the same capacity. The watering drinking system consists of an external tank equipped with a booster pump and supplied by water from the aqueduct. The tank is also used for the administration of antimicrobial products. The breeding environment consists of 4 rows of cages, in each one there is a tray ahead used to regulate the pressure. Each cage is equipped with one watering point. The watering line ends blindly. Within this site, just after the conclusion of the breeding cycle, a series of water withdrawals from different critical points of the drinking lineswere taken, in duplicate (Figure 1). The "static washing" was then carried out i.e. coarse cleaning of the lines by short-term introduction of forced water. A solution containing 2% of AQUA CLEAN® from Kanters based on hydrogen peroxide was then added. After 24h, this solution was removed, the watering systems was rinsed with forced running water and then left empty. Seven days post weaning, in the presence of breeding animals, and without having done any administration

Table 1: molecules researched with LC-HRMS analysis

GROUP OF ANTIBACTERIALS	ANALYTE
AMPHENICOLS	Florphenicol, Florfenicoloamine, Thiamphenicol
PENICILLINS	Ampicillin, Amoxicillin, Cloxacillin, Dicloxacillin, Nafcillin, Oxacillina, Penicillin G, Penicillin V, Piperacillin
SULPHONAMIDES	Sulfadiazine, Sulfadimethoxin, Sulfadoxine, Sulfamerazine, Sulfamethazine, Sulfamethoxazole, Sulfamonomethoxin, Sulfatiazole, Sulfapyridine, Sulfacloropyridazine, Sulfachinossalina, Sulfamethoxypyridazine
DIAMINOPYRIMIDINE	Trimethoprim
TETRACYCLINE	4-Epichlortetracycline, Chlortetracycline, 4-Epitetracycline, Tetracycline, 4-Epoxytetracycline, Oxytetracycline, Doxycycline, Metacycline
LINCOSAMIDES	Lincomicyn
QUINOLONE	Oxolinic Acid, Nalidissic Acid, Ciprofloxacin, Danofloxacin, Difloxacin, Enoxacin, Enrofloxacin, Flumequina, Marbofloxacin, Norfloxacin, Levofloxacin, Orbifloxacin, Sarafloxacin
MACROLIDES	3-O-acetylethylosin, Erythromycin A, Gamithromycin, Josamycin, Kitasamycin, Neospiramycin I, Oleandomycin, Spiramycin I, Tildipyrosine, Tilmicosin, Tulathromycin A, Tulatromycin marker, Tylosin A, Tylvalosin
PLEUROMUTILINES	Thiamulin, 8-alpha-hydroxymutilin, Valnemulin
RIFAMYCINS	Rifaximin

of antimicrobials, a similar set of samples, as before, was taken. The second site was equipped with enriched cages (WRSA type) or dual-purpose cages (W 38-L102-H 65). In this case also, after weaning, the does are removed. The breeding environment consists of six rows. The watering system is substantially identical to that of site 1 and sampling was done following the same protocol. Subsequently, the "dynamic washing" was carried out: water recycling for each row was ensured by using a pump, (Evolution 40 AQUAMAX Srl). After a first rough cleaning, with forced emptying of the lines, the ring was closed, and the disinfecting product was added (5% Acquaskill 827® Adriawater Srl). This solution was forced to circulate in the created ring cycle after being filtered through a filter with a washable cartridge at 50µm before reintroduction. This operation lasted about an hour per row. Once the washing was finished, the lines were rinsed and forced emptied. Again, 7 days after the introduction of breeding animals, the second set of samples was taken from the same critical points reported in figure 1.

Sample preparation and analysis

A total of 42 samples from site 1 and 83 from site 2 were examined. They were contained in bottles hermetically closed and identified with the relative sampling point's code. Samples collected along two lines for each site were analysed. Table 1 show the molecules researched. The sampling points, the samples analysed and the concentrations detected for each analyte found in the two sites are shown in Figure 1 and 2. In order to remove particulates, aliquots of each water sample were centrifuged at 4000 *rpm* for 10 min at room temperature. Then, 1 mL of supernatant was diluted with 1 mL of aqueous working solution (formic acid 0.1% in water/methanol 95:5 v/v). The solution was vortexed and transferred into vials for LC-HRMS analysis, carried out using a UHPLC chromatography system (Ultimate 3000 Dionex, Thermo) coupled to a high-resolution mass spectrometer system (Q-Exactive Focus Orbitrap, Thermo). Sample aliquots of 5 µL were injected into the chromatography system and compounds were separated using a UHPLC C18 column (2.1 x 100 mm, 1.6 µm particle size from Waters) in linear gradient mode mixing an aqueous phase (containing 0.1% of formic acid) and an organic phase (methanol). The flow rate was 0.3 mL/min; the run time of 30 min allowed the separation of all the analytes. Qualitative results data were calculated using the acquisition in full scan mode where the exact mass of compounds was extracted within a mass range of 200-1200 m/z. For quantitative purpose, the mass spectrometer acquisition was achieved in full scan/dd-MS2 mode monitoring two or more fragment ions for each analyse.

