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Poster Communications

Session 1: Antimicrobials & Antibiotic resistance

1.1.

Time-kill assay for determining the *in vitro* activity of an efflux pump inhibitor with three antimicrobials against *Escherichia coli*

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Overexpression of nonsespecific active efflux pumps in Gram-negative bacteria, is an ubiquitous multidrug resistance (MDR) mechanism. There is increasing evidence that RND efflux pumps, as acrAB-TolC in *Escherichia coli* plays an important role in virulence expression by Gram-negative pathogens[1]. The design of new therapeutic strategies such as inactivation of efflux pump systems by inhibitor drugs is a possible alternative to face the problem[2]. The aim of our study was to evaluate the effect of the pump inhibitor 1-(1-naphthylmethylpiperazine) – NMP – in association with florfenicol (FLF), tetracycline (TET) and ciprofloxacin (CIP) against isogenic *E. coli* MDR phenotype, by time killing curves.

The time-kill method of synergy testing was performed by the broth macrodilution technique[3]. Two isogenic *E. coli* bacteria with known genotype were used: AG112 strain (overexpressing RND type efflux pumps), and AG100 (wild type). The antimicrobials tested were FLF, TET, CIP. Three groups of five LB broth tubes were used for each antimicrobial tested. In the first one, the time-killing curve was determined against the antimicrobial alone. In the second and the third groups, the antibiotic was combined with 50 and 100 μg ml^{-1} of the efflux pump inhibitor respectively. Antimicrobial concentrations evaluated were the CIM, 2, 4 and 8 times the MIC; and a fifth tube without antimicrobial, as control. Duplicate samples from every tube were obtained at 0, 2, 4, 8, 24 and 48 h after incubation for 24 h at 37°C, and colony counts were determined. The activity of the antimicrobials alone and in combination was determined by plotting Log_{10} colony counts (CFU ml^{-1}) against time.

The results were corroborated by statistical analysis (ANOVA). No significant differences ($P < 0.05$) were found in the effectiveness percentage of antimicrobial alone or with NMP (50 μg ml^{-1} or 100 μg ml^{-1}). Nevertheless, antimicrobial concentrations assayed were 4 to 16 times lower with NMP (50 μg ml^{-1} or 100 μg ml^{-1} respectively), than in the group of tubes without the efflux pump inhibitor.

These results mean that it was possible to preserve the kinetics of death in the group of tubes with NMP (50 μg ml^{-1} or 100 μg ml^{-1}). The use of NMP is promising; it may be possible not only to change bacterial susceptibility, but also to infer the abilities of bacteria to successfully infect the host.

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1.2.

Experimentally-induced colibacillosis in piglets: effect of age and size of the inoculum

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Colibacillosis is a severe disease in pigs with outbreaks occurring at weaning worldwide that cause fatal diarrhea. Two infectious models were developed, differing by the age of piglets at inoculation and the inoculum size. A first model using piglets just after weaning was previously developed and a new model with older animals has been implemented.

Post-weaning diarrhoea was induced in conventional piglets by administrating on two consecutive days an *Escherichia coli* K88 strain. Administration of the inoculum via a gastric feeding probe was preceded by drenching of tryptic soy broth containing 1.2% bicarbonate. Piglets were randomly allocated according to body weight and sex to four groups. The animals were inoculated within 1 week or 2 weeks after weaning using different sizes of inoculum (log9 or log10 CFU) at each age depending on groups.

The animals were clinically examined daily for 2 weeks. Clinical examination included rectal temperatures and scoring of general and digestive clinical signs. Body weight and feed intake were measured. Fecal samples were taken for total *E. coli* and haemolytic *E. coli* flora enumeration. The animals were euthanized 2 weeks after inoculation and gross examination of the gastro-intestinal track was conducted.

Inoculation produced characteristic clinical signs of colibacillosis within 1 day whatever the age at inoculation and the size of the inoculum. Abnormal faeces were frequently observed the days following the inoculation, mainly during the first week in all groups, and progressively decreased thereafter. Severity of clinical signs was higher in younger animals but did not differ significantly between groups. Mortality remained limited and occurred shortly after the inoculation. Time courses of mean faecal total and haemolytic counts were similar between groups and showed an increase of the counts just after the challenge and return to basal values the week after for the total counts. No obvious gross lesions were observed at necropsy 2 weeks after the challenge.

Both models succeeded in producing a stable clinical colibacillosis. The age at inoculation and the size of the inoculum did not significantly impact the outcome of this model. Inoculation of piglets 2 weeks after weaning allow a sufficient time period for possible preventive treatment before challenge in experimental efficacy studies.

†The text was changed following initial online publication.

1.3.
Changes in pharmacokinetics and metabolism of florfenicol during the acute phase response induced by *Escherichia coli* lipopolysaccharide, in rabbits

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INTRODUCTION

In veterinary medicine dosage drug recommendations are often based on experimental data obtained from healthy animals, beside marked alterations in drug disposition in disease states may have profound implications on efficacy and/or toxicity under clinical conditions. Thus, identifications of changes in antibiotic disposition during an acute inflammatory response induced by *E. coli* LPS as a model of systemic gram-negative infection may allow for development of more rational therapeutic approaches to the treatment of infections. In the current study we determined the effect of an *E. coli* LPS–induced APR on the pharmacokinetics and biotransformation of FFC in rabbits.

MATERIALS AND METHODS

Six rabbits [3.0 ± 0.08 kg body weight (bw)] were distributed through a cross-over design with 4 weeks of wash-out. Pairs of rabbits similar in bw and sex were assigned to experimental groups: Group 1 (LPS) treated with three intravenous doses of 1 μg kg^{-1} bw of *E. coli* LPS at intervals of 6 h; Group 2 (Control) treated with an equivalent volume of saline solution (SS) at similar intervals and frequency of Group 1. At 24 h after first injection of LPS or SS, an IV bolus of 20 mg kg^{-1} bw of FFC was injected. Blood samples were drawn from the auricular vein before and after FFC administration. FFC and FFC-amine (FFC-a) were extracted from plasma and their concentrations were determined by HPLC. Data were analysed by a non-compartmental pharmacokinetic model and compared by a paired Student *t*-test.

RESULTS

The FFC mean values of $AUC_{0-\infty}$ in endotoxaemic rabbits (26.3 ± 2.7 μg*h ml^{-1}) were significantly higher ($P < 0.05$) than values observed in healthy rabbits (17.2 ± 0.97 μg*h ml^{-1}). The total mean plasma clearance (CL_T) decreased from 1228 ± 107.5 ml*h kg^{-1} in control group to 806.4 ± 91.4 ml*h kg^{-1} in LPS-treated rabbits. The mean values of C_{max} and $AUC_{0-\infty}$ for FFC-a in LPS-treated rabbits were lower ($P < 0.05$) than those observed in SS-treated animals, whereas the values of elimination half-life and MRT were higher for the endotoxaemic rabbits. The ratio AUC-FFCa/AUC-FFC of the LPS group was 22.0% ± 5.3%, which was significantly lower than that observed in the control group (57.8% ± 9.2%).

DISCUSSION

The administration of repeated doses of 1 mg kg^{-1} *E. coli* LPS induced an APR in rabbits, producing significant modifications in plasma concentrations of FFC associated with increases in the AUC, terminal half-life and mean residence time, leading to a significant decrease in CL_T of the drug. As a consequence of the APR induced by LPS, there was a reduction in the metabolic conversion of FFC to their metabolite FFC-a, suggesting that the mediators released during the APR induced significant inhibitory effects on the hepatic drug-metabolising enzymes.

ACKNOWLEDGMENTS

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1.4.
Effects of flouroquinolone resistance determinants on the mutant prevention concentration, mutant selection window, mutant frequency and bactericidal activity of enrofloxacin in *Escherichia coli* isolated from animals

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INTRODUCTION

The aims of this study was to investigate the effects of flouroquinolone resistance determining region (QRDR) mutations (gyrA, parC and parE) and transmissible quinolone resistance genes (aac (6')-Ib-cr, qnrA1 qnrS1 and oqxB) on the mutant prevention concentration (MPC), mutant selection window (MSW), mutant frequency (MF) and bactericidal activity of enrofloxacin (ENR) in *E. coli* isolated from animals.

MATERIALS AND METHODS

The five *E. coli* isolates, three transformants and two control strains (*E. coli* ATCC 25922 and *E. coli* AG100) were used in the study. Both microdilution test was used to determine susceptibility of ENR. Presence of QRDR mutations and variants of PMQR genes were determined by PCR amplification and sequencing. PCR amplification, HindIII digestion and pUC19 plasmid was used for cloning of qnrS1 and aac(6′)-Ib-cr genes. Transformations were performed by using a commercial kit. The transformants were selected on ampicillin containing agar plates. Agar dilution method was used to determine MPC, MSW and MF, time-kill experiments were used to evaluate bactericidal activity of ENR.

RESULTS AND CONCLUSION

MICs of ENR were 0.032 μg ml^{-1} for control strains, ranged from 1 to 128 μg ml^{-1} for *E. coli* isolates. The transfer of aac (6′)-Ib-cr and qnrS1 to the control strain increased the MIC of ENR from 0.032 μg ml^{-1} to 1 μg ml^{-1}. MPC values were 0.128 μg ml^{-1} for control strains, 4 μg ml^{-1} for transformants and and ranged from 2 to 512 μg ml^{-1} for *E. coli* isolates. MPC/MIC were 4 for control strains and transformants and ranged from 2 to 8 for *E. coli* isolates. MF ranged from 2.5×10^{-14} to 7.5×10^{-8}. ENR displayed a concentration-dependent bacterial killing on all *E. coli* isolates. The maximal bacterial reduction within 24 h was 7 log10 cfu ml^{-1} at all concentrations greater than 8xMIC for the control strains and 16xMIC for the transformants and *E. coli* isolates. The results of this study showed that QRDR mutations and transmissible quinolone resistance genes may have an effect on the survival of *E. coli* by increasing MSW and MF.

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1.5.
Trends in Piedmont Region (Italy) of the use of veterinary drugs in bovine farms

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INTRODUCTION
Since the year 1988, the Italian National Residues Plan for food (PNR) [1] provides for the analysis of chemical residues in food of animal origin. The aim of this study was to compare the effective use of drugs in a specific kind of livestock and those molecules which are reported on the PNR, and therefore checked by the official laboratories.

MATERIALS AND METHODS
The use of drugs in fattening veals in three Piedmont areas was investigated by checking prescriptions made by veterinarians in farms. Eighteen Charolaise and Limousine beef farms with more than 100 veals born in France and fattened in Italy were considered. 10 months old Cattle were fattened until they were 18 months and 620 kg average weight. Both parenteral and oral administrations (drinking water) were examined throughout the fattening period.

RESULTS AND CONCLUSIONS
An average of 30 different molecules were usually prescribed in farms, to be used in parenteral administrations, while just a few drugs were administered orally. 24 different antibiotics or associations, 7 anti-inflammatory molecules and 6 anti-parasitic drugs were listed. 25 out of the 37 different molecules used are currently checked by the official laboratories.
Despite the great effort of the official laboratories to keep up with the wide range of different molecules used in bovine therapy, a consistent number of medicinal products are still not routinely checked. In Italy, the Istituti Zooprofilattici Sperimentali are the official laboratories designed by the competent authority. They are in charge of performing controls to ensure safety of animal-derived food products. This lab network should contrast more efficiently the use and the abuse of drugs in farms, checking the respect of withdrawal periods. An effective interaction with the local Veterinary Service and laboratories could play a key role in determining an up-to-date list of molecules that should be investigated, taking into account that all lab tests have to be accredited according to ISO 17025. Nevertheless, a new multiresidue method for beta lactam residues is on the way.

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1.6.
In vitro/ex vivo release and antibacterial effect of enrofloxacin-containing bone cement

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INTRODUCTION
The combination of polymethylmethacrylate (PMMA) with antibiotics like vancomycin or gentamicin is widely used to protect from infections after orthopedic implantations. The aim of this study was to analyze the release of antibacterials from PMMA *in vitro* and *ex vivo* as well as to determine their antibacterial activity. Enrofloxacin was used as test compound. Furthermore, the isolated perfused bovine udder model was used to perform *ex vivo* experiments. Besides, the influence of PMMA on the biocompatibility of murine keratinocytes (MSC) and fibroblasts (L929) cell lines was analyzed.

MATERIAL AND METHODS
Enrofloxacin-loaded PMMA cylinders (25 mg g^{-1} and 50 mg g^{-1} PMMA) were incubated in phosphate buffered solution (PBS, incubation medium) up to 40 weeks. Incubation medium was analyzed by measuring the antibiotic concentration at different time points by high-performance liquid chromatography. The antibacterial activity of released enrofloxacin was tested by a brilliant-black reduction test with *Geobacillus stearothermophilus var. calidolactis C95* after 3 and 24 h as well as 20 weeks. The isolated bovine udder was used to analyze the enrofloxacin-release from PMMA. For this purpose, udders from healthy cows, all of which were obtained from the slaughterhouse, were perfused via the left and right external pudendal arteries with tyrode solution. A PMMA-cylinder was positioned in the gland tissue and the diffusion of enrofloxacin from PMMA was determined by microdialysis technique. For biocompatibility testing, MSC and L929 were used to determine the proliferation-rate and viability by BrdU- and MTS-assay.

RESULTS
In vitro, PMMA releases the highest amount of enrofloxacin during the first 24 h. Afterwards, the enrofloxacin-release decreases and a plateau becomes visible. These results are confirmed *ex vivo* by the experiment performed in the isolated bovine udder. The incubation media containing enrofloxacin are antimicrobially active during the first hours. After 24 h, the antibacterial effect decreases. PMMA shows no influence on biocompatibility of MSC or L929.

CONCLUSION
During the first 24 h, the amount of released enrofloxacin seems to be sufficient to ensure an antibacterial activity. Afterwards, the concentration and thus the antimicrobial activity decrease. It has to be questioned if the low antibiotic-concentration could result in resistance development which has to be studied in further investigations.

1.7.

The increase in LEAP-2 mRNA suggests a synergistic probiotics-antibiotic interaction in chickens

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INTRODUCTION

The gastro-intestinal tract is the most important reservoir of microorganisms and an extensive interaction between gut microbiota and the innate immune system exists [1]. Pathogenic bacteria, probiotics and antibiotics have a significant impact on the intestinal microbiome and they can modulate the expression of antimicrobial peptides such as LEAP-2 in chickens [2]. Therefore, our aim was to investigate the influence of *Lactobacillus spp.* commonly used as probiotics and the effect of an administration of antibiotics on LEAP-2 mRNA expression in the gastro-intestinal tissues of broiler chickens.

MATERIALS AND METHODS

24 Ross and 24 Duc one-day-old chicks were included in two experiments: with enrofloxacin and doxycycline, respectively. They were allocated to the following groups for the experiments with each antibiotic: Group 1 control (without treatment); Group 2 treated with the probiotics for 15 days; Group 3 treated with a combination of probiotics (for 15 days) and antibiotic (10 mg kg^{-1}, via drinking water for five days); Group 4 given the antibiotic only. Samples from liver, duodenum and jejunum were collected after the end of the treatment. Expression levels of LEAP-2 mRNA were determined by qRT-PCR and were statistically evaluated by Mann–Whitney test.

RESULTS

LEAP-2 mRNA expression levels remained similar in all four groups of Ross chickens from the experiment with enrofloxacin. Doxycycline, administered alone or in combination with probiotics, provoked a statistically significant up-regulation of the antimicrobial peptide in the liver and in the duodenum ($P < 0.05$). Administration of doxycycline without probiotics caused only a moderate induction of LEAP-2 mRNA, which was much lower ($P < 0.05$) if compared to the treatment with the combination of probiotics and doxycycline.

CONCLUSIONS

A synergistic effect in the activation of innate defence mechanisms was observed when chickens received a combination of *Lactobacilli* and doxycycline. Doxycycline, given alone, is able to stimulate the LEAP-2 expression to a lesser extent. Such an up-regulation of the studied antimicrobial peptide might be beneficial in terms on host protection [3]. Enrofloxacin, administered alone and in combination, does not stimulate LEAP-2. Finding combinations between probiotics and antibiotics that work synergistically in the stimulation of the host immune system would be a great relevance for the clinical practice in poultry husbandry.

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1.8.

Intestinal concentrations of sulfadiazine-trimethoprim in pigs after (non-)conventional oral and intramuscular treatment

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INTRODUCTION AND AIM

Dosage regimens of antimicrobials in animal husbandry commonly show considerable variability, even between manufacturers. This can have consequences on the occurrence of resistance selection. Hence the objective of this study is, first, to develop and validate a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to quantify sulfadiazine and trimethoprim in plasma and intestinal content, and secondly to evaluate intestinal concentrations of sulfadiazine-trimethoprim combination after oral (P.O.) as well as intramuscular (I.M.) administration in pigs, using conventional dosing and dose alteration. This data will give insight into the exposure of gut flora to these active compounds and will be further used to assess the selection pressure exerted by the various concentrations in the intestinal content.

MATERIALS AND METHODS

Sample preparation of the plasma and intestinal samples consisted of liquid-liquid extraction with ethyl acetate. Next an animal study was conducted using 64 piglets, randomly divided into four different treatment groups, each counting sixteen animals. The groups were set up in order to evaluate and compare the effect of administration route (P.O. versus I.M.) and (non-) conventional dosage schemes (ranges for both administration routes were between 15–45 mg kg^{-1} bodyweight), on intestinal and plasma concentrations. Twelve hours post-administration, eight animals in each group were euthanized. This time-point was selected as it was expected that concentrations of both active substances would be maximal in the cecum-colon section, of which samples were taken. After five consecutive days of treatment, the eight remaining piglets in each group were also euthanized and intestinal concentrations were determined in the same manner. Fecal and plasma samples were also collected daily during this five day period.

RESULTS AND CONCLUSIONS

The LC-MS/MS method was validated using matrix-matched samples for feces and intestinal content. Linearity was achieved for concentrations from 25–100 000 ng g^{-1} for both sulfadiazine and trimethoprim. The limit of quantification was

25 ng g^{-1} for both compounds and limits of detection varied between 1.59–15.97 ng g^{-1}. Furthermore the method proved to be accurate and precise by evaluation of within- and between day quality control samples. Any matrix effects were compensated by use of internal standards trimethoprim-d9 and sulfadiazine-(phenyl-13C6).

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1.9.
Effects of the administration of colistin on the susceptibility of commensal *Escherichia coli* in the porcine intestinal microbiota

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As bacterial resistance increases in both human and veterinary medicine, the interest in the polymyxin colistin, is reawakened. Colistin is a protected antibiotic in human medicine for the treatment of multi drug resistant bacteria e.g. *Acinetobacter baumanii*. It has a good bactericidal effect against gram-negative bacteria, including *Enterobacteriaceae*. In veterinary medicine colistin is widely used in the treatment of livestock. Resistance against colistin can be developed through mutation or adaptation mechanisms. Between colistin and polymyxin B exists an almost complete cross- resistance.
The aim of the present study was to determine the influence of intramuscular administration of colistin on the susceptibility of *E. coli*, chosen as a model for the commensal intestinal microbiota of pigs.
In this study two groups, each of them composed of 4 piglets, were housed in the same stable but in two different bays with a 3 meter distance in between. Group A received the recommended therapeutic dosage (2.5 mg kg^{-1}) of colistin sulfate injected on day 1–5 and day 22–26. At weekly intervals the rectal faeces of every piglet were taken for analysis. Ten *E. coli* colonies per animal and sampling day were isolated and the particular minimal inhibitory concentration (MIC) was determined using epsilometertests. With the same microbiological procedure the MIC of airborne *E. coli* isolates were determined, which were caught with Endo- Agar plates positioned at specific spots in the stable. Sedimentation dust and air filters were used to measure the amount of colistin in the environment.
Throughout the study, treatment with colistin sulfate administered for two periods of five days, produced MICs that did not exceed 2 mg l^{-1}, which marks the clinical breakpoint as well as the epidemiological Cut- Off. The MIC did not significantly change. Presumably the stability of the MIC during this experiment can be explained by the poor passage of colistin through the intestinal wall. Consequently the commensal *E. coli* have no contact with the colistin after intramuscular administration. Therefore, they have no need to develop any form of resistance, which would be detectable by a MIC shift.
The study was supported by the German Federal Office of Consumer Protection and Food Safety (BVL).

1.10.
Plasma concentrations of florfenicol in koalas (*Phascolarctos cinereus*) with naturally occurring systemic chlamydial disease following intravenous and subcutaneous administration

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INTRODUCTION
Florfenicol (FFC) is being investigated as an alternative treatment of chlamydial disease in koalas.
Koala populations are listed as vulnerable across most of their natural range. Chlamydiosis is the most important infectious disease of koalas. [1] Infection with chlamydia causes painful keratoconjunctivitis and blindness, chronic urinary tract inflammation and infertility. The most recent review of outcomes following treatment of chlamydial disease in koalas reported a mortality rate of 50% following treatment. A significant percentage of these treated patients returned with active signs of chlamydial disease following return into their home range.[2]
The aim of this study was to assess the tolerance of FFC in koalas and to obtain basic pharmacokinetic data to aid determinations of likely efficacy. Systemic chlamydiosis is a chronic intracellular disease with current treatment periods of 2–4 weeks, thus preliminary pharmacokinetic data is helpful before undertaking prolonged clinical trials in this iconic species.

MATERIALS AND METHODS
A new method of sample handling for koala plasma has been developed for validation of a HPLC-UV assay. A modified liquid-liquid extraction technique using methyl tert-butyl ether (tBME) facilitated removal of endogenous peaks at the retention times of interest for FFC and internal standard phenacetin.
Florfenicol was administered to koalas with naturally occurring systemic chlamydial disease that failed to respond to conventional therapies.

RESULTS AND CONCLUSIONS
Poor absorption was noted following a single subcutaneous (SC) injection. The mean maximum concentration of FFC in plasma (C_{max}) of 3 koalas given 20 mg kg^{-1} SC was 1.2 μg ml^{-1} at 4 h with concentrations <LLOQ by 24 h.
Surprisingly, following intravenous (IV) dosing at 10 mg kg^{-1}, FFC was noted to persist at clinically useful concentrations for a practical dosing interval (mean of 3 koalas = 19 μg ml^{-1} at 24 h).
Maintaining plasma concentrations of >1–2 μg ml^{-1} has been set as the pharmacodynamic target, based on previously reported minimum inhibitory concentrations of FFC for chlamydia *in-vitro*.[3] Preliminary tolerance data from the field suggests FFC is unlikely to be useful in treating chlamydiosis via the SC route, however IV dosing may prove efficacious, however the authors may have observed an anaphylactoid reaction via IV administration, so caution is warranted when administering the drug via this route.

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1.11.
Evolution of cefquinome minimum inhibitory concentrations against commensal bovine *Escherichia coli* strains after *in vitro* serial passages exposure

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INTRODUCTION/OBJECTIVE

The emergence of *Enterobacteriaceae* strains producing Extended-Spectrum Beta-Lactamases is a major issue for both human and animal health, as these enzymes confer resistance to last generation cephalosporins. Among the uses of these cephalosporins in cattle, the particular use by intramammary route at drying off should be considered. As for intramammary use in lactation, no induction of resistance is expected on mastitis pathogens nor on cow digestive flora due to intramammary route features (infusion in a gland unfavourable to genetic exchanges between bacteria and low if any elimination in digestive tract). Nevertheless in case of intramammary use at drying off, the possible impact of colostrum residues on calf gut flora should be assessed. This study was performed to adress this issue for cefquinome according to an *in vitro* serial passages model on *Escherichia coli*.

MATERIALS AND METHODS

Ten *Escherichia coli* strains were tested among a collection of isolates from the digestive tract of healthy cattle sampled at slaughter in Europe. They were selected to be representative of the cefquinome minimum inhibitory concentrations (MICs) distribution (0.03–0.12 μg ml^{-1}). Cefquinome MICs of the 10 isolates were determined before and after 10 serial passages using a microdilution method. Serial passages were performed in Cation-Adjusted Mueller Hinton Broth supplemented or not with cefquinome at the concentration of 15 μg l^{-1} (maximal concentration assayed in colostrum after regular use of a cefquinome-containing intramammary dry cow formulation). The MICs after 10 passages with or without cefquinome exposure were compared by the paired Student's *t* test after logarithmic transformation.

RESULTS

No significant differences were seen between MICs of isolates before the 10 passages or after these passages either in broth medium alone or supplemented with cefquinome, MIC_{90} being equal to 0.06 μg ml^{-1} before or after the passages.

CONCLUSION

According to this model, no change of *Escherichia coli* susceptibility to cefquinome was observed after repeated exposure to the maximal expected concentration in colostrum. These data are consistent with the lack of colostrum concentrations superior or equal to milk maximal residue level (20 μg l^{-1} for cefquinome) defined as safe for a long term human consumption, including possible effects on digestive flora.

1.12.
Antimicrobial drug penetration into the pulmonary epithelial lining fluid and muscle measured by microdialysis in anesthetized pigs

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OBJECTIVE

The aim of this study was to determine drug penetration of different antimicrobial drug classes into the pulmonary epithelial lining fluid (PELF) and to examine the relationship between drug penetration and molecular weight, lipophilicity (Log P, Log D), charge, and polarity (hydrogen bond donors/acceptors, polar surface area (PSA)) of the drugs. Because PELF has been described as a discrete anatomical compartment, drug concentrations in PELF were also compared with concentrations in extracellular fluid (ECF) of muscle to study whether ECF of muscle can serve as easy accessible surrogate tissue for estimation of PELF concentrations.

METHODS

Microdialysis probes (proximal bronchi, *M. gluteobiceps)* were calibrated by retrodialysis by drug, followed by a wash-out period. Gentamicin, sulfadiazine, cefquinome, minocycline and danofloxacin were infused to steady state plasma concentrations. Samples from PELF, muscle and plasma were collected every 20 min during 2 h, and for another hour after the infusion were terminated. Each drug was tested in two experiments (pigs 24 $\pm$ 3 kg.). Plasma was corrected for protein binding by ultrafiltration or equilibrium dialysis and samples were analysed by HPLC. The $AUC_{PELF/PLASMA}$ and $AUC_{ECF/PLASMA}$ of free drug were calculated from samples acquired under steady state conditions.

RESULTS

The $AUC_{PELF/PLASMA}$ was for gentamicin 0.8, sulfadiazine 1.1, cefquinome 1.3, minocycline 1.6 and danofloxacin 2.2. Drug penetration into PELF was positively correlated to LogD ($r^2 = 0.4183$, $p = 0.0433$) and negatively correlated to PSA ($r^2 = 0.4613$, $p = 0.0308$) and charge ($r^2 = 0.4114$, $p = 0.0456$), suggesting that liphophilicity of the drug facilitates penetration, whereas increasing polarity and charge reduces drug penetration into PELF. Drug concentrations in PELF and ECF declined almost in parallel with plasma concentrations.

$AUC_{ECF/PLASMA}$ was for gentamicin 0.7, sulfadiazine 0.7, cefquinome 1.5 and minocycline 0.7. Concentrations of PELF and ECF of muscle were similar for gentamicin, sulfadiazine and cefquinome, whereas minocycline and danofloxacin accumulated in PELF. Both drugs are known substrates of the PgP-drug transporter, which is present in the respiratory epithelium.

CONCLUSION

Albeit different size, liphophilicity and charge, none of the drugs had an inferior penetration to PELF compared to ECF, suggesting that the respiratory epithelium is highly permeable to drugs and that PELF may be considered a part of the ECF.

1.13.
Quorum sensing inhibitory and antibacterial activity of *Nymphaea tetragona*

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INTRODUCTION/OBJECTIVE

Quorum sensing (QS) inhibition became a novel strategy in combating infections, pathogenesis and resistance of bacteria (Hong *et al*., 2012). QS inhibition of *Nymphaea tetragona (water lily) 50% methanol extract (NTME)* has been screened out in our previous study (Hossain *et al*., 2014). So, in the present study, we further confirmed NTME concentration-dependent QS inhibitory activity and several pharmacological activities.

MATERIALS AND METHODS

QS inhibitory and antibacterial activities of NTME were examined against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Chromobacterium violaceum*. Qualitative and quantitative inhibitions of violacein pigment were determined with *C. violaceum*. Effects of NTME on swarming motility, biofilm, pyocyanin and LasA protease activity were checked against *P. aeruginosa*. Finally the safety of NTME was verified by acute oral administration in rats.

RESULTS

The extract showed concentration-dependent antibacterial activity *and* sub-MIC (2.5 mg ml^{-1}) of the extract significantly lowered the violacein of *C. violaceum* compared to control and lower concentration (0.63 mg ml^{-1}) treated. Swarming motility was inhibited by 50% when treated with (0.31–5.00 mg ml^{-1}) extract. The confocal micrograph of 24 h biofilm of *P. aeruginosa* exposed to (5.00–20.00 mg ml^{-1}) of *NTME* exhibited lower live cell than control. Pyocyanin production and LasA protease activity were significantly inhibited in overnight culture supernatant of *P. aeruginosa* treated with (1.25–2.50) mg ml^{-1} of NTME compared to control. The extract exhibited no toxic effects after a single oral administration in rats.

CONCLUSIONS

NTME inhibited the QS mediated virulence factors in bacteria. Therefore, it would be a great opportunity to overwhelm the resistance of bacteria, together with conventional antimicrobial agents.

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1.14.
Characterization and growth inhibition against fish pathogenic bacteria of a novel *Lactobacillus plantarum* PL13

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INTRODUCTION

The rapid expansion of aquaculture production has led to the outbreaks of infectious diseases caused by bacteria, viruses or parasites. The widespread use of antibiotics to control these fish diseases has developed antibiotic-resistant bacterial strains and may cause water pollution in the aquaculture environment. Lactic acid bacteria (LAB) (e.g. *Lactobacilli* spp.) are a strong potential candidate of probiotics to inhibit the growth of pathogenic fish bacteria. Therefore, the purpose of the present study is to characterize a novel *Lactobacillus plantarum* PL13 (*L. plantarum* PL13) screened in our laboratory and evaluate inhibitory activity of *Streptococcus. Iniae (S. iniae*) and *Edwardsiella tarda (E. tarda)* by *L. plantarum* PL13.

MATERIALS AND METHODS

The strain *L. plantarum* PL13 used in this study had been previously isolated from the intestinal content of fish. The acid tolerance test and bile tolerance test were performed to *L. plantarum* PL13 for obtaining the possibility as probiotics, comparing with *L. plantarum* KCCM 12116. In order to evaluate an antagonistic the growth property of *L. plantarum* PL13 against fish pathogenic bacteria, *S. iniae* and *E. tarda*, co-culture system for obtaining inhibition concentration (IC_{50}) (Prism program, GraphPad software, USA) was used and the number of bacterial strains were calculated by plating on MRS and BHI agar plates.

RESULTS AND CONCLUSION

L. plantarum PL13 could serve as a potential probiotics with acid and bile tolerance, production of digestive enzymes, antibacterial activity, resistance of gastrointestinal protease, and inhibition of fish pathogen adhesion to intestinal mucus. During the acid tolerance test, *L. plantarum* PL13 and *L. plantarum* KCCM 12116 were grown at pH 3 only for the first 6 h, but continued to grow at adjusted pH 4, 5, and 7 for 24 h. In bile tolerance, two strains were grown more than 10^6 CFU ml^{-1} with and without bile salt in MRS broths. The growth of *S. iniae* at initial level of 10^3 or 10^5 was inhibited by *L. plantarum* PL13. It has been shown that *L. plantarum* PL13 concentrations (IC_{50}; 8.80×10^3 CFU ml^{-1}) have antagonistic action over 24 h from the co-culture incubation of *S. iniae* (10^5 CFU ml^{-1}) and *E. tarda* (in work). In conclusion, it has been established that *L. plantarum* PL13 tolerates acid and bile and effectively inhibits the growth of fish pathogenic bacteria.

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1.15.

The impact of pharmacokinetics on emergence of *in vitro* bacterial resistance to cefovecin

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INTRODUCTION
Antibiotic resistance is a global issue in both human and veterinary medicine with substantial social and economic impact. Sub-inhibitory antibiotic cultures were used to investigate the interaction of antimicrobial concentration and time on the development of *in vitro* bacterial resistance. Cefovecin, a third-generation cephalosporin antibiotic has a half-life of approximately 166 h in the cat. This contrasts with the significantly shorter two-hour half-life of the oral first-generation cephalosporin cephalexin. We hypothesized that the long half-life of cefovecin would cause an increased emergence of bacterial resistance.

MATERIALS AND METHOD
Clinical isolates of *Escherichia coli* from the University of Melbourne Veterinary Hospital (UMVH) laboratory collection were used for these cultures. *Escherichia coli* NCTC 10418 was used as a reference stain. Minimum inhibitory concentrations (MIC) of cefovecin and cephalexin to the isolates of *E. coli* were determined by broth microdilution techniques as described by the Clinical and Laboratory Standards Institute guidelines. Antibiotic concentrations of 0.2, 0.4 and 0.8 × MIC were used as sub-inhibitory cultures in Mueller-Hinton cation adjusted broth (CAMHB). The cultures were incubated on an orbital shaking platform at 37°C for a total period of 144 h. The antibiotic media was refreshed and bacteria diluted every 24 h.

RESULTS
Results from the sub-inhibitory antibiotic culture experiments demonstrated that when an *E. coli* isolate was incubated with 0.8 × MIC concentration of cefovecin in CAMHB there was up to an 8-fold increase in the MIC after 72 h. When 0.8 × MIC cephalexin was incubated with the same *E. coli* isolate there was a four-fold increase in the MIC after 96 h. There was no change in MIC when the *E. coli* isolates were incubated in CAMHB alone. This research provides preliminary evidence that cefovecin should be used with prudence to limit *in vivo* resistance emergence. Future DNA sequencing will investigate the mechanisms of the evoked cefovecin-resistance.

1.16.

Penetration of the antibiotic fosfomycin into swine intestinal mucosa colonized with *Lawsonia intracellularis*

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INTRODUCTION/OBJECTIVE
Fosfomycin (FOS) is an antibiotic (ATB) used for the treatment of lung and enteric infections of pigs. Intracellular fluids of enterocytes can act as biophase for *Lawsonia intracellularis* (LAW), causative agent of porcine proliferative enteropathy (PPE), which presence could modify ATB penetration. The aim of this study was to determine FOS penetration into swine intestinal mucosa colonized with LAW.

MATERIALS AND METHODS
Four healthy pigs in grow-finish stage; live attenuated vaccine (Enterisol Ileitis®, Boehringer-Ingelheim); primers for LAW PCR detection (GATAATCTACCTTCGAGACGG; TGACCTCAGTGT-CAGTTATCGT, Invitrogen); calcium FOS. Explants were produced from ileum of euthanized animals. As a positive control for the PCR, LAW DNA was obtained from the vaccine. Explants (0.5 ml of vaccine; 24 h of incubation; 37°C) were incubated with 580 μg ml^{-1} of calcium FOS (0.5–6 h). Then, they were washed to remove extracellular FOS, deproteinized (1 ml of methanol) and sonicated (30 min) to release intracellular ATB. Tubes were centrifuged (6 min; 4°C; 10 000 rpm), supernatants were evaporated to dryness (60°C), dry extracts were dissolved in 200 μl of HPLC water and 1 ml of Folch reagent (hexane-ethanol; 1:0.2) was added for lipids removal. Samples were shaken (20 min), centrifuged (6 min), hexane phase was discarded, 40 μl of each sample were taken and carried to 800 μl with HPLC water, filtered and analyzed by HPLC MS/MS.

RESULTS
Intracellular concentration of FOS ranged between 3.75 and 24.81 μg ml^{-1} (T_{max}: 4 h). On previous studies on healthy swine intestinal explants, we have found that a low concentration of ATB enters into the enterocytes (5.84–12.99 μg ml^{-1}; T_{max}: 2 h), which could be attributed to the soluble nature of FOS. When comparing intracellular concentrations of FOS found in explants with LAW versus those found on healthy pigs intestinal explants, a higher proportion is present in explants with the bacteria (4%) than in those explants without LAW (2%). However, differences were not statistically significant ($p > 0.05$).

CONCLUSIONS
Although FOS concentrations are not too high, they exceed the MIC_{90} for *E. coli* (0.5 μg ml^{-1}) and *Salmonella* (4 μg ml^{-1}). There are no studies indicating FOS MIC_{90} for LAW. Nevertheless, MIC_{90} of various ATB for LAW ranges between 0.125 and 128 μg ml^{-1}. Further studies should be carried out to determine FOS MIC_{90} for LAW to discern the usefulness of this ATB in the treatment of PPE.

1.17.

Intracellular activity of penicillin G against *Staphylococcus aureus* in a ploymorphonuclear leukocytes bovine model

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Intracellular infections treatment remains a medical challenge, given the inability of some antimicrobial (ATM) to penetrate and act in the intracellular environment. Bovine mastitis caused by *S. aureus*, continues to generate great concern in all areas of health.[1,2] One possible factor that explains their persistence in the mammary gland after ATM treatment may be its ability to survive inside phagocytes and mammary epithelial cells[3]. ATMs do not always penetrate in host cells, and when they do it, their concentration may be too low to effectively destroy hidden bacterias[3]. So, efficacy data *in vitro* to ATM against *S. aureus* cannot be applied to intramammary infections. Our objective was to evaluate the penicillin G activity (PenG) on *S. aureus* phagocytosed by polymorphonuclear leukocytes (PMN) bovines.

S. aureus strains isolated from clinical mastitis cows and *S. aureus* ATCC 25923 as control were used. MIC was determined by microdilution method at pH 7.4, 6.5 and 5.0, to emulate the acidic conditions of subcellular structures. Blood samples were collected from healthy lactating cows and were processed immediately to obtain the PMN samples by density gradient centrifugation method using Histopaque 1077 and 1119. Trypan blue method was used to estimate the cell isolation yields in the PMN suspensions, after this, the cells were counted in a Neubauer chamber under the microscope. PenG was used at a concentration of 4 × MIC. PMN (5×10^6 ml^{-1}) were incubated with PenG. Cells of the extracellular (EC) solution were filtered off. The pellet was incubated in glycine buffer and intracellular (IC) PenG was released for its quantification in EC and IC fluid by microbiological methods using *Kocuria rhizophila* ATCC 9341 as control. Subsequently IC PenG activity was evaluated, for which it was added *S. aureus* (5×10^7 CFU ml^{-1}) to the association PMN/PenG, after phagocytosis, washed with phosphate buffer and centrifuged to remove EC bacteria. Cells were lysed and plated on agar for subsequent counting of colonies of *S. aureus*. The MIC at pH 7.4 was consistent with the CLSI (0.125 μg ml^{-1}). In contrast, the decrease in pH over the same range increased massively and almost linearly the PenG activity (~20 times). PenG showed a slow and poor IC uptake, the IC/EC ratio after 3 h of exposure was 0.42. However PenG showed activity as the CFU decreased IC 2 log after the contact time. This can be explained by their greater bactericidal activity against S. aureus at acidic pH.

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1.18.

Occurrence of cephalosporin resistant and ESBL-producing commensal *Escherichia coli* in the microbiota from swine treated with ceftiofur

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INTRODUCTION

The emergence of extended-spectrum-lactamases (ESBL)-producing *Enterobacteriaceae* has become a serious health problem in human and veterinary medicine. An association between antimicrobial consumption of ceftiofur and the appearance of *E. coli* with reduced susceptibility to third-generation cephalosporins has been reported. The present study aimed (i) to monitor a development or spread of resistance in commensal porcine *E. coli* after treatment with ceftiofur, (ii) the influence of treatment on untreated animals in neighbouring bays as well as (iii) the distribution of desfuroylceftiofur (DFC, first metabolite) in the surrounding.

MATERIALS AND METHODS

Twelve pigs were housed in two bays in the same stable. One group was treated intramuscularly with the recommended dosage for three days while the other one remained untreated. Physical contact between both groups was prevented by spatial separation. Four weeks later a second treatment followed. After seven weeks the initially untreated group got also therapeutic treatment to determine a possible influence of bacterial transfer from treated to untreated animals or environmental contamination on the microbiota. The therapeutic treatment aimed putting selective pressure onto the initially untreated intestinal flora.

Faecal samples were obtained to isolate commensal *E. coli* and to determine the particular minimal inhibitory concentration (MIC) by microdilution assay. Additionally a qualitative investigation of the susceptibility of *E. coli* was carried out by agardilution assay. Furthermore, a determination of the resistant *E. coli* by PCR, to detect Extended spectrum β-lactamases (ESBL)-encoding genes, followed.

In another experiment the therapeutic treatment was administered to six pigs. Plasma, urine and sedimentation dust were analyzed for DFC by UPLC MS/MS.

RESULTS

Within three weeks epidemiological resistant *E. coli* (MIC > 1 mg 1^{-1}) was determined in treated and untreated

animals. ESBL-producing *E. coli* were identified, its ESBL-encoding genes belonged to the plasmid located CTX-M family.
The metabolite DFC was found in all samples.

CONCLUSIONS
The administration of ceftiofur selects for ESBL-producing *E. coli* in treated and untreated animals presumably based on horizontal gene transfer. It has to be investigated whether the occurrence of ESBL-*E. coli* in untreated animals is based on bacterial transfer between the groups or on selection pressure exerted by oral intake of leftovers of DFC.