RESULTS AND DISCUSSION

Figure 1: Antimicrobial residues and concentrations (µg/L) detected in the samples of site 1, before and after the "static washing" using AQUA CLEAN®; ND: not detected

	PRE CLEANING FASE				POST CLEANING FASE			
	TANK	START OF LINE	HALF LINE	END LINE	TANK	START OF LINE	HALF LINE	END LINE
Enrofloxacin	81,1	5,7	8,4	14,3	63,2	9,3	13,1	10,0
Ciprofloxacin	5,5	ND	ND	3,3	2,4	3,0	3,2	3,1
Trimethoprim	6,3	ND	ND	ND	3,6	4,4	1,9	12,9
Sulfadimethoxin	12,4	3,4	14,7	20,0	4,3	30,0	20,3	34,8
Oxytetracycline +4-epi	7,3	1,6	ND	ND	7,4	2,0	2,1	2,4
Flumequina	3,4	ND	ND	ND	4,3	1,2	ND	ND
	X	X	X	X	X	X	X	X
	LINE A				LINE A			
Enrofloxacin	53,4	13,5	4,1	3,2	47,0	26,0	13,1	4,9
Ciprofloxacin	1,8	3,2	ND	ND	1,1	ND	ND	ND
Trimethoprim	5,9	0,9	ND	1,4	7,9	2,9	1,6	2,8
Sulfadimethoxin	12,8	8,5	2,9	5,6	10,9	63,0	11,1	45,6
Oxytetracycline +4-epi	1,9	2,1	ND	ND	3,8	0,7	0,6	0,6
Flumequina	4,7	ND	ND	ND	5,8	ND	ND	ND
Tiamulin	ND	ND	ND	ND	ND	ND	1,8	ND
	X	X	X	X	X	X	X	X
	LINE B				LINE B			

The results obtained with the static washing are indicated below and reported in Figure 1; the trend of the concentrations detected in the lines A and B for each site were generally superimposable.

Site 1 – line A and line B. Before static washing, higher concentrations of antimicrobial residues were detected into the external tank of the watering line. Enrofloxacin concentration was 81.1 µg/L in line A and 63.2 µg/L in line B,

Sulfadimethoxin concentration was respectively 12.4 µg/L and 12.8 µg/L. Ciprofloxacin, Trimethoprim, Oxytetracycline with its 4-epimer, Tilmicosin and Flumequine were also detectable. From the beginning to the end of lines, the other concentrations found were lower than the first samples taken in the tank and these had a different trend. Concentrations of Enrofloxacin increased along the watering line until the end in line A but decreased in line B. Sulfadimethoxin increased along the watering line A whose ranged from 3.4 µg/L to 20.0 µg/L but it had a similar concentrations along line B. Ciprofloxacin was detected at the end of first line and at beginning of the second line. Traces of Oxytetracycline, its 4-epimer and Trimethoprim were detected only at the beginning of the line. Flumequine wasn't detected along both lines. After static washing: the trend did not change after the washing operation: into the external tank higher concentrations of analytes were detected. The concentration of Enrofloxacin was 63.2 µg/L and 47 µg/L respectively in line A and B; the concentrations of Sulfadimethoxin were respectively 4.3 µg/L and 10.9 µg/L and were detectable also other molecules detected before washing. Along the lines the sample points with residues were more numerous than before washing. When detectable, Ciprofloxacin and Oxytetracycline were almost distributed with the same concentration level along the entire lines. Concentrations of Trimethoprim were higher at the end of line A (12.9 µg/L) and were steady along the line B. Flumequine was present in the beginning of the line A (1.2 µg/L) and wasn't detected along lines B. Tiamulin was detected at half line B (1.8 µg/L).