1.19.
Intracellular activity and postantibiotic effect of danofloxacin against *Staphylococcus aureus*
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Antibiotic treatment of *S. aureus* infections is often problematic due to the slow response to therapy and the high frequency of infection recurrence. The intracellular (IC) persistence of staphylococci has been recognized and could offer a good explanation for these treatment difficulties. Fluoroquinolones generally have good IC uptake with an intracellular /extracellular (IC/EC) ratio 5–10[1]. *S. aureus* is capable of surviving in alveolar cells, neutrophils and macrophages of bovine mammary gland for long time. Our objectives were: (i) evaluate the penetration and IC activity of danofloxacin (DAF) in polymorphonuclears (PMN) of dairy cows, (ii) investigate its *in vivo* PAE against *S. aureus* –using thigh infection model in neutropenic mice-. *S. aureus* strains isolated from clinical mastitis cows and *S. aureus* ATCC 25923 as control were used. MIC was determined by microdilution method at pH 7.4, 6.5 and 5.0, to emulate the acidic conditions of subcellular structures. Blood samples were collected from healthy lactating cows and were processed immediately to obtain the PMN samples by density gradient centrifugation method. Trypan blue method was used to estimate the cell isolation yields in the PMN suspensions. DAF mesylate was used at $10 \times$ MIC. PMN (5×10^6 ml^{-1}) were incubated with DAF. Cells of the EC solution were filtered off. The pellet was incubated in glycine buffer and IC DAF was released for its quantification in EC and IC fluid by HPLC with fluorescence detection after liquid/liquid extraction. Subsequently IC DAF activity was evaluated, for which it was added *S. aureus* (5×10^7 CFU ml^{-1}) to the association PMN/DAF, after phagocytosis, washed with phosphate buffer and centrifuged to remove EC bacteria. Cells were lysed and plated on agar for subsequent counting of colonies of *S. aureus*. For PAE evaluation, C57/h3 mice were rendered neutropenic by administration of cyclophosphamide at 150 and 100 mg kg^{-1} on days 0 and 3, respectively. After that, the experimental mice were inoculated into the thigh with *S. aureus* (10^6–10^7 CFU ml^{-1}) according Craig[2]. DAF exhibits good IC penetration. The IC/EC ratio obtained after 5 h was more than 10 times to that obtained after 2.5 h contact, indicating an IC accumulation of DAF in function of time. The IC CFU decreased 2 log after 5 h of contact, 99% of *S. aureus* IC died. DAF shows a moderate PAE against *S. aureus*. Its ability to attack the *S. aureus*, makes it a viable alternative for the treatment of intracellular infections.

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1.20.
Putative drug and vaccine target protein identification using comparative genomic metabolic pathways of *Salmonella enterica serovar* Typhimurium
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INTRODUCTION
The availability of genome sequences leads to rapid analysis for drug discovery. Comparative and reductive genomics using computational approaches would be very beneficial for searching for a new antimicrobial agents compared to conventional methods which are time consuming and labor-intensive. In the present study, a computational comparative and subtractive genomics/proteomics analysis aimed at the identification of putative therapeutic targets and vaccine candidate proteins from Kyoto Encyclopedia of Genes and Genomes (KEGG) annotated metabolic pathway of S. Typhimurium was performed for drug design and vaccine production pipelines.

MATERIALS AND METHODS
This study employs a comparative genomics and metabolic pathways analysis is followed by stepwise additional prioritizing parameters of drug and vaccine candidates with a predefined computational systemic workflow. The approach works by subtracting the genes or proteins homologous to both host and the pathogen and identify those set of gene or proteins which are essential for the pathogen and are exclusively present in the pathogen. For this systemic workflow, bioinformatics tools such as NCBI BLAST search engine, database of Essential Genes (DEG), subCELlular LOcalization predictive system (CELLO), Topcon, transmembrane helices (TMHMM) and Universal Protein Resource (Uniprot) were used in the present study.

RESULT AND CONCLUSION
A total of 5132 annotated metabolic pathways form the whole genome of *S.* Typhimurium SL1344 were extracted from KEGG. A total of 1411 proteins were identified to be involved in these metabolic pathways. From which 158 were found both non-homologous showing no similarity to the host and predicted essential at the same time. Among these, 329 non-homologous and essential proteins were identified and could serve as a potential drug targets and vaccine candidates. 19 proteins predicted subcellular localization and transmembrane characteristics were prioritized as vaccine candidate. Evalua-

tion of the druggability of each of the identified proteins by the DrugBank database is under study. Results from this study could facilitate selection of *S.* Typhimurium proteins for entry into drug design and vaccine production pipelines.

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1.21.

Mutant prevention concentration and genetic mechanism of resistnace to fluoroquinolone in clinical isolates and *in vitro*-derived mutants of *Salmonella enteria serovar* Typhimurium from pig

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INTRODUCTION

Salmonella enterica (*S. enterica*) is a ubiquitous pathogen that infects both animals and humans. Fluoroquinolones (FQs) have been used for the treatment against *Salmonella* infection but therapeutic failure currently emerges due to bacterial resistance. The resistance breakpoint ciprofloxacin is MIC 4 mg l^{-1} (the high-level FQ resistance) but remains rare among clinical *Salmonella* isolates worldwide while the decreased susceptibility (MICs 0.12–1 mg 1^{-1}) phenotype is now prevalent worldwide. Therefore, it is important to monitor and understand of the low-level FQ resistants to prevent poor treatment outcomes for infection. In this study, we investigated the resistance to FQs of *S.* Typhimurium isolates of pig origin by determination of the mutant prevention concentrations (MPCs) and underlying mechanisms of selected mutant.

METHODS AND MATERIALS

29 strains of *S.* Typhimurium provided from Gyeongsangbuk-do Veterinary Service Laboratory were used in this study. The MICs of marbofloxacin were determined in the presence/absence of efflux pump inhibitor (PAβN) by a broth microdilution method (CLSI 2013 guidelines). The MPC values were determined for 15 isolates and ATCC14028 using agar plates containing drug (1–16 × MIC) for 72 h incubation. In addition, single-step mutants were selected from plate containing the highest concentration below MPC. Amino acid substitutions in the quinolone resistance determining regions (QRDRs) were further analyzed.

RESULTS AND CONCLUSION

The MICs of marbofloxacin for *S.* Typhimurium isolates ranged from 0.03 to 1 mg l^{-1} (MIC_{50} 0.25 mg l^{-1} and MIC_{90} 1 mg l^{-1}). For 16 isolates, the MPCs of marbofloxacin were ranged 0.13–5 mg l^{-1}, showing the range of 2.5–8 of MPC/MIC ratios. Higher MPC values (5 mg l^{-1}) were observed only in two isolates. The MIC change between parent and one-step mutants was shown in the range from 1 to 16-folds. The single mutations in only *gyrA* (D87Y, H or S83F) were exhibited in 9 parental isolates and 12 one-step mutants. Interestingly, one one-step mutant showed the double mutation in *gyrA* (D87H and S83F), while amino acid alterations in 3 one-step mutants was not found. MICs values were decreased (1–9 × MIC) after treatment with PAβN indicating resistance involved in an efflux pump. Given the possibility of development of FQs resistance, continuous monitoring of the emergence of resistant isolates and responsiveness of animals to FQs treatment would be required.

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1.22.

Fosfomycin residues in colostrum: Impact on morpho-physiology of suckling piglets

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INTRODUCTION/OBJECTIVE

Ingestion of colostrum containing antimicrobial residues can alter the proper development of piglet's intestine, causing morpho-physiological changes which would negatively impact on its future productive life. Irrational use of antibiotics can bring about imbalance on microbiota diversity causing diarrhea and even death. The aim of this study was to determine the effects of fosfomycin residues found in colostrum on intestinal morpho-physiology and microbiota of suckling piglets.

MATERIALS AND METHODS

Farrow was induced in 18 sows at 114 days of gestation. 9 received 15 mg kg^{-1} BW disodium fosfomycin (Fosbac®, Bedson S.A., Argentina) via IM; and 9 were used as control. Piglets were monitored during the first 24 h of life at maternity room (PPS, Pro Surveillence System®). Colostrum production and intake were calculated using the equation developed by Devillers *et all.* (2007). 8 piglets were selected at random from treated sows and divided into 2 groups: A: euthanasia was done after 12 h of lactation and B: euthanasia was done after 24 h of lactation. Likewise 8 piglets were selected from control sows and divided into groups C and D where euthanasia took place at 12 and 24 h respectively. Intestine samples were collected to determine bacteriology (CFU *Lactobacillus* and *Enterobacteria*) and histology (absorption surface area). For statistical analysis software PROC MIXED and GLM del SAS V9.3 was used.

RESULTS
Colostrum/milk production by the sows and its intake by the litter were 2921 and 294.2 mL accordingly. Fosfomycin average ingestion per piglet was 0.27 mg kg^{-1} BW. No significant interactions between *Enterobacteria* were observed for the different groups (($P > 0.05$). Bacterial count for *Lactobacillus* was greater at 24 h than at 12 h (7.55 ± 0.19 y 6.64 ± 0.3 respectively). No significant interactions between groups were detected by histological studies ($p > 0.05$). Measured absorption surface areas were between 10.30 and 6.30 μm^2 in all groups.

DISCUSSION AND CONCLUSIONS
Results show that ingestion of colostrum containing fosfomycin residues would not have an impact on intestinal microbiota balance of neonatal piglets. This can be explained by phisicochemical properties of this antibiotic and its low distribution to mammary fluids. Therefore fosfomycin can be considered to be safe for treatment of gestating sows during farrowing and lactation.

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1.23.
Characterization of antimicrobial use in animal production: medicated feed in swine

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INTRODUCTION/OBJECTIVE
The rapid emergence of bacterial resistance becomes crucial for the reduction and, especially, the prudent use of antibiotics in human and veterinary medicine. However, data on the use of antibiotics in livestock production, which are not currently available in Portugal, are needed in order to assess and confirm the appropriate practices in terms of animal husbandry and treatment of animals.
This study characterizes qualitatively and quantitatively the use of antibiotics through medicated feed produced in Portugal, which consists on 70% of total the antibiotic consumption at national livestock level.

MATERIALS AND METHODS
To meet the objectives three surveys had been send to medicated feed authorized manufacturers, either industrially or self-production mode, and to pig farms of the Lisbon and Tagus Valley region (LVT). The surveys were addressed to the veterinarians responsible for each establishment.
R® program was used to perform simple descriptive analysis and for inferential statistical analysis toestablished cause-effect relationships between variables.

RESULTS AND CONCLUSIONS
In 2012, Portugal produced 395 102 tonnes of medicated feed, with a total of 64.9 tonnes of antibiotic incorporated. The most commonly used classes of antibiotics were tetracycline (22.3 tonnes), followed by macrolides (9.5 tonnes) and β-lactams (8.0 tonnes).
Pig production is the sector that consumes more medicated feed in a total of 314 528 tones, followed by poultry, rabbits and cattle farming. In 2012, the pig industry, essentially used tetracyclines (10.5 tonnes), macrolides (5.7 tonnes) and pleuromutilins (3.8 tonnes). The comsuption of these substances in medicated feed was more important in rearing and fattening phases with 7010 and 8723 kg, respectively.
Pig farming follows the reduction of antibiotic use because in 2012 the amount of antibiotics in medicated feed by the average number of animals was 155 kg against 173 kg in 2010, representing a decrease of 10.4% in the use of these substances.

KEY-WORDS
medicated feed, antibiotics, pig production, prudent use, reducing.

1.24.†
ABSTRACT DELETED

1.25.

Oral fluconazole therapy (10 mg kg^{-1}) with and without amphotericin B in koalas with subclinical and symptomatic cryptococcosis

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INTRODUCTION/OBJECTIVE

The *Cryptococcus neoformans* species complex is responsible for a continuum of manifestations ranging from colonisation, subclinical infection to life-threatening respiratory and/or neurological disease in a variety of species including koalas. *Cryptococcus gattii* is responsible of a spectrum of disease in koalas ranging from transient colonisation to widely disseminated disease, often with central nervous system involvement.[1]

MATERIALS AND METHODS

Fluconazole (Diflucan; Pfizer, West Ryde, Australia) was administered (10 mg kg^{-1} orally b.i.d. in artificial milk) to three subclinical or asymptomatic koalas serologically positive for cryptococcosis and two symptomatic koalas. An additional symptomatic koala was treated concurrently using fluconazole and amphotericin B deoxycholate (0.7–0.8 mg kg^{-1} in 350 ml 0.45% NaCl and 2.5% dextrose [warmed to 37°C]) as a subcutaneous bolus infusion twice weekly.[2] Serial bloods were collected over eight hours after the first dose. Plasma fluconazole concentrations were determined by high performance liquid chromatography.[3]

RESULTS AND CONCLUSIONS

The median plasma $AUC_{0-8\ h}$ and C_{max} for fluconazole (10 mg kg^{-1} orally) were 4.9 μg ml^{-1} h and 0.9 μg ml^{-1} in asymptomatic; and 17.3 μg ml^{-1} h and 3.2 μg ml^{-1} in symptomatic koalas. During on-going therapy with amphotericin B, the fluconazole $AUC_{0-8\ h}$ and C_{max} were 25.8 μg ml^{-1} h and 3.7 μg ml^{-1}. For all groups, the C_{max} never reached 16 μg ml^{-1} – the *in-vitro* MIC_{90} for *C. gattii* viz. (http://www.mycology.adelaide.edu.au/Fungal_Descriptions/Yeasts/ Cryptococcus /C_gattii). This fluconazole dose rate ± amphotericin was inadequate for effective therapy. Based on this data and the results of sporadic therapeutic monitoring of numerous clinical cases subsequent to the present study, our current recommendation for therapy involves (i) dosing with fluconazole (20–25 mg kg^{-1} orally b.i.d.) in conjunction with amphotericin B (0.7–0.8 mg kg^{-1} in 350 ml 0.45% NaCl and 2.5% dextrose via a subcutaneous bolus twice weekly) until the antigen titre becomes negative; (ii) monitoring of trough plasma fluconazole concentrations during therapy (iii) culturing each clinical isolate and undertaking broth microdilution antifungal susceptibility, as some strains of *C. gattii* (especially VGII, VGIII and VGIV) have shown intrinsic resistance to fluconazole *in vitro* and likely *in vivo.*

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Session 2: Pharmacokinetics

2.1.
Pharmacokinetic profile of enrofloxacin and the metabolite ciprofloxacin after intracoelomic administration in tortoises (Testudo hermanni)

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INTRODUCTION

Enrofloxacin (E) belongs to the fluoroquinolone class of antibiotics. It is commonly used in a variety of reptile species due to its wide spectrum of efficacy, partly due to its formation of an active metabolite ciprofloxacin (C). The pharmacokinetics of E following various routes of administration have been investigated in different species of turtles and tortoises with plasma concentrations of E and C showing a wide disposition variability [1, 2]. This underlines the importance of conducting pharmacokinetic studies for individual animal species. Several PK/PD indices such us C_{max}/MIC and AUC_{0-24}/MIC have been included in the present study to evaluate the clinical efficacy of E.

The aim of this study was to evaluate the pharmacokinetics of E and C after a single intracoelomic injection of 10 mg kg^{-1} of E in 9 tortoises (*Testudo hermanni*).

MATERIAL AND METHODS

The study protocol was approved by the University of Pisa's ethics committee for animal welfare and transmitted to the Italian Ministry of Health. E as the commercial injectable solution (Baytril® 25 mg ml^{-1}, Bayer, Milan Italy), was diluted with saline to 10 mg ml^{-1} and given as a 10 mg kg^{-1} bolus by intracoelomic injection in the left prefemoral fossa.

RESULTS AND CONCLUSION

No adverse effects at the point of injection and no behavioural or health alterations were observed in the animals during or after the study. Blood samples were collected at scheduled times and analyzed using a validated HPLC method. Plasma concentration of E was quantifiable in all subjects up to 240 h, while C was detected in all subjects up to 120 h. The C_{max}(s) of E and C were 8614 ± 1116 ng ml^{-1} obtained at 2.19 h and 605 ± 43 ng ml^{-1} obtained at 4.23 h, respectively. In the present study considering a *bacterium* with a MIC value of 0.5 μg ml^{-1}, the C_{max}/MIC ratio of E was 17.23 and the average AUC_{0-24}/MIC ratio was higher (132.78) than the requested therapeutic value. In contrast, C_{max}/MIC ratio and AUC_{0-24}/MIC ratio of C were below the target ranges. These results could be due to the limited extent to which C is produced in reptiles (<15%). It has been postulated that this minimal presence of C could be due to the slow metabolism of turtles and tortoises. In fact, cytochrome P450 3A, the enzyme that metabolizes E to C, has been found to be poorly expressed in reptiles [3].

In conclusion, administration of 10 mg kg^{-1} of E via the intracoelomic route in *Hermann's* tortoises appeared safe and produced optimal pharmacodynamic parameters.

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2.2.
Flupirtine: intravenous and oral pharmacokinetics in the donkey

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INTRODUCTION

Flupirtine (FLU) is a non-opioid analgesic drug with no antipyretic or antiphlogistic effects labelled for humans. It does not induce the side effects associated with the classical drugs used as pain relievers (NSAIDs and opioids). The aim of this study was to evaluate the pharmacokinetic profiles of FLU after IV and PO administration in healthy donkeys.

MATERIALS AND METHODS

Six Amiata breed adult jennies were randomly assigned to two treatment groups using an open, 2 × 2 Latin-square crossover study design. Group 1 (n = 3) received a single dose of 1 mg kg^{-1} of FLU injected IV (Katadolon® 100 mg 3 ml^{-1} vial, FLU D-gluconate) into the jugular vein. Group 2 (n = 3) received FLU (5 mg kg^{-1}) via nasogastric tube (Efiret® 100 mg hard capsules, FLU maleate). The wash out period was 1-week. Blood samples (5 ml) were collected at 0.083, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 10, 24, 36 and 48 h and plasma was then analysed by a validated HPLC method. Drug plasma concentration versus time curves were modeled for each subject using a two-compartment open model.

RESULTS AND CONCLUSIONS

No behavioral changes or alterations in health parameters were observed in the IV and PO groups of animals during or after (up to 7 days) the drug administration. Physiological signs and parameters were normal. After IV and PO administrations, FLU was detectable in plasma for up to 24 h. The mean elimination half-life was longer after PO (10.81 h) than after IV (0.90 h) administration. The T_{max} found in this study (0.33 h) was shorter than the T_{max} reported for dogs (1.42 h) (1), humans (range 1.6–1.8 h) (2), and cats (2.78 h) (3) showing a faster rate of absorption of the drug in donkeys. A number of factors may be responsible for this difference: the large variation in this parameter in the donkey, different absorption, gastric emptying, transit time or other species-specific factors. The clearance was 4812.8 ml h kg^{-1} and the AUC was small, findings consistent with a low oral bioavailability of about 20%. The pharmacokinetic trend of FLU in donkeys was different from those earlier reported in cats and dogs where the oral bioavailability was 40%. Surprisingly, the oral bioavailability of FLU in donkeys was much smaller than that reported in horses (70%) (M. Giorgi personal communication). This might be triggered by the faster clearance value in donkeys compared to horses (411 ml h kg^{-1}) rather than a poor drug absorption (C_{max} 937 versus 1639 ng ml^{-1}). Further studies are needed to understand if this active ingredient may be used in donkeys.

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2.3.

Pharmacokinetics of meloxicam in turtles (*Trachemys scripta scripta* spp) after single oral, intracoelomic and intramuscular administrations

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OBJECTIVE

To obtain pharmacokinetic information after single intramuscular, intracoelomic and oral administration of meloxicam in turtles (*Trachemys scripta scripta* spp).

METHODS

Eighteen turtles equally divided in three groups were treated with a single dose of meloxicam (0.2 mg kg^{-1}) given via intramuscular, intracoelomic and oral administration, respectively. Blood samples were collected at predetermined time points (before administration and at 0.5, 2, 4, 8, 12, 24, 48, 72, 96 and 120 h post administration) from the subcarapacial vein, and plasma meloxicam concentrations were determined by HPLC (Chinnadurai *et al.*, 2014). Pharmacokinetic parameters were calculated from the resultant concentration-time curves.

RESULTS

In all subjects in all treated groups, meloxicam appeared in the bloodstream at the first time point (30 min). It was detectable up to 24 h post treatment in all subjects after intracoelomic treatment and in 5 out of 6 turtles following intramuscular and oral administration. Forty-eight hours post-administration, meloxicam was still detectable in 4 out of 6, 3 out of 6 and 1 out of 6 turtles after intracoelomic, intramuscular and oral administration, respectively. At the sampling time on the third-day (72 h), the drug was only detectable in 1 subject treated via the intracoelomic route. Following intramuscular administration, the C_{max} was reached at 1.17 ± 0.45 (mean ± SD) hours indicating a faster absorption of meloxicam with respect to oral treatment (T_{max} 5.23 ± 3.80 h, $P = 0.004$) and the intracoelomic route (T_{max} 2.82 ± 1.39 h), although this last difference was not statistically significant. The intramuscular group accounted for the highest plasma peak of meloxicam (1590.03 ± 1845.32 ng ml^{-1}), the intracoelomic group for the largest AUC (12621.04 ± 6203.79 h*ng ml^{-1}). The oral group had the smallest drug plasma concentrations, meloxicam concentrations were always below 100 ng ml^{-1}, indicating a poor absorption through this administration route.

CONCLUSIONS

From the data obtained, oral administration of meloxicam seems unsuitable for turtles (*Trachemys scripta scripta* spp), due to the very low drug concentrations in the blood. Conversely, the intramuscular and intracoelomic routes lead to higher blood concentrations of the drug. Further studies are warranted to establish the effective plasma concentration of meloxicam in turtles, and, consequently, the most suitable route of administration and the dosage regimen.

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2.4.

Validation of models describing the relation between hemodynamics and pharmacokinetics of metronidazole in turkeys

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INTRODUCTION

In our previous pharmacokinetic (PK) studies on turkeys weighing 1.4, 2.7, 5.5, and 10.7 kg administered intravenously with metronidazole (MTZ) at the dose of 25 mg kg^{-1} we observed

significant changes in the majority of pharmacokinetic parameters, especially mean residence time (MRT) and body clearance (Cl_B). These correlations were significant for both parent drug and its major metabolite (hydroxymetronidazole, MTZ-OH) (1). A parallel study of hemodynamics (HD) performed on turkeys belonging to similar body weight (BW) groups provided with the knowledge on age dependent changes in the heart rate (HR), stroke volume (SV) and cardiac output (CO). The aim of the present work was to investigate the correlations between the PK parameters (of the parent drug and its metabolite), and HD parameters together with the BW changes in the animals. The resulting models were subjected to validation.

MATERIALS AND METHODS

PK calculations were performed with the use of Phoenix™ WinNonlin® 6.3. Hemodynamic study was carried out by means of echocardiography (Aloka α -7). Following relations were subjected to validation – HD:BW; HD:PK_{MTZ} and HD:$PK_{MTZ\text{-}OH}$. The 'leave one out' method was used for model cross validation. Squared cross-validated correlation coefficient (Q^2), coefficient of determination (R^2), total sum of squares (SS) and predicted residual sums of squares were analyzed (PRESS). Results: It was found that the HD:BW relation is best described by the following equations: $1/SV = BW^{-0.7}$; $1/CO = BW^{-0.47}$, and $CO^{1/SV} = BW^{-0.01}$. All of them met the validation criteria. The ratio $Cl_{MTZ}/MRT_{MTZ\text{-}OH}$ correlated well with the HD parameters and BW ($R^2 \geq 0.99$, Q^2-$R^2 < 0.01$, SS < 5, PRESS < 0.01). The relation between PK and HD was described by following equations: $Cl_{MTZ} = 0.0013 \times HR\text{-}0.2035$; $Cl_{MTZ}/MRT_{MTZ\text{-}OH} = 0.0078 \times [\sqrt{HR}+Log(CO+SV)] - 0.1164$ and $Cl_{MTZ}/MRT_{MTZ\text{-}OH} = 0.0002 \times (HR+\sqrt{BW}) - 0.0369$. It was also found that the derivative $[1/MRT_{MTZ}]/HR$ is independent of the BW.

CONCLUSIONS

It was shown that in turkeys the relations between HD, BW and PK parameters for both MTZ and MTZ-OH are linear and they meet validation criteria. It was proven that the ratio $Cl_{MTZ}/MRT_{MTZ\text{-}OH}$ is the derivative that binds the PK processes of MTZ and its metabolite with the HD parameters in the experimental turkeys. It was also found that some PK parameters tend to become constant when normalized by the value of HR (RSD% < 10%), independently of the BW.

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2.5.

Use of physiologically based pharmacokinetic (PBPK) models to support canine drug product development

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OBJECTIVE

To demonstrate the use of PBPK models to support canine therapeutic drug product development.

METHODS

Using danazol (DNZ, high permeability & low solubility, BCS II) as an example, the Simcyp canine PBPK module (Simcyp Dog Version 14) was used to predict intestinal segmental permeability and systemic clearance in a 10 kg beagle dog. Using published information on danazol, we explored the relationship between drug solubility, intestinal segmental permeability and the *in vitro* dissolution characteristics necessary to achieve the targeted drug product profile. *In vivo* plasma concentration-time profile after IV administration to beagle dogs[1] was used to estimate systemic clearance using the parameter estimation module in Simcyp. The Berezhkovskiy corrected Poulin & Theil method, in combination with DNZ physico-chemical parameters, was used to predict the steady state volume of distribution (V_{ss}, l kg^{-1}). The effective intestinal permeability ($P_{eff} \times 10^{-4}$ cm s^{-1}) was predicted using the inbuilt 'Mech-Peff' model. Predicted (P) and observed (O) plasma concentration-time profiles (IV and Oral solution) were compared to support the validity of the model parameter estimates. The resulting model and *in vitro* parameters (e.g., intrinsic solubility and dissolution profiles) were used to predict plasma profiles for a conventional DNZ formulation and a DNZ nanosuspension.

RESULTS AND CONCLUSION

The predicted pharmacokinetic (PK) parameters were as follows: V_{ss} = 3.54 l kg^{-1}; systemic clearance = 158 ml min^{-1}; segmental intestinal permeability ($P_{eff} \times 10^{-4}$ cm s^{-1}): Duodenum = 0.635, Jejunum = 0.633; Ileum = 0.66–0.36, and Colon = 0.826. The P versus O plasma profiles after IV and Oral solution dosing were in good agreement [AUC_{0-t}; IV (μg h ml^{-1}) = 2.83 (O) and 3.36 (P)]; Oral Solution AUC_{0-t} (μg h ml^{-1}) = 20.4 (O) and 18.27 (P); C_{max} (μg ml^{-1}) = 3.94 (O) and 3.44 (P)]. Using similar model inputs, *in vivo* PK profiles were predicted for a conventional and nano- suspension formulation, and model robustness tested by comparing predictions with other published datasets. All P versus O PK parameters were within 2 fold margin of error. These results demonstrate that PBPK models can be used to predict *in vivo* product performance and showcase their utility as a cost-effective tool for integrating available information to support the development of safe and effective animal drug products.

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2.6.

Pharmacokinetics of Ketoprofen in Ruminant and Pre-Ruminant calves after oral administration

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Ketoprofen (KTP) is a NSAID drug widely used in cattle by oral, intravenous or intramuscular routes. However, no data about the influence of physiologic development on pharmacoki-

netics of KTP is available in calves. Therefore, we studied the pharmacokinetic behaviour of KTP racemic formulation to ruminant (RA) and pre-ruminant (PRA) animals, administered orally or intravenously.

Six Frisian calves 3–5 months old (149.3 kg) were used as RA group and six Frisian calves 4–8 weeks old (73.7 kg) as PRA group. A two period cross-over design, with a wash-out period of 5 days (RA) or 2 days (PRA) was used. In each period, animals were treated with a single (oral or IV) dose of 3 mg kg^{-1} b.w. of KTP (Dinalgen®/Danidol® Laboratorios Dr. Esteve S.A.) RA was treated by gastric tube and PRA mixed with the morning milk ration. Intravenous administration was performed in the jugular vein. Blood samples were collected and drug determined by a validated HPLC method (LOQ 0.025 μg ml^{-1}). Non-compartmental analysis, using the WinNonlin TM Professional 2.0 (Pharsight Scientific) software was conducted.

After IV administration, both groups showed bi-exponential pharmacokinetics with similar $t_{½}$. However, PRA exhibited higher plasma levels and significantly lower V_d (0.24 versus 0.43 l kg^{-1}) and Cl (0.068 versus 0.15 l h^{-1} kg^{-1}), as well as higher MRT (3.0 versus 1.7 h) and AUC_{0-t}. (44.6 versus 22.4 μg h ml^{-1}). After oral dosing, PRA also showed higher plasma levels (C_{max} 5.5 versus 3.7 μg ml^{-1}) with a longer absorption period (2–3 h) and a peak plasma concentration delayed (T_{max} 2.5 versus 1.2 h) compared to the RA.

The differences in the elimination pattern between PRA and RA can be attributed to immaturity of metabolic pathways and underdevelopment of the excretion processes [2]. The higher V_d in RA are explained by the presence of functional rumen. Higher C_{max} and longer T_{max} in PRA are related with anatomical (esophageal leak) and physiological (pH) differences, as well as type of feeding (milk replacer versus solid feed), which can modify the absorption process [1].

In this experiment, levels of KTP were well above those required to induce 50% of maximal effect in calves (0.00029–0.118 μg ml^{-1}) [3]. However, the much higher blood levels of KTP in PRA suggest that these young animals could benefit of a lower therapeutic dose, which would yield similar efficacy and could improve the safety margin thus minimizing any potential intolerance in suckling calves.

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2.7.

Preliminary kinetic data on tylosin after oral administration to rainbow trout [*Oncorhynchus mykiss* (Walbaum, 1792)]

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INTRODUCTION

Tylosin, an antibiotic of the macrolides group, is produced by fermentation from a strain of the soil microorganism, *Streptomyces fradie*. The compound is active against Gram-positive bacteria, mycoplasma and certain Gram-negative bacteria. Like other macrolide antibiotics, tylosin inhibits protein synthesis by inhibiting aminoacyl-tRNA and peptidyl-tRNA binding to the ribosomes (Hamill *et al*., 1961; Gaynor and Mankin, 2005). Tylosin consists of a mixture of the macrolides Tylosin A, Tylosin B, Tylosin C and Tylosin D. Tylosin A is by far the major component (usually about 90% and not less than 80%). The Regulation (EC) No 37/2010 (Annex I) listed the active substance tylosin to be used in all food producing species including fish with a maximum residue limit established. Because limited information is available on disposition of tylosin in fish, the present study reported here a preliminary kinetic study of tylosin after a single oral administration in rainbow trout (*Oncorhynchus mykiss*).

MATERIAL AND METHODS

Rainbow trout (*Oncorhynchus mykiss*) ranging in weight from 170 to 220 g were used. Fish which were treated at the single oral dose of 40 mg kg^{-1} bw received the feed mixed with tylosin at the concentration of 4 g kg^{-1} feed. Fish were kept in 2000 l tanks with an aerated continuous flow of water (O_2 concentration, 6.6 mg l^{-1}). Water temperature was 18°C. After treatment, the fish were anesthetized with 2-fenoxy-etanol (0.15 ml l^{-1} water) and heparinised blood samples were collected at several times (7 fish/time). Plasma samples were stored frozen at −45°C until analysed. Plasma concentrations of tylosin were measured by HPLC-MS system equipped with a column ACE C18-AR (100 × 2.1 mm I.D. 5 μm). The mobile phase consisted of a mixture of ammonium acetate 10 mM – 0.5% formic acid – methanol (35:10:55). Recovery rate was 90–95%. Mean plasma concentration versus time curve were analyzed using WinNolin program. The study was undertaken in accordance with the ethics requirements.

RESULTS AND CONCLUSIONS

After a single oral administration of tylosin (40 mg kg^{-1} bw) in rainbow trout the pharmacokinetic parameters obtained were $t½a$ 1.53 h, C_{max} 3.59 μg ml^{-1}, T_{max} 2.99 h, AUC 36.75 mg h l^{-1}, and distribution and elimination half-lives, $t½\alpha$ 2.02 h and $t½\beta$ 5.90 h, respectively. In rainbow trout, oral administration of tylosin (40 mg kg^{-1} bw) results in potential therapeutic plasma concentrations against Gram-positive bacterial pathogens such as *Streptococcus iniae* and *Lactococcus garvieae* as well as against Gram-negative bacteria such as *Yersinia ruckeri.*

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2.8.
Comparative pharmacokinetics and allometric scaling of carboplatin in different avian species

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INTRODUCTION
The empirical use of chemotherapeutics as a possible treatment strategy in avian oncology is steadily increasing over the last years. Despite, literature reports regarding dosing strategies and pharmacokinetic behaviour of chemotherapeutics in avian species are lacking. The aim of present study was to investigate the pharmacokinetic characteristics of carboplatin in a representative species of the order of *Galliformes, Anseriformes, Columbiformes* and *Psittaciformes*.

MATERIALS AND METHODS
Eight chickens, ducks and pigeons and twenty-eight parakeets were administered carboplatin intravenously (5 mg kg^{-1} BW). A specific and sensitive liquid chromatography-tandem mass spectrometry method was developed and validated for quantification of free carboplatin in plasma of the four species (limit of quantification: 50 ng ml^{-1} for chicken, duck and pigeon and 100 ng ml^{-1} for parakeets). Non-compartmental pharmacokinetic analysis was performed and allometric scaling was applied on $T_{1/2el}$, Vd and Cl based on the following power function:
$Y = a.W^b$ where Y is the value of the respective PK parameter, a is the coefficient of the intercept of the trend line, W is the body weight and b is the slope of the trend line.

RESULTS AND CONCLUSIONS
Non-compartmental pharmacokinetic analysis and allometric scaling demonstrated a significant correlation ($R^2 = 0.9769$) between body weight and elimination half-life ($T_{1/2el}$). $T_{1/2el}$ ranged from 0.41 h in parakeets (BW: 61 ± 8 g) to 1.16 h chickens (BW: 1909 ± 619 g). In contrast, the Cl and Vd displayed a low to moderate correlation, namely R^2 of 0.08 and 0.79, respectively. $T_{1/2el}$ seems to be a good parameter for dose optimization of carboplatin in other avian species, since also the previously reported $T_{1/2el}$ in cockatoos (average BW: 769 g) of 1.00 h (Fillipich *et al.*, 2004) corresponds to the results obtained in the present study.

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2.9.†
ABSTRACT DELETED

2.10.
Potential influence of ketoprofen on the ceftiofur kinetic profile

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INTRODUCTION/OBJECTIVE
A GLP study was carried out in order to assess the bioequivalence of ceftiofur, between two veterinary medicinal products, E (ceftiofur, 50 mg ml^{-1}, EXCENEL RTU, Zoetis) and C (ceftiofur 50 mg ml^{-1} + ketoprofen 10 mg ml^{-1}, CURACEF DUO, Virbac).

MATERIALS AND METHODS
16 Angus heifers were allocated at random to group E or C. Animals were then dosed on two occasions following a standard cross-over design with a 26 days wash-out period between doses. 1 mL per 50 kgBW of either E or C was delivered to animals at the side of the neck, via the SC route for medicine E or IM for C, according to product labelling. Injection sites were observed daily up to 96 h after each dosing occasion. Blood samples were regularly collected up to 96 h post-dosing from a jugular vein on a periodical basis from each animal. Plasma samples were analyzed for ceftiofur-derived concentrations as a single analyte (including ceftiofur and all desfuroylceftiofur-related metabolites) using a validated method that involved solid phase extraction followed by LC-MS/MS analysis. The limit of quantification was 50 ng ml^{-1}.
PK parameters C_{max}, AUC_{0-t} were calculated using a non-compartmental approach. Bioequivalence of the two formulations was defined as: the 90% confidence interval of the AUC_{0-t} and C_{max} ratios (C/E) being within the acceptance range of 0.80 to 1.25. For C_{max} a bioequivalence range of 0.70 to 1.43 was also deemed to be acceptable.

RESULTS
There were no signs of intolerance (local or systemic) following the administration of C while 7 of animals treated with E were noted to have swellings at the injection site. A sufficient number of samples was collected to adequately describe the plasma concentration-time profile. Mean concentrations versus time plots for the two formulations were largely superimposed between approximately 12 h and 96 h and mean elimination half-lives estimated from these data were comparable. Mean AUC_{0-t} and C_{max} estimates were higher after C than after E administration.

CONCLUSIONS
Assessment of bioequivalence with respect to AUC_{0-t} and C_{max} estimates concluded that the two combinations of formulations and routes of administration (IM for C and SC for E) could not be considered bioequivalent. These differences observed in the ceftiofur kinetic profiles can be either due to the route of administration or to the presence of ketoprofen.

2.11.
Impact of ceftiofur on the ketoprofen kinetic profile

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INTRODUCTION/OBJECTIVE
A GLP study was carried out in order to assess the influence of ceftiofur on the pharmacokinetics of ketoprofen by comparing the bioavailabilities and kinetic profiles obtained after IM administration of one veterinary medicinal product, C (CURACEF DUO®, ceftiofur 50 mg ml^{-1} + ketoprofen 150 mg ml^{-1}, Virbac), and an experimental formulation CWC (product C without ceftiofur). Intravenous administration of a veterinary medicinal product K (KETOFEN® 10%, ketoprofen, 100 mg ml^{-1}, Merial) was done for obtaining absolute bioavailability of ketoprofen in C and CWC products.

MATERIALS AND METHODS
18 young cattle of both genders were allocated into three groups (K, C and CWC) at random based on their body weight, allowing homogenous groups of treatment (3♀ and 3♂). Animals were then dosed once: 1.5 ml per 50 kg BW of product K, or 1.0 ml per 50 kg BW of either product C or CWC. Treatments were delivered via the IM route for C and CWC and via the IV route for K. Injection sites were observed after injection. Blood samples were regularly collected from a jugular vein for 12 h (IV) or 24 h (IM) from each animal. Plasma specimens were analyzed to determine ketoprofen concentrations using a validated HPLC/UV method, with a lower limit of quantification (LOQ) of 100 ng ml^{-1}.
Pharmacokinetic analysis was done using a non-compartmental model. With respect to the route of administration, various PK parameters were calculated, including absolute bioavailability (F) of ketoprofen in product C and CWC.

RESULTS
There were no signs of intolerance (local or systemic) following the administration of treatments but a small hematoma (one animal in group K) or slight bleeding (2 animals in group C), were observed at the injection site. The following C_{max}, t_{max}, AUC_{last} and apparent $t_{1/2}$ values (±SD) were obtained after IM administration of C and CWC, respectively: 5.55 (±1.58) versus 6.16 (±2.03) μg ml^{-1}, 2.12 (±1.25) versus 0.72 (±0.23) h, 27.17 (±4.68) versus 19.57 (±3.70) μg ml^{-1} h, 3.75 versus 2.42 h. Differences between AUC_{last} values are significant ($P < 0.01$). The absolute bioavailabilities of ketoprofen were $F = 99.6\%$ and $F = 72.3\%$ for C and CWC products respectively.

CONCLUSIONS

Results of this study suggest that ceftiofur could have an influence on the pharmacokinetics of ketoprofen after IM administration to cattle allowing a complete bioavailability in ketoprofen (99.6%) with the combination C. Indeed, AUC_{last} parameter was significantly increased in the presence of ceftiofur.

2.12.
Influence of the administration of omeprazole on the oral absorption of cephalexin: differences between adults and aged dogs

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INTRODUCTION

Cephalexin, a first generation cephalosporin is frequently used in dogs. Studies in humans have shown that co-administration of oral cephalexin and omeprazole, a proton pump inhibitor, delayed absorption of the antibiotic with negative consequences on antibiotic efficacy (Madaras-Kelly, 2004).

OBJECTIVE

The aim of this study was to evaluate the impact of previously administered omeprazole between adults and aged dogs on cephalexin oral pharmacokinetic.

MATERIALS AND METHODS

Ten dogs, five between 5–6 years old (group A), and five between 10–14 years old (group B) were used. The trial was divided into two stages (I and II). Stage I: A and B received a single dose of cephalexin tablets (25 mg kg^{-1}, oral). Stage II: both groups received omeprazole (1 mg kg^{-1}, oral) for 5 days and a single dose of cephalexin (25 mg kg^{-1}, oral) on day 5. After cephalexin administration, blood samples were taken at predetermined times. Cephalexin plasma concentrations were determined by a microbiological assay. Pharmacokinetic parameters were analysed using a computer program (Phoenix® WinNonlin® 6.3, Certara, LP).

RESULTS

Plasma concentrations were best described by a one-compartmental model. Main pharmacokinetic parameters (mean ± sd) obtained for Group A (stage I/stage II) were: $AUC_{(0-inf)}$ (mg/h*mL) 197.17 ± 48.48/171.14 ± 29.35; C_{max} (mg ml^{-1}) 38.49 ± 7.44/28.56 ± 5.90; $t_{½abs}$ (h) 0.70 ± 0.49/1.21 ± 0.62; $t_{½}$ (h) 2.14 ± 0.72/2.06 ± 0.62; T_{max} (h) 2.31 ± 0.57/2.68 ± 0.75. For Group B the results were: $AUC_{(0-inf)}$ (mg h^{-1}*ml^{-1}) 185.52 ± 37.52/187.65 ± 39.83; C_{max} (mg ml^{-1}) 28.66 ± 5.63/27.07 ± 5.08; $t_{½abs}$ 0.91 ± 0.36/0.84 ± 0.46; $t_{½}$ (h) 2.63 ± 0.76/3.08 ± 1.28; T_{max} (h) 2.37 ± 0.61/2.19 ± 0.58.

CONCLUSIONS

Significant differences in C_{max} of adult animals were found between stage I and II. In humans, significant differences were only observed in T_{max}. This could be explained by the reduced effectiveness of omeprazole in older patients where stomach pH is naturally higher than in younger ones. The T>MIC was not affected between stages or groups; so, the observed differences would not affect clinical efficacy of cephalexin in dogs.

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2.13.
Integrated assessment of ivermectin kinetics, metabolism and tissue residues in laying hens

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INTRODUCTION

Ivermectin (IVM) is reported to be effective against nematode parasites in poultry (Ibarra-Velarde *et al.* 2011), but is not approved for use in avian production. The extralabel use of this drug has been reported (Bennett and Cheng, 2012) mainly to control endo and ectoparasites. The available information on the pharmacokinetic behaviour of IVM in poultry is scarce. The aim of the current work was to investigate the IVM plasma disposition kinetics, liver microsomal metabolism and tissue and egg residues profiles following its administration to laying hens.

MATERIALS AND METHODS

One hundred *Plymouth Rock Barrada* laying hens were used in three experiments. Experiment 1: Eight animals were intravenously treated (0.4 mg kg^{-1}) with IVM. Blood samples were taken at different times. Experiment 2: Eighty-eight hens were treated with IVM administered daily in water (0.4 mg kg^{-1}, for 5 days). Forty hens were kept and their daily produced eggs collected until 20 days post-treatment. Forty-eight hens were sacrificed in groups of eight at different times (1, 3, 5, 7, 10, and 15 days post-treatment) and blood, muscle, liver, kidney, and skin+fat samples taken. Samples were frozen until analysis by HPLC. Experiment 3: The *in vitro* enzymatic biotransformation of IVM was studied in liver microsomes obtained from not treated hens (n = 4).

RESULTS

After its IV administration, IVM plasma concentration decreased from 739.6 to 0.38 ng ml^{-1} (10 days). Pharmacokinetic parameters were: AUC 85.1 ng·day ml^{-1}; Vdss 4.43 L kg^{-1}; Cl 4.8 l day^{-1} kg^{-1}; $T_{½el}$ 1.73 days; MRT 0.95 days. Low IVM tissue residues were quantified after its oral administration in water. The highest IVM concentration was measured in liver tissue, followed by skin+fat, kidney, plasma and muscle. Although IVM residues were not found in egg white, significant residues were quantified in yolk. After 30 min of microsomes incubation, IVM concentrations decreased 27.8 ± 4.4%.

CONCLUSIONS

IVM pharmacokinetic behaviour in laying hens is characterized by larger apparent volume of distribution and higher plasma clearance compared to mammalian species. IVM half-life was shorter and plasma exposure lower than in other species, probably associated to a higher excretion/metabolism. IVM tissue residues were below the MRLs established for mammalian species. Residues quantified in eggs were greater than some MRLs values, suggesting that a withdrawal period would be necessary for eggs after IVM oral administration in laying hens.

KEY-WORDS

ivermectin, pharmacokinetics, *in vitro* metabolism, residues, laying hens

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2.14.

Pharmacokinetic and optimal dosage of marbofloxacin in Hanwoo, Korean native cattle

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INTRODUCTION/OBJECTIVE

Marbofloxacin (MRFX) is a fluoroquinolone that exhibits concentration-dependent bactericidal activity against gram-positive and gram-negative bacteria (Aliabadi *et al*, 2002). Owing to this broad spectrum of bactericidal activity, MRFX has been indicated in the treatment of bacterial infections in animals (Thomas *et al*, 2001). The pharmacokinetics of MRFX has been investigated in different animal species. However, there are no reports that describe pharmacokinetics of MRFX in Hanwoo, Korean native cattle. Hence, investigating MRFX pharmacokinetics in Hanwoo is important to establish optimal dosage for treatment of bacterial infection. Therefore, the study aimed to characterize the pharmacokinetics of MRFX and to determine the optimal dosage on the basis of PK/PD parameters against susceptible and intermediate pathogenic bacteria.

MATERIALS AND METHODS

Six male Hanwoo weighing 300 ± 10 kg were carried out in a two-period crossover manner with animals randomly divided into two groups of three Hanwoo. In two phases, 2 mg kg^{-1} body weight intravenous and intramuscular dose (2 mg kg^{-1}) of marbofloxacin was interchangeably administered for each animal. Blood samples were collected before and at 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 12 and 24 h after i.v. and i.m. administration and then centrifuged at 2000 × g for 15 min. Serum concentrations of MRFX were assayed using Agilent *1100* series HPLC *system* comprising 4.6 × 250 mm, 5 μm column. The limit of detection and quantification were 0.012 and 0.062 μg ml^{-1}, respectively. Pharmacokinetics analysis was performed using Phoenix WinNonlin 6.0 (Pharsight Corp., St. Louis, MO.) software program.

RESULTS

After i.v. administration, the AUC, $t_{1/2}$ and CL were 6.87 h μg ml^{-1}, 2.44 h and 0.29 L kg^{-1} h^{-1}. After i.m. administration, the AUC, $t_{1/2}$ and CL were 5.07 h μg ml^{-1}, 2.44 h and 0.39 L kg^{-1} h^{-1}. The optimum dosage for i.v. and i.m. administration required to achieve target $AUC_{0-24\ h}$/MIC ratio of 125 h against susceptible (MIC ≤ 1 μg ml^{-1}) and intermediate (MIC ≤ 2 μg ml^{-1}) pathogenic bacteria in the present study indicates that the administered dose (2 mg kg^{-1} day^{-1}) is inadequate to achieve target end point associated with efficacy of fluoroquinolones. Due to this, 2.9 and 5.8 mg kg^{-1} day^{-1} (i.v.) and 3.9 and 7.8 mg kg^{-1} day^{-1} (i.m) doses are suggested to achieve target PK/PD indices ($AUC_{0-24\ h}$/MIC = 125 h) against susceptible (MIC ≤ 1 μg ml^{-1}) and intermediate (MIC ≤ 2 μg ml^{-1}) pathogenic bacteria, respectively.

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2.15.

Pharmacokinetic parameters of amoxicillin against Streptococcus spp. in olive flounder (*Paralichthys olivaceus*)

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INTRODUCTION

The olive flounder (*Paralichthys olivaceus)* is the most common flatfish species raised in aquaculture in East Asia, including Korea, Japan and China. Amoxicillin (AMX) is a beta-lactam antibiotics largely used in veterinary medicine for its broad spectrum, and has been reported to provide good results in the specific field of fish antimicrobial therapy. *Streptococcus iniae (S. iniae)* and *Streptococcus parauberis (S. parauberis) h*ave been reported as major causes of economic damages on the fish farming. There is rarely reported pharmacokinetics (PK) of amoxicillin after intramuscular (IM) administration in olive flounder. Therefore, the present study was carried out to obtain amoxicillin PK parameters and minimal inhibitory concentration (MIC) values against *S. iniae and S. parauberis* in olive flounder and then recommended optimal dosage of AMX.

MATERIALS AND METHODS

AMX was injected to flounder with the accurate dose of 12.5 mg kg^{-1} and 125 mg kg^{-1} via IM administration in order to calculate PK parameters in healthy olive flounder. The bodyweight of the fish and water temperature were 150 ± 10.4 g (100 ± 10d) and 23 ± 1°C. The blood samples

were collected at 0, 1, 2, 4, 8, 12, 24, 48 and 168 h after IM treatment. The concentration analysis of AMX in plasma was done by HPLC (HP1100 Series). The calculated AMX concentration in plasma was simulated by Winnonlin program (Pharsight Co., Inc., USA) to obtain PK parameters. We also are studying on MIC against *S. iniae* and *S. parauberis* were measured according to Clinical Laboratory Standards Institute (CLSI, 2007) Guidelines.

RESULTS AND CONCLUSION

Two-compartment model was observed with the low dose (AMX 12.5 mg kg^{-1}) after a single IM administration. However, zero-order kinetics was observed for the high dose (AMX 125 mg kg^{-1}) by 12 h, and then changed to first-order kinetics. The concentration of AMX in plasma was between 1 $\mu g\ ml^{-1}$ and 2 $\mu g\ ml^{-1}$ by 48 h after IM administration at low dose. And the average elimination half-life ($T_{1/2el}$) was calculated by 23 h and the peak plasma concentration (C_{max}) was 17.8 $\mu g\ ml^{-1}$, respectively. According to the results, MIC ranged from 0.007 to 0.062 $\mu g\ ml^{-1}$ for *S. iniae* and 0.0015 to 0.25 $\mu g\ ml^{-1}$ for *S. parauberis*. AMX concentration in plasma was maintained more than MIC level against *S. iniae* and *S. parauberis* after IM administration. Taken together, we can suggest that that IM administration of AMX 12.5 mg kg^{-1} was useful for bacterial infections in flounder.

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2.16.

Pharmacokinetics of tramadol and its metabolite M1 following intravenous administration of tramadol at two dosing rate in sheep undergoing spinal surgery

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OBJECTIVE

To assess the pharmacokinetics of tramadol (T) and its metabolite O-desmethyltramadol (M1) after IV administration of T in sheep undergoing experimental lumbar spine surgery.

METHODS

Twelve Brogna breed, approximately 3-year-old, female sheep were equally/randomly divided into two groups. Once the target level of general anaesthesia was achieved, 4 or 6 mg kg^{-1} T, were IV administered. Blood samples were collected at scheduled times (0, 5, 15, 30, 45, 60, 90, 120, 240 and 300 min). T and M1 quantification in plasma was carried out by a HPLC validated method. The pharmacokinetic analysis was performed by WinNonlin 5.3.

RESULTS

Pharmacokinetic parameters of T and M1 were determined by a bi-compartmental and non-compartmental analysis, respectively. The plasma concentrations of T after administration of both doses dropped down rapidly. T was detectable in all the sheep up to 2 h from the drug administration. After administration of 4 and 6 mg kg^{-1} of T, the main parameters of the parental drug were: $T_{1/2elim}$ 0.99 ± 0.46 and 0.68 ± 0.20 h; Cl 2.49 ± 0.28 and 3.24 ± 0.39 l h^{-1} kg^{-1}; Vd 772.72 ± 149.47 ml kg^{-1} and 734.36 ± 265.53 ml kg^{-1}, respectively. M1was found in all the animals but its concentrations were very low. The C_{max} was 0.09 ± 0.04 and 0.10 ± 0.10 $\mu g\ ml^{-1}$ achieved at T_{max} of 0.98 ± 0.50 and 0.58 ± 0.71 h after administration low and high dose of T, respectively.

DISCUSSION AND CONCLUSIONS

After the administration of the two doses of T, the concentration versus time curves of T and M1, were similar. An earlier study (Bortolami *et al.*, submitted) where non-anesthetized sheep received T at 4 and 6 mg kg^{-1} showed lower concentrations of the parental drug than those reported in the present study. The diverse value of clearance (smaller in the present study) seemed to trigger this difference. It might be due to the blood flow modification that occurs during anesthesia. This is in line with the findings reported in a former pharmacokinetic study in anesthetized/non-anesthetized dogs (Buhari *et al.*, 2013).

In the present study, the $AUC_{M1/T}$ ratio after the low dose was similar to that obtained with high dose, suggesting that T metabolism is not dissimilar (saturated) at 4 and 6 mg kg^{-1}.

Further studies are warranted to establish the efficacious blood concentration of T and M1 in sheep.

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2.17.
Pharmacokinetics of the selective COX-2 inhibitors celecoxib and mavacoxib in cockatiels

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INTRODUCTION

Celecoxib (CELE) and mavacoxib (MAVA) are structurally related selective cyclooxygenase-2 inhibitors registered for the treatment of osteoarthritis in humans and dogs, respectively[1]. Besides, CELE is frequently used off-label in the treatment of proventricular dilatation disease (PDD) in parrots. PDD is a fatal neurologic disease that affects the enteric nervous system. The inflammatory characteristics of the neurologic lesions suggest a beneficial effect of the use of nonsteroidal anti-inflammatory drugs[2]. A clinical improvement of birds with clinically diagnosed PDD after oral treatment with CELE was demonstrated[3]. Despite its frequent usage in clinical setting, no pharmacokinetic (PK) data of CELE are available for birds. Furthermore, MAVA is known to be a long acting NSAID, however, its PK behaviour in birds is unknown as well as its therapeutic effect on PDD. It might offer the advantage of less frequent dosing compared to CELE. Therefore, the objective of this research was to study the comparative PK of CELE and MAVA in cockatiels (*Nymphicus hollandicus*).

MATERIALS AND METHODS

In a first study, cockatiels were administered either CELE ($n = 34$) or MAVA ($n = 40$) analytical standard (Clearsynth, Mumbai, India) in a vehicle containing PEG400: physiological saline (75:25 v/v), both orally (PO) and intravenously (IV) in a two-way cross-over design. Additionally, the PK of a commercially available formulation of both drugs (Celebrex®, Pfizer and Trocoxil®, Zoetis) was evaluated after PO administration to 22 and 26 birds, respectively. CELE and MAVA were administered at a dose of 10 mg kg^{-1} bodyweight (BW) and 4 mg kg^{-1} BW, respectively. A sparse sampling PK strategy was used, namely a maximum of three sampling points for each bird. Plasma samples were analysed using an in-house developed and validated high-performance liquid chromatography-tandem mass spectrometry method. PK parameters were calculated using WinNonlin 6.3 (Pharsight, IBM, USA).

RESULTS AND CONCLUSIONS

Preliminary results show a high absolute oral bioavailability of CELE and MAVA in cockatiels, 100% or 67–86%, in birds administered analytical standard or the commercial formulation, respectively. Clearance (Cl) of MAVA was 80 times lower compared to CELE, and the volume of distribution (Vd) of MAVA was 1.4–2.7 times higher compared to CELE. This results in a 110–218 times longer elimination half-life ($T_{1/2el}$) of MAVA compared to CELE. These PK data suggest less frequent dosing of MAVA compared to CELE in cockatiels.

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2.18.
Analysis of penicillins and penicilloic acids in bacterial isolates using LC-MS/MS for determination of antimicrobial resistance

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A fast, easy and sensitive detection method was developed and validated by liquid chromatography tandem mass spectrometry for the simultaneous determination of 3 penicillins and penicilloic acid forms of each penicillin G, ampicillin and amoxicillin in bacterial isolates from Korean farms. In order to prepare the sample, the microorganism (9 log CFU ml^{-1}) which were spiked 3 penicillins and 3 penicillioic acids was mixed with 1 ml acetonitrile, followed by centrifuged for 5 min at at 2000 × *g*. About 1 ml of supernatant was filtered by 0.2 μm PVDF filter. Finally, 10 μl of the extract was injected into the LC-MS/MS system. The analysis was carried out mobile phase gradient consisting 0.1% formic acid in D.W. (A) and 0.1% formic acid in ACN (B) with C18 reverse phase column. Mass spectrometry was performed using the positive ion mode and the selected ion monitoring (MRM). The method validation was performed in the sample matrix. Good linearity ($R^2 > 0.99$) was observed and the quantified average recovery was 95–100% at level of 10 μg ml^{-1}. The percent of coefficient of variation (CV) for the described method was less than 10% over the range of concentrations studied. The limits of detection (LOD) and quantification (LOQ) were detected 0.03 and 0.1 μg ml^{-1}, respectively. This method has been applied successfully to analyze the level of ß-lactams antibiotic resistance by the change of their pattern and level of penicillins and penicilloic acids with the comparision of MICs (Minimum inhibition concentrations) in *St. aureus*, *E. coli* and *S. typhimurium*.

2.19.
Determination of marbofloxacin, bromhexine and tolfenamic acid in pig plasma using LC-MS/MS and its application to the pharmacokinetic studies

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Marbofloxacin is a new fluoroquinolone antimicrobial agent developed exclusively for veterinary use. As comparing with enfloxacin, its oxadiazine cycle has shown some advantages, which is supposed to give the molecule some pharmacokinetic advantages such as an elimination half-life, a large volume of distribution and a high bioavailability after i.m. and oral administration. Recently, several investigations to development complex formulation of marbofloxacin containing other effective agents were carried out world widely. A fast, easy and sensitive detection method was developed and validated by liquid chromatography tandem mass spectrometry for the simultaneous determination of marbofloxacin, bromhexin, and tolfenamic acid in pig plasma, which was further applied to study the pharmacokinetics of marbofloxacin. In this study, we have conducted pharmacokinetics study of marbofloxacin containing bromhexine HCL and tolfenamic acid and developed simultaneous analysis of three drugs from serum samples.

In order to prepare the serum sample, the 500 ul of plasma contained each drugs was mixed with 1.5 ml of 0.1% formic acid in acetonitrile (ACN), followed by centrifuged for 15 min at 5000 × *g*. About 1.5 ml of supernatant was concentrated up to 500 μl by nitrogen gas. Finally, 10 μl of the extract was injected into the LC-MS/MS system. The LC-MS/MS analysis was carried out mobile phase gradient consisting 0.1% formic acid in D.W. Chromatographic analysis was carried out mobile phase gradient consisting 0.1% formic acid in D.W. (A) and 0.1% formic acid in ACN (B) with C_{18} reverse phase column. Mass spectrometry was performed using the positive ion mode and the selected ion monitoring (MRM).

The method validation was performed in the sample matrix. Good linearity ($R^2 > 0.999$) was observed and the quantified average recovery of marbofloxacin was 87~92% at level of 10 ng g^{-1}~100 ng g^{-1}. The percent of coefficient of variation (CV) for the described method was less than 10% over the range of concentrations studied. The limits of detection (LOD) and quantification (LOQ) were 2 and 5 ng g^{-1}, respectively.

This method has also been applied successfully to pharmacokinetic analysis of single dose of marbofloxacin and complex dose of marbofloxacin with bromhexin, and tolfenamic acid after intramuscular (IM). The mean peak plasma concentration (C_{max}) of single and complex dosage of marbofloxacin was 1663 ± 101 ng g^{-1} at 2.1 h, 1433 ± 64 g^{-1} at 2.3 h, respectively. The area under the curve (AUC_{0-t}) and was 23 574 ± 900 h ng ml^{-1} (single) and 19 100 ± 330 h ng ml^{-1} (complex) and the elimination half-life ($T_{1/2}$) was 10.5 ± 0.3 h (single) and 15.5 ± 1.5 h (complex), respectively. In summary, pharmacokinetic analysis of marbofloxacin administered single and complex dosage showed that combined formulations of marbofloxacin do not affect to the pharmacokinetics of marbofloxacin alone. Furthermore, in the *in vitro* efficacy such as antimicrobial effect using MIC test, marbofloxacin complex dosage also have shown the similar effect compared with single dosage of marbofloxacin.

2.20.
Population analysis of a physiologically-based model of intra-mammary pirlimycin residues in bovine milk.

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INTRODUCTION

Milk discard times are set using small experimental trials. Variability in pharmacokinetics in cows treated in the field, because of disease and management factors, may lead to violative residues. Conversely, statistical assumptions made in small scale trials may result in overly conservative milk discard times and resulting economic losses. A population pharmacokinetic model, incorporating a novel physiologically-based model of intra-mammary drugs, was developed to describe the pharmacokinetics of the lipophilic pirlimycin after intra-mammary infusion, and to evaluate the effects of clinical mastitis.

MATERIALS AND METHODS

The physiologically-based, structural pharmacokinetic model was adapted from a previous study of lipophobic beta-lactam intra-mammary antibiotics, and implemented in MATLAB. A 3 level, hierarchical statistical model was defined, with population-level fixed effects, individual-level random effects, and error models. The milk volume and drug concentration were considered as simultaneous dependent variables. Presence of clinical mastitis was included as a categorical covariate, and its influence on the fixed effects was estimated. Optimisation of the statistical model was performed using the stochastic-approximation expectation-maximization (SAEM) method, as implemented in Monolix 4.3.3 and MATLAB. The model was optimised using an existing dataset, describing bulk milk concentrations of pirlimycin in 194 cows, 117 with clinical mastitis.

RESULTS/DISCUSSION

The physiologically-based model, developed using beta-lactam drugs adequately described pirlimycin pharmacokinetics. Covariate analysis indicated that mastitis changed the fixed effects between groups, with higher alveolar compartment transfer of pirlimycin (50.8:58.1%), and higher systemic absorption rate (0.071:0.092 per h), indicating higher drug exposure both in milk and systemically then healthy cows. However, random effect variance of these parameters was large, indicating substantial inter-individual variability.

Variability in milk production, and unexplained variability in milk drug concentrations, were greater in the mastitis group, with a tendency for over-prediction of drug concentrations. This suggests that the normal cow may be unsuitable for efficiently predicting milk discard times. We showed that the physiologically-based pharmacokinetic model can be applied to hierarchical models, and to drugs of different physicochemical classes.

2.21.
Oral pharmacokinetics of diclofenac in holstein cattle

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INTRODUCTION

It is well recognized that oral drug administration might not be suitable for ruminants, because of long residence of drugs in rumen or slow drug absorption. Since slow absorption does not always result in long $T_{max,}$ there are drugs for which an oral route is appropriate even in ruminants. We previously found a rapid antipyretic effect of diclofenac (DF) in dairy cows with infectious disease following its oral administration in a preliminary trial. We also reported that DF which have high lipophylicity may be substantially absorbed from the forestomach of goats (Elbadawy *et al*., 2015). In this study, therefore, we examined oral pharmacokinetic profiles of diclofenac (DF) in cattle.

MATERIALS AND METHODS

Total 5 Holstein oxen, weighing 670–810 kg, were used in this study. DF were intravenously and orally administered at a dose of 1.0 mg kg^{-1} using cross over design with a 4-week washout period. Plasma concentration of DF were determined by HPLC with UV-detection. Plasma concentration-time data after intravenous injection and oral administration were simultaneously analyzed using a curve fitting program (MULTI) to calculate pharmacokinetic parameters. The several parameters were calculated by non-compartmental analysis.

RESULTS

The DF kinetics after iv injection was best described by a two-compartment model. After oral administration, plasma concentrations of DF rapidly increased and reached maximum at 2 h of administration and followed by similar elimination profile with iv injection. C_{max} of DF after oral administration was 6.93 ± 2.60 μg ml^{-1}. The calculated mean absorption time (MAT) was 1.61 ± 0.63 h. The half-lives of absorption and elimination (β phase) were 1.51 ± 0.38 h and 5.69 ± 0.55 h, respectively. The oral bioavailability (F) for DF was 102%. The volume of distribution at steady state (V_{dss}) was 0.086 ± 0.027 l kg^{-1}.

CONCLUSIONS

This finding suggests a rapid absorption of DF from the gastrointestinal tract. The MAT (1.61 h) of DF in this study may be due to the fact that DF was partly absorbed from stomach because DF is much more lipophilic. Therefore, oral administration can be used for some drugs such as DF in cattle and therefore the problem of both tissue damage and presence of local residues, as is often the case for drugs administered by IM and SC injection, can be avoided.

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2.22.
Pharmacokinetic study of the antiviral ribavirin in Atlantic salmon (Salmo salar)

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INTRODUCTION

ISA virus is a pathogenic agent which mainly affects Atlantic salmon (*Salmo salar*) with devastating consequences for the salmon farming industry. To date, there is no effective treatment for the virus, although there are studies which prove effectiveness of ribavirin as antiviral, reducing notoriously mortality among infected salmons. Ribavirin is currently used in human medicine and although its effectiveness has been proven against viral diseases on laboratory animals, this molecule is not authorized for its use on veterinary medicine. The present study is the first to show ribavirin's pharmacokinetics administered by pellet on salmons.

MATERIAL AND METHODS

A commercial ribavirin formula was used, administered by pellets to 80 smoltified fish. Salmons were separated in two groups: A) which received a single dose of 1.6 mg kg^{-1} of body weight (bw); and B) as control group, receiving fodder without ribavirin. Sampling times were carried out, at hours 1, 3, 6, 8, 12, 18, 24, 36, 48 and 72 after medicated pellet ingestion, and on each sample, 6 fish from group A and 2 from group B were tested. Blood plasma samples were extracted from each animal. Extraction was carried out according to the method described by Yeh *et al*. (2005). Rivabirin was analyzed using a HPLC Agilent 1200 system (Agilent, Waldbronn, Germany) and the chromatographic separation was reached with a Chromolith RP-18E column (4.6 mm × 100 mm diameter; Merck, Darmstadt, Germany) and a Chromolith RP-18E precolumn (4.6 mm × 10 mm; Merck, Darmstadt, Germany). A mass spectrometer Sciex API 4000 (AB Sciex, Concord, ON, Canada) was used for detection. The mean values of the concentration of ribavirin in plasma, versus time data were sequentially fitted to 1-, 2- and multiple-compartment models, using the computer program WinNonlin (Version 6.3; Pharsight Corporation, Mountain View, CA, USA). The two-compartment model was the best fit for the plasma concentration-time curve and was used to establish kinetic characteristics.

RESULTS AND CONCLUSION

Some of the pharmacokinetics values calculated for ribavirin were $t_{1/2}$ β = 81.61 h; K_{10} = 0.0421 (h^{-1}); AUC = 21.394.01 μg h l^{-1}; C_{max} = 413.57 ng ml^{-1}; and T_{max} = 6.96 h. This study shows a great AUC value reached with the dose of 1.6 mg kg^{-1} bw of ribavirin in salmon, an extensive permanence in the organism and a delayed absorption reflected by the belated T_{max}.

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Session 3: Toxicology Clinical/Farm Animals

3.1.
Poisoning in Farm Animals: Breakdown of the inquiries dealt with by the CAPAE-Ouest

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The CAPAE-Ouest is animal Poison Center of veterinary school of Nantes, which has provided information to the veterinarians and public since 1991.
Breakdown of the inquiries registered in the database gives an indication of the types of poisoning incidents encountered in veterinary practice. In farm animal, most of them concern plants, feed and agrochemicals.

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1. Intoxication par les produits de nettoyage du robot de traite L.Gratius *et al*. Journée nationale des GTV 2011

3.2.
Albendazole treatment in breeder hens: evaluation of drug effects on egg fertility and hatchability

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INTRODUCTION

The benzimidazole (BZD) compounds are broad-spectrum anthelmintics widely used in veterinary medicine. Although flubendazole is the only BZD approved for parasite control in poultry, albendazole (ABZ) is frequently extra-label used for this purpose in avian species. Toxicological studies in both farm and laboratory animals have shown ABZ and its active sulphoxide metabolite (ABZSO) to be embryotoxic/teratogenic (Delatour *et al.*, 1984, Teruel *et al.*, 2009). The goal of the work reported here was to evaluate the effect of ABZ treatment on the fertility and hatchability of eggs collected from treated breeder hens.

MATERIALS AND METHODS

Forty six (46) *Plymouth Rock Barrada* breeder hens were randomly divided into four groups and treated with ABZ at either 10 (group ABZ_{10}), 40 (group ABZ_{40}) or 80 (group ABZ_{80}) mg kg^{-1} day^{-1} in medicated food over seven days. Hens in group C remained as untreated control. Eggs produced during the trial period were identified and incubated under controlled conditions. Ovoscopic evaluation was made at the beginning of incubation to determine fertility. Hatchability, determined at the end of the incubation period (21 days), was assessed according to the number of chicks born (Ricaurte, 2005).

RESULTS

While fertility was not affected by ABZ administration, the hatchability values decreased inversely with the administered ABZ dose level. A statistically significant ($P < 0.05$) reduction of egg hatchability was observed when the breeder hens were treated with ABZ at the highest doses tested here (40 and 80 mg kg^{-1} day^{-1}).

CONCLUSIONS

ABZ administration to breeder hens did not affect the egg fertility at the assessed dose rates. Furthermore, ABZ treatment at a therapeutic dose (10 mg kg^{-1} day^{-1}) in medicated food did not alter egg hatchability. However, egg hatchability decreased when the administered dose was 4–8 times higher than that required to obtain satisfactory antiparasitic efficacy. Altogether, these results should be considered when ABZ is used for deworming breeder hens.

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Session 4: Pharmacodynamics, General

4.1.
Effect of valproate, sodium benzoate and dextromethorphan in hyperglycinemic captive bred Vervet monkeys

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INTRODUCTION

The Primate Unit and Delft Animal Centre (PUDAC) of the SAMRC maintain the only research colony of captive bred Vervet monkeys (*Chlorocebus aethiops*) in South Africa. A small percentage (7%) of this colony has congenital cataracts and was later established that the affected individuals had high levels of glycine in their plasma and cerebrospinal fluid (CSF). Although cataracts have been documented for a variety of primate species, hyperglycinemia as well as this rare and unusual association of conditions have not been reported in the literature before, and clearly need elucidation. The study aimed to investigate the effects of nonketotic hyperglycinemia (NKH) therapy in induced and spontaneous cataract individuals.

MATERIALS AND METHODS

Twelve animals were selected for a three months study and assigned into two groups (induced and spontaneous) and a control. Blood, urine and cerebrospinal fluid (CSF) were collected in order to determine glycine levels for baseline, induction (phase 1), treatment (phase 2) and washout period. The induction was achieved by valproate whereas sodium benzoate and dextromethorphan were used as treatment to reduce glycine levels.

RESULTS

In phase 1, 50 mg kg^{-1} of valproate only induced a non-significant ($P = 0.55$) increase in glycine levels. However, platelets, bicarbonate, LDL and total protein biochemistry changes were clinically significant ($P < 0.05$). In phase 2, reduction of CSF and plasma glycine levels were observed in both groups, but significant change was only seen in the spontaneous group ($P = 0.01$).

CONCLUSION

A dose of 50 mg kg^{-1} valproate showed no significant effect on the glycine concentrations in Vervet monkeys. However, the combination of sodium benzoate and dextromethorphan showed a beneficial effect in reducing glycine levels in the spontaneous group.

4.2.
Effect of nebivolol treatment during pregnancy on the genital circulation, fetal growth and postnatal development in the Wistar rat

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ABSTRACT

The aim of study was to evaluate the effects of nebivolol, a cardioselective $beta_1$-adrenergic receptor blocker of the 3rd generation with vasodilatory properties, versus bisoprolol on the genital circulation, uterine vasculature, fetal growth and postnatal development in pregnant Wistar rats. Non-invasive measurements of systolic and diastolic blood pressure (SBP and DBP) and heart rate (HR), with invasive measurement of genital blood flow (GBF) were taken in pregnant rats, by tail cuff and transonic probe methods respectively, after an oral treatment by gastric gavage with nebivolol (8 mg kg^{-1} day^{-1}) or bisoprolol (10 mg kg^{-1} day^{-1}) from day 11 to day 18 of pregnancy. Other morphometrical and histological studies were performed on the ovarian and uterine arteries to evaluate the effect of nebivolol on the uterine vasculature. Furthermore, postnatal mortality and pup growth were recorded. The data demonstrated that nebivolol (compared with bisoprolol) induced a significant decrease in SBP, HR and GBF while DBP remained unchanged. Moreover, nebivolol increased the diameter (and length) of ovarian and uterine arteries and the number of uterine artery segmental branches. The results also showed that the body weight gain of newborns in the nebivolol group was significantly lower: versus bisoprolol and versus control with a higher mortality rate. The action of nebivolol is not only limited to its favorable hemodynamic effects represented by a decrease in blood pressure, but it also produces adverse effects on fetal growth and postnatal development which may limit its therapeutic use during pregnancy.

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4.3.

A 0.05% hypochlorous acid hydrogel significantly reduces scratching behavior in an allergic dermatitis mouse model

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INTRODUCTION

Allergic skin diseases like atopic dermatitis in humans and dogs are characterized by chronic relapsing skin lesions and severe itch. There is still a need for the optimal treatment of the inflammatory symptoms as well as itch with formulations lacking typical side effects associated with the use of e.g. glucocorticoids. It has been reported that a topical hypochlorous acid formulation leads to relief of itch in human atopic dermatitis patients. It is however not clear whether this is a secondary effect due to reduction of bacterial load by administration of this antiseptic. Thus, we tested a 0.05% commercial hydrogel formulation supplied by PuriCore, Inc., in a mouse model of allergen induced itch and inflammation.

MATERIAL AND METHODS

Female BALB/c mice were sensitized and challenged with the strong Th2 response inducing hapten toluene-2,4-diisocyanate (TDI)[1]. After sensitization, mice were treated topically on the ears with hypochlorous acid gel 24 h and 2 h before as well as 1 h and 6 h after TDI challenge. Control mice were treated with the vehicle gel. Ear thickness was measured before and 24 h after TDI challenge and cytokines were determined in skin homogenate. Scratching bouts were video monitored for one hour after repetitive TDI challenge onto the rostral neck.

RESULTS AND CONCLUSIONS

Although there was only a slight reduction of ear swelling, the concentration of IL-4 was significantly reduced in hypochlorous acid treated ears (IL-6 concentration was not altered compared to vehicle treated ears).

When mice are challenged repetitively with TDI onto the rostral neck a robust itch-scratching response can be established. In mice treated topically with hypochlorous acid gel 24 h, 2 h before as well as immediately after TDI challenge, scratching bouts measured in the hour after TDI challenge were significantly reduced (> 30%). This could be exactly reproduced in a second experimental setting.

Taken together, although there is only a slight reduction in the allergic inflammatory response by topical treatment with hypochlorous acid, the reduction of itch was robust and significant and obviously not related to reduction of bacterial load. This study lays ground for mechanistic studies to elucidate the mechanism of itch reducing potential of hypochlorous acid in allergic skin diseases.

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This study was supported by a grant from Puricore, PA, USA.

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4.4.

Effects of anti-beta1- and beta3-adrenergic receptor antibodies on reactivity of rat aorta and mesenteric arteries

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INTRODUCTION

The presence of functional autoantibodies (AABs) directed against β_1– and β_3–adrenoceptors (ARs) has been detected in sera of human patients with dilated cardiomyopathies (DCM) [1–2]. Abnormalities in the vascular reactivity were found in these patients and β_1–AABs removal by immunoabsorption has shown a decrease of mean arterial pressure and systemic vascular resistance [3]. Although β–ARs are located in both myocardial and vascular tissues, most of the work on effects of the β_1– and β_3–AABs has focused on the heart and their influence on vascular reactivity has received less attention. The aim is to evaluate whether active immunization producing both β_1– and β_3–antibodies (ABs) has deleterious effects on reactivity of thoracic aorta and mesenteric arteries in Lewis rats.

MATERIAL AND METHODS

Lewis rats were immunized for 6 months with peptidic sequences corresponding to the second extracellular loop of β_1– and β_3–ARs. During immunization, systolic blood pressure (SBP) was monitored using the tail plethysmography. The vascular reactivity of immunized rats was assessed by *ex vivo* studies on isolated thoracic aorta and mesenteric arteries using various β– and α-AR agonists and antagonists (isoproterenol, dobutamine, salbutamol, nebivolol) and phenylephrine.

RESULTS AND DISCUSSION

The immunizations producing functional β_1– and β_3–ABs did not affect the SBP. However, in β_1–AR-immunized rats, the relaxation mediated by isoproterenol, dobutamine and salbutamol were significantly impaired, but nebivolol-induced relaxation was not modified in comparison to control rats. Moreover, phenylephrine-mediated contraction was improved in these rats ($P < 0.0001$). In contrast, immunization with β_3–AR peptide led to the improvement of relaxation induced by isoproterenol and dobutamine ($P < 0.0001$) but did not change those induced by salbutamol, nebivolol and phenylephrine-induced contraction. Surprisingly, in the mesenteric artery, for both rats immunized with β_1– or β_3–peptides, the isoproterenol- and salbutamol-mediated relaxations and phenylephrine-mediated contraction were impaired. Our study shows for the first time that β_1– and β_3–ABs can affect vascular reactivity. β_1–ABs

lead to vasoconstriction of conductance and resistance arteries, potentially leading to deleterious effects, whereas β_3–ABs could have a beneficial effect on aorta reactivity.

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4.5.
Vasorelaxant effects of camel and bovine casein tryptic hydrolysates in rat thoracic aorta and their antihypertensive effect in awake spontaneously hypertensive rats.

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INTRODUCTION
In our previous study performed in Wistar Kyoto (WKY) rat thoracic aorta, we have demonstrated that camel casein tryptic hydrolysates (CTH) induced a more potent endothelium-dependent relaxation than that induced by bovine casein tryptic hydrolysates (BTH). However, CTH and BTH-induced relaxations and antihypertensive effect in spontaneously hypertensive rats (SHR) isolated thoracic aorta have not studied yet.
The aim of this study is to evaluate the vasorelaxant effect of both CTH and BTH in SHR thoracic aorta and to determine their effect following long-term oral intake on blood pressure and vascular reactivity.

MATERIAL AND METHODS
Vasorelaxant effect was studied with SHR thoracic aorta rings mounted in isolated organ bath and pre-contracted with phenylephrine (0.3 μM). Then, cumulative concentration–response curves were established for CTH (1 ng–100 μg ml^{-1}) and BTH (1 ng–100 μg ml^{-1}) under different experimental conditions. To study the antihypertensive effect of CTH and BTH, 36-week-old male SHR were surgically implanted with telemetric transmitters for blood pressure monitoring. Then, during 15 days, 800 mg kg^{-1} day^{-1} of CTH and BTH were administered by oral gavage daily at the same time to rats assigned randomly to three groups: untreated (control) and treated with BTH or with CTH.

RESULTS
Similar to the result observed in WKY rats, CTH and BTH produced a relaxation in SHR thoracic aorta. However, the comparison of the response obtained between both rat strains showed an alteration of CTH and BTH-induced relaxation in SHR rats. This relaxation was endothelium-dependent and mediated through the activation of the NO pathway in both rat strains. Oral administration of CTH was able to decrease blood pressure and heart rate only during the first week of treatment in aged SHR. The same dose of BTH did not modify blood pressure and heart rate throughout the 15 days of treatment.

CONCLUSION
We demonstrated that NO-dependent relaxation induced by CTH and BTH was impaired in SHR thoracic aorta and was partly restored by an endothelium-independent pathway. Only the oral administration of CTH was able to decrease blood pressure and heart rate during the first week of treatment in awake SHR. This decrease was too weak to be considered as a health benefit and further studies are necessary with higher dose of CTH and BTH to evaluate their antihypertensive effect.

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4.6.
Eicosapentaenoic acid effect on cholesterol gallstone formation in C57BL/6J mice

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PURPOSE
The present study investigated the preventive effect of ω3 fatty acids against cholesterol gallstone (CG) formation.

METHODS
CG formation was induced in C57BL/6J mice using a lithogenic diet (LD). The mice were divided into four treatment groups: i) LD, ii) LD plus eicosapentaenoic acid (EPA), iii) LD plus docosahexaenoic acid (DHA) and iv) LD plus EPA plus DHA. Subsequent to feeding the mice the LD for four weeks, EPA and/or DHA (both 70 mg kg^{-1} day^{-1}) were orally administered for eight weeks.

RESULTS
The mice in the EPA treatment group exhibited significantly less gallstone formation than those in the LD group. In contrast, DHA treatment only slightly suppressed gallstone formation. The expression of mucin 2, 5AC, 5B and 6 was significantly decreased in the gallbladders of mice in the LD plus EPA (70% to 90%) and LD plus DHA (30% to 50%) groups, compared with mucin expression in the mice in the LD group. In addition, the mRNA expression of 3-hydroxy-methylglutarylcoenzyme A reductase was significantly decreased in the livers of mice in the EPA treatment group compared with that in the livers of mice in the LD group.

CONCLUSION
In conclusion, EPA was found to have a dominant anti-lithogenic effect in C57BL/6J mice.

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4.7.
Long-term atorvastatin treatment decreases mitochondrial vulnerability to reactive oxygen species in a model of hereditary hypercholesterolemia

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INTRODUCTION
Early primary prevention of atherosclerosis in high-risk patients is still a major challenge to decrease the burden of cardiovascular diseases. Treatment with statins is part of the strategies for prevention either by lowering LDL-CT or by their pleiotropic effects. Indeed, statins improve endothelial function, enhance the stability of atherosclerotic plaques, decrease inflammation and reactive oxygen species (ROS) production (at least in the myocardium). Our aim was to determine the long-term effect of atorvastatin on myocardial mitochondrial function focusing on ROS susceptibility, using the Watanabe rabbit hereditary hypercholesterolemia animal model.

MATERIALS AND METHOD
Thirty-three Watanabe rabbits were randomly assigned to two groups: a control group without treatment and a group treated with atorvastatin (*per os* 2.5 mg kg^{-1} day^{-1}) from the age of 3 months. Blood was sampled monthly from the median artery of the ear for lipid analysis. The myocardium was sampled at the age of 3, 6, 9 and 12 months in both groups. Cardiac fibers were then permeabilized (using saponin). Maximal mitochondrial oxygen consumption was measured *in vitro* after incubating or not fibers with ROS (Fenton reaction) in order to assess the susceptibility of the mitochondrial function to ROS.

RESULTS
Decrease in blood lipids showed the efficiency of the atorvastatin treatment. After exposure to ROS, decrease in maximal mitochondrial oxygen consumption was significantly less pronounced in treated than in control rabbits from the age of 9 months (respectively $24.8 \pm 1.5\%$ versus $40.9 \pm 2.8\%$, $P = 0.0006$). No difference was observed in younger rabbits.