Site 2 – Line A and line B. Lower concentrations of analytes were detected in drinking lines compared to the samples of site 1. Figure 2 shows molecules and their concentrations detected at least once after "dynamic washing". Before dynamic washing: only low traces of Enrofloxacin and Sulfadimethoxin (and Trimethoprim for line A) residues were detected in the external tank. The highest concentrations of Enrofloxacin were found at the beginning (14.5 µg/L line A, 28.9 µg/L line B) and in the middle of drinking lines (22.6 µg/L line A, 10.2 µg/L line B) against the concentrations to the end lines (2.8 µg/L line A, 7.6 µg/L line B). Sulfadimethoxin showed the same trend but the concentrations were lower of about 80% than Enrofloxacin. Trimethoprim residues were founded at the beginning and at the middle of both lines. Ciprofloxacin was present at the beginning of both lines, at the middle of line A and at the end of line B. Oxytetracycline was present at the end of line B only. After dynamic washing: traces of Enrofloxacin in the inlet tank and at the end of both lines (and at the beginning of line A) and traces of Tilmicosin (1.7 µg/L) at the end of line B only were detectable.

Figure 2: Antimicrobial residues and concentrations (µg/L) detected in the samples of site 2, before and after the "dynamic washing".

	PRE CLEANING FASE				POST CLEANING FASE			
	TANK	START OF LINE	HALF LINE	END LINE	TANK	START OF LINE	HALF LINE	END LINE
Enrofloxacin	1.2	14.5	22.6	2.8	2.2	1.0	ND	2.0
Ciprofloxacin	ND	3.7	4.2	ND	ND	ND	ND	ND
Trimethoprim	3.7	3.7	1.1	ND	ND	ND	ND	ND
Sulfadimethoxin	0.6	3.2	1.7	0.8	ND	ND	ND	ND
Tilmicosin	ND	ND	ND	ND	ND	ND	ND	1.7
	X	X	X	X	X	X	X	X
	LINE A				LINE A			
Enrofloxacin	5.4	28.9	10.2	7.6	1.7	ND	ND	2.0
Ciprofloxacin	ND	1.7	ND	3.1	ND	ND	ND	ND
Trimethoprim	ND	4.0	4.4	ND	ND	ND	ND	ND
Sulfadimethoxin	0.7	4.0	2.3	0.6	ND	ND	ND	ND
Oxytetracycline + 4-epi	ND	ND	ND	2.0	ND	ND	ND	ND
Tilmicosin	ND	ND	ND	ND	ND	ND	ND	1.7
	X	X	X	X	X	X	X	X
	LINE B				LINE B			

CONCLUSIONS

In this study, two rabbit farms with different method of watering and cleaning of the lines were exanimated and different result were obtained. In both sites almost the same molecules were detected. The residues of antimicrobial belonged to different classes and derived from previous treatments, thus showing that cleaning operations were often ineffective. In site 1, where a hydrogen peroxide-based product was used, there wasn't cleaning effect of the washing. Enrofloxacin was detected particularly in the inlet tank and in the beginning of the lines, i.e. it remains more easily in the tray and not along the watering line. Sulfadimethoxin, instead, remains mainly in the middle or in the end of the line and lower concentrations remains at the beginning of line. Furthermore, higher concentrations of Sulfadimethoxin were found even after washing. In site 2, where a specific product was used, higher concentrations of Enrofloxacin were detected in the middle of lines than at the beginning or the end. The same trend of site 1 was observed for Sulfadimethoxin. The study confirmed the lower effectiveness of static cleaning method compared to the dynamic one. In fact, concentrations on site 1 were higher than on site 2. In site 1, concentrations after first cleaning method in both lines decreased, but a complete cleaning never occurred, whereas in site 2 there weren't antimicrobials residues anymore after cleaning. Finally, the higher concentrations of antimicrobials residues detected in the middle and end points of the lines can be considered the effect of an accumulation due to a diminished water flow. If this occurs during the pharmacological treatment, animals could likely intake different levels of antibiotics, an hypothesis that should be further investigated in the future.

REFERENCES

Agnoletti F., Brunetta R., Bano L., Drigo I., Mazzolini E. Longitudinal study on antimicrobialconsumption and resistance in rabbit farming. *International Journal of Antimicrobial Agents Vol 51, Issue 2 February 2018 pages 197-205*

Grilli, G., Frabetti A., Pedicone R., Zuffellato A., Lavazza A., La Gestione del fármaco ed ni risultati del Piano Nazionale Antibiotici, *Convegno Asic 2017*

http://www.salute.gov.it/portale/documentazione/p6_2_2_1.jsp?lingua=italiano&id=2660

http://www.salute.gov.it/portale/news/p3_2_1_1_1.jsp?lingua=italiano&menu=notizie&p=dalministero&id=3486