CONCLUSION
Our results show that long-term atorvastatin treatment decreases mitochondrial vulnerability to ROS (6 months) in Watanabe heritable hyperlipidemic rabbits. This effect may be related to an increase in antioxydant capacities (measurements in progress) and/or decrease in ROS production (by mitochondria or NADPH oxidase) and/or lower potential ROS target.

4.8.
Effect of nebivolol treatment during pregnancy on the genital circulation, intra-uterine fetal growth and postnatal development in L-NAME-induced hypertensive rats

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INTRODUCTION
The present study was carried out to evaluate the effect of nebivolol, a highly selective beta-1 adrenoceptor blocker of the 3rd generation with vasodilatory properties, versus bisoprolol, a cardioselective beta-1 adrenoceptor blocker of 2nd generation without vasodilatory properties, on the intra-uterine fetal growth, mortality and postnatal development of newborn rats in N^{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME)-induced hypertensive pregnant rats.

MATERIALS AND METHODS
Hypertension was induced in normotensive pregnant Wistar rats by a daily administration of L-NAME (100 mg kg^{-1} day^{-1}, in the drinking water) for the period of pregnancy. After 9 days of L-NAME treatment, rats with systolic and diastolic blood pressure (SBP and DBP) more than 140/90 mmHg were considered hypertensive and treated from day 11 to day 18 of pregnancy with nebivolol (8 mg kg^{-1} day^{-1}) or bisoprolol (10 mg kg^{-1} day^{-1}) via oral gavage. SBP, DBP and heart rate (HR) were re-evaluated by tail cuff method on day 19 of pregnancy and morphometrical or histological studies were performed on the day 20. In addition, the mortality and postnatal development of newborn pups were assessed in all groups.

RESULTS
The L-NAME administration during pregnancy induced an increase in SBP and DBP without change in HR. Both nebivolol and bisoprolol completely prevented the elevation of SBP and DBP induced by L-NAME with a reduction in HR of treated rats. The intra-uterine fetal growth and the postnatal development in nebivolol hypertensive rats were significantly lower versus control and higher versus bisoprolol group with a higher mortality in both types of treatments versus control rats.

CONCLUSION
Nebivolol administration during pregnancy in hypertensive rats produced adverse effects on fetal growth and postnatal development that may limit its therapeutic use in females during pregnancy, even in order to prevent hypertension.

4.9.

Valvular density and pharmacological reactivity of the veins in the distal part of thoracic and pelvic limbs in Horses

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INTRODUCTION
The valve apparatus is an essential component that can modulate the digital venous hemodynamics in the horse. The present study was designed to investigate the distribution of venous valves in the veins of horse limbs and their possible influence on digital venous reactivity.

MATERIALS AND METHODS
We firstly investigated the distribution of venous valves in palmar proper digital, coronary and tori digital veins of 18 front and 18 hind feet from 9 healthy horses. Data were expressed as means ± SD. Unpaired Student's *t* test was used to compare the valvular density between the thoracic and pelvic limbs. Secondly, cumulative concentration-response curves (CCRC) to various agonists were established in the coronal veins isolated from 14 front and 14 hind feet of 7 adult healthy horses. Data were reported as mean ± SEM. The results were compared using NLME models. A P value < 0.05 was considered to be significant.

RESULTS
significant difference was found in the valvular density of the coronal veins between the thoracic and pelvic limbs (0.08 ± 0.02 and 0.04 ± 0.01, respectively, $P < 0.05$). It was not significant in the other veins in the front and hind feet. The pharmacological study showed that phenylephrine induced a similar vasoconstriction in pelvic ($pD_2 = 6.37 \pm 0.09$, $E_{max} = 13.14 \pm 0.45$) and thoracic limb ($pD_2 = 6.20 \pm 0.11$, $E_{max} = 14.03 \pm 0.63$). Moreover, no significant difference was observed in response to sodium nitroprusside, an endothelium-independent vasorelaxant agent, ($pD_2 = 7.35 \pm 0.15$, $E_{max} = 100 \pm 4.45\%$ in thoracic limb, $pD_2 = 7.16 \pm 0.16$, $E_{max} = 96.83 \pm 4.26\%$ in pelvic limb), and to acetylcholine, an endothelium-dependent vasorelaxant agent, ($pD_2 = 7.24 \pm 0.07$, $E_{max} = 69.19 \pm 2.24\%$ in thoracic limb, $pD_2 = 7.47 \pm 0.11$, $E_{max} = 71.78 \pm 2.95\%$ in pelvic limb) between the thoracic and pelvic limbs.

CONCLUSION
We showed, for the first time, that valvular density of coronal veins was higher in thoracic limb than pelvic limb of healthy horses. This difference does not seem to influence the coronal vein reactivity of the two limbs that exhibit a similar pattern of vasoconstrictor and vasorelaxant responses. However, it remains to be established whether valvular disturbances occurring in laminitic horses may affect differently the coronal veins reactivity of both forelimb and hind limbs.

Session 5: Drug Residues

5.1.
Discovery of the new marker residue of olaquindox in swine, broiler and carp

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Olaquindox (OQD) is one of quinoxaline-1,4-dioxides with antimicrobial and growth-promotional activities that have been used in food animals in some countries for decades. 3-methyl-quinoxaline-2- carboxylic acid (MQCA) was recommended by the Joint of FAO/WHO Expert Committee on Food Additives (JECFA) as the marker residue, the target compound for the detection of OQD residues in edible tissues from animals treated with OQD, although an acceptable daily intake (ADI) for olaquindox could not be established due to the absence of data on the carcinogenic potential of the drug and its metabolites. However, MQCA was not the longest persisting compound of OQD in the edible tissues in treated animals. To explore the new marker residue of OQD in food animals, the mass balance, metabolism, distribution and tissue depletion of OQD in swine, chicken and carp were studied with the synthesized [3H]OQD with ≥98% of both chemical purity and radiochemical purity and the developed method of an high performance liquid chromatography combined with ion trap time-of-flight mass spectrometry (LC/MS-IT-TOF) for the identification of metabolites and on-line liquid scintillation counting (LSC) for the quantification of metabolites. It was found that eight, eight and four metabolites were detected in pigs, chickens and carps, respectively. Pigs and chickens had the same metabolites (O1-O8) while carps had O1, O2, O4 and O7 (seeing Fig. 1). OQD and its metabolites were distributed widely in the animals. Higher radioactivities were detected in bile, kidney and liver of the animals at 6h after last administration of [3H]OQD in feed. Trace amounts of radioactivity could be detected in the liver and kidney of pigs and carps at 14d after administration. In pig, O2, O5 and O8 were detected in liver, O1, O2, O3, O4, O5, O6, O7 and O8 in kidney, O0, O1, O2, O3, O6 and O7 in muscle, and O0, O1, O2, O3, O4, O5, O7 and O8 in fat. In chicken, O1, O2, O3, O4, O5, O8 and O9 were detected in liver and kidney, O0, O1, O2, O3 and O8 in muscle and O0, O1, O2, O3, O5, O7 and O8 in fat. In carps, O0, O1, O2, O4 and O7 were found in liver and skin, O1, O2, O4, O5 and O7 in kidney and O0, O1, O2, O6 and O7 in muscle. O2 is the major residue of OQD in all edible tissues of pig and chicken while O1 and O2 were the major in carps. The total residues in liver and kidney were eliminated slower than those in other organs with half-times of 2.87d and 4.94d, 3.13d and 3.27d, 3.28d and 3.22d in pig, chicken and carp, respectively, suggesting that kidney be the target organ of OQD residues in the treated animals. As O2 was persist longest in the kidney with the half-life of 4.60d in pig, 3.59d in chicken and 3.52d in carp, respectively, it is recommended that O2 is the marker residue of OQD in the treated animals. The present study provides scientific data that are not only useful for the determination of OQD residues in edible tissues but also helpful for the risk assessment of OQD uses in food animals.

5.2.
Investigation of veterinary drug residues in sea water, sediment, and wild fishes captured around fish farms in the Aegean Sea: Sulfonamides (Sulfamerazine, SMR; Sulfadimidine, SMT; Sulfamethoxazole, SMXZ; Sulfadimethoxine, SDMX)

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INTRODUCTION

Aquaculture is one of the major causes of antibiotic residues to the environment. Every year, millions of tons of antibiotics are released to the aquatic environment. Also antibiotic contamination may cause resistance in microorganisms. It is essential to conduct monitoring programs to maintain a sustainable model for aquaculture, and to minimize the undesirable effects to the environment.

In this research, residues of certain veterinary drugs Sulfonamides (Sulfamerazine, SMR; Sulfadimidine, SMT; Sulfamethoxazole, SMXZ; Sulfadimethoxine, SDMX) were screened in natural fish, sediment, and seawater samples of the Aegean Sea.

MATERIALS AND METHOD

Samples were collected from fish farming cages in selected coordinates (Bodrum, Salihli Region, Turkey) on September, October 2011 and March, April 2012. Analyses were carried out using High Pressure Liquid Chromatography (HPLC) followed by the validation the method for each matrix and each drug.

RESULTS

For the sea water samples the lowest temperature was recorded as 14°C in March 2012 but the highest was 28.3°C in October 2011. According to the analysis results sea water dissolved oxygen (mg L^{-1}) were found to be 0.59–11.19/7.78–8.64 and 9.49–12.29/10.78–11.55 for September and October (2011) samples; and 5.15–6.50/6.05–6.44 and 7.37–9.59/8.11–10.32 for March–April (2012) samples; The pH values slightly varied month by month; varied between 7.96 and 8.68. Limit of detection (LOD) values for the sea water, sediment and fish (red mullet) samples for Sulfamerazine-SMR, sulfadimidine (sulfametazine)-SMT; Sulfametoxazole- SMXZ; sulfadimetoxine-SDMX respectively are as follows 20.41, 20.92,19.45, 21.08; 19.37, 20.20, 18.03, 19.43 ve 28.81, 29.16, 28.03, 28.54 ppb and LOQ values were found as follows 61.85, 63.39, 58.96, 63.88; 58.71, 61.22, 54.64, 58.89; 87.30,

88.39, 84.95, 86.50 ppb. For all samples the regression lines between 50–800 ppb were found to be lineer (r^2 = 0.997–0.999), recovery values (%) for the same concentration for seawater, sediment and fish samples were found in no relation within the increased dose and are as follows 94.44–104.30, 105.11–110.82, 64.30–76.49; 92.29–104.66, 97.08–106.61, 76.30–64.77; 85.61–98.77, 95.86–105.78, 58.50–70.75; 91.50–100.81, 108.63–100.35, 62.29–74.32. This research revealed that dissolved oxygen and pH values of samples were in accordance with the normal limits. No residues were found to be above the LOD in the screened samples. To conclude; no contamination were recorded for the screened veterinary drugs. In order to understand the possible risk of veterinary antibiotics, especially for low dose accumulation, to the ecosystem for sustainable aquaculture, more screening analysis are expected to be conducted by local and international authorities.

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5.3.

Fosfomycin residues and withdrawal time in eggs after oral administration to laying hens

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INTRODUCTION/OBJECTIVE
Fosfomycin is a bactericidal broad spectrum antibiotic widely used in poultry production in South and Central America and Asia. Data on fosfomycin residues accumulation in eggs is not available so far and withdrawal times stated in labels of commercial products greatly differ from each other and lack of scientific support. The goal of the present study was to determine fosfomycin withdrawal time from egg white and yolk after administration of different formulations (in drinking water and feed) to laying hens.

MATERIALS AND METHODS
Two homogeneous groups of 20 Lohmann laying hens each, were treated with 160 mg kg^{-1} day^{-1} of a commercial formulation containing 25% calcium fosfomycin (Fosbac, Bedson SA, Argentina), administered for seven days in drinking water or premix accordingly. Besides a control group of untreated animals was also considered. Fosfomycin administration took place 1 h after egg laying in order to synchronize it with ovulation (which occurs 30 min after egg laying), since the dynamic process of yolk formation significantly influences incorporation and storage of drug residues. After the end of the treatments, eggs were collected daily and fosfomycin concentrations were quantified by HPLC-MS/MS. Analytical method development and validation was carried out following international guidelines. For withdrawal time calculation EMEA WT 1.4 software was used, and an MRL of 0.5 μg g^{-1} was considered (established by Japan for animal tissues other than eggs, but it is the only MRL value given by an official agency so far).

RESULTS
Calculated withdrawal times were 0.46 and 4.02 days for white and yolk respectively when the antibiotic was administered in drinking water, and 1.37 and 4.6 days for white and yolk respectively when the antibiotic was administered in premix.

DISCUSSION AND CONCLUSIONS
Differences found in yolk and white may be due to the longer period of yolk formation which undergo a later stage of rapid growth, approximately two weeks before ovulation. Probably residues were incorporated and stored along dosing period in preovulatory yolks. Conversely, egg white is built in the latter period of whole egg development, just before eggshell formation, decreasing contact time with the antibiotic. We conclude that even though withdrawal time from eggs is more than 4 days, it can be reduced to two days or less (when administered in premix) when only white is considered, enabling its use as raw material for food industry.

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5.4.

Development of a direct competitive ELISA for the detection of semicarbazide in foodstuff of animal origin

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INTRODUCTION
Nitrofurazone (NFZ) belongs to a class of synthetic broad spectrum antibiotics and mainly uses for livestock, aquaculture and bee colonies in the prophylactic and therapeutic treatment of bacterial and protozoan infections. The use of NFZ has been prohibited completely in food animal production in the European Union (EU) since 1995 due to its carcinogenicity and mutagenicity; however, it is still widely used in Russia. The MRPL for nitrofuran metabolites residues is 1 μg kg^{-1}.
NFZ is characterized by its rapid metabolism to semicarbazide (SEM) *in vivo* in less than a few hours. Furthermore, the SEM residues are stable in tissues and persists for at least 6 weeks in pig tissues after drug withdrawal. As a result, effective ana-

lytical detection of these compounds could be carried out by the determination of bound NFZ metabolite. To avoid using labor-intensive instrumental methods to detect NFZ metabolite residues in food, a simple direct enzyme-linked immunosorbent assay (ELISA) method for SEM determination was developed in this study.

MATERIALS AND METHODS
Polyclonal antibody production was achieved through repeated immunisation of male Chinchilla rabbits inoculated with 0.1 mg of CPSEM immunogen as an emulsion in Freund's adjuvant. Booster injections (0.02–0.05 mg) were administered every month during 16 months. Antisera were taken 7 days after each immunisation and screened for the presence of antibodies to CPSEM using a direct competitive ELISA. Sample preparation were performed by derivatization of the homogenized tissues with 3-carboxybenzaldehyde and the following ethyl acetate extraction.

RESULTS AND CONCLUSION
Polyclonal antiserum raised using CPSEM-KLH exhibited the highest sensitivity of binding to CPSEM after 8 immunization cycles. The 50% inhibition values (IC_{50}) ranged from 2 to 4 $\mu g\ l^{-1}$ for CPSEM (which corresponded to 0.6–1.2 $\mu g\ l^{-1}$ for SEM).
The limits of detection (LOD) calculated from the analysis of 20 known negative honey samples were 0.4 $\mu g\ kg^{-1}$ for SEM. Recoveries of SEM fortified ranged from 76 to 110% in honey. The coefficients of variation were less than 30%. All results were submitted by HPLC-MS.

CONCLUSION
The developed analytical technique can be used as first step in routine analysis of NFZ residues substances in honey samples. This ELISA technique is simple and sensitive and can be applied by residues laboratories and the food industry in dealing with the nitrofurazone problem.

5.5.
Should we establish the maximum residue limit for thiouracil in liver?

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INTRODUCTION
The use of thyreostats in food animal production is banned in many countries due to potential risks to human health and production of meat of low quality. In spite of this, data from annual residue reports of EU and Canada authorities show positive results for thiouracil in urine and liver samples at concentrations ranging from 1–10 $\mu g\ l^{-1}$, 5–50 $\mu g\ kg^{-1}$, respectively, which is attributed to the cruciferous diet. To monitor thyreostats in liver, we have developed and validated the LC-ESI-MS/MS method for the determination of thiouracil, methylthiouracil, propylthiouracil, phenylthiouracil, and mercaptobenzimidazol.

MATERIALS AND METHODS
Thiouracil, methylthiouracil, propylthiouracil, phenylthiouracil, mercaptobenzimidazol were purchased from Sigma. The HPLC system was Eksigent UltraLC-100 (Eksigent, USA) consisted of a binary pump, a vacuum degasser and an autosampler. The separation of thyreostats was achieved on ACE3 C_{18} column (150 mm × 2.1 mm, particle size 2 μm, ACE). The triple quadrupole mass spectrometer QTRAP 5500 (AB Sciex, Canada), operated in negative multiple-reaction monitoring mode (MRM), was coupled to HPLC through an electrospray atmospheric pressure ionization interface. The injection volume was 20 μl and the analysis was carried out with gradient elution using eluent A (water) and eluent B (methanol) at a flow rate of 0.2 ml min^{-1} in 23 min.

RESULTS
A rapid liquid chromatography/tandem mass spectrometry method has been developed and validated to determine 5 thyreostats in liver. Sample preparation involves derivatisation with 3-iodobenzylbromide and solid phase extraction using silica gel cartridges. The method was validated in the range of 2–30 $\mu g\ kg^{-1}$ for thiouracil, methylthiouracil, propylthiouracil, phenylthiouracil, and 0.4–30 $\mu g\ kg^{-1}$ for mercaptobenzimidazol. Recovery, repeatability, within-laboratory reproducibility lie in the range of 87–119%, 8–22%, and 7–23%, respectively.
32 samples were analyzed and thiouracil was found in beef and deer liver samples at concentrations ranging from 2–32 $\mu g\ kg^{-1}$, and 2–10 $\mu g\ kg^{-1}$, respectively.

CONCLUSIONS
Our study shows that some liver samples contain high levels of natural thiouracil (up to 50 $\mu g\ kg^{-1}$), which makes us think that risk assessment for this potentially carcinogenic and teratogenic drug in liver should be performed.

5.6.
Screening evaluation of growth-promoter abuse in veal calves using serum biomarkers

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INTRODUCTION
Growth-promoter (GP) abuse is monitored in the EU in both live animals and carcasses by chemical analysis. Based on the wide availability of anabolic substances on the black market and the results of histological screenings on target organs the official results reveal only a very limited number of non compliances (1). Therefore, the design of a battery of screening tests based on the biological effects of GP has been proposed (2). Recently, a class modeling strategy (unequal dispersed classes, UNEQ) based on five serum biomarkers reflecting the exposure to the main classes of GP (sexual steroids, corticosteroids, and β-agonists) has been set up as a possible screening test to reveal illegal treatments in veal calves (3). The aim of this study was to analyze the results obtained from UNEQ in order to evaluate the reliability of the model.

MATERIAL AND METHODS

The study was carried out on 215 five-month-old (Group A) and 565 six/seven-month-old (Group B) veal calves collected from 95 farms and reared according to standard programmes. Serum cortisol, ir-inhibin, osteocalcin, antioxidant capacity (SAC), and urea were measured using commercially available kits. Biomarker values (medians with interquartile ranges) were compared using the Mann Whitney test.

RESULTS AND CONCLUSION

After UNEQ model application, 24.2% ($n = 52$) Group A-, and 55.3% ($n = 284$) Group B calves were classified as non compliant (NC). In both age groups, median values of serum cortisol, ir-inhibin, and osteocalcin were significantly lower in NC than in compliant animals (Cortisol: Group A, 9.83 ng ml^{-1} versus 16.41 ng ml^{-1}, $P < 0.0001$; Group B, 10.18 ng ml^{-1} versus 16.37 ng ml^{-1}, $P < 0.0001$. Ir-inhibins: Group A, 0.98 ng ml^{-1} versus 1.19 ng ml^{-1}, $P = 0.03$; Group B, 1.01 ng ml^{-1} versus 1.16 ng ml^{-1}, $P = 0.01$. Osteocalcin: Group A, 8.55 ng ml^{-1} versus 10.22 ng ml^{-1}, $P = 0.0009$; Group B, 8.61 ng ml^{-1} versus 9.43 ng ml^{-1}, $P < 0.0001$). In Group A calves no differences in SAC and urea levels were observed between compliant and NC, whereas in Group B SAC values were higher in NC than in compliant animals ($P = 0.011$); urea was lower in NC than in compliant calves ($P = 0.033$). The recorded changes in biomarker values from calves classified as NC reflect those expected after the exposure to the different GP classes (1), suggesting that UNEQ model based on the selected serum biomarkers may represent a good approach to identify possible GP abuse in veal calves.

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5.7.

Evaluation of stress-related prednisolone biosynthesis in cows participating to 'Batailles des Reines'

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INTRODUCTION

Natural corticosteroids include two families of substances: mineralocorticoids and glucocorticoids. Several molecules simulating their structure and behaviour, with increased pharmacological activity, have been synthesized and are widely used in the clinical practice. Beside legal treatments, these drugs can also be misused. One of the more abused 'corticosteroid' is prednisolone. Until few years ago, it was considered exclusively synthetic, but nowadays a debate on its possible endogenous origin is under way. Many researchers have worked to ascertain the relation between stressful conditions, such as transportation and slaughterhouse environment, and endogenous production of prednisolone. In order to verify further allegedly stressful conditions, our laboratory analyzed urine samples collected from the cows participating to the 'Batailles des Reines' (a traditional contest based on ritual and spontaneous fights of pregnant cows), to verify if an endogenous prednisolone production may occur in these animals.

MATERIALS AND METHODS

We developed and validated a LC-MS/MS method for the simultaneous determination of cortisol, cortisone, prednisolone, prednisone, 20α-dihydroprednisolone, 20β-dihydroprednisolone, 20β dihydroprednisone and 6β-hydroxyprednisolone. Sample preparation includes an enzymatic deconiugation, followed by a SPE. The method was applied for the analysis of urine samples from 2012 and 2013 'Batailles des Reines' competitions, for a total of 114 samples.

RESULTS

The analytical method was validated following the 2002/657/CE Decision. Cortisol and cortisone were found in all but one urine samples, with average values of 8.35 ± 5.17 ng ml^{-1} and 4.93 ± 3.10 ng ml^{-1}, respectively, and no significant differences between 2012 and 2013. Prednisolone was found in only one sample, at a concentration of 1.45 ng ml^{-1}, accompanied by cortisol and cortisone concentrations at the highest values found in these urine samples, 35.5 and 18.1 ng ml^{-1} respectively. Traces of prednisolone were found also in three other samples. In these urines cortisol and cortisone concentrations were around average values.

CONCLUSIONS

The stress produced by the 'Batailles des Reines' fight appears to be present but lower than that caused by both transportation and slaughterhouse environment, as evaluated from cortisol and cortisone urine concentrations. This stress level is probably not sufficient to induce endogenous prednisolone biosynthesis, which was observed in only one case.

5.8.

Illicit administration of estradiol in cattle: case report

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INTRODUCTION

Estrogens, of which 17β-estradiol is the most active molecule, constitute a group of steroid compounds known for their importance in the estrous cycle. Besides their function in the

reproductive cycle, they also play an important role in a number of other physiological processes, including mineral, fat, sugar and protein metabolism and sodium and water retention. Owing to their wide-reaching systemic effects, estrogens are also illegally administered to stimulate growth in calves and boost meat production.
In February 2014, a police investigation was conducted for alleged illicit cattle treatments, within a livestock in Piedmont, northern Italy. During the operations of judicial investigation, an undeclared animal treatment was observed by hidden cameras. The subsequent day, four anonymous liquids were impounded and so also serum of ten animals was rapidly collected and transferred to our laboratory.

MATERIALS AND METHODS
The anonymous liquids were properly diluted and injected into a triple quadrupole mass spectrometer API 4000 (ABSciex), equipped with an electrospray ionization source. Serum samples were analysed with a procedure validated according to 2002/657/CE Decision, using a LC-MS/MS (QQQ) equipped with a H-ESI, operating in negative multiple reaction monitoring (MRM) mode (Thermo Fisher). Sample preparation involved liquid/liquid extraction followed by centrifugation and SPE clean up.

RESULTS
In all four anonymous liquids 17β-estradiol benzoate, a typical prodrug of 17β-estradiol, was found at concentration ranging from 12.0 mg ml^{-1} to 14.4 mg ml^{-1}. Nine out of ten serum samples were found positive to 17β-estradiol at concentrations comprised between 0.059 $\mu g\ l^{-1}$ and 0.208 $\mu g\ l^{-1}$, which are much higher than physiological values.

CONCLUSIONS
The illicit drug treatment was found thanks to a very good collaboration between Police and the laboratory staff. The requisition of anonymous liquids permitted to identify the analyte to look for. The direct observation that an animal treatment had been carried out permitted to collect the animal serums immediately thereafter, making it possible to find strong evidence of the exogenous treatment. It is also interesting the finding of one compliant animal out of ten controlled. This practice of keeping one untreated animal in the same box with treated veals is quite typical in cattle breeding: it is suggestive of the corruptive intent of the breeder, addressed to obtain dishonest veterinary controls focused only on the untreated animal.

5.9.
A deltamethrin nil milk withdrawal time and good local tolerance after treatment of ewes with Deltanil® pour-on solution

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INTRODUCTION/OBJECTIVE
A GLP study was carried out according to EMA Guideline in order to follow the residue depletion of deltamethrin in ewe's milk and to estimate the milk withdrawal period after treatment with Deltanil® $1\%_{w/v}$ deltamethrin pour-on solution. The local tolerance at the application site was also followed.

MATERIALS AND METHODS
Ten high and ten low yielding milking ewes were included in this study. Five mL of the product were applied once, topically on each animal, equivalent to about 50 mg of deltamethrin per animal. Clinical signs and application sites were observed daily for local tolerance up to 8 days post-treatment. Animals were milked twice daily (every 12 h ± 30 min). Milk samples were regularly collected before treatment, 6 h, 8 h and 12 h post-treatment and twice daily until 8 days post-treatment for deltamethrin analysis. Deltamethrin levels in milk were determined by an adequate and validated routine analytical LC-MS/MS method according to criteria defined in Vol. 8. The lowest limit of quantification (LLOQ) was 10 $\mu g\ kg^{-1}$, half the milk MRL set by the EMA for all ruminants. The withdrawal period was calculated using the EMA software MELK14 and a statistical method.

RESULTS
A good general and local tolerance was observed for the product. All deltamethrin individual concentrations were below the LLOQ (10 $\mu g\ kg^{-1}$), with no difference between low and high yielding ewes from 6 h (first milking) after the treatment, except for one ewe which showed quantifiable values from 8 h to 24 h after treatment, but always under the MRL (20 $\mu g\ kg^{-1}$). Using the SCPM approach, the statistically calculated withdrawal period was 0 days.

CONCLUSIONS
The results of this study allow a nil milk withdrawal time to be determined and show the good local tolerance of the Deltanil® Pour-on Solution when topically applied at the recommended dose in milking ewes for the control of ectoparasites.

5.10.
Residues of decoquinate in eggs after feeding hens with compliant cross-contaminated feed

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INTRODUCTION
Decoquinate is a quinolone derivative that can be used as feed additive or in-feed medication for the treatment or prevention of coccidiosis in poultry and ruminants. It is also regarded as an effective aid in the toxoplasmosis and cryptosporidiosis in ruminants. In EU countries decoquinate is authorised for chicken broilers as feed additive.
As cross-contamination of feeds for non-target animals with decoquinate is unavoidable, its maximum levels (ML) in feed and in food of animal origin have been established. It is not known however if undesirable residues of decoquinate in hen eggs may occur after contaminated but compliant feed is administered to hens.

MATERIALS AND METHOD
Twenty laying hens received feed containing 0.34 ± 0.081 mg kg^{-1} of decoquinate (ML for feed = 0.40 mg kg^{-1}) during 14 days. Than, for the next 14 days decoquinate-free diet was applied. The eggs were collected daily during the whole experiment and stored in 6 ± 4°C. The

whole eggs (6 eggs per day), and white and yolk separately, were analysed individually using LC-MS/MS technique. Limit of detection of decoquinate in eggs was 0.1 μg kg^{-1}.

RESULTS

The plateau of decoquinate in whole eggs (8.9 ± 2.89 μg kg^{-1}) was reached at eight day of experiment and was far below the maximum level established for eggs (20 μg kg^{-1}). The plateau lasted until 4th day after cessation of decoquinate-contaminated feed. Afterwards, the concentrations of decoquinate in whole eggs decreased to the level of 0.3 μg kg^{-1} on the last day of experiment. Residues of decoquinate were deposited mainly in egg yolks. Concentration in yolks during plateau level was 26.2 μg kg^{-1} and did not deplete completely during 14 days of administration of decoquinate-free feed (concentration 2.1 μg kg^{-1} in the last day of observation). Concentrations of decoquinate in egg whites were negligible (1.2 μg kg^{-1} during plateau).

CONCLUSION

The results of the study confirm that it is highly unlikely that the residues of decoquinate in whole eggs exceed maximum level (20 μg kg^{-1}), when hens are fed with a compliant cross-contaminated feed (<ML 0.4 mg kg^{-1}). The residues of decoquinate are found mainly in egg yolks and deplete slowly.

5.11.
Detection of residual metoclopramide and scopolamine in pork by liquid chromatography tandem mass spectrometry

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A liquid chromatography-tandem mass spectrometry (LC-MS/MS) method has been developed and validated for the simultaneous determination of metoclopramide and scopolamine in pork. Metoclopramide and scopolamine were determined based on the LC-MS/MS after solid-phase extraction. A C_{18} column with 0.1% formic acid and 0.1% formic acid in acetonitrile gradient was used. Electrospray ionization was used to ionize the analytes. The quantification was performed by LC-MS/MS detection mode of multiple-reaction monitoring. For metoclopramide, the ion transition (parents ion→products ion) was 300.03 m/z→227.10 m/z and for scopolamine was 305.13 m/z→138.00 m/z. The limits of quantitation (LOQ) for metoclopramide and scopolamine were 0.2 ng g^{-1} and 10 ng g^{-1}. The calibration curves of metoclopramide and scopolamine showed a good linearity ($r \geq 0.99$). The mean recoveries were ranged from 90.92 ± 2.93 to 98.78 ± 12.10 and 70.92 ± 3.75 to 78.78 ± 6.89 (Mean ± RSD, %) for metoclopramide and scopolamine, respectively. Six replicates at 2 concentrations (1, 2 times the LOQ) were analyzed. This method had an acceptable precision and recovery. The procedure was simple and allowed the determination of the residues of metoclopramide and scopolamine in pork with high sensitivity.

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5.12.†
ABSTRACT DELETED

5.13.
Histological biomarkers to reveal low-dose dexamethasone illicit administration in veal calves: results from a three years experimental study

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INTRODUCTION

Despite the proven efficacy as therapeutical remedies in a wide range of pathologies, glucocorticoids (GCs) are also illegally used as growth promoting agents in animal breeding. In particular in the last few years dexamethasone has been shown tempting characteristics in the field of illicit treatments in food producing animals, due to its high glucocorticoid potency associated to a fast kinetics, as well as for the synergic effect when used in association with estrogens or β-agonists.

OBJECTIVE

The first aim of the study was to establish the applicability of the histological method in regard to morphological changes in

thymus following anabolic treatments with GCs as a screening method in calves. For this reason we analysed the fat infiltration and the cortex-medulla ratio in dexamethasone treated calves and control calves; the second aim was to test the potentiality of these two methods to better discriminate between treated and untreated animals.

MATERIALS AND METHODS
One hundred and seventy-two male Friesian veal calves were farmed under controlled condition and divided into two groups: Group A (n = 106 calves) was given dexamethasone for twenty consecutive days (0.4 mg day^{-1}) during the sixth month, Group B (n = 66) was used as control. At the end of the experimental treatment, the calves were slaughtered and the thymus of each animal was sampled for histological examination. The presence of fat infiltration, as an indirect indicator of the degree of atrophy, was evaluated and a grading from 1 to 3 (mild, moderate, severe) was attributed by an expert histopathologist. Moreover the thymic cortex-medulla ratio was calculated for each animal. Fisher's exact test and Wilcoxon–Mann–Whitney test have been performed respectively to study the differences of the thymic atrophy and of the cortex-medulla ratio between the groups.

RESULTS AND CONCLUSIONS
The results of the present study demonstrated that the thymic atrophy grading was significantly increased in group A (P = 0.006), whereas the cortex-medulla ratio was decreased (P < 0.004) when compared to group B. Our data suggest that fat infiltration and cortex-medulla ratio are reliable tools to discriminate between treated and non treated animals. These results confirm that the morphometric examination of thymus represents a robust strategy to assess the effective prevalence of misuse of GCs in food producing cattle.

5.14.

Seven plate test, for the screening of antimicrobial residues in bovine meat

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INTRODUCTION
The control of antimicrobial residues in meat is important, due to these residues can affect the consumer's health. In Colombia, the vigilance of the Maximum Residue Limits MRLs of veterinary antimicrobials in cattle meat is limited, because the analytical capacity is insufficient to monitor a large number of samples and a wide range of antibiotics. One of the methods of choice for the detection of residues is the microbiological bioassay, described for the United States Department of Agriculture.

OBJECTIVE
As first time in Colombia, It was evaluated preliminary this bioassay for the detection of four antimicrobials in diaphragm muscle of bovine, slaughtered in the principal processing plant in Bogotá, as contribution to the Food Safety.

MATERIALS AND METHODS
The standardization protocol used fortified muscle with varying concentrations of four families of veterinary antibiotics, penicillin G potassium, oxytetracycline, erythromycin and streptomycin as representatives, preliminarily evaluated under guidelines of the European Decision 2002/657.

RESULTS AND CONCLUSIONS
The test showed excellent specificity (without false positives). Limits of Detection (LOD) were determined for the four antibiotics in relation to their respective MRLs: The LOD of penicillin, oxytetracycline and erythromycin meet with the MRLs in bovine muscle according to *Codex Alimentarius*. However, the sensitivity for streptomycin was unsatisfactory. The relative accuracy for LOD was appropriate. Stability of the analytes in the matrix was adequate during the period evaluated. With the standardized bioassay method, were evaluated 104 samples, not detecting residues breaking out the permit limits. In parallel, samples were analyzed by swab microbiological methods and the commercial test Premi®Test, determining concordant results among all tests. Additionally, incurred tissues with a product containing penicillin and streptomycin were evaluated. Muscle samples analyzed by bioassay showed penicillin violatory levels of residues, but were negative for streptomycin, which confirms the low sensitivity of the method for aminoglycosides detection. This method improved analytical capability of one official authority, 'Instituto Colombiano Agropecuario', and will help the implementation of the 'Programme for Control of Veterinary Drugs' in our country, contributing to ensure food safety to protect the health on consumers and enhance the competitiveness of meat.

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5.15.

Factors affecting withdrawal times of antimicrobials in eggs

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INTRODUCTION
The antimicrobials administered to laying hens can lead to the presence of drug residues in eggs. To ensure the safety of these

products, only eggs produced after completion of an adequate withdrawal time (WDT) can be used for human consumption. Today, there is no consensus about what factors are the main involved in the magnitud of the disposition of drugs in eggs. The aim of this study was to determine the WDT in eggs of three antimicrobials with different characteristics and associate the values of these parameters with the pharmacokinetics and physicochemical properties of the drugs.

MATERIAL AND METHODS

Three commercially available formulations for oral administration were used: a hydrochloride oxytetracycline (OTC) 80% powder formulation, a 10% powder formulation of tylosin (TYL) and a 10% powder of florfenicol (FF). Thirty-nine 25-week-old Leghorn hens were used. They were randomly allocated in one of four experimental groups: Groups A, B and C ($n = 12$) were treated with 40 mg kg^{-1} of body weight (bw) of OTC for 10 days, 35 mg kg^{-1} bw of TYL for 7 days and 40 mg kg^{-1} bw of FF for 7 days, respectively. Group D ($n = 3$) remainded as untreated control hens. When the drug administration was completed, eggs were collected daily for a 15-day period. Egg whites and yolks were analyzed together each day. The extractions were carried out according to the methods described by Cristofanie *et al.* (2009) for OTC, Civitareale *et al.* (2004) for TYL and by Xie *et al.* (2012) for FF. OTC and TYL were analyzed using an Elite LaChrom HPLC system coupled to a diode array detector (Hitachi High Technologies America, Inc.). FF concentrations were determined using an Agilent 1290 Infinty HPLC system (Agilent, Waldbronn, Germany). WDTs were calculated using the software WT1.4 as recommends the EMA, with a 95% upper one-sided tolerance limit and a 95% confidence.

RESULTS AND CONCLUSION

Considering MRLs established by the European Union for OTC and TYL for eggs, the WDTs were of 9 days for OTC, 4 days for TYL and 11 days for FF. These values are consistent and very probably related to three main factors: the molecular weight of 910, 460 and 358 g/mol for TYL, OTC and FF respectively; the absorption of 32%, 70% and 87% for TYL, OTC and FF respectively; and a volume of distribution of 0.69, 1.5 and 3.3 for TYL, OTC and FF respectively. Therefore, these three factors could be used to predict WDT lengths of drugs which have not determined this parameter for eggs.

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5.16.

Modulation of tyrosine aminotransferase activity by natural and synthetic glucocorticoids in a rat hepatoma cell line

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INTRODUCTION

The illicit use of glucocorticoids (GCs) as growth-promoting agents in cattle is still practiced within the EU. The use of cocktails of different active principles at very low dosage, or of new drugs not included in the list of the molecules subjected to chemical monitoring, results in the need to develop new biological approaches to detect GCs exposure, including cell-based *in vitro* systems (1). In this study we characterized the modulation by different GCs of the tyrosine aminotransferase (TAT) activity, a gluconeogenic enzyme known to be induced by GCs and well expressed in the H4-II-E-C3 rat hepatoma cell line.

MATERIALS AND METHODS

The H4-II-E-C3 cells were seeded at $3x10^6$ in 10-cm dishes and allowed to attach for 24 h. After replacement with fresh medium, cells were treated with DMSO alone (control) or with increasing logarithmic concentrations (from 10^{-12} to 10^{-6} M) of GCs, and harvested after 16 h. The tested compounds were dexamethasone (DEX), betamethasone (BETA), flumethasone (FLU), prednisone (PRED), prednisolone (PRDNSL), methylprednisolone (METHYL), cortisone (CORT) and cortisol (CORTL). TAT activity was assayed using a method previously described (2), and expressed as nmol/min/mg protein (mean ± SEM). EC50 values were calculated using Graphpad Prism.

RESULTS AND CONCLUSIONS

Dose-response curves for each compound showed that FLU exhibited the greatest TAT inducing potency (EC50 = 0.132 ng ml^{-1}), whereas CORT and PRED did not substantially affect enzyme activity. In good agreement with their glucocorticoid potency, BETA, METHYL, DEX, and PRDNSL caused a 4.6 to 66 fold lower induction compared to FLU. The endogenous GC CORTL displayed an inducing potency 2.5 times lower than PRDNSL. Interestingly, apart from PRDNSL and CORTL, the EC50 values of the investigated GCs were below the cut-off level of the official screening tests for corticosteroids (2 ng ml^{-1}). Such findings are in line with the results of a recently published report based on a CALUX bioassay (3). The GC-mediated increase in TAT activity in H4-II-E-C3 cells may represent a cost-effective and sensitive method for detecting synthetic GCs in biological fluids. Further investigations are underway to confirm the applicability of such bioassay under field conditions.

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Session 6: PK/PD Modeling

6.1.
Pharmacokinetic/pharmacodynamic relationships of a combination of amoxicillin and clavulanic acid against *Streptococcus suis, Pasteurella multocida* and *Actinobacillus pleuropneumoniae*.

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INTRODUCTION

Recently, a combination water soluble product containing amoxicillin (AMX) and the beta-lactamase inhibitor, clavulanic acid (CA) (Strenzen® – Elanco Animal Health) was approved in the European Union (EU) for the treatment of respiratory infections in pigs caused by *Streptococcus suis (Ss), Pasteurella multocida (Pm)* and *Actinobacillus pleuropneumoniae (App)*. The purpose of this paper was to compare the pharmacokinetics (PK) of AMX with the pharmacodynamics (PD) of AMX and CA in combination against various respiratory pathogens.

MATERIALS AND METHODS

Pharmacokinetic study (1) – The product was administered to give 10 mg of AMX/kg bodyweight (bwt) and 2.5 mg CA kg^{-1} bwt twice daily, via the drinking water for 5 days to two groups of 4 male and 4 female pigs of about 15 weeks of age. Blood samples were taken on the first and fifth day at 3 hourly intervals over each day. The plasma samples were assayed using LC-MS for CA and LC-MS/MS for AMX. *Pharmacodynamic studies* (2) – the minimum inhibitory concentrations (MICs) of AMX/CA against 182 EU isolates of *Ss*, 230 isolates of *Pm* and 220 isolates of *App,* using the CLSI broth microdilution methods, were recorded. *PK/PD relationships* – Time greater than the MIC50 and MIC90 (T >MIC) was used for comparison.

RESULTS

Pharmacokinetics – The mean C_{max} for AMX was 0.83 and 1.06 $\mu g\ ml^{-1}$ for day 1 and 5, respectively; the T_{max} was 9.8 and 9.0 h respectively and the AUC 24 h was 8.09 and 7.43 $\mu g\ h\ ml^{-1}$. *Pharmacodynamics* – the MIC50, MIC90 and MIC range for AMX/CA against *Ss* was 0.06, 0.125 and 0.06–0.25 $\mu g\ ml^{-1}$, respectively; against *Pm* 0.25, 0.25 and 0.12 – 4.0 $\mu g\ ml^{-1}$ and against *App* 0.25, 0.5 and 0.06–0.5 $\mu g\ ml^{-1}$, respectively. No resistance was reported in all the isolates tested (2) but one isolate of *Pm* showed reduced susceptibility to AMX/CA. *PK/PD relationships* – The T >MIC for *Ss* was 100% and 83%, respectively; for *Pm* was 63% and 63% and for *App* was 63% and 8%, respectively.

CONCLUSIONS

From these results, the combination should have an excellent therapeutic effect against *Ss* and *Pm* and up to the MIC50 of *App* (81% of the isolates) as T >MIC of 40% is considered the required minimum effective level. AMX is very rapidly absorbed and excreted (3) when given by gavage at 20 mg kg^{-1} bwt, the C_{max} was 3.14 $\mu g\ ml^{-1}$ and T_{max} was 1.19 h. This is in contrast to the findings in this PK study (1) when given in the drinking water. The mean C_{max} was comparatively low at 1.0 $\mu g\ ml^{-1}$ and the T_{max} was exceptionally long at 9 h based on a mean of 8 figures, or the population average. The sampling at 3 h intervals per day could easily have missed the individual peaks and troughs seen with gavage dosing or individual water intake and could underestimate the T >MIC for the MIC 90 of *App*.

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6.2.
Pharmacokinetic/pharmacodynamic and clinical relationships of valnemulin for the metaphylaxis of epizootic rabbit enteropathy

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INTRODUCTION

Epizootic rabbit enteropathy (ERE) is a complex gastrointestinal disorder of farmed rabbits, usually occurring after weaning and is characterised by inanition, bloating of the stomach, borborygmy, diarrhoea and mortality (1), which can reach 50% in untreated herds. *Clostridium perfringens* seems to be the dominant pathogen (2) and it is thought that toxin production may be the cause of the abdominal distension etc. Valnemulin hydrochloride (Econor® - Elanco Animal Health) has recently been approved for use against this disease.

MATERIALS AND METHODS

The concentration of valnemulin was determined in the caecal contents using a validated LC MS/MS method following administration of valnemulin for 35 days in feed at 60 ppm. The minimum inhibitory concentration (MIC) of valnemulin was determined against 74 rabbit isolates of *C. perfringens* from Italy, France and Spain. A number of clinical trials were carried out for prevention, early treatment and later treatment, when clinical signs of disease had already developed. Valnemulin at 20, 35 and 60 ppm was administered via the feed and compared with untreated controls.

RESULTS

Pharmacokinetics – the concentration of valnemulin in the caecal contents was 3.8 $\mu g\ g^{-1}$ at 60 ppm, equivalent to 2.2 $\mu g\ g^{-1}$ for 35 ppm and 1.3 $\mu g\ g^{-1}$ for 20 ppm. *Pharmacodynamics* – the MIC 50 was 0.125 $\mu g\ ml^{-1}$, MIC 90 was 0.5 $\mu g\ ml^{-1}$ and MIC range 0.031–64 $\mu g\ ml^{-1}$ (9.5% resistance – MIC $\geq$ 4.0 $\mu g\ ml^{-1}$). *Clinical effect* – in an artificial challenge prevention study, the rabbits were given the medication before infection and completely prevented mortality in the 20, 35 and 60 ppm valnemulin treated groups but was associ-

ated with ERE mortality in 19% of the untreated rabbits. In a treatment study, where the rabbits were breaking down with a natural infection there was little difference between the treated and untreated rabbits with a mortality of 22%. In those rabbits that were not showing clinical signs when treatment started, mortality was reduced to 6% in the 20 and 35 ppm groups but 12% in the 60 ppm group in comparison with the untreated controls with 14% mortality. In a further early curative treatment study, the mortality in the 20 and 35 ppm groups was 11% and 7.5%, respectively, which were significantly better than the non-medicated controls at 23%.

CONCLUSION

Valnemulin at 20 ppm and 35 ppm (approximately 3 mg kg^{-1} bwt day^{-1}) was highly effective in controlling ERE when given before the clinical signs of the disease had developed (metaphylaxis). Higher inclusion levels showed no additional benefits.

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6.3.

Comparison of iohexol and endogenous creatinine clearance as estimators of glomerular filtration rate in piglets at different age categories

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INTRODUCTION

The glomerular filtration rate (GFR) is considered to be a very important parameter of the renal function. In clinical routine, equations based on serum and urinary creatinine concentrations are still commonly used to determine the GFR despite their disadvantages. These disadvantages include confounding variables such as high protein intake and inability to detect mild kidney disease. Especially in the paediatric subpopulation, validated methods and adapted formulas to accurately determine the GFR function are lacking (Gretz *et al*., 2007). Over the last decade, iohexol has proven to be a sensitive and selective marker for GFR. Iohexol is a non-radiolabeled contrast medium, which is cleared solely by glomerular filtration. It has already been successfully applied in both human and veterinary medicine and seems to be of particular interest in the paediatric population as well.

Since the renal function of pigs and humans display great similarities, the aim of this study was to assess the gender and age related differences in GFR in piglets based both on creatinine and iohexol clearance. These results might then have a predictive value for GFR determination in a human paediatric setting.

MATERIALS AND METHODS

The GFR was measured in 16 male and 16 female piglets at 8 days, 4 and 8 weeks of age. Each animal received an intravenous injection of 64.7 mg kg^{-1} BW iohexol (Omnipaque 300®). Blood and urine samples were taken before and at different time-points after injection. Creatinine concentrations in plasma and urine were obtained with an enzymatic assay. Endo- and exo-iohexol concentrations were measured by an in-house validated high-performance liquid chromatography method with ultraviolet detection (De Baere *et al*., 2012). The area under the plasma concentration time curve from time 0 to 24 h ($AUC_{0-24\ h}$) was determined by WinNonlin 6.3. Whereafter, the clearance of endo- and exo-iohexol was calculated.

RESULTS AND CONCLUSION

The results will be presented at the EAVPT congress.

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6.4.

A quantitative approach to analysing hydrocortisone response in the horse

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INTRODUCTION

The hydrocortisone response to glucocorticoid intervention has in spite of extensive investigation in horses not been fully characterized with regards to the determinants of onset, intensity and duration of response.

MATERIALS AND METHODS

Robust and quantitative pharmacodynamic information on hydrocortisone response were derived from a study of constant rate infusion regimen of dexamethasone (control, 0.17, 1.7 and 17 $\mu g\ kg^{-1}$) to six Standardbreds. Plasma was analysed for dexamethasone and hydrocortisone concentrations using UPLC-MS/MS (LLOQ for dexamethasone: 25 ng l^{-1}). A two compartment model was simultaneously fitted to dexamethasone data from all dose levels. The derived parameters then served as constants 'driving' the hydrocortisone response turnover model.

RESULTS

The turnover model captured the oscillatory behaviour in data well when simultaneously fitted to all dose groups. The system (turnover rate and fractional turnover rate) and the drug (potency efficacy and sigmoidicity factor) properties were quantified for all horses. The amplitude parameter was in the range

6.8–24 μg l^{-1}, R_0 34–57 μg l^{-1}, k_{out} 0.47–1.5 h^{-1}, I_{max} 0.77–0.97, IC_{50} 6–65 ng l^{-1} and the sigmoidicity factor 0.7–30. The model predicted a high and between individuals variable concentration-response relationship.

DISCUSSION AND CONCLUSIONS

The pharmacodynamic drug parameters (IC_{50} and I_{max}) and system parameters (R_0, k_{out} and amplitude parameter) were estimated with acceptable precision. This analysis has demonstrated a better understanding of factors governing the time-course of hydrocortisone response. This includes factors behind baseline variability within and between horses (frequency and amplitude) and determinants of the equilibrium concentration-response relationship.

6.5.

Pharmacokinetic and pharmacodynamic (PK/PD) evaluation of cefazolin in dog

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OBJECTIVE

Cefazolin is a first generation cephalosporin that is frequently used prophylactically to prevent infections in small animal surgical patients. The aim of the study was 1) to evaluate the pharmacokinetics (PK) of cefazolin in dogs undergoing gonadectomy, 2) to correlate the PK parameters with literature data of minimum inhibitory concentration (MIC) for microbial strains commonly present in surgical environment and 3) to verify the efficacy of the dosage regimen used by a PK/PD correlation.

MATERIALS AND METHODS

9 dogs (22 ± 7.5 kg and 1.3 ± 0.7 years old) were IV treated with 25 mg kg^{-1} of cefazolin soon after induction. Blood samples were taken at prefixed times from 0 to 8 h after treatment. Cefazolin concentrations were quantified by a validate HPLC method with UV detection [1] and were analysed by dedicated software. A two-compartmental model best described the pharmacokinetic profile of IV cefazolin in dog. Available literature for MIC_{50} against canine *Pasteurella* spp, *Staphylococcus* spp., *Streptococcus* spp. ranged from 0.25 to 0.5 mg ml^{-1} [2].

RESULTS AND CONCLUSIONS

Area under the curve was 182.3 ± 50.6 h*μg ml^{-1}, half-lives of distribution and elimination were 0.3 ± 0.2 h and 3 ± 1.6 h, respectively. Clearance and volume of distribution at steady state were 150.2 ± 49.8 ml h kg^{-1} and 383.5 ± 58.1 ml kg^{-1}, respectively. The PK/PD correlation, calculated by time above MIC (T>MIC), resulted for the 100% of 8 h observation period from 2 to 6 times higher the highest MIC value of 0.5 μg ml^{-1}. The PK/PD index for beta-lactams efficacy is T>MIC for 60% of the dosing interval with values at least 4 times higher the MIC. Our results showed a good efficacy of cefazolin against all the strains considered. A dosage regimen of 8 h would represent a valid therapeutic option for the limitation of microbial diffusion during surgery. Nevertheless, to limit the spread of resistance and to monitor the efficacy of the treatment, it is advisable to periodically evaluate the MIC values of strains isolated in the surgical facilities.

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6.6.

In vitro Pharmacokinetic/Pharmacodynamic testing of three oral administration of Marbofloxacin at a dose of 7 mg kg^{-1} against *Bordetella bronchiseptica* strains isolated from rabbit respiratory disease

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INTRODUCTION

Marbofloxacin is a fluoroquinolone antimicrobial agent with a broad spectrum of activity used in veterinary medicine for the treatment of respiratory infections in companion animals. The aim of this study was to evaluate the activity of marbofloxacin against pathogenic *Bordetella bronchiseptica* strains, isolated from rabbit respiratory infections, using an *in vitro* dynamic system (Vallé 2011) reconstituting the drug concentration-time profile obtained *in vivo* in rabbit plasma, after three oral administrations of marbofloxacin, every 12 h, at a dose of 7 mg kg^{-1}. The impact of an oral marbofloxacin treatment on the potential development of resistance was also evaluated.

MATERIALS AND METHODS

Pharmacokinetics were determined in 6 rabbits after an oral dose of 7 mg kg^{-1}, twice a day during 7 days, using the 1% solution (Marbocyl® FD). The concentration-time profile of the drug was reconstituted after 3 oral administrations, given 12 h apart in the *in vitro* system. *In vitro* dynamic killing curves were obtained against two susceptible isolates of *B. bronchiseptica* with a marbofloxacin MIC of 0.25 (modal class) and 1 μg ml^{-1} (close to the MIC_{90}) respectively (Rougier 2006). The dose of 7 mg kg^{-1} given twice a day resulted from a PK/PD integration using a MIC against *B. bronchiseptica* of 0.25 μg ml^{-1} (modal class).

RESULTS

For the *B. bronchiseptica* strain with a MIC of 0.25 μg ml^{-1}, after the first administration a decrease of total bacterial count was observed with level reaching ≈3 Log_{10} CFU ml^{-1} at 12 h. The second administration of marbofloxacin controlled the inoculum size although a slight regrowth occurred with a bacterial load of 4.71 Log_{10} CFU ml^{-1} at 24 h. After the third administration, the inoculum size was stable during the next 12 h.

A bacteriostatic effect of marbofloxacin was observed against *B. bronchiseptica* strain with a MIC of 1 μg ml^{-1} over the all three administrations.

No resistant *B. bronchiseptica* isolates appeared for both strains during the *in vitro* dynamic testing simulating three oral administrations of marbofloxacin.

CONCLUSION

A repeated oral administration of marbofloxacin at a dose of 7 mg kg^{-1} twice a day for several days is likely to control the bacterial burden of *B. bronchiseptica* during respiratory infections in rabbit without promoting the emergence of resistance. This kind of treatment coupled with animal immune activity and sputum system, may be effective.

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6.7.

Pharmacokinetics and effects of methadone after intravenous administration in horses

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INTRODUCTION

In treatment of nociceptive pain opioids are highly effective and the full μ-agonists are known to have highest efficacy. Methadone is a synthetic μ-agonist/NMDA-antagonist used in horses. The aims of this study were to investigate the pharmacokinetics and effects of methadone after intravenous administration in order to gain more knowledge and to optimize analgesic treatment in horses.

MATERIAL AND METHODS

The study was a randomized blinded placebo controlled with *cross over* design. Eight Standardbred horses were treated with methadone IV (0.2 mg kg^{-1}) or placebo (saline) given in a total volume of 20 ml during 5 min. Blood samples were obtained at fixed intervals up to 22 h after administration and the plasma concentrations of methadone were quantified with liquid chromatography–electrospray ionization–tandem mass spectroscopy (LC–ESI–MS/MS). The pharmacokinetics and the effects of methadone on behavior, respiratory rate, heart rate and hematocrit were examined. Analgesia was evaluated using a thermal threshold testing system adopted for use in horses (Topcat Metrology).

RESULTS

The mean terminal half-life was 1.43 ± 0.58 h, the apparent volume of distribution 0.95 ± 0.27 l kg^{-1}, and total body clearance 0.71 ± 0.16 ml h kg^{-1}. The horses displayed behavioral changes such as staggering for a short period, head tremors and looking vigilantly around after methadone administration but never when treated with saline. The behaviors licking, nodding head, picking hay, tail flapping, skin twitching and scraping with their front leg were more frequent after treatment with methadone compared to control. There were no differences between treatments in respiratory rate, heart rate or hematocrit. The thermal threshold was elevated during the first hour when treated with methadone compared to saline ($P = 0.001$). At one hour after administration the methadone plasma concentration was 88 ± 21 ng ml^{-1}.

DISCUSSION/CONCLUSION

The methadone plasma half-life and duration of the analgesic effect were short. This indicates a short dosing interval or a constant rate infusion to maintain analgesia over an extended period of time.

6.8.

Methadone pharmacokinetics and effects in combination with detomidine in horses

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INTRODUCTION

Methadone is a synthetic opioid that is used to treat pain in horses. Opioids are effective in treatment of nociceptive pain but may also cause non-wanted effects such as excitement or respiratory depression. The α_2-agonists are often used in horses for sedation and analgesia both separately and in combination with opioids. A combination of methadone and the α_2-agonist detomidine may be useful in pain management but the influence of detomidine of the pharmacokinetics of methadone is not well studied. The aims of this study were to investigate the pharmacokinetics and effects of methadone IV in combination with detomidine IM in order to optimize analgesic treatment in horses.

MATERIALS AND METHODS

The study was a randomized blinded placebo controlled with *cross over* design. Eight Standardbred horses were treated with methadone IV (0.1 mg kg^{-1}) in a total volume of 20 ml over 5 min together with detomidine IM (0.01 mg kg^{-1}) or equivalent volumes placebo (saline) IV and IM. Blood samples were obtained at fixed intervals and the plasma concentrations of methadone were quantified with LC–ESI–MS/MS. The pharmacokinetics and the effects of methadone together with detomidine on behavior and respiratory rate were examined. Analgesia was evaluated using a thermal threshold testing system adopted for use in horses.

RESULTS

The mean terminal half-life of methadone was 1.42 ± 0.97 h, the apparent volume of distribution 0.69 ± 0.21 l kg^{-1}, and total body clearance 0.59 ± 0.16 ml h^{-1} kg^{-1}. During the

first one to three hours after methadone/detomidine administration the horses showed drowsiness. They snored and the head was dropped or supported against the wall or the crib and a few horses were sweating. Those effects were not detected when treated with saline. The respiratory rate was lowered between one and three hours after administration of the drugs compared with placebo ($P = 0.0002$–0.02). The thermal threshold was elevated for up to two hours when combining methadone/detomidine compared to control ($P = 0.001$–0.02). At one hour after administration the mean methadone plasma concentration was 46 ± 19 and at two hours 21 ± 7 ng ml^{-1}.

DISCUSSION

The methadone plasma half-life was short but duration of the analgesic effect seems to be extended by combination with detomidine compared with methadone *per se* indicating that analgesia could be prolonged by detomidine. The combination prevented excitement caused by opioid exposure but induced decreased respiratory rate.

6.9.

PK/PD modelling of oxytetracycline for pneumonia pathogens

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INTRODUCTION

Tetracyclines are remarkable drugs in that they: (1) have remained in widespread use in farm animal medicine for more than 50 years; (2) possess broad spectrum of antimicrobial activity; (3) may act additionally to reduce bacterial pathogenicity and/or virulence; (4) exert other potentially beneficial actions (immunomodulatory, anti-inflammatory) on the host. Clinical and bacteriological efficacy may depend on several of these properties. We reported on the microbiological (Minimum Inhibitory Concentration, Minimum Bactericidal Concentration, time-kill profiles) of oxytetracycline for calf and pig pneumonia pathogens at the AAVM 2014 Congress. MICs were some 25-fold greater in serum than in Mueller Hinton Broth (MHB) and time-kill profiles suggested a co-(concentration and time) dependent killing action.

METHODS

Based on the sigmoidal E_{max} equation, PK/PD modelling of oxytetracycline time-kill curves for six isolates each of the calf pneumonia pathogens, *M. haemolytica* and *P. multocida*, was conducted in MHB and calf serum to establish $AUC_{0-24\ h}$/MIC breakpoint (BP) values for growth inhibition. BPs were used with: pharmacokinetic (clearance and bioavailability) data; serum protein binding data and; literature MIC distributions in Monte Carlo simulations to predict 50 and 90% Target Attainment Rate (TAR) dosages for single dose administration and for daily dosing at steady state. Parallel studies were conducted on pig pneumonia pathogens.

RESULTS AND CONCLUSIONS

The MIC literature distributionfor calf pathogens was bimodal. Mean serum protein binding was 53% and independent of concentration. $AUC_{0-24\ h}$/MIC serum BPs for *M. haemolytica* were 19.1 ± 18.3 h bacteriostatic and 27.5 ± 16.0 h bactericidal, slope = 8.2. Corresponding values for *P. multocida* were 28.0 ± 3.4 h bacteriostatic and 60.9 ± 12.7 bactericidal, slope = 4.6. Inter-isolate variability was greater for *M. haemolytica*. For calf pathogens, the predicted 90% TAR dosages were very high for both bacteriostatic and bactericidal actions, due to the bimodal MIC distribution. Even for 50% TAR dosages, it is concluded that the clinical efficacy of oxytetracycline in severe infections and for an empirical antimicrobial therapy may not solely depend on its direct killing actions. Efficacy of recommended doses in mild infections and the contribution of the host's immune mechanisms to therapeutic outcome will be discussed.

Session 7: Endocrine Disruptors

7.1.

4 week oral toxicity study of a combination of two pesticides in rats: comparison with the toxicity of the individual compounds

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INTRODUCTION

Exposure to pesticides affects many body organs including reproductive system. Disorder of the reproductive system leads to infertility and therefore has been in the center of attention within the recent decades. Adverse effects of pesticides on the male reproductive systeme specially semen characteristics are an important health problem in all the world. Several international studies have been conducted on causes of endocrine disrupters, one of the most famous are pesticides, showing evidence of reduction in semen quality due to agricultural pesticides. The aim of this study was to determine the toxicity of two widely used fungicides propiconazole and propineb both separately, and in combination in the possible reproductive adverse effects and oxidative damage induced in an animal model.

MATERIAL AND METHODS

Twenty eight male Wistar rats were used and divided into four groups of seven each: group I served as control and received distilled water as vehicle, group II rats were treated with propiconazole at a dose of 60 mg kg^{-1} body weight (1/50 of the oral LD50), group III was treated with 100 mg kg^{-1} body weight propineb (1/50 of the oral LD50), Group IV was treated with both propiconazole 30 mg kg^{-1} day^{-1} b.w and .pPropineb 50 mg kg^{-1} day^{-1} b.w. All the animals were treated orally by gavage daily. At the end of the experimental period (28 days) the animals were sacrificed, semen was collected and desired organs (testes and epididymis) were removed and weighted.

RESULTS AND DISCUSSION

The results indicated that the fungicide and their mixture were toxic in the treated animals and revealed a significant reduction in the weight of testes, epididymis and also in the number and mobility of sperm, accompanied with a significant decrease in morphological changes of flagellum in the treated groups compared to control group, Histological changes were observed in the testis and epidydimis in the groups treated especially those exposed to propiconazole and the mixture. Reduced glutathione (GSH) and Glutathione peroxidase (GPx) level was decreased in testicle in all treated groups. In conclusion we think that the repeated administration of fungicides used alone or in combination with the used doses in the same conditions by gavage may cause structural and functional disorders in the hormonal system.

Session 8: Workshop Pharmacovigilance

8.1.

Veterinary Pharmacovigilance: drugs traceability and antimicrobial consumption. A pilot study in Piedmont Region

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INTRODUCTION

The development of antimicrobial resistance in the last decades has led to an intensification of discussion about the prudent use of antimicrobial agents, both in veterinary and human medicine. To preserve the usefulness of antimicrobials in people, the WHO recommends avoiding the use of antimicrobials considered to be 'critically important' for human medicine (fluoroquinolones, 3rd and 4th generation cephalosporins) in food animals [1]. This recommendation derived from the observation that both humans and animals seem to demonstrate a positive association between antimicrobials consumption and the increasing of antibiotic resistance of bacteria.

MATERIAL AND METHODS

Antimicrobials sales data are continuously collected through the ESVAC project, which annually collects harmonized data about veterinary drugs [2]. However, it is now considered that the sales data are not a suitable indicator, because it seems that they do not correspond to the real drug use. The Regional Center of Veterinary Pharmacovigilance has conducted a pilot study in the Piedmont Region to trace veterinary recipes for food-producing animals. The voluntary project, named TO-BE, involved veterinarians, pharmacists, wholesalers, and feed producers.

RESULTS

The stakeholders entered veterinary recipes data in a computer software created for this purpose. During 10 months of testing, data were collected, plotted and extrapolated from 24 000 recipes for veterinary drugs and 3200 recipes for medicated feeds. Data were classified by antimicrobial class or sub-class. In order to standardize the antimicrobial consumption, with a specific correlation with animal population data, the Population Correction Units (PCU) method was used.

DISCUSSION

There are several reasons for tracing drugs and monitoring antimicrobial resistance: 1) to obtain data that will help the practitioner to choose the right drug for the patient 2) from a public health point of view, to attempt to protect consumers 3) long-term surveillance data are necessary to evaluate the impact of any intervention 4) finally, there is a need to harmonize and standardize the surveillance methods within the European Union (EU). Such these objectives are not easy to realize due to the lack of financial resources and differences in the health services of each country in EU, but the method proposed in the present project could be a useful tool.

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8.2.

Retrospective survey on adverse events of veterinary drugs in animal in the regions of Dosso, Niamey, and Tillabéri (Niger)

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INTRODUCTION/OBJECTIVES

In Niger, there is no veterinary pharmacovigilance system to survey and evaluate adverse drug events. The aim of this study is to inventory suspected cases of veterinary drugs adverse reaction and lack of efficacy in this country.

MATERIALS AND METHODS

This study consisted of a retrospective survey (1990–2013) by questionnaire with 23 of the 46 animal health professionals in three regions of Niger (Niamey, Dosso and Tillabéri). The questionnaires were presented and answered from August to November 2013.

RESULTS

The survey allowed to inventory 90 cases of suspected adverse reactions (24%; $n = 22$) and lack of efficacy (76%; $n = 68$) of veterinary drugs occurring only in food-producing animals, reported by 18 health professionals. The therapeutic classes receiving the most complaints are antibiotics (46%; $n = 41$) and antiparasitics (36%; $n = 32$). Among the 2586 treated animals concerned by these 90 cases, 66% were reacting.

CONCLUSIONS

In view of these results, it is necessary to establish a system of pharmacovigilance in Niger for continuous monitoring and analysis of adverse events of veterinary drugs.

8.3.

A 24-year retrospective survey on suspected adverse reactions in animals of veterinary medicines in Lomé and Dapaon, Togo

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INTRODUCTION/OBJECTIVE

Veterinary pharmacovigilance concerns monitoring, evaluating and improving the safety of veterinary medicines, with particular reference to adverse events in animals and human beings

related to the use of these medicines. But there is no veterinary pharmacovigilance system to survey and evaluate adverse events of veterinary products occurring in Togo. This study aimed to inventory suspected adverse reactions in animals of veterinary medicines in two town of this country.

MATERIALS AND METHODS

This study consisted of a retrospective survey (1990–2013) by questionnaire with 15 animal health professionals in Lomé and Dapaon, two towns of Togo. The questionnaires were presented and answered from August to October 2013.

RESULTS

Only Twenty cases of suspected adverse reactions of veterinary medicines in animals were reported by 11 veterinarians. Of the 20 cases of suspected adverse reactions, 16 were for companion animals and 4 for food-producing animals. Among the 79 treated animals concerned by these cases, 25 were reacting. The suspected adverse reactions have been reported especially in dog (canine). The most complained veterinary medicines were Alimentary tract and metabolism (QA), according to the veterinary anatomical therapeutical chemical (ATCvet) coding system of products.

CONCLUSIONS

It is necessary to establish veterinary pharmacovigilance system for continuous monitoring and evaluating adverse events in animal of veterinary products in Togo.

Session 9: Contaminants

9.1.

Food-toxicological assessment of heavy metal content in the muscle of Roe deer (*Capreolus capreolus*)

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Heavy metals emitted from industry, vehicles or other sources can contaminate the soil and thereby the plants that will be consumed by humans and animals. The feedstuff given to food-producing animals like cattle can be effectively controlled for the presence and concentration of heavy metals. But for wild animals it is more difficult or even impossible to control the whole diet to estimate the intake of unwanted substances. It seems that the best option for controlling the safety of the feed of wild animals is to investigate their edible tissues that are used for human consumption. The aim of this study was to determine the concentrations of the heavy metals such as arsenic, mercury and lead in the muscle tissue of Roe deer.

The study was performed on 20 (10 males, 10 females) Roe deer (*Capreolus capreolus*). The muscle samples were taken from *m. biceps femoris* after shooting during the regular hunting season on a hunting area close to Eger in Hungary, then they were immediately removed from each deer without external contamination. The determination of heavy metal contents was carried out by ICP-MS. The statistical analysis was performed by SPSS 11.0.

Based on our results the measured levels (mg kg^{-1}) of arsenic (0.271 ± 0.201), mercury (0.867 ± 0.402), lead (0.480 ± 0.212) in the meat do not pose any health risk for the human consumers according to PTWI and other official regulations. Some values were above the European average, but the concentrations were as high that would cause any harmful effect. However, due to man-made hazards (e.g. industrial pollution), the environment in which the Roe deer reside can be contaminated and this could lead to accumulation of heavy metals in their tissue. The increased concentrations of heavy metals in the tissue of the Roe deer may derive from their elevated levels in the feed (e.g. mosses, fungi; 1, 2) at the site where the samples were collected. Similarly, the use of lead-based ammunition by hunters can lead to high concentration of lead in the tissue of the hunted animal, and the hunters must take extra precautions to avoid ingestion of tissues contaminated from the bullet (3). The prolonged intake of the highly contaminated foods of animal origin (muscle, liver and other edible tissues) can lead to accumulation of heavy metals in the human body and can induce latent alterations or even chronic toxicosis.

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9.2.

Cadmium and lead in Great Cormorants from the Cuneo area, Northern Italy

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INTRODUCTION

Lead (Pb) and cadmium (Cd) are nutritionally non-essential elements, and are not controlled *in vivo* by homeostasis. These elements have a long biological half-life so accumulate in the body with age, as a result of increasing levels of exposure in the environment. The common cormorant (*Phalacrocorax carbo*) habitats are inland rivers and lakes, and coastal regions, but some cormorant groups live near urban areas. We measured Cd and Pb in feathers and livers of common cormorants from a Piedmont (North Western Italy) province where they are local breeding residents. Differences in gender and age were considered.

MATERIALS AND METHODS

A total of 44 great cormorants (27 adults and 27 juveniles) were collected under license from the province of Cuneo, close to rivers and lakes and near to fisheries. Detection of Cd and Pb was performed using GFAAS. Accuracy of analysis was tested using certified reference material NRCC-DORM-2 Dogfish muscle.

RESULTS

Cd and Pb levels were detected in livers with the following concentrations: Cd 0.16 mg kg^{-1} in juveniles, 0.20 mg kg^{-1} in adults; Pb 0.25 mg kg^{-1} in juveniles, 0.66 mg kg^{-1} in adults. In feathers: Cd 0.04 mg kg^{-1} in juveniles, 0.06 mg kg^{-1} in adults; Pb 4.49 mg kg^{-1} in juveniles, 3.36 mg kg^{-1} in adults. Liver Cd levels were higher in juvenile males while liver Pb levels were higher in juvenile females. In feathers, there were no differences between genders for Cd, while Pb was higher in juvenile males although in adults, males (2.45 mg kg^{-1}) had lower lead values than females (4.67 mg kg^{-1}).

CONCLUSIONS

There were no age or gender differences in cadmium in feathers, due to the similar fish diet. In the liver, juvenile males had higher levels than females, probably reflecting differential pathways of metal excretion. Significant age differences were

found in liver Pb contents, which show that cormorants accumulated this heavy metal during their lifetime, despite the fact that Pb can be partially eliminated during the process of seabird feather growth (Jerez *et al.* 2011). Therefore, the maximum Pb levels were recorded in feather samples (5.66 mg kg^{-1}). In fact, some birds exceeded the feather Pb level of 4 mg kg^{-1}, which is known to cause adverse effects and is associated with delayed parental and sibling recognition, impaired thermoregulation, locomotion, and feeding behavior, as well as reduced chick survival (Burger *et al.*, 2008).

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9.3.
Heavy metal levels in griffon vulture feathers from the North Western Italian Mountains

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INTRODUCTION

Contamination is a global phenomenon, and transmission of persistent pollutants may even occur in remote areas (Markowski *et al.,* 2013). Recently, there has been an increasing interest in the use of sentinel organisms for pollution monitoring studies (Burger *et al.,* 2008). Among these, birds have been a particular focus of interest (Roux and Marra, 2007) because they are highly exposed to heavy metals, both by environmental exposure and diet. Feathers provide a potentially useful biomonitoring option in studies regarding pollution exposure in avian species. Here, data are presented for two individuals of large scavenging raptor species, the griffon vulture (*Gyps fulvus*), found dead and collected from the Italian North Western Mountains (Val di Viù).

MATERIALS AND METHODS

Surface lipids and contaminants were removed from the feathers that were subjected to microwave digestion with 7 ml of HNO_3 and 1.5 ml of H_2O_2. Multi-elemental determination was performed using ICP-MS. The quantification limit (LOQ) for all elements was set at 0.01 mg kg^{-1}.

RESULTS

The following relationships between trace elements were observed: Al>Fe>Mn>Zn>Pb>Cu>Cr>Ni>As>Se>Sn>Tl. Mean concentrations were the following Al: 4694.7 mg kg^{-1}; Fe: 3271.8 mg kg^{-1}; Mn: 89.0 mg kg^{-1}; Zn: 79.2 mg kg^{-1}; Pb: 60.7 mg kg^{-1}; Cu: 7.3 mg kg^{-1}; Cr: 7.2 mg kg^{-1}; Ni: 4.7 mg kg^{-1}; As: 1.7 mg kg^{-1}; Se: 0.8 mg kg^{-1}; Sn: 0.4 mg kg^{-1}; Cd: 0.1 mg kg^{-1}; Tl: 0.1 mg kg^{-1}.

CONCLUSIONS

There is usually a high correlation between metal levels in the bird's diet and detected metal levels in their feathers. This is related to the relatively high proportion of the body burden of certain metals being excreted to feathers. The griffon vulture is a large bird of prey, a scavenger that feeds mostly on carcasses of dead domestic livestock and, to a lesser extent, on wild species found dead in the environment. We found elevated concentrations of aluminum and iron in griffon feathers in comparison with the little data in the literature that is available for the same species. Many historical mines were located in the surrounding valley and their presence could have resulted in an increase of the levels certain contaminants, such as iron and aluminum in the environment. We suggest that the measured concentrations are indicative of environmental exposure to persistent contaminants, which may pose a threat to raptors in the studied area.

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9.4.
The incidence of non-dioxin-like polychlorinated biphenyls (NDL-PCB) in fat tissues of great cormorants from the River Roja, Northern Italy

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INTRODUCTION

Wild birds are exposed to pollutants in their habitats. A recent EFSA report showed that y high levels of non-dioxin-like PCBs (NDL-PCBs) can be found in fish and fishery products (EFSA, 2010). Great cormorants are piscivorous predators at the top of the food chain, and therefore they tend to accumulate high levels of contaminants. In 2012, the presence of NDL-PCBs exceeding the legal limit in some fish samples was reported by the French authorities in the Roja River, and resulted in a ban of fishing at the French side of the river. We investigated the residue levels of NDL-PCBs in fat tissues of four great cormorants from an Italian branch of the River Roya (Airole district).

MATERIALS AND METHODS

The quantification of NDL-PCBs in fat tissues was performed by adapting the method of Perugini (2004). The six indicators 28, 52, 101,138, 153 and 180, and their cumulative analytical concentration (Σ6 PCBs) were quantified by GC/MS coupled to a DSQ single quadruple mass spectrometer. In line with European regulation (EU 1259/2011), Σ_6 NDLPCB was expressed as 'upper bound' (UB) concentrations, on the assumption that all values of the different congeners below the LOQ are equal to the LOQ.

RESULTS

NDL-PCBs were detected in all the analyzed samples. The average concentration of the sum of the six indicator PCBs was 3997.73 ng g^{-1}. The individual NDL-PCBs followed the pattern: PCB-153 > PCB-138 > PCB-180 > PCB 101 > PCB-28 > PCB-52, with the following mean values: 1753.00 ng g^{-1}; 1621.00 ng g^{-1}; 537.20 ng g^{-1}; 51.35 /ucodep>ng g^{-1}; 28.55 ng g^{-1}; 6.50 ng g^{-1}. These results are in line with findings reported in other studies, which have demonstrated that PCB-153 has an average contribution of approximately one third to the sum of the six indicator PCBs (EFSA, 2005; Squadrone *et al.*, 2013).

CONCLUSIONS

Toxicological data indicate that NDL-PCBs alter a number of physiological processes that are important during the development of the species, in particular in the nervous and endocrine systems. The European Union has undertaken short- and long-term actions, aimed at reducing environmental contamination and human exposure, which have recently been extended to incorporate NDL-PCBs. Our analyses of NDL-PCBs in fat tissues of great cormorants from the River Roya assess the presence of these organic compounds in this area and the biomagnification phenomena that occurs in major predator of the freshwater food chain.

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9.5.

Effect of lanthanum and cerium on the growth of colorectal and hepatic cancer cell lines

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INTRODUCTION

Lanthanides are a unique group of rare earth elements (REE) with extensive applications in industrial and agricultural fields. In view of the increasing application of lanthanides for improving animal growth and medical practices, it is becoming necessary to obtain in-depth information on their environmental toxicity in impact on humans (Dai *et al.*, 2002; Xue *et al.*, 2009). We explored the effects of different dosages of lanthanum (La) and cerium (Ce) on cell viability and proliferation in human cancer cells.

MATERIALS AND METHODS

Human colorectal (HT-29) and hepatocellular (HepG2) cancer cell lines employed in our study. Cells were plated in 96-well plates, and treated with increasing concentrations of lanthanides (0.1 μM–10 mM), alone or in combination, for 24, 48 and 72 h. Effects on cell proliferation were measured using MTT colorimetric assay. Cell mortality and type of cell death was determined by Annexin V binding to phosphatidyl serine at the cell surface of apoptotic cells using flow cytometry.

RESULTS

Concentrations of La and Ce between 200 μM and 10 mM significantly inhibit cell growth. In HT-29 cells, Ce was able to completely abolish cell proliferation after 24 h, while La exerted a dose-dependent inhibitory effect; in HepG2 cells, both elements significantly reduced cell viability at high doses (500 μM, 1 mM) at all the times considered. Conversely, at lower concentrations an improvement in the proliferation rate in both cell lines was observed. When the cells were incubated with a mixture of La and Ce, in HT-29 cells, high concentrations (500 μM, 1 mM) of lanthanides increased the inhibitory effects; in HepG2 cells, low concentrations of the mixture exerted a strong growth-promoting effect, while at 200 μM concentration, the inhibitory effect was significantly reinforced.

CONCLUSIONS

The effects of La and Ce on cell growth may depend upon the doses rather than the type of lanthanides applied. The dosage represents the pivotal factor for switching the biological effects of lanthanides from down-regulation to up-regulation of cell growth; thus, low concentrations are able to promote cell survival and proliferation, but when concentrations increased, the drugs exert anti-proliferative and cytostatic/cytotoxic effects. The molecular mechanisms underlying these effects at still not well defined and further analysis of the mechanisms which result in inhibition or induction of cell proliferation is crucially important.

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9.6.

Pig jejunal explants: an *ex-vivo* model reducing animal experimentation (3Rs) for studying the effects of feed contaminants on the intestinal mucosa, alone and in combination

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INTRODUCTION

In the context of implementing the 3Rs by reducing the numbers of animals used, we developed pig jejunal explants for studying the effects of mycotoxins. Deoxynivalenol (DON) and nivalenol (NIV) are type B trichothecenes fusariotoxins, contaminating cereals worldwide, and targeting intestinal mucosa (Pinton *et al*, 2009, 2012). Pig jejunal explants were used to characterize the effects of DON and NIV, alone or in combination, on the intestinal tissue of pig, the most sensitive animal species.

MATERIALS AND METHODS

Crossbreed weanling piglets of 4–5 week-old (n = 6) were used for explanting jejunal tissue (Kolf-Clauw *et al*., 2009). Explants were exposed to DON, NIV, and the mixture DON+NIV (1:1) for 4 h, at 0.1 to 30 μM for each mycotoxin or for the mixture 1:1. Mucosal lesions were assessed by using histopathological scores. Realistic *in vitro* concentrations compared to pig *in vivo* digestive exposure to contaminated feed were used.

RESULTS

The individual treatment with the mycotoxins DON and NIV resulted in a significant impact on histopathological scores from doses of 3 μM and 1 μM, respectively. The main morphological and lesional changes were flattening of epithelial cells, villi fusion, apical denudation of villi with the highest dose of NIV, villi with absence of epithelia were observed. The interaction effects were evaluated by the isobologram method. The DON+NIV combination demonstrated synergism for IC_{50} whereas antagonism was observed at the lower doses. Taken together, the present data provide strong evidence that NIV and DON mycotoxins alone or in combinations at low exposure alter the intestinal health. Our results are in accordance with previous investigations on intestinal and non-intestinal cells lines, showing less severe toxicity of DON compared to NIV.

CONCLUSION

Pig explants represent a sensitive model to assess the digestive barrier alterations following toxins exposure, allowing analysis of interactions between toxins.

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9.7.

Nivalenol has a greater impact than deoxynivalenol on intestinal mucosa in pig jejunal explants and in loops

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INTRODUCTION

Deoxynivalenol (DON) and nivalenol (NIV), two fusariotoxins and worldwide cereal contaminants, raise safety concerns for animal and human health. The intestinal mucosa is the first target following exposure to contaminated food or feed. The aim of this study was to investigate and compare the impact of DON and NIV on intestinal mucosa after acute exposure, *in vitro* and *in vivo*.

MATERIALS AND METHODS

Two alternative models from pig, the most sensitive species, were used, to reduce the number of animals, in order to analyze the histological changes in intestinal mucosa following DON and NIV exposure. Jejunum explants (from 6 pigs) and jejunum loops (from 3 pigs) were exposed to DON and NIV for 4-h *in vitro* (0, 1, 3 and 10 μM) and *in vivo* (10 μM), respectively.

RESULTS

On explants, dose-dependent increases in the histological changes were observed, from 3 μM for DON and from 1 μM for NIV. An almost two-fold increase in lesion severity compared to that of control explants was observed with 10 μM NIV, and more severe microscopic changes than with DON. On loops, NIV exposure had a greater impact on the mucosa than DON. The overall proliferative cells in the mucosa showed a 30% decrease after NIV exposure (13% decrease for DON), and the proliferative index of crypt enterocytes was significantly increased. Apoptosis at the top of villi increased after DON and NIV exposure, and the proliferative/apoptotic cell ratio was reduced by almost half for NIV.

CONCLUSION

Our study shows that NIV exposure had a greater impact on the intestinal mucosa than DON, both *in vitro* and *in vivo*. Our *in vivo* results also show that *lamina propria* cells (mainly lym-

phoid cells) are more sensitive than enterocytes (epithelial cells) to apoptosis induced by acute NIV exposure.

9.8.
Dose additivity for a mixture of six Type II pyrethroids

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INTRODUCTION

Pyrethroids are increasingly used in a wide array of insecticide applications, including agriculture, medical, veterinary, aquatic systems and home pest control. Despite the widespread use of pyrethroid insecticides that led to common exposure in the population, few studies have been conducted to quantitatively assess dose-additive effects of pyrethroids using a funcional measure involved in the common toxic mode of action. The aim of this study was to evaluate the potency and efficacy of 6 Type II pyretroids to evoke induction of both nitric oxide and lipid peroxide levels measured as malondialdehyde (MDA) in an *in vitro* model as well as to test the hypothesis of dose additivity for mixtures of these same 6 pyrethroids.

MATERIAL AND METHODS

Human hepatoma HepG2 cell line was used as *in vitro* model in this study.

MTT assay and cell viability. Human HepG2 cell viability was measured by quantitative colorimetric assay with MTT as described previously (Denizot and Lang, 1986). The ED30 values for the pyrethroids α-cypermethrin, cyfluthrin, l-cyhalothrin, deltamethrin, cyphenothrin and esfenvalerate were calculated.

Determination of lipid peroxidation and nitrite measurement. Intracellular MDA as an indicator of lipid peroxidation was quantified using a thiobarbituric acid reactive substance (TBARS) assay kit (Cell Biolabs Inc., San Diego, CA). Changes in NO production were measured indirectly as the accumulation of nitrites (the end-product of NO metabolism) in the medium using Griess assay as previously described (Bauche *et al.*, 1998).

RESULTS AND CONCLUSIONS

MDA and NO production in human hepatoma HepG2 cells induced after the incubation with 4 mixtures of 6 pyrethroids, where 100% is the mixture of the ED30 of each pyrethroid (λ-cyhalothrin, α-cypermethrin, deltamethrin, cypermethrin, cyfluthrin, esfenvalerate), and the others are subsequent dilutions of this mixture (66%, 50% and 33%).

Malondialdehyde (μmol l^{-1}):

- Control →18.1 ± 0.78
- 33% → 24.0 ± 1.75*
- 50% → 40.4 ± 1.15***
- 66% → 41.7 ± 1.73***
- 100% → 52.9 ± 2.14***

Nitrite (μmol l^{-1}):

- Control: 0.98 ± 0.17
- 33% → 2.0 ± 0.21
- 50% → 2.3 ± 0.21*
- 66% → 2.9 ± 0.39***
- 100% → 5.0 ± 0.39***

Our results showed that the effects of mixtures of 6 Type II pyrethroids were additive on MDA and NO production, being the human hepatoma HepG2 cell line a sensitive *in vitro* model. Dose addition can be used as a means to predict the effects of pyrethroid mixture composed of low-level, equitoxic doses of individual chemicals, on MDA and NO production. These findings may provide a valuable contribution for risk assessment of these pesticides.

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9.9.
Deoxynivalenol in turkey poults: toxicokinetic study, absolute oral bioavailability and comparative biotransformation with broiler chickens

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INTRODUCTION

Deoxynivalenol (DON) is one of the most frequently occurring Fusarium mycotoxins. Although poultry are considered to be quite resistant to the effects of DON, differences in sensitivity between poultry species exist for DON (Girgis and Smith, 2010) and other mycotoxins (Gambrione *et al.*, 1985). The first aim of present research was therefore to study the toxicokinetic behavior and absolute oral bioavailability of DON in turkey poults. Secondly to determine whether chickens and turkeys might have a different sensitivity to DON based on phase II biotransformation differences of this mycotoxin.

MATERIALS AND METHODS

Six turkey Hybrid Converter poults (BW = 1.27 ± 0.07 /ucodep>kg BW) and three Ross 308 broiler chickens (BW = 1.17 ± 0.11) were administered 0.75 mg DON kg^{-1} BW per os (PO) and intravenously (IV) in a two-way cross-over design. DON was quantified by a validated LC-MS/MS method, whereas its metabolites were determined by HR-MS.

RESULTS AND CONCLUSIONS

Based on non-compartmental toxicokinetic analysis, DON was absorbed rapidly (T_{max} = 0.57 h) but incomplete, as the abso-

lute oral bioavailability was only 20.9%. DON was rapidly eliminated as well, both after PO (T1/2el PO = 0.86 h) as well as IV (T1/2el IV = 0.62 h) administration. Furthermore, HR-MS analysis revealed that DON-3α-sulfate is the major metabolite of DON in turkeys, with DON-3α-sulfate/DON ratios ranging between 1.3–12.6 and 32.4–140.8 after IV and PO administration, respectively. Glucuronidation of DON to DON-3α-glucuronide is a minor pathway in turkey poults, with DON-3α-glucuronide/DON ratios between 0.009–0.065 and 0.020–0.481 after IV and PO administration, respectively. Only trace amounts of other metabolites were found including 10-DON-sulfonate, de-epoxydeoxynivalenol and 10-de-epoxydeoxynivalenol-sulfonate. In broiler chickens, the major metabolite of DON was DON-3α-sulfate as well, with even higher DON-3α-sulfate/DON ratios, ranging between 243–453 and 1365–29624 after IV and PO administration, respectively. However, in contrast to turkey poults only trace amounts of DON-3α-glucuronide could be detected. In conclusion, the differences in biotransformation of DON between turkey poults and broiler chickens might attribute to different sensitivity of both animal species to this mycotoxin.

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9.10.
Influence of a decontamination protocol on the blood redox status of dioxin-like PCB naturally contaminated heifers

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INTRODUCTION
Exposure to dioxin-like (DL) compounds promotes reactive oxygen species (ROS) production and depression of several ROS quenching systems, leading to oxidative stress. We previously reported that dioxins impair the plasma antioxidant defence system (ADS) of lactating buffalos (1), and that the extent of damage to plasma proteins and lipids in dairy cows is correlated with the concentration of DL-PCBs in bulk milk (2). Aim of the study was to evaluate in naturally exposed heifers the effect of a decontamination procedure, based on the removal of animals from the polluted area and the feeding of a controlled diet, on specific markers of blood redox homeostasis.

MATERIALS AND METHODS
Eight one-year old DL-PCB exposed heifers were removed from a contaminated area and reared in an experimental facility under controlled conditions and diet. From each animal perirenal fat biopsies and blood samples were collected bimonthly 4 times (A-B-C-D). Fat PCB content was measured by GC-HRMS with a validated method. Serum Retinol (Ret), alpha-Tocopherol (Toc), Ascorbate (Asc), the total antioxidant capacity (TAC), and the glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities were used as indices of the ADS. Protein and lipid damage were assayed by serum Nitro-tyrosine (N-Tyr), protein-bound carbonyls (PC), and lipid hydroperoxides (LPO) measurement. Values were expressed as mean±SEM and statistically analyzed by one-way ANOVA followed by Tukey's test. Correlation was calculated through the Pearson's coefficient.

RESULTS AND CONCLUSIONS
Initial DL-PCB TEQ values of each animal was higher than 20 pg g^{-1} fat, and rapidly decreased in sampling B (7.71 ± 0.32 pg g^{-1} fat), complying with legal limits in samplings C and D (below 5 pg g^{-1} fat) (3). According to previous data (2), sampling A displayed significantly lower ($P < 0.001$) Ret, Toc and Asc concentrations, as well as the TAC and the SOD and GPx activities, and significantly higher ($P < 0.001$) N-Tyr, PC and LPO levels, compared to the decontaminated samples. A correlation between TEQ values and TAC, N-Tyr, and PC was also observed in sampling A. Our results confirm the DL-PCB mediated impairment of the ADS, associated with a higher extent of oxidative protein and lipid modifications, and demonstrate the restoration of the redox homeostasis by a decontamination procedure. Finally, TAC, N-Tyr and PC could be suitable monitoring biomarkers, as their levels are strongly affected by the extent of contamination.

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9.11.
Changes in serum low molecular weight proteins from DL-PCB contaminated heifers

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INTRODUCTION
Dioxin-like (DL) compounds are persistent and highly toxic organic pollutants. Due to their high lipophilicity, they accumulate along the food chain and contaminate animal products, which are by far the most important non-professional source for humans. Official methods for DL-compounds detection in foodstuffs are expensive and time consuming. Thus, there is a demand for faster and cost effective screening methods based on the identification of biomarkers of exposure in easily collectable samples. Proteomic techniques are of growing interest in this respect (1). Aim of this study was to

investigate the changes in low molecular weight (MW) protein profile in serum samples from heifers accidentally exposed to DL-PCBs and then subjected to a decontamination protocol.

MATERIALS AND METHODS

Eight one-year-old DL-PCB accidentally exposed heifers were reared in a non-contaminated experimental facility under controlled conditions for six months. Four serum and fat samples were collected from each animal at two-month intervals (A, B, C, D). DL-PCB content was assayed in fat using GC-HR-MS with a validated method. Serum samples were extracted and analyzed in the 2–20 kDa range using a linear MALDI-TOF. Mass spectra were statistically processed using ClinProTools and analyzed by Wilcoxon and Kruskal-Wallis test. Peak identification was performed through separation by liquid chromatography, structure characterization with a MALDI-TOF/TOF-MS and/or nanoLC-ESI-linear ion trap (LIT)-MS/MS, and analysis using MASCOT search engine.

RESULTS AND DISCUSSION

Exposed heifers displayed very high DL-PCB TEQ values (26.22 ± 2.17 pg g^{-1} fat) in sampling A, undergoing a rapid decontamination in the following two months (B), showing values complying with legal limits in C and D samples. Protein profiling revealed 48 statistically significant peaks ($P < 0.05$), which were selected to be identified according to their increasing or decreasing intensity among the 4 samplings. Fibrinogen β-chain, apolipoprotein A-II, and apolipoprotein C-III raised throughout the decontamination, whereas apolipoprotein C-II short-form, Complement C4, amyloid A-4 protein, and hemoglobin subunit-α declined. Interestingly, similar changes in serum apolipoprotein and Complement C4 have been reported in TCDD-exposed humans (2), while amyloid A, a known inflammatory marker, is associated with PAH exposure (3). Further studies are needed to build a predictive model able to identify contaminated animals.

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9.12.
Comparison of polychlorinated dibenzo-dioxins/furans and dioxin like-PCBs profiles in sheep and bovine liver sampled in Piedmont Region, Italy

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INTRODUCTION

Polychlorinated dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs) and polychlorinated biphenyls (PCBs) are persistent organic environmental contaminants of food and feed, representing a significant and constant threat to consumer health. Being food of animal origin the main source of exposure for humans, EU has fixed maximum levels (MLs) to reduce their intake (EC, 2011). Recently, it has been shown that sheep livers frequently exceeded the expected maximum levels (Rose *et al.*, 2010). Thus, EFSA evaluated the levels reported by member states (EFSA, 2011), concluding that consumption of sheep liver may be a potential health concern. Following Recommendation 2013/711/UE, a monitoring program was performed on liver from sheep and cows reared in the same areas of Piedmont Region (North-Western Italy) in order to investigate possible differences in accumulation patterns. Moreover, during the analysis the MLs for sheep liver were revised (EC, 2013) and expressed on a wet weight (ww) base. A comparison between old and current MLs was then considered.

MATERIALS AND METHODS

Liver samples from 30 sheep and 10 cows were collected between May 2012 and June 2013. Animals were selected on the basis of three main criteria: age > 7 years, multiparous and reared only in North-Western Italy to provide data on dioxin and DL-compound contamination in a specific area.
Extraction, purification on liver fat fraction was followed by GC–HRMS determination of dl-PCBs and PCDD/Fs.
Statistical significance of comparison study was assessed by *t* test, corrected for multiple comparison using Holm-Sidak method ($P < 0.05$).

RESULTS AND CONCLUSIONS

Accumulation profiles recorded in analyzed samples proved significant differences between sheep and cows for both PCDD/DFs (mean value 0.34 ± 0.2 pgTEQ per g ww in sheep, 0.074 ± 0.02 pgTEQ per g ww in cows), and sum of PCDD/DFs and dl-PCBs liver contents (mean value 0.8 ± 0.38 pgTEQ per g ww in sheep group, 0.16 ± 0.036 pgTEQ per g ww in cow group). Assuming a similar basal exposition to DL-compounds in sampled specimens, our data confirm high accumulation rates in sheep liver when compared to bovine ones. Regarding MLs shift, previous fat related MLs resulted more

precautionary than current MLs: 6 ovine samples would be not compliant for PCDD/DF and/or for sum of PCDD/DF + dl-PCB; but considering effective legislation they all result below existing wet weight based MLs.

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9.13.†
ABSTRACT DELETED

9.14.
Cadmium concentrations in tissues of red deer from northern Italy

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INTRODUCTION
Cadmium (Cd) is a heavy metal and its increased concentration in the environment is mostly caused by anthropogenic activity like industrial emission and urban pollution. Game species, like red deer, are known to be good bioindicator of Cd pollution. This game species is part of the human food chain and, therefore, the presence of this contaminant in muscle and organ meats can pose a serious threat to human health, particularly hunters and their family, more exposed to general population for higher consumption of meat from game animals (1,2). The aim of this study was to evaluate Cd contamination in muscles, livers and kidneys samples of red deer collected in northern Italy from 2009 to 2011.

MATERIALS AND METHODS
A total of 210 muscles (masseters), 201 livers and 152 kidneys were collected from red deer hunted during 2009–2010 and 2010–2011 hunting seasons. All animals were aged based on the tooth eruption pattern. According to the age determined, the animals were assigned to two categories: young (<12 months) and adult (>12 months). Muscles, livers and kidneys of each animal were separately sampled. The samples were homogenized and subsequently mineralized in a closed system, with a mixture of HNO_3 at 70% and H_2O_2 at 30% and with the aid of microwaves. After mineralization the samples were cooled at room temperature and diluted with MilliQ water. Atomic absorption measurements (AAS) were performed by an Agilent AA240Z.

RESULTS
The mean values of Cd concentration in red deer kidney, liver and muscle tissues were 1.02 mg kg^{-1}, 0.07 mg kg^{-1} and 0.006 mg kg^{-1}, respectively. Cd concentrations were found to significantly increase ($P < 0.05$) with age in kidneys, whereas no difference ($P > 0.05$) between young and adult red deer was observed as regards Cd concentrations in the liver and muscles. Comparing sampling years, meaningfully results were obtained for Cd concentration in the liver that was significantly higher in season 2009–2010 than in 2010–2011.

CONCLUSIONS
Results from this study show that Cd concentrations in the kidneys of red deer were higher than those found in the liver and muscle tissues, and the results indicate a positive correlation

between renal Cd levels and age of the animals, confirming data previously reported (3).

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Session 10: Antiparasitics Internal & External

10.1.
Pyrethroid disposition in brain and changes in serotoninergic and dopaminergic systems

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INTRODUCTION

Pyrethroids act primarily on the nervous system. Acute symptoms in rats administered Type I pyrethroids include aggression and hypersensitivity, general and fine tremor, convulsive twitching, coma and death. Type II pyrethroids elicit salivation, coarse tremor, increased extensor tone, writhing convulsions and death. The site of action of pyrethroids is the voltage-dependent sodium channels, but the chloride, calcium, and other channels may also be targets. For pyrethroids, few studies have been conducted to analyze the toxicokinetic properties, as well as the toxicokinetics (TK) – toxicodynamics (TD) relationship. The objective of this work was to correlate pyrethroid brain disposition with neurochemical effects for the pyrethroids Type II, λ-cyhalothrin and deltamethrin.

MATERIAL AND METHODS

The study was undertaken in accordance with the ethic requirements and authorized by the official ethical committee of our university. Two experiments were carried out:
(1) Male Wistar rats treated with λ-cyhalothrin or deltamethrin (20 and 26 mg kg^{-1}, per os) were killed at different time period after treatment and plasma samples and brain regions were collected, homogenized and extracted with *n*-hexane to determine pyrethroid levels by HPLC-UV using a Shimadzu HPLC LC-10AD with an UV/VIS photodiode array detector SPD-M10A VP and a C-18 column. UV detection at 266 nm. The mobile phase acetonitrile-water (80:20, v/v) was pumped at 1 ml/min. Kinetic profiles of λ-cyhalothrin and deltamethrin in plasma and nervous tissues were determined.
(2) Male Wistar rats treated with λ-cyhalothrin or deltamethrin (8 and 9 mg kg^{-1}, per os, 6 days) and with corn oil (vehicle) (control animals) were killed 24 h after dosing, brain regions isolated and contents of DA (dopamine), and 5-HT (serotonine) quantified by HPLC-ED. The brain regions analyzed were striatum, frontal cortex, hippocampus and hypothalamus. Levels of 5-HT and DA determined using a Shimadzu HPLC LC-9A, a C_{18}-Nucleosil column and electrochemical detection. The mobile phase consisted of 0.1 M $Na_2HPO_4.2H_2O$, 0.1 M citric acid (pH 3.5) and 10% (v/v) methanol pumped at 1 ml/min. The working electrode potential was set at 0.8 V.

RESULTS AND CONCLUSION

Brain regions Kinetic parameters:

Cyhalothrin: C_{max} ($\mu g\ g^{-1}$); AUC (mg h^{-1} l^{-1}); $t_{1/2}$ β (h)
- Hippocampus 12.1 $\mu g\ g^{-1}$; 212.7 mg h^{-1} l^{-1}; 23.1 h
- Striatum 18.1 $\mu g\ g^{-1}$; 258.2 mg h^{-1} l^{-1}; 17.3 h
- Frontal cortex 17.4 $\mu g\ g^{-1}$; 276.7 mg h^{-1} l^{-1}; 18.7 h
- Hypothalamus 25.6 $\mu g\ g^{-1}$; 442.1 mg h^{-1} l^{-1}; 34.6 h

Deltamethrin
- Hippocampus 10.5 $\mu g\ g^{-1}$; 10.1 mg h^{-1} l^{-1}; 38.5 h
- Frontal cortex 1.27 $\mu g\ g^{-1}$; 59.2 mg h^{-1} l^{-1}; 28.9 h
- Hypothalamus 30.01 $\mu g\ g^{-1}$; 1975.8 mg h^{-1} l^{-1}; 40.8 h

DA and 5-HT depleting effect:

DA (% control) and 5-HT (% control)
Cyhalothrin
- Hippocampus −34%; −26%
- Striatum −39%; −31%
- Frontal cortex −53%; −35%
- Hypothalamus −54%; −45%

Deltamethrin
- Hippocampus −26%; −14%
- Frontal cortex −42%; −26%
- Hypothalamus −52%; −46%

Hypothalamus presented the higher $t½\beta$, C_{max}, and AUC. Both pyrethroids caused a statistically significant decrease in the DA and 5-HT levels in the brain regions. The major depleting effect was observed in the hypothalamus. These findings may provide a valuable contribution for risk assessment of these pesticides.

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10.2.
Oral safety of a dinotefuran – pyriproxyfen combination (Vectra Felis®) in adult and young cats

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INTRODUCTION

Vectra Felis® (DP) is an ectoparasiticide spot-on combining two active ingredients: dinotefuran (423 mg) and pyriproxyfen (42.3 mg). A single unit dose spot-on (0.9 ml), is recommended for cats from 0.6 to10 kg of body weight. The oral safety of DP was studied in adult and young animals to evaluate accidental oral ingestion and oral exposure through licking which is a common behavior in cats.

MATERIALS AND METHODS

Ten cats (5/sex; 7–8 months old; 3.2–5.5 kg) and 12 kittens (6/sex; 7-week old; 648–895 g) were included in two indepen-

dent GLP studies. Each study was performed in two successive steps. During the first step, two animals (one per sex) received escalating doses of DP to determine the maximal dose which can be administered without inducing serious adverse effects. Considering the animal's weight, the maximal dose tested in this preliminary phase corresponded approximately to the entire volume of a pipette. Observations included clinical observations, food consumption and body weight. During the second step, the highest dose was administered as a single oral dose to four animals per sex. Animals were observed during 7 days as for the preliminary phase. In addition blood and urine samples were collected for clinical pathology at three different occasions.

RESULTS

Adults: No treatment-related effects were observed during the first step. The highest dose (0.3 ml kg^{-1}) was administered during the second step. This dose corresponded to a volume higher than the volume of a single unit spot-on (up to 1.8X). No significant treatment related effects were detected.

Kittens: Transient signs (abnormal feces, salivation and emesis) were observed during the first step. As these clinical signs were reversible, the highest dose (1.5 ml kg^{-1}) was administered during the second step. This dose corresponded to a volume higher than the volume of a single unit spot-on (up to 1.5X). Abnormal feces, salivation and/or emesis, generally reversible within 3–4 h, were observed after administration of 1.5 ml kg^{-1}. Thereafter, no significant treatment-related effects were detected.

CONCLUSION

Accidental oral ingestion of a full single unit spot-on of Vectra Felis® does not induce serious adverse effects in young cats or kittens. Transient and limited reactions may be observed in very young animals. These reactions resolved spontaneously. Vectra Felis® is therefore considered as sufficiently safe even in case of oral ingestion of the entire dose.

10.3.
In-vitro assessment of dinotefuran and pyriproxyfen diffusion through human skin after topical administration of Vectra® Felis

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INTRODUCTION

Vectra® Felis is a unique ectoparasiticide topical formulation combining two active ingredients, dinotefuran and pyriproxyfen, and is used on cats to treat and prevent flea infestations. Pet owners could experience accidental skin contact with this formulation, leading to potential exposure to the active ingredients. This study was therefore designed to assess *in-vitro* the kinetic of diffusion through human skin of dinotefuran and pyriproxyfen.

MATERIALS AND METHODS

Human skins (abdominal skin) were collected from five different donors. Franz cell devices were used as dynamic diffusion cell. The cell diffusion was maintained during 24 h in a water bath at about 32°C in a non-occlusive system with continuous magnetic agitation. The diffusion model was validated using caffeine as a control. Vectra® Felis (20 μl) was dropped uniformly on the area of skin (about 2 cm^2). Samples (300 μl) of the receptor fluid (saline + phosphate buffer, pH 7.4) were collected 1, 2, 4, 8 and 24 h after administration using an automatic sampling device. After 24 h of contact, the skins were washed and the rinsed fluids were collected for analysis. The quantification of the two active ingredients was performed in the receptor and the donor fluids, using a specific LC/MS/MS method with LLOQ of 2.5 μg ml^{-1} and 0.25 μg ml^{-1} for dinotefuran and pyriproxyfen, respectively.

RESULTS

For dinotefuran and pyriproxyfen, less than 1% (0.9% and 0.14% respectively) of the applied dose had diffused through the human skin within the first 8 h post dosing. After 24 h of contact, less than 4% of the applied dose (3.3% for dinotefuran and 1.5% for pyriproxyfen) had diffused through the human skin. Over 85% of the dose remained above the skin surface for both compounds (85% and 89% for dinotefuran and pyriproxyfen, respectively). Thus, less than 12% of the dose (11.5% and 9.8% for dinotefuran and pyriproxyfen, respectively) could remain in the adjacent tissues or in the skin.

CONCLUSION

This study demonstrates that, after 24 h of contact with Vectra® Felis, less than 3.5% for dinotefuran and less than 1.5% for pyriproxyfen diffused through the human skin. The proportions are reduced to 0.017% and 0.041% for dinotefuran and pyriproxyfen, respectively, when exposure is limited to 1 h. In realistic situations of accidental skin exposure, contact should not exceed a few minutes since the pet owners are advised to wash their hands after administration.

10.4.
Determination of dislodged residues upon petting following topical administration of Vectra® 3D on dogs

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INTRODUCTION

Vectra® 3D, is a unique ectoparasiticide topical formulation dedicated to dogs combining permethrin, dinotefuran and pyriproxyfen. Since companion animals can live in close contact with their pet owners and family, the exposure to active ingredients through petting must be evaluated. This study aimed to determine drug residues potentially dislodged upon petting after topical administration of Vectra® 3D to dogs.

MATERIALS AND METHODS

Healthy male and female adult dogs (body weigh from 7.45 to 9.97 kg) were included in 3 groups (Group 1: 6/sex; Group 2: 2/sex; Group 3: 2/sex). Dogs were housed indoor. Dogs were administered 0.4 mL/kg of Vectra® 3D, on Day 0, by parting the hair and applying the product directly onto the skin in the middle of the neck between shoulder blades. Dislodged residues

were measured by petting dogs, with cotton glove, at different times (Group 1: Day −1, Day 1 at 4 h & 8 h, Days 2, 3, 7, 14, 21 & 30 after application; Group 2: Day −1, Day 3; Group 3: Day −1, Day 30). Petting was performed using a standardized procedure. The sampler stroked down, with uniform medium pressure, the specific body surface as follows: 1 stroke on the right and left side of the ventral zone, 1 stroke on the right and left flank and 1 stroke on the back line. Cotton glove was removed and concentrations of active ingredients were determined using a specific LC/MS/MS method. Maximal and average dislodged residues were calculated for dinotefuran, pyriproxyfen & permethrin at each time point.

RESULTS

Temperature during the experiment ranged between 17 and 22°C. Each compound was recovered in low proportions whatever the time of petting. The maximum of residues dislodged after petting were observed 4 h after application for all active ingredients, which represented about 0.8% of the applied dose in average (range: 0.24–1.87% for dinotefuran, 0.22–2.01% for permethrin and 0.24–1.88% for pyriproxyfen). Residues dislodged decreased all over the times after application for all compounds: 0.3% of the applied dose was recovered on D3 and less than 0.02% on D30.

CONCLUSION

Very low amounts of the 3 active ingredients of Vectra® 3D were dislodged from treated dogs by petting from 4 h until 30 days after administration. Drying speed of the product on the dog's coat, driven by temperature and/or air flow, is expected to influence the time interval between application and petting. There was no influence of the petting frequency on the amount dislodged.

10.5.

In-vitro assessment of dinotefuran, pyriproxyfen and permethrin diffusion through human and dog skin after topical application of Vectra® 3D

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INTRODUCTION

Vectra® 3D (DPP) is a unique ectoparasiticide topical formulation dedicated to dogs, combining three active ingredients: dinotefuran (D), pyriproxyfen (PY) and permethrin (PE). In case of accidental skin contact with this formulation, dermal exposure to the active ingredients could occur. This study aimed to assess the *in-vitro* percutaneous absorption of dinotefuran, pyriproxyfen and permethrin on human and dog skins after administration of DPP.

MATERIALS AND METHODS

Skins were collected from five human donors and from 2 Beagle dogs. Franz cell devices were used as dynamic diffusion cell with diffusion maintained for 24 h in a water bath at 32.0°C, in a non-occlusive system with continuous magnetic agitation. The diffusion model was validated using [4-^{14}C]-testosterone as a control. DPP (25 μl cm^{-2}, corresponding to 44 μl) was dropped on the area of skin (2 cm^{-2}). Samples of the receptor fluid (saline + 5% of BSA, pH 7.4) were collected at pre-dose, 0.5 h, 1 h, 2 h, 4 h, 8 h and 24 h after administration. After 24 h of contact, the skins were washed and the rinses were collected for analysis. The three active ingredients were quantified in the receptor and the donor fluids, using a specific LC/MS/MS method (LLOQ of 40 ng ml^{-1}, 10 ng ml^{-1}, 80 ng ml^{-1} and 120 ng ml^{-1} for D, PY, *cis* and *trans*-PE, respectively).

RESULTS

D diffused through the dog skin from 1 h to 24 h of contact while no other ingredient had diffused until 24 h of contact. After 1 h of contact less than 4.5% of the applied dose of D had diffused through the skin. After 24 h of contact, about 57% of the applied dose of D and negligible proportions of the other ingredients (<0.1%) had diffused through the skin. After 30 min of contact with human skin, no ingredient had diffused. D diffused through skin between 1 h and 24 h of contact while no PY had diffused before 24 h after contact. After 24 h of contact, <19% of the applied dose of D and 0.05% of PY had diffused. No PE (*cis*-/*trans*-) was recovered through the skin, after 24 h of contact.

CONCLUSION

This study demonstrated that systemic exposure to the active ingredients after topical administration on dogs is moderated. Further studies were performed to investigate such exposure of the target species *in vivo*. Moreover, when exposure of human skin is limited to 30 min, no ingredient diffused through human skin. In realistic situations of accidental skin exposure, contact should not exceed a few minutes since the pet owners are advises to wash their hands after administration.

10.6.

Determination of dislodged residues upon petting following a topical treatment with Vectra® Felis on cats

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INTRODUCTION

Vectra® Felis is a unique ectoparasiticide topical formulation combining two active ingredients: dinotefuran (D) and pyriproxyfen (PY), used on cats and kittens to treat and prevent flea infestations. This study was designed to determine drug residues potentially dislodged upon petting after topical administration of Vectra® Felis to cats.

MATERIALS AND METHODS

Healthy cats (males and females: 32–70 months, 2.3–4.5 kg) were included in 3 groups (G1: 6/sex; G2: 2/sex; G3: 2/sex). One pipette of Vectra® Felis (0.9 ml) was applied, on day 0, to each cat, by parting the hair and applying the product from a pipette directly onto the skin in the midline of the neck between shoulder blades. Dislodged residues were measured by petting cats with cotton glove at different times (Group 1: Day −1, Day 1 at 4 h & 8 h, Days 2, 3, 7, 14, 21 & 30 after application, Group 2: Day −1, Day 3; Group 3: Day −1, Day 30).

Petting was performed using a standardized procedure. The sampler stroked down, with uniform medium pressure, the specific body surface as follows: 1 stroke on the right and left side of the ventral zone, 1 stroke on the right and left flank and 1 stroke on the back line. Cotton glove was removed and concentrations of each active ingredient were determined with a specific LC/MS/MS method. Maximal and average dislodged residues were calculated for each AI at each time point.

RESULTS

Temperature during the experiment ranged between 17 and 26°C. Low proportions of applied dose of dinotefuran and pyriproxyfen were recovered whatever the time of petting. The maximum of residues dislodged after petting were observed 4 h after application for all compounds, which represented about 2.74% in average (range: 0.13–9.56%) of the applied dose for dinotefuran and 1.90% in average (range: 0.12–4.33%) of the applied dose for pyriproxyfen. Residues dislodged decreased all over the times after application for both active ingredients: 0.51% and 0.27% of the applied dose were recovered for dinotefuran and pyriproxyfen, respectively on D3 and 0.07% and 0.05% were recovered on D30.

CONCLUSION

There was no influence of the petting frequency on the amount dislodged. Very low amounts of the 2 active ingredients of Vectra® Felis were dislodged after petting. Drying speed of the product on the cat's coat, driven by temperature and/or air flow, is expected to influence the time interval between application and petting.

10.7.

Distribution in the dog fur of Fipronil and Permethrin after single topical treatment with Effitix® spot-on

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INTRODUCTION/OBJECTIVE

A GLP study was carried out in order to investigate the hair coat distribution of fipronil and permethrin (*cis* and *trans* forms) after a single topical treatment with Effitix® spot-on (Virbac, Carros, France) to dogs. Fipronil sulfone, the major active metabolite of fipronil was also followed.

MATERIALS AND METHODS

Six healthy adult Beagle dogs (males, mean BW 11.7 kg) were treated with a pipette (2.2 ml) of Effitix® corresponding to a minimum dose of 6.7 mg of fipronil (mean dose of 11.2 mg kg^{-1}) and 60 mg of permethrin (mean dose of 101 mg kg^{-1}) per kg BW. The treatment was applied in two spots: one between the shoulder blades and one at the lumbar area (base of tail). Licking was not impeded in order to be in normal conditions of use. Hair samples (neck, shoulder, back, thigh) were collected before treatment, at 6 h post-treatment and then on Days 1, 3, 7, 14, 21, 28 and 35 days post-treatment. Fipronil, fipronil sulfone and *cis*- and *trans*- permethrin were assayed in dog hair using validated HPLC-MS/MS methods. The limit of quantification (LOQ) was of 100 ng g^{-1} in hair for both fipronil and fipronil sulfone, and 5 μg g^{-1} in hair for both *cis* and *trans*-permethrin.

RESULTS

The product was well tolerated during the study. In hair, the proportion of *cis* and *trans*-permethrin was always the same throughout the sampling period. The sum of both isomers values were used for permethrin analysis. Permethrin concentrations were quantifiable in all sample areas as from 6 h post application and up to Day 35. A peak concentration was observed at Day 3 with mean concentration from 648 to 1371 μg g^{-1}. The highest concentration was, in most cases, found on the back. As from 6 h post-treatment, fipronil concentrations were equal to or higher than 100 ng g^{-1} for all animals and for all sampling areas. A peak was observed in all the sampled areas on Day 3 with the highest mean concentration observed on the shoulders (130 μg g^{-1}). Fipronil and fipronil sulfone were detected on hair up to Day 35 but in comparison with fipronil evolution, a delay in the increase of fipronil sulfone concentration was observed. A peak was detected on Day 14 in all the sampled areas with highest concentrations (11 μg g^{-1}) observed on the back.

CONCLUSIONS

Fipronil and permethrin, the two active ingredients of Effitix® spot-on are rapidly well-distributed in the dog hair and persist in the dog hair during the one-month treatment duration.

10.8.

Anthelminthic efficacy in England – results of multi-site faecal egg count reduction study

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INTRODUCTION/OBJECTIVE

The Anthelmintic Resistance (AR) situation in UK cattle is unclear; existing reports differ in dosing accuracy, sample handling, egg-counting technique sensitivity and statistical analysis, preventing clear conclusions being drawn. A three grazing season investigation was conducted to evaluate effective protocols for the early detection of anthelmintic resistance in cattle, and the results from 2014 are presented.

MATERIALS/METHODS

Forty groups of co-grazing first-season animals in geographically distinct English regions had their faecal egg counts (FEC) monitored throughout the grazing season, 24 groups (937 animals in total) were subjected to a Faecal Egg Count Reduction Test (FECRT) once a pre-defined composite FEC threshold (≥150 epg) was reached, with the remainder either housed without reaching threshold (6 groups), or withdrawn for other reasons (10 groups). Macrocyclic Lactone (ML; ivermectin (IVM) and doramectin (DOR) pour-on & injectable) and fenbendazole (FBZ; drench) products were used; two products were used simultaneously on 3 groups. Treatment was administered by veterinarians or technicians based on individual bodyweights (estimated by weighband or scales), following manufacturers recommended dose rates. FEC were performed using a

combination of the McMaster (McM) method (15 epg sensitivity) on all samples and the Sensitive Centrifugal Salt Floatation (SCSF – 1 epg sensitivity) on samples measuring ≤60 epg by McM. Larval speciation was conducted on cultured pre- and post-treatment samples (bulked by treatment). Percentage reductions in egg counts were calculated, by group, using pre- and post-treatment group means (Kochapakdee *et al.*, 1995), and 95% confidence limits (CL) constructed. Thresholds of >95% efficacy and >90% lower 95% CL were used to indicate efficacy (Coles *et al.*, 1992).

RESULTS/CONCLUSIONS

Seven FECRTs demonstrated efficacy (2/12 DOR injection, 1/3 DOR pour-on, 4/7 FBZ, 0/4 IVM injection, 0/1 IVM pour-on). When interrogating these data, 14 of 27 'Lack Of Efficacy (LOE)' groups (9/12 DOR injection, 2/3 DOR pour-on, 3/7 FBZ) could be defined as 'inconclusive' using more recent interpretation guidance (Levecke *et al.*, 2012). Caution is necessary in interpreting farms showing 'LOE' under current WAAVP guidelines due to the imprecision of this testing system, and clear industry guidance on statistical methodologies for efficacy calculation is required to avoid misclassifying 'inconclusive' farms as 'LOE'.

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10.9.

Evaluation of the efficacy of flubendazole, albendazole and ivermectin against gastrointestinal parasites in goats

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INTRODUCTION

Albendazole (ABZ) and ivermectin (IVM) are anthelmintics drugs widely used to control gastrointestinal parasites in domestic animals. However, resistance to albendazole was observed in several countries including in Mozambique (Atanasio *et al.*, 2002). As strategy to improve the benzimidazole (BZD) efficacy, and due to intensive use of ABZ, FLBZ was experimental tested in goats from extensive production farms in order to introduce it in the national market. Also the efficacy of ivermectin, an important drug used in the country, was tested.

MATERIAL AND METHODS

This assay was conducted in the district of Magude, south of Mozambique, where the climate is tropical. Fifty six goats from 4 months age were divided in 4 groups. Animals were treated with 5 mg kg^{-1} of ABZ – Group A, 5 mg kg^{-1} of FLBZ – group B and 0.2 mg kg^{-1} of IVM – group C, respectively. Group D was used as control. Before treatment and at day 7 and 14, feces from all animals was analysed using Mcmaster test. The efficacy in all treated drugs was evaluated according to *Coles et al (1992)*. Gastrointestinal parasites were also assessed in all samples. Attending that FLBZ and ABZ are benzimidazole compounds, we applied the same dose in this study.

RESULTS AND DISCUSSION

Haemonchus spp, Oesophagostomum spp, Trichostrongylus spp, are the main parasites identified in samples from treated animals. Fourteen days after treatment, the parasite reduction observed was 98.66%, 96.89% and 93.48% for IVM, ABZ and FLBZ, respectively. The results demonstrated that benzimidazole (BZD) compounds have effects against gastrointestinal parasites. On the other hand, FLBZ efficacy showed in this study confirm higher efficacy of BZD compounds in small animals. Further studies using different doses and formulation should be done to perform its efficiency. The higher efficacy of IVM compared to other drugs suggest higher potency compared to BZD compounds. The results presented herein indicate that studies with different doses and from different formulation of FLBZ including ABZ equimolar dose in different farms should be performed to confirm the really efficacy of this drug. Compared to BZD compounds, IVM is more potent but not statistically difference was observed. The similarity in the efficacy observed in this study, suggest that all anthelmintic can be used in studied area.

CONCLUSION

Albendazole, FLBZ and IVM present efficacy against gastrointestinal parasites and FLBZ can be improved to use in goats, but further studies is necessary.

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10.10.

Comparative assesment of hepatic and ruminal metabolism of the novel anthelmintic monepantel in sheep and cattle

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INTRODUCTION

Monepantel (MNP) is a novel anthelmintic compound with activity against a wide range of gastro-intestinal nematodes including those resistant to the macrocyclic lactones, benzimidazoles and levamisole. The plasma disposition kinetics, distribution to target tissues and metabolism of MNP were recently characterized in sheep. The current work assessed the comparative hepatic sulphonation of MNP in sheep and cattle. The chemical stability of both MNP and its main metabolite monepantel sulphone ($MNPSO_2$) in ruminal fluid from both ruminant species was also investigated.

MATERIAL AND METHODS

Liver microsomes from sheep ($n = 5$) and cattle ($n = 5$) were obtained by differential ultracentrifugation. The microsomal biotransformation of MNP (40 μM) to $MNPSO_2$ was evaluated after the inactivation of flavin-monooxygenase (FMO) system and in the presence of metabolic inhibitors such as methimazole (MTZ) (FMO inhibitor) and piperonyl butoxide (PB) (cytochrome P450 inhibitor). MNP and $MNPSO_2$ were incubated under anaerobic conditions during 3, 6 and 24 h in ruminal contents collected from untreated sheep and cattle. The partition of both molecules between the solid and fluid phases of ruminal contents was assessed. MNP and $MNPSO_2$ concentrations were measured by HPLC.

RESULTS

MNP and $MNPSO_2$ showed a high chemical stability without evident metabolism and/or degradation in ruminal contents of sheep and cattle. Both molecules were extensively bound (>85%) to the solid material of ruminal content. Significant higher ($P < 0.05$) hepatic metabolic rate of $MNPSO_2$ formation was observed in sheep compared to cattle. Whereas the FMO inactivation and the presence of MTZ affected the formation of $MNPSO_2$ in sheep liver, no significant changes were observed in cattle. When the incubation of MNP was done in the presence of PB at 4 μM, the reduction of the sulphone formation was between 59% (sheep) and 100% (cattle).

CONCLUSIONS

The current work corroborated that both FMO and cytochrome P450 are involved in the conversion of MNP into its sulphone metabolite. However the MNP sulphonation activity seems to be mainly cytochrome P450 dependent in cattle. Since MNP is currently available to be used only in sheep, the observed differential metabolic pattern should be corroborated under *in vivo* conditions to evaluate the potential use of MNP in cattle.

ACKNOWDGEMENTS

The authors thank Novartis Animal Health for the donation of MNP and $MNPSO_2$ pure analytical standards.

Session 11: Drug Transporters & Biotransformation

11.1.

Most significant changes in ABCC2 mRNA registered following probiotics and enrofloxacin challenge in chicken

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INTRODUCTION

Poultry feed is often supplemented by *Lactobacilli* probiotics which may alter drug bioavailability by affecting the expression of intestinal ABC efflux transporters (1). ABCB1, ABCC2 and ABCG2 were recognized as determinants of fluoroquinolone pharmacokinetics. Therefore the aim of the present investigation was to evaluate the effect of probiotics, administered alone or in combination with enrofloxacin, on mRNA expression of these transporters in the duodenum, jejunum and liver of the chicken.

MATERIALS AND METHODS

24 one-day-old Ross chicks were divided in four groups (each consisted of $n = 6$). Control group was not treated. Five days after hatching the second group was treated with *Lactobacillus brevis, L. plantarum and L. bulgaricus* for 15 days. Third group received probiotics as described above plus enrofloxacin (at age of 15 days,10 mg kg^{-1}, via drinking water for 5 days). The last group received enrofloxacin at age of 15 days (10 mg kg^{-1}, via drinking water for 5 days). Samples from liver, duodenum and jejunum were collected after the end of drug administration. Expression levels of ABC transporters were determined by qRT-PCR and were statistically evaluated by ANOVA test.

RESULTS

ABCC2 and ABCG2 mRNAs were down-regulated ($P < 0.05$) in the liver of chickens treated with enrofloxacin when compared to the groups that received probiotics plus enrofloxacin and probiotics, respectively. ABCC2 mRNA expression was decreased in the duodenum in all three groups of treated animals. ABCG2 mRNA in the duodenum was up-regulated ($P < 0.05$) in enrofloxacin treated group. ABCB1 mRNA was up-regulated ($P < 0.05$) in the jejunum of chickens treated with enrofloxacin in comparison to the both groups that received probiotics.

CONCLUSIONS

Expression of the studied ABC efflux transporters in chickens showed organ specific changes in the liver and in the intestines after enrofloxacin treatment. Down-regulation of ABCG2 mRNA in the liver, up-regulation of ABCG2 mRNA in the duodenum and ABCB1 mRNA in the jejunum can be attributed to enrofloxacin. The observed significant decrease of expression of ABCC2 mRNA in the duodenum and in the liver can be associated with enrofloxacin administration and to a lesser extend to supplementation of *Lactobacilli* in the feed (2). *Lactobacilli* probiotics did not influence the expression of ABCB1 and ABCG2 mRNAs and changes in the pharmacokinetics of concomitantly administered drugs, substrates for these transporters, cannot be expected due to alteration of their expression.

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11.2.

Effects of malachite green exposure on liver drug metabolizing enzymes and oxidative stress in untreated versus β-naphthoflavone pre-treated rainbow trouts (*Oncorhyncus mykiss*)

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INTRODUCTION

Despite the ban imposed by EU, the triphenylmethane dye malachite green (MG) is still illegally used in aquaculture to treat fungal infestations and parasitic diseases. Previous *in vitro* studies indicate that in the trout MG could inhibit a number of liver drug metabolizing enzymes (DME) and suggest that it may be a CYP1A substrate (1). It is believed that the oxidative metabolism of MG results in the formation of a number of reactive species which may participate in the MG-mediated oxidative stress (OS) (2). This study was designed to characterize the effects of a short MG exposure on DME and OS in the rainbow trout and to study their modulation by the pretreatment with β-naphthoflavone (β-NAF), a model cytochrome P450 (CYP) 1A inducer.

MATERIALS AND METHODS

Thirty-two rainbow trouts (weight range 100–150 g) were allotted to 4 groups ($n = 8$ each): one control group (K), one MG group (3 mg l^{-1} for 30 min), one β-NAF group (100 mg kg^{-1} bw i.p.), and one β-NAF+MG group. Pretreatment with β-NAF occurred 48 h prior to MG exposure; all fishes were sacrificed 24 h after MG exposure, and livers and plasma were collected. The following parameters were determined in liver subfractions with standard procedures: CYP content, NADPH-CYP reductase, EROD, ECOD, MG N-demethylase, as well as UGT 1-napthol, GST (CDNB), and GSH. Plasma activities of LDH as well as plasma antioxidant capacity (PAC) and reactive oxygen species content (ROS) were assayed with

commercial kits. Results (mean ± SEM) were analyzed by ANOVA followed by Tukey-Kramer *post-hoc* test.

RESULTS AND CONCLUSIONS

The short exposure to MG resulted in an overall reduction (20–40%, $P < 0.05$ or less) of the tested monooxygenase activities, without affecting CYP content or NADPH-CYP reductase activity. No statistically significant changes were observed in the examined phase II enzymes and in the GSH content, nor in plasma ROS and PAC. Conversely, there was a marked increase (+44%) of plasma LDH. An enhancement of the MG-mediated inhibition of ECOD and a sharp decline in GST were detected in β-NAF–pretreated trouts along with a greater increase in plasma LDH and a rise in plasma ROS ($P < 0.05$). It is concluded that β-NAF pretreatment did not significantly increase the rate of MG N-demethylation but worsened the MG-dependent inhibition of some monooxygenases and GST, and apparently increased the extent of tissue damage and OS (3).

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11.3.

Relative contribution of Cytochrome P450 3A to midazolam oxidation in cattle liver microsomes

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INTRODUCTION

In humans and rodents, members of the cytochrome P450 3A subfamily (CYP3A) are major contributors to midazolam (MDZ) biotransformation into 1-hydroxy-MDZ (1-OHMDZ) and 4-hydroxy-MDZ (4-OHMDZ), and 1-OHMDZ activity is commonly used as a surrogate marker for CYP3A in humans. In veterinary species, it is still crucial to identify isoform- and species-specific CYP substrates, to better characterize drug biotransformation and potential for drug–drug-interactions. The aim of this study was to characterize MDZ oxidation in cattle liver microsomes.

MATERIALS AND METHODS

Pooled microsomes were prepared from the liver of male Piedmontese beef cattle, and the formation of 1-OHMDZ and 4-OHMDZ was evaluated using a slightly modified HPLC-UV method; all the incubations were carried out under linear conditions of metabolite formation, with respect to incubation time and microsomal protein concentration. A confirmatory immunoinhibition study was performed by pre-incubating the pooled liver microsomes with increasing amounts of a polyclonal antibody raised against rat CYP3A1. Finally, MDZ hydroxylation was evaluated in 300 single-donor Piedmontese cattle liver microsomes, and analyzed for correlation with 6β-hydroxylation of testosterone (TST).

RESULTS AND CONCLUSIONS

Under the adopted chromatographic conditions, 4-OHMDZ, 1-OHMDZ and MDZ were eluted and well separated; the retention times were 13.9, 15.3 and 20.1 min, respectively. Formation of both metabolites *conformed* to *single-enzyme Michaelis*-Menten kinetics; V_{max} and K_m values were 665 pmol min^{-1} mg^{-1} protein and 6.16 μM for 4-OHMDZ, and 64 pmol min^{-1} mg^{-1} protein and 10.08 μM for 1-OHMDZ. The anti-rat CYP3A1 polyclonal antibody inhibited 4-OHMDZ formation up to 94%; however, only a 50% inhibition was noticed for 1-OHMDZ. The rates of formation of 4-OHMDZ and 6β-OHTST in single-donor liver microsomes were poorly correlated. In conclusion, cattle liver microsomes are capable of metabolizing MDZ to 1-OHMDZ and 4-OHMDZ. Furthermore, the immunoinhibition results indicate a major contribution of cattle CYP3A to 4-OHMDZ formation, while other CYPs might be involved in drug oxidation to 1-OHMDZ. Finally, the observed poor relationship between 6β-OHTST and 4-OHMDZ deserve further investigation to clarify the specific role played either by individual cattle CYP3A isoforms or other CYPs in MDZ and TST hydroxylation.

ACKNOWLEDGEMENTS

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Session 12: Pharmacodynamics 2 Pain/Inflammation

12.1.
Effect of Benazepril (Fortekor®) and Robenacoxib (Onsior®) on glomerular filtration rate in cats and dogs

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INTRODUCTION

Angiotensin-converting enzyme (ACE) inhibitors and non-steroidal anti-inflammatory drugs (NSAIDs) are sometimes used in combination which might lead to pharmacodynamic interactions, notably the induction of acute renal insufficiency (ARI). The risk of ARI with benazepril and robenacoxib was hypothesized to be relatively low due to the mixed biliary and urinary excretion of benazeprilat and the short residence time of robenacoxib in the central compartment[1,2]. The objective of this study was to determine the effect on glomerular filtration rate (GFR) and tolerability of benazepril, robenacoxib and their combination in healthy cats and dogs.

MATERIALS AND METHODS

Separate non-blinded, parallel group design studies were conducted in 32 healthy cats and dogs (16 female and 16 male). Animals were randomized to one of four treatment groups. Treatments were administered orally once daily in the morning on days 1–7: placebo; 0.5–1.0 mg kg^{-1} benazepril hydrochloride (cat and dog); 1.0–2.4 mg kg^{-1} (cat) or 1.0–2.0 mg kg^{-1} (dog) robenacoxib; and the combination of 0.5–1.0 mg kg^{-1} benazepril hydrochloride (cat and dog) and 1.0–2.4 mg kg^{-1} (cat) or 1.0–2.0 (dog) mg kg^{-1} robenacoxib. GFR was estimated from the plasma clearance of iohexol during baseline and again 1 h after the last administration of the treatments at the approximate maximal blood concentration. Changes from baseline and differences between groups were evaluated statistically using analysis of covariance. Two-tailed P values less than 0.05 were considered significant.

RESULTS

All test treatments were well tolerated. Incidents of mild gastrointestinal signs were judged unrelated to the treatments. No significant or biologically relevant differences between groups were observed in clinical condition, body weight, food intake, clinical chemistry, hematology or coagulation variables. In cats, the GFR was significantly higher post-treatment in the benazepril group compared to the control, and also significantly higher in the benazepril plus robenacoxib group compared to the other groups. In dogs, there were no differences in GFR between the groups.

CONCLUSIONS

No safety concerns, including on GFR, were identified for the concomitant short term administration of the ACE inhibitor benazepril and the NSAID robenacoxib in healthy cats and dogs.

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12.2.
Safety of intravenous Robenacoxib (Onsior®) in cats

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INTRODUCTION

The non-steroidal anti-inflammatory drug (NSAID) robenacoxib is registered in the EU for the treatment of pain and inflammation associated with surgery in cats, administered by subcutaneous (SC) injection prior to or after surgery. Administration of NSAIDs by intravenous (IV) injection may be more convenient in hospitalized animals and should result in a faster onset of action and a higher exposure compared to SC or oral routes. The objective of this study was to evaluate the tolerability of IV robenacoxib in healthy cats.

MATERIALS AND METHODS

A total of 32 healthy European shorthair cats were randomized into 4 parallel groups ($n = 4$ females and $n = 4$ males per group). All cats were anesthetized with ketamine and medetomidine and then received, approximately 15 min later, a single injection either of 2 or 4 mg kg^{-1} robenacoxib intravenously (IV), 2 mg kg^{-1} robenacoxib SC (reference), or saline solution IV (control). The following variables were measured prior to and following administration of the test items: body weight, clinical observations (baseline plus 4 and 8 h post-dosing), electrocardiogram (ECG, baseline plus 5 and 60 min), feed consumption, blood clinical chemistry, hematology and coagulation parameters (baseline plus 1–2 h).
Changes from baseline and differences between the treatment groups were evaluated statistically using analysis of covariance. Two-tailed P values less than 0.05 were considered significant.

RESULTS

All the test treatments were well tolerated with no biologically relevant or significant changes from baseline recorded. Incidents of vomiting and significant body weight changes were judged unrelated to the test treatments. There were no significant changes from baseline or differences between groups for other variables including food consumption, clinical chemistry, hematology, coagulation or ECG parameters.

CONCLUSIONS
Single administration of robenacoxib by IV bolus injection at 2.0 or 4.0 mg kg^{-1} was well tolerated and had no detected effect on cardiovascular parameters in healthy cats.

12.3.
Safety of intravenous Robenacoxib (Onsior®) in dogs

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INTRODUCTION
The non-steroidal anti-inflammatory drug (NSAID) robenacoxib (Onsior®) is registered in the European Union in dogs for the treatment of pain and inflammation associated with surgery, administered by single subcutaneous (SC) injection prior to surgery. Administration of NSAIDs by intravenous (IV) injection may be more convenient in hospitalized animals, and should result in a faster onset of action and higher exposure compared to SC or oral routes.
The objective of this study was to evaluate the tolerability of IV robenacoxib in healthy beagle dogs.

MATERIALS AND METHODS
A total of 8 beagle dogs (4 female and 4 male) received once in a non-blinded randomized four-phase crossover design a single administration of: IV robenacoxib (2 mg kg^{-1} and 4 mg kg^{-1}); IV administration of isotonic saline (control); and SC robenacoxib at 2 mg kg^{-1} (reference). For each treatment the animals were monitored clinically including body temperature over 6 h post-dose, an 8-h post-dose buccal mucosa bleeding time (BMBT) and blood clinical pathology evaluation (clinical chemistry, hematology and coagulation parameters) and an 8- to 24-h post-dose urinalysis. Arterial blood pressure, heart rate and electrocardiogram (ECG) were assessed via telemetry in four dogs over 8 h post-dose.
Changes from baseline and differences between the treatment groups versus control were evaluated statistically using analysis of variance for repeated measures. Two-tailed P values less than 0.05 were considered significant.

RESULTS
No biologically relevant or significant changes from baseline or differences between groups were detected for any cardiovascular variables including: arterial blood pressure, heart rate, cardiac conduction times (e.g. QT interval) and arrhythmias incidence; body temperature; body weight and food consumption; clinical signs; hematology, coagulation, plasma clinical chemistry and urine variables; and BMBT.

CONCLUSIONS
Single administration of robenacoxib by IV bolus injection at doses of 2 or 4 mg kg^{-1} was well tolerated including no effect on cardiovascular parameters in healthy dogs.

12.4.
Antihistaminic effect of cetirizine after oral administration in the dog

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INTRODUCTION
Cetirizine is a non-sedative antihistaminic drug used in dogs, but plasma concentrations in relation to effect after oral administration are not well studied. The aims of this study were to investigate the cetirizine exposure and the plasma cetirizine concentration-antihistamine response relation in the dog after oral administration of cetirizine.

MATERIALS AND METHODS
Cetirizine dihydrochloride (4 mg kg^{-1}) was administered *per os* once daily for three days to 4 female beagles at the age of 4–7 years and weighing 8–12 kg. Blood samples were drawn at hour 0 (premedication), before drug administration at hours 24, 48 and additional samples at hours 50, 51, 52, 55, 57, 59, 72, 76, 81 and 96. Plasma was analysed for cetirizine with Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry (LLOQ 0.1 ng ml^{-1}). Histamine (7 μg per site) was injected intradermally prior to blood sampling. The antihistaminic effect was evaluated by measuring the wheal area formed after histamine injection. The plasma exposure of cetirizine was explored with the use of a one-compartment model with lag time and the response was quantified with the use of an I_{max} model.

RESULTS
Cetirizine significantly inhibited wheal formation compared to premedication baseline. Maximum inhibition of wheal formation after treatment with cetirizine *per os* was 100% compared to premedication wheal area. The median (range) maximum effect appeared 6.5 h (2–11) after drug administration. The wheal area 24 h post last drug administration was 18 (13–28) % of baseline. The median potency value (IC_{50}) was 1.1 μg ml^{-1} (0.3–2). The C_{max} the t_{max} and the plasma half-life of cetirizine was 4.6 μg ml^{-1} (4.1–5.2), 4.7 h (2.8–8.2) and 11.7 h (10.7–16.4), respectively.

DISCUSSION
Cetirizine prevented wheal formation and no adverse effects were observed. The results indicate that a once daily dosing regimen of 4 mg kg^{-1} cetirizine *per os* clearly provides a sufficient antihistamine effect. At 48 h after last administration only one dog had returned to baseline wheal area. That, together with observed plasma concentrations considerably above the IC_{50} 24 h after last administration suggests that the dose was higher than necessary for the 24 h administration interval. Cetirizine may be an alternative when treating histamine mediated inflammation in the dog but additional clinical studies are required.

12.5.

Allergic inflammation can be augmented via histamine H_4 receptor activation: the role of natural killer cells *in vivo* and *in vitro*

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INTRODUCTION

Natural killer cells (NK cells) accumulate in dermal and epidermal infiltrates not only in hypersensitivity reactions, but also in atopic dermatitis [1] and have the potential to modulate dendritic cell functions [2]. Histamine release in the skin induces NK cells and dendritic cells to react in a bidirectional manner [3] and this crosstalk may be modulated by the histamine H_4 receptor (H_4R). The objective was to determine if the H_4R is involved in NK cell chemotaxis and does influence the interplay between NK cells and dendritic cells in a model for allergic inflammation.

MATERIAL AND METHODS

In vitro: Highly pure NKp46$^+$ NK cells were isolated from the spleen and real time PCR was used to detect H_4R expression. Chemotactic function of the H_4R was determined in a transwell migration assay using a selective H_4R agonist ST-1006 [(N^4-(2,6-dichlorobenzyl)-6-(4-methylpiperazin-1-yl)pyrimidine-2,4-diamine] and the H_4R antagonist JNJ7777120. A co-culture of NKp46$^+$ cells and bone marrow derived dendritic cells was stimulated for 24 h with lipopolysaccharide (1 μg ml^{-1}) and CXCL10/IP-10 was selected as a specific target from a cytokine array and quantified by ELISA. *In vivo*: NK cell migration in the skin was further characterized in the TDI (toluene 2,4-diisocyanate) model of allergic contact dermatitis inducing an acute Th2 immune response by staining for NKp46$^+$ cells.

RESULTS

ST-1006 (10 μM) induced NKp46$^+$ cell chemotaxis *in vitro* and the selective H_4R antagonist JNJ7777120 (10 μM) blocked the migration. Co-culture experiments showed that addition of NKp46$^+$ cells significantly increased the CXCL10/IP-10 release from bone marrow derived dendritic cells. This increase was slightly reduced by ST-1006 (10 μM), but could not be reversed by JNJ7777120 (10 μM). *In vivo*, ears topically treated with TDI in combination with ST-1006 (100 nmol per 20 μl i.d.) resulted in significant ear swelling and increase in NKp46$^+$ cells in the skin 8 h after treatment compared to PBS injected, TDI challenged. Numbers of T cells and dendritic cells remained unaffected at that early time point.

CONCLUSIONS

These results identify the H_4R as a new target controlling NK cell migration into sites of allergic inflammation. Blocking the H_4R in the skin and therefore reducing inflammation could help improve diseases like atopic dermatitis or psoriasis.

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12.6.

Effects of systemic treatment with H1 and H4 receptor antagonists and betamethasone in a murine ovalbumin-induced atopic dermatitis model

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INTRODUCTION

Atopic dermatitis (AD) is a chronic skin disease characterized by skin lesions and pruritus. The histamine H4 receptor (H4R) is currently evaluated as a new therapeutic target for the treatment of AD[1]. Glucocorticoids such as betamethasone are commonly used to treat AD.

Aim of the study was to test the effects of H1R and H4R antagonists as well as betamethasone in a murine ovalbumin (OVA) – induced model of allergic dermatitis.

MATERIALS AND METHODS

BALB/c mice were sensitized by epicutaneous application of OVA to induce AD-like lesions[2]. The H1R antagonist mepyramine (30 mg kg^{-1}) and the H4R antagonist JNJ39758979 (20 mg kg^{-1}) were given two times daily intraperitoneally (i.p) during the challenge phase. JNJ39758979 (20 mg kg^{-1} and 50 mg kg^{-1}) was also given orally three times a day. Betamethasone (2 mg kg^{-1}) was administered i.p. once a day. To evaluate treatment success clinical skin score, scratching behavior, serum level of OVA-specific IgE, epidermal thickness, epidermal infiltration of inflammatory cells and total cell count in spleen and axillary lymph nodes were analyzed. The cellular profile in the lymph nodes was determined by FACS. Lymphocytes and splenocytes were re-stimulated *in vitro* with OVA and concentrations of IL-4, IL-10 and INF-y were quantified by ELISA.

RESULTS

Betamethasone could elicit a slight improvement of the dermatitis severity, whereas none of the histamine receptor antagonists could improve clinical symptoms. Mean scratching behavior in vehicle treated mice was 45 scratching bouts in 30 min, which was reduced to 35 by JNJ39758979 and to 30 by betamethasone ($P < 0.05$). All treatments modulated secondary parameters: JNJ39758979 reduced the number of splenocytes, mepyramine and betamethasone decreased weight

and total cell count in the axillary lymph nodes and modulated the cellular profile.

CONCLUSIONS

A previous study demonstrated a clear improvement of clinical signs in the OVA model in H4R knockout mice. Thus it was hypothesized, that pharmacological blockade of the H4R would also improve severity of AD-like lesions in the OVA model. It should be clarified, whether pharmacological aspects are responsible for the lack of treatment effect. Furthermore, it might be necessary to block the H4R during sensitization and challenge phase. Additionally it should be examined, if systemic application can reach an effective drug level in the skin or if additional topical treatment is necessary.

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Session 13: Evidence-Based Veterinary Medicine & Innovative Learning

13.1.
'Learning by doing': news gathering on Toxicology
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Within the new teaching methodologies, and on the frame of the European Higher Education Area, a broad spectrum of tools are available. In fact, professors must motivate our students to eagerness to better themselves, to have a spirit of scientific inquiry . . . and to be connected with the surrounding reality. In this sense, the collaboration of different educational areas involving critical, reflective and ethical approach, together with collaborative methods, should enable them to develop their future professional activity with a social commitment.
Toxicology is a living science, with an evident social relevance according to its presence in the media. Therefore, the aim of the activity here presented is intended to help students to establish a clear link between toxicological knowledge and real life through the development and presentation of a collection of news concerning toxics. This fact makes students discover the importance of this specialty, thus connecting public news concerning toxics and poisons with what they already know, and acting as future specialists.
The objective of such teaching methodology was to introduce a complementary source to transmit both scientific knowledge and topical issues from a different approach, thus allowing a good connection to a variety of audiences, especially young people, by motivation. Also this activity helps develop and reinforce values and competencies of the Veterinary degree (to improve oral expression, to promote teamwork, to increase empathy, . . .).
Through mentoring activities, students show, criticize and discuss their individual work. The group collects the individually selected news to layout their own newspaper/magazine, describing different sections of Toxicology, even including hobbies (e.g., 'toxicological crossword') or curiosities ('toxic cinema', 'poisonous books'). Complete freedom is given to the group, so that the design is free, with the only condition that all the news must concern toxic substances and situations.
During these years of activity, a mean of 10–15 groups (each one of 2–3 students) have been involved each academic year. A user survey is sent to the students at the end of the activity, and it must be emphasized than only 10–12% stated that it took longer than it was planned. However, in all cases they conclude that 'those toxics you taught us, they really exist, and we and our animals, we are all surrounded by these dangerous compounds'.

13.2.
Abrupt withdrawal of oclacitinib leads to rebound phenomenon in a chronic mouse model of allergic dermatitis
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INTRODUCTION/OBJECTIVE
The janus kinase-inhibitor, oclacitinib has recently been developed as a new therapeutic for allergic skin diseases for dogs and we are trying to clarify the mechanism of its anti-itch potency. In the process of this study, we observed some rebound phenomenon in itch after abrupt withdrawal of oclacitinib. Therefore, the primary objective of the study reported here was to demonstrate the rebound phenomenon of oclacitinib by using a chronic mouse model of allergic dermatitis.

MATERIALS AND METHODS
Chronic mouse model of allergic dermatitis is conducted by repetitive toluene-2,4-diisocyanate (TDI) sensitization and challenge in female BALB/c mice. After TDI sensitization, oclacitinib was orally applied BID at 45 mg kg^{-1} for 7 days, and treatment of oclacitinib was then discontinued abruptly. Scratching bouts were monitored frequently until day 15 and each monitoring was conducted for 1 h after TDI challenge onto the rostral neck. In order to examine pruritogen evoked Ca^{+} signals in neurons and cytokine profiles in the affected skin, in a second setting the dorsal root ganglia as well as rostral neck skin were isolated from each mouse 24 h after last oclacitinib treatment and 30 min after last TDI challenge (day 8).

RESULTS AND CONCLUSIONS
Whereas mice treated with oclacitinib showed a significant decrease in scratching behaviour throughout oclacitinib treatment, scratching bouts after withdrawal of oclacitinib was significantly enhanced compared to vehicle treatment group. Corresponding to higher scratching behaviour, both TNFα and IL-31 evoked Ca^{+} signals in more dorsal root ganglia neurons in the oclacitinib treatment group (TNFα 7.19%, IL-31 10.18%) compared to vehicle treatment group (TNFα 1.62%, IL-31 2.45%). Cytokine levels in skin revealed that oclacitinib treatment led to a significant increase of TNFα and TSLP whereas concentration of IL-31 was comparable in both groups.
In conclusion, oral treatment with oclacitinib reduced itch dramatically throughout the treatment, however a significant rebound phenomenon in itch was shown after abrupt withdrawal of oclacitinib. Although this phenomenon has already been demonstrated for immunomodulators like cyclosporine A and glucocorticoids [1, 2], the exact mechanisms remains to be elucidated. Our findings indicate a peripheral sensitization of the neurons by repetitive administration of oclacitinib under allergic inflammatory conditions.

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13.3.

Effect of combined application of prostaglandins and oxytocin on the duration of parturition and number of newborn piglets of sows

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INTRODUCTION

Process of farrowing in sows on farms represents the most delicate stage in the production of piglets. It is best to finish the delivery as soon as possible, because in this way sows recover as soon as possible, and allows the piglets to suck colostrum. In order to achieve the shortest duration of parturation, in the control farrowing most often are applied uterotonics, such as oxytocin in combination with drugs for induction of parturition (prostaglandin analogues, PGF_2-alpha).

The aim of this study was to examine the extent to which prostaglandins F_2-alfa (applied alone or in combination with oxytocin) influence on the duration of parturition, and the number of liveborn piglets.

MATERIALS AND METHODS

The experiments were performed *in vivo* on 133 pregnant sows, breeds Landrace-Yorkshire, which were divided into nine groups. The animals of the first three groups were administered prostaglandin F_2-alfa (Dinoprost), i.m. at a single dose of 2 ml, at 112 days of gestation and once (after farrowing fifth piglet-second group) oxytocin (Oxytokel), i.m., at a dose of 2 ml per animal (eq. 20 units per animal) or twice (after farrowing fifth and tenth piglet-third group) oxytocin, i.m., at a dose of 2 ml per animal (eq. 20 units per animal) first time and then 1.5 ml per animal (eq. 15 units per animal) second time. All of this was done at 113 days (groups IV, V, VI) and at 114 days of gestation (groups VII, VIII, IX).

RESULTS

The obtained results showed that average duration of farrowing was the shortest (4.56 h) in sows which is applied only prostaglandin at 114 days of gestation, and the longest (7.17 h) in sows treated with prostaglandin at 112 day of gestation with twofold application of oxytocin. The largest number of newborn piglets (20, 47) have been reported in sows which were treated with prostaglandin at 113th day of pregnancy in combination with twofold application of oxytocin.

CONCLUSION

On the base of all results it may be concluded that the best effect is achieved (duration of partus and number of newborn piglet) when prostaglandin applied in combination of oxytocin (twofold) at 113th day of pregnancy.

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13.4.

Legal status regarding distribution/dispensing and administration of veterinary medicines in Republic of Macedonia

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INTRODUCTION

Animal medicines play an important role in the control and prevention of disease but have the potential to cause harm if not used properly. The use of veterinary medicines (VM) can sometimes result in residues in foods taken from the treated animals and can seriously endangered the health of people as potential consumers. Therefore, the significance of control of the VM in these animals is exceptionally high. These include statutory controls on the authorisation, distribution and use of such medicines. The aim of this paper is to show legal status regarding distribution/dispensing and administration of VM in Macedonia (RM) in order to identify legal weaknesses.

MATERIALS AND METHODS

National Law on VM (Article 47) provides legal basis for distribution of VM in categories. Following evaluation of scientific data provided by the MAH, for each VM is granted a specific distribution category by the Food and Veterinary Agency (FVA) when it is for first time authorised. The data was collected from the web site of sector for Public Health in FVA and was compared with Veterinary Medicines Regulations in other countries.

RESULTS

All VM in the RM are assigned into one of six distribution categories. Only veterinary surgeons (VS) are entitled to prescribe VM and they must be dispensed from registered premises. The highest level of control is the VM intended for food production animals which can be used only in veterinary premisses by the VS or under their direct responsibility. This would include VM containing controlled drugs and those intended for administration only following a diagnosis and clinical assessment of the animal(s). VM which can be dispensed in veterinary pharmacies only by written prescription is intended for food production animals but is not required a clinical assessment. VM intended for non-food production animals may be supplied by any retailer without any restrictions, or provision of advice.

CONCLUSIONS

Distribution categories provide controls on the supply of veterinary medicines to help ensure that appropriate advice is given at the point of sale so that products can be used safely and effectively. Also it is a practical tool for identification of different groups of VM for the veterinary practitioners as well as all subjects involved in production, trade and distribution of VM. The results obtained given an overall picture of trends in the use of VM in RM and allows comparison of such trends in other countries.

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13.5.

Treatment of bovine retained fetal membranes: the opinion of vets and breeders confronted with evidence-based veterinary literature

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INTRODUCTION

This study was designed to examine the possibility for veterinarians to locate and to apply data of literature for evidence-based practice in the field of cattle reproduction[1]. Treatment of retained fetal membrane was chosen as an example.

METHODS AND RESULTS

In a first step, an internet survey was sent to 639 cow breeders and veterinarians, asking them how they did manage the treatment of retained fetal membranes after Calving. It appears that manual removal of placenta is the main treatment employed by 89% of the vets motivated by the request of the breeders and the fear of endometritis. Only 10% consider that it's not useful to remove manually the placenta. Their answers are motivated on what they did learn in their veterinary school curriculum or previous trainings.

In a second step, the treatment was looked for after literature reading with evidence-based veterinar medicine (EBVM) methods. The study of CAB Abstracts, Science Direct and Medline databases using specific key-words found 271 articles, but only 6 original studies fulfilled EBVM criteria [2]. Manual removal of the placenta seems to be disadvised. The best solution would be to identify the animals that retained their placenta and to treat systemically with antibiotics only febrile cows.

CONCLUSION

Although the randomized controlled trials are likely lacking concerning the treatment of cows with retained fetal membranes, our study revealed that the use of appropriate database and combination search terms are important to help clinicians for locating relevant EBVM literature. However, only the critical analysis of full-text papers can allow them to find better evidence to support their therapeutic decision.

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13.6.

Randomized comparative study of two products for the treatment of otitis externa in dogs

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Otitis externa is one of the most prevalent diagnoses in canine practice. Small inflammatory changes in the fragile microclimate of the skin in the external ear allow abnormal proliferation of commensal bacteria (*Staphylococcus pseudintermedius*) and yeast (*Malassezia pachydermatis*) or opportunistic invaders. The majority of cases of otitis externa can be treated successfully with topical medication administered into a clean, dry external ear canal. The efficacy and safety of Posatex (MSD Animal Health, Boxmeer, NL – mometasone furoate, orbifloxacin and posaconazole) was compared with Aurizon (Vétoquinol, Lure, France – dexamethasone acetate, marbofloxacin and clotrimazole) in a non-blinded, controlled, randomized and blocked, multicentre clinical field study in dogs with otitis externa.

Dogs (>4 months old, $n = 152$) were enrolled based on clinical signs, cytology and culture. Affected ear canals were cleaned with saline, dried and then treated once daily for 7 days. Treatment success (excellent/good/moderate/poor) was assessed by both veterinarians and owners.

Total clinical scores decreased from 7.5 (95% CI [7.1; 7.9]) and 7.7 [7.3; 8.1] on Day 0 in the Posatex ($n = 76$) and Aurizon ($n = 76$) groups, respectively to 2.5 [2.1; 3.0] and 2.7 [2.3; 3.2] on Day 7. Non-inferiority was confirmed using a confidence interval approach for clinical success. More dogs had normal scores for pain, redness and swelling on Day 7 in the Posatex (67.1%, 32.9% and 61.8%, respectively) than in the Aurizon (55.3%, 28.9% and 60.5%, respectively) group. Both products were well tolerated. The overall assessment by the owner was significantly better for Posatex (38.2% excellent, 51.3% good, 9.2% moderate and 1.3% poor) than for Aurizon (25.0%, 47.4%, 25.0% and 2.6%, respectively) (Mann–Whitney 0.61 [0.53; 0.69], Wilcoxon $P = 0.0085$). Overall treatment success was significantly better for Posatex ($P = 0.0083$) than for Aurizon.

The present study provided further confirmation that Posatex was safe and effective in the treatment of canine otitis externa associated with yeast (*Malassezia pachydermatis*) and bacteria when administered once daily for 7 days (1). Antibiotics, particularly fluoroquinolones, should be used with prudently so as to minimize the selection of resistant pathogens. The high topical potency of mometasone furoate may have contributed to the resolution of ear inflammation (pain, redness and swelling) and thus the better overall evaluation of this product 1–3).

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Session 14: Toxicology 2 Clinical/Small Animals & Regulatory

14.1.

Lidocaine plus adrenaline in dogs: pharmacokinetic profile and toxicity evaluation after intra-articular (IA) administration

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OBJECTIVE

To evaluate the safety in terms of cardio- and neurotoxicity and of chondrotoxicity of the IA administration of lidocaine plus adrenaline in anaesthetised dogs undergoing arthroscopy of the elbow.

METHODS

Twelve dogs were recruited in the study. Six subjects (LA group) were injected IA different volumes of a solution of lidocaine 1.98% plus adrenaline 1:100 000, while other six dogs (S group) received saline 0.9% via the same route. Heart rate, electrocardiogram, respiratory rate, non-invasive systolic, diastolic and mean arterial blood pressure were monitored during the entire arthroscopic procedure. As eventual neurotoxic signs could have been masked by the general anesthesia, blood samples were withdrawn at scheduled time points (before and after the IA administration of lidocaine plus adrenaline), in order to determine whether levels of lidocaine and its active metabolite monoethylglycinexylidide (MEGX) responsible for neurotoxicity were reached. Primary cultures of canine chondrocytes were used to assess condrotoxicity, following exposure to different concentrations of lidocaine alone and lidocaine plus adrenaline 1:100 000.

RESULTS AND CONCLUSIONS

No bradyarrhythmia, hypotension, electrocardiographic modifications, or severe respiratory rate variations were observed during the entire procedure, either in the LA or the S group. No neurological side effects, such as muscle tremors, excitation, convulsions or depression of the CNS, were reported during/after recovery from general anaesthesia. The C_{max} of lidocaine and MEGX were between 0.188 and 2.188 $\mu g\ ml^{-1}$ and between 0.023 and 0.408 $\mu g\ ml^{-1}$, respectively. The C_{max} of lidocaine was lower than that indicated by Lemo *et al.* (2007) as responsible for the appearance of neurotoxic effects, and also lower than that obtained in a similar study were the sole lidocaine was administered (Di Salvo *et al.*, in press). This data suggest that adrenaline reduces the absorption of lidocaine, not allowing the achievement of neurotoxic blood concentrations. As regards to the *in vitro* chondrotoxicity, lidocaine proved to have a dose- and time-dependent effect on the viability of chondrocytes. However, the presence of adrenaline appeared to be capable of reducing the chondrotoxicity of lidocaine by 1%, following an exposure of up to 30 min. Pending further investigation, veterinarians should be advised to use lower concentrations of lidocaine when an IA route is contemplated.

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14.2.

Confirmed case of cocaine poisoning in a young dog

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INTRODUCTION

Cocaine is the natural alkaloid of the shrubs *Erythroxylon coca* and *Erythroxylon monogymum*, originally from South America. This compound is an illicit drug with intense psychostimulant effects and complex pharmacological properties, being estimated as the second most problematic illegal drug after heroin. Giving the scale of the problem in human medicine, clinical veterinary reports of cocaine toxicosis are surprisingly sparse. This exposition is more likely to affect dogs (particularly police dogs) but also, for example, athletic horses which may be dosed with cocaine to improve their competition performance. The LD_{50} for dogs is 3 mg kg^{-1} IV, but carnivores in general can tolerate 2–4 times that dose given PO.

MATERIAL AND METHODS: THE CLINICAL CASE

A case initially suspicious of cocaine poisoning was referred to the Toxicology Unit. A 2 year-old Schnauzer male dog was presented to the veterinary clinic with a history of two hours of vomiting and hyperexcitability; during physical examination, unsteadily march, bilateral mydriasis, ataxia and focal muscular tremors were observed. Rectal temperature was 39.8°C (hyperthermia). When monitoring cardiac and respiratory functions, tachycardia was clearly observed, but thoracic auscultation was normal. The owner referred to have witnessed the dog ingesting 'cocaine, from my home mate', which is a quite interesting situation, as in general the pet owner may be reluctant to admit it.

The treatment was associated to digestive decontamination (with limited effect, as the drug is absorbed extremely rapidly) by means of gastric lavage. Seizure control was made with Diazepam, and body temperature was controlled with cool environment, cool IV fluids and close monitorization.

DISCUSSION: DIAGNOSIS AND EVOLUTION

According to these facts, analysis of this 'white powder' (brought in a plastic bag by the owner) was performed for

identification of cocaine and its metabolites by means of GC-MS, and the alkaloid was clearly confirmed. The signs were compatible with cocaine ingestion and a final diagnosis of cocaine poisoning was established.
In less than 24 h, all the symptoms completely disappeared, and the patient was sent back home two days later.

14.3.
Pet poisoning in Spain: some regional differences
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INTRODUCTION
The use of poisoned baits is an illegal, massive and non-selective method that affects many species, thus rendering criminal poisoning a serious threat for both domestic and wild animals. In fact, all domestic species are potential victims of accidental or deliberate poisoning, according to the experience of the Diagnostic Toxicology Laboratory at the Faculty of Veterinary Medicine of Cáceres, where suspected cases of animal poisoning are analyzed. However, some substantial differences can be observed when a comparison between cases from different geographical regions of Spain is considered. Domestic animals constitute approximately half of the total cases referred from Northern Spain (Asturias and Galicia regions), whereas they constitute no more than 20% of the total cases in the West (Extremadura region).

MATERIAL AND METHODS
Epidemiological data from the Diagnostic Toxicology Lab of Caceres have been considered in order to determine the major chemical agents involved in pet poisoning. Although samples from all of Spain are analyzed in our lab, only data from the North (Galicia and Asturias) and the West (Extremadura) were considered for this study. Most of the samples received in our lab comes from these regions/areas. Chemical analysis was developed in tissue samples (mainly gastric content) and suspected baits (when available), in order to establish the relationship between those two relevant samples.

RESULTS AND DISCUSSION
Aldicarb and Carbofuran, two carbamate pesticides, were banned by the EU legislation in 2003 and 2008, respectively. Similarly, Strychnine was banned in Spain in 1994. Some interesting differences can be observed between both geographical areas. Samples from the North are very often associated to Strychnine and Chloralose, whereas those poisons have hardly been identified (frequency <1%) in Western samples during the last 5 years. Conversely, both carbamates showed a high prevalence in samples from Extremadura, where other toxic compounds, as for example Metaldehyde, Bromadiolone or Phenobarbital, were also detected. These geographical differences show us a different access and availability to poisons, but in both cases a criminal mentality, which seriously affects our domestic animals.

14.4.
Oral toxicity of isotretinoin, misoprostol, methotrexate, mifepristone, and levonorgestrel as pregnancy category X medications in female mice
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An oral toxicity study of several pregnancy category X drugs was performed in female ICR mice. The drugs were given orally once daily for 3 days at doses of 1, 10, and 100 mg kg^{-1} for isotretinoin; 6.7, 67, and 670 μg kg^{-1} for misoprostol; 83, 830, and 8300 μg kg^{-1} for methotrexate; 3.3, 33, and 330 mg kg^{-1} for mifepristone; and 25, 250, and 2500 μg kg^{-1} for levonorgestrel. During the test period, clinical signs, mortality, body weight, hematology, serum biochemistry, and necropsy findings were examined. After dosing of methotrexate at 8300 μg kg^{-1}, many animals showed decreased spontaneous activity, and one animal died. In hematological analysis, compared to the controls, the animals treated with the drugs showed similar significant decreases in the number of granulocytes and granulocyte differentiation and increases in lymphocyte differentiation. In serum biochemical analysis, animals receiving high doses of the 5 drugs showed significant changes in uric acid, glucose, alkaline phosphatase, total bilirubin, lipase, total cholesterol, and calcium. At necropsy, intestinal redness was frequently observed in animals that received the high dose of methotrexate. Uterus enlargement and ovarian dropsy were also detected in the groups receiving mifepristone and levonorgestrel. Despite the short-term exposure, these drugs exhibited significant side effects, such as white blood cell toxicity, in the mouse model. Category X drugs can be traded illegally via the internet for the purpose of early pregnancy termination. Thus, illegal abuse of the drugs should be further discouraged to protect young mothers.

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14.5.
Case report: intentional endosulfan poisoning of domestic animals

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INTRODUCTION
Endosulfan is a chlorinated insecticide used in many Countries. It is usually provided as a mixture of two steroisomers, α- and β-endosulfan, that are partly metabolized and excreted in urine and feces as oxidation products, such as endosulfan-sulphate, endosulfan-alcohol, endosulfan-ether and endosulfan-lactone. Endosulfan presents moderate toxicity for mammal, but is frequently in malicious poisonings. It is highly toxic to fish and some bird species and was reported to have oestrogenic effects on humans. Two indipendent cases are reported here involving a cat and a dog, both treated for severe seizure symptoms. In both cases, they died within few hours. Liver and gastric content samples were sent to our laboratory in order to investigate over possible poisoning.

MATERIALS AND METHODS
The analysis were performed by gas-chromatography-mass spectrometry (GC-MS). After sample preparation. 4 g of each homogenised liver and gastric content were treated with the QuEChERS approach. All the extracts were evaporated under nitrogen and dissolved in 100 μl methanol. 1 μl was then injected in the GC-MS system.

RESULTS
Cat's gastric content resulted positive to α-endosulfan and β-endosulfan, while liver sample contained endosulfan-sulphate and endosulfan-ether (the former more abundant than the latter). No endosulfan-lactone was formed from its precursor endosulfan-sulphate (probably because of the short time elapsed from ingestion to death),
The dog's gastric content contained the same quantities of α- and β-endosulfan. Unlike in cat's sample, the same compounds were present also in the liver, where β-endosulfan concentration was about one fifth of the α-form. In the liver sample, also endosulfan sulphate and endosulphan ether were identified.

CONCLUSIONS
Ingestion of endosulfan by animals leads to its identification in the gastric content only when their death occurs shortly after ingestion. On the other hand, unmodified endosulfans can be detected in liver samples only at low concentration, because of its biotransformation. Endosulfan-sulphate and endosulfan-ether can therefore represent useful target analytes to disclose fatal conditions of endosulfan poisoning.

14.6.
Dog susceptibility to drug toxicosis and MDR1: the contribution of veterinary pharmacovigilance

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INTRODUCTION/OBJECTIVE
P-glycoprotein (PgP) is a membrane efflux pump. As PgP is ubiquitary, it minimizes the body exposure to potentially toxic xenobiotics. In some dog breeds, a mutation in the MDR1 gene results in a non-functional PgP, rendering them susceptible to some drug adverse effects. A lot of drugs interact with PgP (as substrates, activators or inhibitors) while susceptibility reactions are not systematically described in dogs with the gene deletion. Guidelines and warning comments on the use of some drugs in dogs with the MDR1-1Δ mutation were developed on www.collie-online.com. If toxicity is well documented for a few drugs (eg. avermectines) in breeds known to have the MDR1 gene mutation, there are few bibliographic data to support recommendations on a lot of other drugs (e.g. metoclopramide). In this context, data from veterinary pharmacovigilance concerning these drugs may provide additional information on the susceptibility to drug in connection with the mutation in the MDR1 gene.

MATERIALS AND METHODS
We have compiled a list of drugs for which the site www.collie-online.com references a susceptibility for dogs with the gene deletion but for which there is no specific mention on the summary product characteristics for corresponding veterinary products. A literature review and analysis of data from veterinary pharmacovigilance were conducted to look for over representation of breeds at risk or more severe symptomatology in dogs of these breeds.

RESULTS
Simple knowledge of type of interaction between a drug and PgP does not allow relevant conclusions to be drawn. Also, all PgP substrates do not cause toxicity in dogs with the MDR1 gene mutation. Some drugs like spinosad induce susceptibility only with some specific substrates of PgP (eg. ivermectin but no milbemycin). For other drugs like metoclopramide, experimental data collected in other species point to a possible susceptibilty to these drugs for breeds known to have the MDR1 gene mutation, but data from veterinary pharmacovigilance do not support this view.

CONCLUSIONS
Data from veterinary pharmacovigilance provide insight into recommendations developed on the web.

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14.7.
Emerging poisonings in small animals

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INTRODUCTION
Small animals may be exposed to an enormous variety of possible toxicants. The present work is a 3-year epidemiological study (2011–2013) of all enquiries received by the human Poison Control Centre of Milan (MPCC) related to small animal poisoning by drugs, pesticides and plants.

MATERIALS AND METHODS
For each enquiry, details of the exposure and the clinical history were recorded in a standard report form and after a complete follow-up entered in the MPCC database. The data were statistically analysed in order to determine trends and identify emerging toxicants.

RESULTS
MPCC recorded 796 cases of poisoning involving dogs (82.8%) and cats (17.2%). Pesticides, drugs and plants accounted for 37%, 25.1% and 9.9%, respectively. Insecticides (42.2%) proved to be the most common cause of pesticide poisoning and exposure to pyrethrins-pyrethroids accounted for the majority of calls (33.3%) followed by anticholinesterase insecticides (20.4%) and neonicotinoids (10.6%). Rodenticides accounted for 28.2% of the pesticide-related calls and second-generation anticoagulant rodenticides such as brodifacoum and bromadiolone were the primary cause of poisoning. MPCC recorded a substantial increase in enquiries about herbicide-related animal poisoning (12.2%) and glyphosate was the main culprit. Accidental ingestion of drugs intended for human use accounted for several poisonings in dogs. NSAIDs and CNS drugs were the most commonly involved. Among NSAIDs, ibuprofen was the main culprit (32%) followed by naproxen and diclofenac (16% each). Misuse of veterinary parasiticides was a common cause of drug poisoning with many cases involving the combination permethrin-imidacloprid in cats. The plants most frequently involved were *Cycas revoluta* (15.4%), *Nerium oleander* (9.2%), *Euphorbia pulcherrima* (7.7%) and *Hydrangea* spp. (3.1%).

CONCLUSIONS
On the one hand the present data point out a reduction in the number of pesticide poisonings, if compared with a previous epidemiological study (1), showing a decrease of anticholinesterase insecticides and a trend of increasing poisonings with neonicotinoids. On the other hand a higher incidence of drug and plant poisonings was observed. These data are relevant to amplify awareness about emerging toxicants that may pose a threat to animal health.

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14.8.
Lead poisoning in a cat

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A 5-year-old male cat, weighing 4.1 kg, was presented with sudden onset of weakness, anorexia, weight loss and episodes of vomiting and diarrhoea since 4–5 days. Clinical examination of this cat confirmed weight loss (0.8 kg since one week) and revealed abdominal pain and neurological signs such as lethargy, muscle tremors, incoordination and disorderly movements of the head. A complete blood count revealed moderate anemia, leukopenia and neutropenia. A biochemical profile only revealed a moderate increase of the activity of the alanine aminotransferase (180 UI/L). After anaesthesia, the cat was equipped with an intravenous infusion system and an esophageal probe in order to rehydrate and feed by enteral route the animal. A symptomatic medical treatment was given by using an anti-emetic to treat vomiting (maropitant – Cerenia® 10 mg ml^{-1} solution for injection for dogs and cats) and a drug for treating potential ulcers of the stomach and the intestines (ranitidine – Azantac® 50 mg per 2 ml solution for injection). The cat died two days later.
The cat owner informs the vet that he moved into an old apartment for about 5 months. Her cat was drinking tap water since moving, knowing that the building would be equipped with lead pipes. Moreover, the cat owner also said he had to make sanding old paint from the apartment about 15 days before the cat does exhibit symptoms.
The epidemiological informations, the clinical symptoms and blood changes are consistent with those of a lead intoxication. A dosage of lead in the liver of the cat revealed a lead concentration of 23 561 $\mu g\ kg^{-1}$ (wet weight), confirming the diagnosis of lead poisoning. Unfortunately, no lead analysis in paint and tap water was done.
An overview is given of the literature on the diagnosis, pathogenesis, and treatment of lead intoxication in cats (1). In this clinical case, the most likely source of lead is the old paint; it is known that poisoning may occur during house renovations when old paint is removed by sanding or scraping and then

dust and chips environment may be both inhaled and ingested during grooming.

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14.9.
Theobromine poisoning in dogs ingesting croquettes

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Cases of dog poisoning in the departments of Aude, Isère and Drôme (France) were reported in early September 2013. Dogs exhibited clinical signs or were found dead a few hours after ingestion of croquettes. When clinical signs were observed in dogs, they were mainly vomiting, diarrhoea, epileptic seizures and cardiac arrhythmias. Necropsy of dogs revealed a congestion of the stomach and of the pancreas.
The origin of the poisoning, sometimes fatal, seemed to be the ingestion of a brand of croquettes made in Spain. Analyses carried out in France on such croquette samples from the Spanish manufacturer, including the remains of servings consumed by dogs died suddenly, gave the following results: 8 of 8 samples contained theobromine at a level higher than the maximum regulatory concentration (from 97 to 3440 mg kg^{-1} feed, or from 1.9 to 68 times greater than the maximum regulatory concentration which is 50 mg kg^{-1} feed). Additional analyses were performed in parallel by the Spanish authorities. They revealed the presence of theobromine in 7 official samples of croquettes produced by the manufacturer: contents ranging from 800 to 3700 mg kg^{-1} (levels approximating those found by official analyses in France). These results suggested a very strong presumption of a causal link between the consumption of croquettes containing high levels of theobromine and the death of dogs. Theobromine is a naturally occurring substance in cocoa and is well known by veterinarians and manufacturers of animal feed as the cause of poisoning in dogs. Cocoa pods were incorporated at a high level in the croquettes manufactured in Spain. An overview is given of the literature on the diagnosis, pathogenesis, and treatment of theobromine poisoning in dogs (1, 2).
The activity of the manufacturer was suspended by the Spanish authorities on 18 September 2013 and all feed from this company were withdrawn from the market by the Spanish authorities on 27 September 2013. In France, without waiting for the results of analyses, the six French distributors concerned had conducted the withdrawal and recall to their customers of all the croquettes bags sold since April 2013.

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14.10.
Baclofen intoxication in dog

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Baclofen is a muscle relaxant wich can be used in veterinary medicine for treatment of urinary retention, but most intoxications in dog are linked to accidental ingestion of drugs for treatment of its owner. During the period 2000–2015, the CAPAE-Ouest (animal Poison Center of veterinary school of Nantes), registered 60 phone calls about baclofen ingestion.
The poster presents a review of the reported clinical picture.
Vomiting are common (50% of cases). Nervous signs depend on the ingested dose. At moderate dose (<5 mg kg^{-1}), behaviour disorders are noted (vocalisations, going in circles…) or listlessness or even drowsiness. At higher doses, gait disorders appear, then decubitus and at dose >10 mg kg^{-1} coma is noted. There is often hypothermia, sometimes tachycardia, but rarely true seizures. No deaths were observed after treatment with diazepam and fluid therapy.
The frequency of baclofen intoxication increases in pets, as a result of additional or broader indications of the drug for human use. Medical management relies on anticonvulsivants and appropriate fluid therapy.

14.11.
Preliminary toxicological study of L-DOPA-coated iron oxide nanoparticles in mice

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INTRODUCTION
Theranostics is an emergent concept, combining imagistic diagnosis and therapy. Superparamagnetic iron oxide nanoparticles (SPIONs) are promising nanostructures for drug carriers and magnetic resonance imagistics.
Objective: preliminary toxicological study on SPIONs stabilised with dopamine in normal mice. Cytostatic-loaded SPIONs will be further developed as theranostic agents for brain tumors.

MATERIALS AND METHODS
SPIONs (>75 emu g^{-1} saturation magnetisation) were synthesized using the laser pyrolysis technique, and were thereafter dispersed in L-DOPA solution as stabiliser. SPIONs-DOPA were administrated in the lateral tail vein of C57 Black mice. Histological investigations were performed on liver, kidney, spleen and brain, at 3 and 30 days after SPIONs-DOPA administration.

RESULTS
Intravenous administration of SPIONs-DOPA (0.08 mg g^{-1} b.w.) induced instant death of mice, and consequently we

lowered the dose to 0.04 mg g^{-1} b.w. Histological signs of toxicity were assessed at 30 days post-inoculation. The brain tissue exhibited marked hyperemia of the meninges and cerebellum hemispheres, moderate edema, and slightly dilated cerebral ventricles. The renal tissue presented frequent atrophy of glomeruli, slightly dilated capillaries with red cells agglutinated in the intraluminal space, and renal tubule epithelium with dystrophic-type changes. The hepatic tissue presented at pericentrolobular level dilated hepatocytes with intracytoplasmic vacuoles, and slightly dilated centrolobular veins, with frequent red blood cells agglutinated in the intraluminal space. The splenic tissue exhibited marked hyperemia and the expansion of the red pulp, while numerous apoptotic bodies were found in the white pulp.

CONCLUSION

Results highlighted the type of histological changes induced by intravenous administration of SPIONs-DOPA in mice, which should be considered for further development of nanotheranostic agents.

ACKNOWLEDGEMENTS

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Session 15: Recent and Innovative Advances

15.1.
Comparison of different pharmaceutic formulations of local anaesthetics on permeation through equine skin *in vitro*

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INTRODUCTION

In this study we used commercially available local anaesthetic formulations, which contain the local anaesthetics (LA) lidocaine, prilocaine or tetracaine. These LA are used for topical treatment before physical injuries to prevent pain during the performance.

The aim of the present study was to find out a formulation which provides the fastest and the highest permeability of the LA trough equine skin. Because it was the first step to the utilization of LA for pain therapy in hot iron branding of horses, equine skin was used.

MATERIALS AND METHODS

The examinations were performed *in vitro* in Franz-type diffusion cells with split skin (700 μm) of horses, all of which were euthanized for reasons not related to the present study. As test formulations commercially available pharmaceutic formulation: Emla® (lidocaine 2.5%, prilocaine 2.5%), Anesderm® (lidocaine 2.5%, prilocaine 2.5%), Pliaglis® (lidocaine 7%, tetracaine 7%) were applied. The studies were performed with and without occlusive conditions (Tegaderm®). One gram of each formulation was applied onto the skin (1.7 cm^2) and samples of the acceptor medium were taken at predefined times (15 min–6 h). To determine the lidocaine (LA) concentration in the treated skin slices of 100 μm, by a cryostat, were performed. After that the slices were homogenized, followed by an extraction. The concentrations of all LA in the acceptor medium and the extracted solution were determined by a validated UV-VIS-HPLC method.

RESULTS

The permeation coefficients (P_{app}-value) showed that prilocaine (1×10^{-5} cm s^{-1}) and lidocaine (1×10^{-5} cm s^{-1}) permeated nearly equally, whereas tetracaine did not reach such a high permeability (2×10^{-6} cm s^{-1}). The permeated amount of tetracaine was the lowest, followed by lidocaine and prilocaine. Liberation, penetration and permeation was best in Emla® under occlusive conditions (Tegaderm®), compared to Anesderm® (occlusive or with added tetracaine) and Pliaglis® (all compositions). For this reason we performed the analysis of the amount contained in the skin with Emla® under occlusive conditions. Prilocaine and lidocaine reached high amounts in the skin (Prilocain: 2580 ± 678 ng mg^{-1}, Lidocain 2511 ± 674 ng mg^{-1}), which are known to be effective in suppressing pain (Rolsted *et al.* 2009).

CONCLUSION

We inferred Emla® combined with Tegaderm® as the best formulation for permeability of lidocaine and prilocaine, reaching sufficient amounts in the skin. Concerning the effectiveness of the tested formulations in the hot iron branding in horses *in vivo* studies are required.

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15.2.
HPLC method validation and quantification of flupirtine in canine plasma

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INTRODUCTION

Flupirtine (FLU) is a non-opioid analgesic drug belonging to the unique class of the 'Selective Neuronal Potassium Channel Openers' without antipyretic or antiphlogistic properties [1]. The pharmacological properties of FLU contribute to its therapeutic benefits, without undesirable adverse effects typical of classic analgesic drugs [2] and that might potentially be useful in veterinary medicine. No analytical method to detect FLU in canine plasma samples through a fluorimetric detector has been published to date. The aim of the study was to develop an analytical method providing a selective and accurate quantification of FLU.

MATERIAL AND METHODS

The mobile phase consisted of ACN: $AcONH_4$ (20 mM) pH 6.8 (60:40, v/v) at a flow rate of 1 ml min^{-1} in isocratic mode. Excitation and emission wavelengths were set at 323 and 370 nm, respectively. Typical retention times for FLU and IS were 4.6 ± 0.2 and 5.8 ± 0.2 min, respectively. The extraction method was performed with 500 μl of plasma sample added to 100 μl of IS (100 μg ml^{-1}) and vortexed for 60 s. Four ml of AcOEt:CH_2Cl_2 (7:3 v/v). Three ml of the organic phase was collected and evaporated under a gentle stream of nitrogen at 40°C and reconstituted with 500 μl of the mobile phase. The described method was validated according to EMA guidelines on the bioanalytical method validation.

RESULTS AND CONCLUSION

The recoveries of FLU and IS (trazodone) were about 89% and 77%. Limits of quantification and detection were 1 and 0.3 ng ml^{-1}, respectively. The applicability of this method has been verified by determining FLU in canine plasma after single oral treatment with 5 mg kg^{-1} of Efiret®. HPLC analysis of the plasma confirmed the presence of FLU in time related amounts. The average FLU concentration in canine plasma ranged

between 4.3 and 760 ng ml^{-1}. The selectivity of the method was also confirmed by HPLC-MS analysis of plasma samples collected in treated dogs. No compounds co-eluting with the analyte of interest were detected by full scan acquisitions in positive ion mode. This is particularly demonstrated from the HPLC–MS extracted ion chromatogram (m/z 305 and 372 Da for FLU and IS, respectively) that exhibited a good correspondence with the HPLC–FL chromatogram. The low LOQ shows that the method could be useful for drug measurement even when administered in sub-clinical doses. As FLU is a drug recently considered for the veterinary medicine application, this method is the most suitable to be used for pharmacokinetic investigations in different animal species.

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15.3.

Efflux of glucocorticoids in the model of the isolated perfused equine distal limb and their dose dependent antiinflammatory effects on cultured equine synoviocytes

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INTRODUCTION

The aim of this study was to analyse the efflux rate of flumethasone, triamcinolone acetonide and dexamethasone phosphate after *intraarticular* injection in the previously described isolated perfused equine distal limb *ex vivo* model (Patan *et al.* 2009; Friebe *et al.* 2013). Furthermore, efficacious antiinflammatory concentrations of the glucocorticoids were examined using an equine synoviocyte culture.

MATERIALS AND METHODS

10 mg triamcinolone acetonide aqueous suspension, 10 mg dexamethasone phosphate and 2 mg flumethasone aqueous solution were administered into the fetlock joint from exarticulated forelimbs from slaughtered horses. Via the *A. mediana* the limbs were perfused with oxygenated tyrode solution for at least 7.5 h. During the perfusion, samples from venous perfusate from the *V. radialis* were taken to analyse the glucocorticoid concentration by high performance liquid chromatography.

Simultaneously, the antiinflammatory potential of flumethasone, triamcinolone acetonide and dexamethasone was analyzed with an equine cell culture model. For 4 h synoviocytes were pretreated with glucocorticoid containing medium (10^{-12} –10^{-8} mol l^{-1}) and then stimulated for 24 h with lipopolysaccharids. Supernatants were collected and the PGE_2 concentration measured using an ELISA.

RESULTS

In the isolated limb, the mean maximum concentration of 0.6 μg ml^{-1} of dexamethasone phosphate was reached after 0.5 h. After *intraarticular* medication, mean maximum concentration of flumethasone (0.08 μg ml^{-1}) and triamcinolone acetonide (0.09 μg ml^{-1}) were reached after 4.5 h perfusion time. In correlation with the flow rate, 8.7 mg dexamethasone phosphate, 1.9 mg triamcinolone acetonide and flumethasone left the joint over 7.5 h of perfusion. Regarding the inhibition of lipopolysaccharide induced PGE_2-production in the cell culture, the three glucocorticoids were comparable with no significant differences.

CONCLUSION

Knowledge about withdrawal times after *intraarticular* medication is useful to avoid positive doping results. The acquired data of the glucocorticoids allow for estimations of their residence time in the joint and furthermore their withdrawal periods. In this model, triamcinolone acetonide had the longest resting time in the fetlock joint after *intra articular* treatment.

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15.4.

Use of a deslorelin implant for the induction of estrus in early anestrus bitches

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INTRODUCTION

Some bitches fail to conceive despite mating or artificial insemination. The aim of estrus induction in such cases is to shorten the anestrus period during which the bitch cannot become pregnant. During embryo transfer, induction is used to synchronize estrus between the donor and recipient bitches. This study was designed to find a minimal period that can induce estrus without causing down-regulation that can be used for all bitches with a deslorelin (*a GnRH agonist*) implant (1–3).

METHODS AND RESULTS

1st experience: Seven mature Beagle bitches were studied over a total of 10 estrous cycles. During the first week of anestrus, each female received a 4.7 mg deslorelin subcutaneous implant (SUPRELORIN®, Virbac), which was removed 5–7 days later. Females in heat were left with an intact male once the progesterone concentration had reached 10 ng ml^{-1} and pregnancy was monitored by ultrasound, 14 days after mating. Cycles were induced in 100% of bitches. 50% of the bitches went on

to estrus, but only one bitch became pregnant and subsequently whelped. The bitches presented a very short estrus period (5 days on average) and no neutrophil granulocytes were observed in metestrus smears. The progesterone concentration did not increase, remaining around 1 ng ml^{-1} in 90% of cases, with the exception of the female that ovulated.

2nd experience: Eight anestral bitches received a 4.7 mg deslorelin subcutaneous implant which was removed after two weeks implantation. Once ovulation was confirmed, natural mating was performed during 72 h. After that, an ultrasound control was carried out each week to detect abortions. All the bitches came into pro-estrus (100%) and into estrus (100%) in the two weeks following implantation. The ovulation rate and gestation rate were high (100 and 87.5% respectively). In all the bitches, no luteal failure was diagnosed during dioestrus or pregnancy and all the puppies were normal and alive.

CONCLUSION

Our study showed for the first time that the withdrawal of deslorelin only after 2 weeks was optimal for fertile estrus induction in early anestrus bitches. The unsuccessful use of deslorelin in a more short-term period (i.e. 5–7 days) is likely related to the insensitivity of the pituitary gland to GnRH in the beginning of anestrus. The impact of the present findings on the bitches fertility will be discussed.

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Session 16: Workshop Analgesics

16.1.
The analgesic efficacy of intraperitoneal administration of bupivacaine in cats

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INTRODUCTION/OBJECTIVE

Intraperitoneal (IP) administration of local anaesthetics (LA) (i.e. IP analgesia) such as bupivacaine reduces early postoperative analgesic requirements, pain scores and time to first intervention analgesia after abdominal surgery in people. The aim of this study was to evaluate the analgesic efficacy of IP bupivacaine in cats undergoing ovariohysterectomy.

MATERIALS AND METHODS

Forty-five cats were included in a randomized, prospective, blinded study after owners' written consent. Anaesthetic protocol included acepromazine-buprenorphine-propofol-isoflurane. A ventral midline incision was made and cats ($n = 15$ per group) were administered either IP saline 0.9% (negative and positive control groups; NG and PG respectively) or IP bupivacaine (2 mg kg^{-1}; bupivacaine group; BG). Cats in the PG received meloxicam (0.2 mg kg^{-1} SC). An ovariohysterectomy was performed and postoperative pain was evaluated using a dynamic interactive visual analog scale (DIVAS), a composite pain scale (MCPS) and mechanical nociceptive threshold (MNT) for up to 8 h after surgery. Rescue analgesia was provided with buprenorphine and/or meloxicam. Repeated measures linear models and a Cochran-Mantel-Haenszel test were used for statistical analysis ($P < 0.05$).

RESULTS

There was a significant effect of treatment on the number of rescue analgesia ($P = 0.002$) (PG, $n = 2$, 13%; NG, $n = 12$, 80%; and BG, $n = 4$, 27%). The prevalence of rescue analgesia was higher in group NG than in groups PG ($P = 0.0004$) and BG ($P = 0.02$). The DIVAS, MCPS and MNT were not significantly different between groups.

CONCLUSIONS

Groups PG and BG produced similar analgesia in terms of pain scores, number of rescue analgesia and MNT.

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16.2.
Effectiveness of ropivacaine blocks in elective ovariohysterectomy in dogs for control of post-operative pain

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INTRODUCTION

Nowadays, pain is an ever-present theme in veterinary practice. The number of available tools to fight painful stimuli has increased and veterinarians are more accurate in recognizing pain in their patients. However, much is still to be discovered and full drug potential has not yet been achieved.

The present study aimed to test the efficacy of the use of ropivacaine as a mean to control post-operative pain, by subcutaneous administration over the incision line, in elective ovariohysterectomies in female dogs.

MATERIALS AND METHODS

In this study, 14 dogs were received at the Veterinary Teaching Hospital of the Veterinary Medicine Faculty of the University of Lisbon for elective ovariohysterectomy. Anesthethic protocol was composed by pre-medication with acepromazine (0.01–0.02 mg kg^{-1}, not given to patients <10 kg) and methadone (0.2 mg kg^{-1}), induction with propofol (2–4 mg kg^{-1}) and maintenance with isoflurane. They were randomly distributed into two groups – Ropivacaine Group (RG) and Saline Group (SG). The observer was blinded to the distribution during the study period. RG was submitted to a subcutaneous infiltration of 1 mg kg^{-1} of ropivacaine, while SG patients were subject to an equivalent volume of saline infiltration. During the procedure, heart and respiratory rate were monitored, with special attention to 5 crucial time-points. Glasgow's Composite Measure Pain Scale was used as pain assessment tool. Pain assessment began thirty minutes post-extubation and was repeated hourly, up until 6 h post-local block (limit of ropivacaine's action). Post-operatively, rescue analgesia (carprofen 4 mg kg^{-1} + buprenorphine 0.015 mg kg^{-1}) were given to either patients whose score was 5 or greater in pain assessment or after 6 h had passed, whichever came first. After the administration of rescue analgesia, the study was ended for that patient. Statistical analysis was done with R software v2.1.2.

RESULTS AND CONCLUSIONS

There were no statistical differences between groups in age, weight, duration of procedure and duration of anaesthesia ($P = 0.743$, $P = 0.318$, $P = 0.796$ and $P = 0.337$, respectively). Comparison between groups was not statistically significant in heart and respiratory rate at the 5 chosen time-points ($P = 0.990$ e $P = 0.529$, respectively) nor in pain scores ($P = 0.638$).

In conclusion, anaesthesia with methadone, acepromazine, propofol and isoflurane provided reliable analgesia during and after the procedure, since no supplemental analgesics were needed in the control group. The pre-incisional administration of 1 mg kg^{-1} SC ropivacaine did not prove effective in lowering post-operatory pain scores.

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16.3.

Comparative study of analgesic efficacy of buprenorphine versus methadone for post-operative pain control in elective ovariohysterectomy in cats

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INTRODUCTION

Pain management in feline patients and its proper assessment, have proved challenging over time. In this context comparison of the analgesic efficacy of two opioid drugs (buprenorphine and methadone) in postoperative pain control in cats undergoing elective OVH, using the UNESP-Botucatu Multidimensional Composite Pain Scale (UNESP-Botucatu MCPS)[1], was performed.

MATERIALS AND METHODS

In this study, 20 feline patients were received at a Veterinary Clinics for elective ovariohysterectomy. The individuals were allocated, randomly, in two study groups: the buprenorphine group (GB; $n = 10$), which received intravenously 0.02 mg of buprenorphine (Bupaq®, Richter Pharma AG)/Kg 30–40 min before surgery, and the methadone group (GMT; $n = 10$) which received intramuscularly 0,5 mg of methadone (Semfortan®, Esteve Farma Lda.) per kg, 15 min before surgery. The observer was blinded to the distribution during the study period. Pain was assessed 1 h (T1), 2 h (T2), 3 h (T3), 4 h (T4) and 24 h (T24) after anesthetic recovery. Rescue analgesia was indicated to animals with pain scores equal to or greater than 8 with consistent clinical assessment. The respiratory rate, heart rate and systolic arterial blood pressure were monitored during the entire procedure. Blood samples were collected to assess changes in the concentration of the serum amyloid A protein (quantification with an EKISA kit (Tridelta Development Limited)), before the OVH (T0) and in the 4th hour of the postoperative period (T4), regarding the analgesic protocol. The statistical analysis (Shapiro-Wilk test, Mann–Whitney test, *t* test, repeated measures ANOVA test and chi-squared test) of results was performed with software R v2.1.2.

RESULTS AND CONCLUSIONS

No statistical significant differences ($P = 0.443$, $\alpha<0.05$) were observed between pain scores of both groups [T1 (GB/GMT $5.5 \pm 1.1/5.3 \pm 2.1$), T2 (GB/GMT $5 \pm 1.4/4.6 \pm 1.6$), T3 (GB/GMT $4.5 \pm 1.7/4 \pm 2.1$), T4 (GB/GMT $3.9 \pm 1.4/3.6 \pm 2.5$), T24 (GB/GMT $4.4 \pm 1.4/3.1 \pm 2.8$)]. Also no statistically significant differences were observed between the two groups for all physiologic variables studied (respiratory rate, heart rate and systolic arterial blood pressure) as stated by other authors[2,3]. The same result ($P = 0.630$, $\alpha < 0.05$) was obtained for serum amyloid concentrations [T0 (GB/GMT $3.46 \pm 3.16/2.96 \pm 2.13$ $\mu g\ ml^{-1}$), T4 (GB/GMT $3.57 \pm 2.29/4.28 \pm 5.86$ $\mu g\ ml^{-1}$)], however the time point used probably wasn't the better to assess this parameter.

For the results obtained it can be concluded that both drugs are effective in pain relief following OVH in cats.

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16.4.

Perception, evaluation and management of pain associated with canine dermatologic disorders by French practitioners: a descriptive survey

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INTRODUCTION

While a considerable amount of work has focused on the management of pain in companion animals, little is known about the attitudes and treatment strategies to deal with pain due to dermatologic disorders. The aim of this study was to describe the attitudes of French practitioners regarding pain for a range of canine dermatologic disorders.

MATERIALS AND METHODS

A questionnaire was sent by email to 216 French practitioners. The practitioners were selected to represent both 'generalist' (50%) and practitioner with advanced skills or diplomate from ECVD (50%). The questionnaire explored pain assessment and treatment for a range of dermatologic conditions in dogs. Descriptive statistics were performed.

RESULTS

The response rate was 64.5%. All the respondents reported that dermatologic affection can be associated with discomfort and/or pain. For 59.9% of them, discomfort is present at least in 50% of cases. For 40.5% of them, pain is present at least in 25% of cases. Fifty percent declared treating specifically pain in 50% of cases. On a pain scale ranging from 0 to 10, the mean threshold leading to implement analgesia was 4. Moreover, 36.6% of the respondents considered their treatment unsuccessful to treat pain in 75% of cases. To treat pain, corticoids are the first option, either topically (91% of respondents) or systemically (92%). Despite their analgesic effect, 14% of respondents never prescribe NSAIDs. The use of morphine or gabapentine was very scarce (respectively 7 and 6%), contrarily to buprenorphine (20%) and tramadol (20%). 83% of respondents frequently prescribe shampoo to manage pain, while bathing is never recommended by 63% of respondents.

DISCUSSION

To our knowledge, this is the first study devoted to pain in dermatology for dogs. The study underlines the need for elaborating an objective and standardized grid to distinguish discomfort from pain. While corticoids are relevant to control inflammation or discomfort, their analgesic effect remains limited. Therefore, the use of NSAIDs, buprenorphine or morphine would deserve to be reconsidered. While the use of topical products is frequent and their composition relevant to treat discomfort, the absence of analgesic/anaesthetic drug could be a limit.

CONCLUSION

This study confirms that the assessment of pain should be included systematically in the clinical examination of dermatologic disorders. In any case, further studies to define the precise regimen for analgesic compounds to treat pain are required.

Session 17: Ecotoxicology

17.1.

A novel method based on ultrasound assisted extraction with low-density solvent and dispersive liquid-liquid microextraction for the determination of selected polychlorinated biphenyls in marine sediments by Gas Chromatography-Mass Spectrometry

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Among persistent organic pollutants, polychlorinated biphenyls (PCBs) are subject to restrictions internationally, due to their adverse health effects including endocrine disruption and concern for their residues in several matrices. Sediment is one of the major sinks of PCBs in aquatic environment. The analysis of sediment samples for PCBs levels requires highly efficient extraction techniques because the analytes tend bind the sample matrix very strongly. A new method has been developed to determine trace levels of selected PCBs in marine sediment samples by using ultrasound assisted extraction with low-density solvent and dispersive liquid-liquid microextraction for gas chromatography-mass spectrometry (GC-MS) analysis.

For method development and optimization procedures, different solvents (acetone, acetonitrile and methanol) were tested at the extraction stage in sediment and the best yield was obtained with 4 ml acetone per 2 g sample. Then a lower density solvent was used, i.e. isooctane along with acetone. The upper layer was collected and analyzed by GC-MS. The optimized procedure was validated. Sediment samples free of PCBs were spiked at six concentration levels (0.25–8.0 ng g^{-1}) of selected PCBs, and used to prepare a series of matrix-matched calibration curves. The samples were measured using this optimized procedure. The linearity was satisfactory in all cases with correlation coefficients between 0.9989 and 0.9995. The limits of detection and the limits of quantification were 0.021–0.057 ng g^{-1} and 0.069–0.190 ng g^{-1}, respectively. The recoveries at 3 spiking levels (0.5, 1 and 4 ng g^{-1}) were in the range of 90.07–100.4% and the relative standard deviations were $\leq$ 7.6%.

The advantage of this extraction method was the use of a less toxic, low-density solvent and the use of a sample syringe as the extraction device and no used any matter to further purified after extraction of sediment. The developed method has been successfully applied to the analysis of selected PCBs in sediment samples with satisfactory results. It provides a sensitive, convenient and ecofriendly process for determining PCBs in sediment samples.

Session 18: Translational Medicine

18.1.
Modulating the immune response to viral infection. Demonstrating immunomodulation using astragalus polysaccharides using a poultry model with infectious bursal disease virus

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ABSTRACT
The immune status of a patient is fundamental to the management of infection, tumours or autoimmune disease. Immunomodulation encompasses the selective up or down regulation of parts of the immune response. Cytokines or their antagonists, gene therapy and vaccination with designer adjuvants have all been proposed and some antibiotics such as macrolides have been shown to have synergistic effects in certain cases. Immune reactions, however, are complex and interdependent with the neurological and endocrine systems. Clearly a better understanding will have far reaching effect: not only on the way pharmacological agents are used but also to help elucidate the impact of psychological, social and environmental factors in the epidemiology of disease. Cost effective and practical short-term immuno-modulation is also possible, yet still poorly understood. In this experiment a poultry model was developed with high replicate numbers, low cost and short turnaround time. Whilst part of a general program of immunological research, the specific application of *astragalus polysaccharides* (APS) on chicken's erythrocyte immune adherence was investigated with application to peri-vaccinational stress.

METHODS
Two hundred and forty Specific Pathogen Free (SPF) Leghorn chickens were randomly divided into four groups and reared in isolated pens. The chickens were tested negative for infectious bursal disease virus (IBDV) at 25 days old. Group 1 was treated with saline, while Groups 2, 3 and 4 were inoculated with 0.3 ml IBDV suspension intranasally the next day. Groups 3 and 4 were also administered APS intramuscularly twice daily at 5 or 10 mg/kg, respectively, until 31 days old. The erythrocyte-C3b receptor rosette rate (E-C3bRR) and erythrocyte-C3b immune complex rosette rate (E-ICRR) were measured at 25, 29, 32, 35 and 38 days old. Duncan's multiple range tests were used to test significance between groups for E-C3bRR and E-ICRR and rank sum analysis was performed on morbidity and mortality.

RESULTS
The results showed that IBDV significantly reduced E-C3bRR and E-ICRR of chickens compared with the control group ($P < 0.05$, respectively), while simultaneous administration of APS with IBDV maintained E-C3bRR at similar levels to control group ($P > 0.05$) and increased E-ICRR compared with control group and the group non-treated with APS ($P < 0.05$). APS treatment also reduced the morbidity and mortality of chickens inoculated with IBDV ($P < 0.05$).

CONCLUSION
APS enhanced immune adherence of chicken erythrocytes by affecting the activity and/or numbers of complement receptor type one on the erythrocyte membrane.

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18.2.
Pharmacology and biomechanics of fracture healing: a translational research approach to the development of human orthopaedic scaffolds

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INTRODUCTION
The musculoskeletal system is well-suited to comparative medicine. Information can be translated across species advancing diagnosis and treatment. Otto Stader, a veterinarian, used this approach to develop the first external skeletal fixation, the Stader splint, to stabilize fractures in dogs. Another veterinarian Jacques Jenny performed one of the first intra-medullary pinning procedures in animals and significantly advanced fracture repair in horses and humans. Sten-Erik Olsson and John L. Marshall, both of whom had medical and veterinary degrees, founded the first dedicated comparative orthopaedic research laboratory at the Hospital for Special Surgery in New York. Comparative orthopaedic laboratories are now located throughout the world and use comparative and translational research approaches to improve diagnostic capabilities and enhance understanding of preventive and therapeutic disease mechanisms. Advances in fracture fixation, total joint replacement and cartilage repair are examples of mutual benefit to human and animal health. Healing of sizeable bone deficits remains a considerable challenge for surgeons typically following trauma or cancer. Several animal models have been developed to investigate bone regeneration. Among these, sheep offer subjects of

similar: body weight; mineral composition of bone; metabolic rate and remodelling rate to human patients. Long bone dimensions are suitable for human fixation implants and prostheses. This model investigated the implantation of synthetic scaffolds and tissue growth stimulators in large bone defects.

METHOD

3 cm and 6 cm ovine tibial bone defects were created in sheep aged 5 or more years and slow biodegradable composite scaffolds comprised of medical grade polycaprolactone and calcium phosphates (hydroxyapatite and tricalcium phosphate) were implanted with or without 1 mm3 rhBMP-2 (recombinant human bone morphogenic protein) or 106 bone marrow-derived mesenchymal precursor cells in 40 ml at 4–6 weeks post implantation.

RESULTS

Both 3 cm and 6 cm bone defects were regenerated by recruitment and stimulation of endogenous cells using a matrix scaffold containing relevant growth factors. The regenerative potential of a scaffold system with BMP outperformed the best available autografts after 12 months of implantation. Improved structural integrity was verified with x-ray, CT and histology, furthermore biomechanical testing revealed improved mechanical strength. The composite scaffold loaded with 40 ml bone marrow-derived mesenchymal precursor cells stimulated more bone formation than the scaffold alone; however, there was significantly less bridging and bone volume than the scaffold plus rhBMP-2 group.

CONCLUSIONS

Animal models allow us to define key steps in bone regeneration and biological and mechanical factors that influence bone healing. Ongoing studies focus on increasing cell implantation number and adapting scaffold design to minimise the invasive injection of cells at 4–6 weeks after the implantation of the scaffold to help avoid the initial inflammatory phase related to surgery and to synchronise implantation with the early vascularization of the implanted matrix.

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18.3.†
ABSTRACT DELETED

Session 19: Experimental Design

19.1.
The effect of propentofylline administered *in vivo* on the splenic regulatory T cells in mice

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INTRODUCTION

Propentofylline is a neuroprotective and vasoactive drug used in veterinary medicine. The drug inhibits the activity of phosphodiesterase (PDE), especially PDE4 (Meskini *et al*., 1994). The selective PDE4 inhibitors exert beneficial effects in the treatment of some autoimmune diseases (Dinter *et al*. 2000). Regulatory T cells (Tregs) promote immunological tolerance and have potential role in the treatment of autoimmunity disorders and transplantation. PDE4 and PDE7 inhibitors increase the expression of Treg marker (Foxp3) and can suppress experimental autoimmune encephalomyelitis in mice (González-García *et al*., 2013).

MATERIALS AND METHODS

The study was conducted on 8-week-old female Balb/c mice, each weighing 20–22 g. Propentofylline (in substance, Sigma), after dissolving in distilled water, was administered orally (by stomach tube) once or six times at 12 h intervals at the dose of 3 mg/kg. Parallely, the control mice received distilled water instead of the drug. Each experimental group consisted of 7 mice.

The absolute number and the percentage of splenic Tregs were determined by a Mouse Regulatory T Cell Staining Kit #1 (eBioscience). These parameters were estimated 12 and 24 h after the last dose of drug administration. Fluorescence was measured using a flow cytometer (BD FACSCalibur) and analysis were performed using a CellQuest Pro software. The data were analyzed statistically using t-Student test. The differences were considered significant at $P < 0.05$.

RESULTS AND CONCLUSIONS

The single administration of propentofylline increased the absolute number and the percentage of splenic Tregs ($CD4^+CD25^+Foxp3^+$ cells). This effect was noted 24 h after a single dose of the drug. Propentofylline administered six times did not change the percentage and the absolute number of splenic Tregs in mice. The stimulating effect of propentofylline on the splenic Treg cells is short-lasting and is balanced during the treatment.

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19.2.
A pilot study on plasma concentrations of buprenorphine following CRI in dogs undergoing ovariectomy

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INTRODUCTION

Management of pain in small animals is essential to improve surgical recovery and general condition of suffering animals. Buprenorphine (BUP) is an opioid μ agonist/k antagonist, widely used in veterinary medicine due to its analgesic efficacy, low risk of respiratory depression and negligible cardiovascular effects. BUP clinical dose in dogs and cats is in the range of 10–20 $\mu g\ kg^{-1}$ administered IV-IM every 6–8 h. Constant rate infusion (CRI) of analgesic agents provides some advantages compared to other modes of administration: it prevents sudden peaks (often associated with adverse effects), maintains a steady state, provides a faster recovery from drug effects (that would depend on the pk of the drug), requires a lower total amount of drug to provide pain control. To date, many studies have been carried out for CRI of many opioids, but not for BUP. This study is part of a wider project, which aim is to investigate the best CRI for BUP in dogs.

MATERIALS AND METHODS

Three dogs (18–26 kg; < 6 years old) undergoing routine ovariectomy received carprofen (4 mg kg^{-1}) IV providing pre-operative analgesia. Animals were monitored through the whole study byusing a Visual Analogue Scale for pain and by using a sedation score. Following surgery, an CRI of 3.2 $\mu g\ kg^{-1}\ h^{-1}$ and 2.0 $\mu g\ kg^{-1}\ h^{-1}$ were administered respectively to 1 dog (A) and 2 dogs (B-C) for 10 h. 1.5 ml blood samples were collected at: 0, 20, 40, 60, 90, 120, 180, 240, 300, 360, 420, 480, 540, 600 min, plasma was separated and kept at −80°C until analysis in UPLC-MS/MS. The number of animals is not yet sufficient for statistical and pharmacokinetic analysis.

RESULTS AND CONCLUSIONS

In all dogs plasma concentration of BUP was above the lower level considered effective (0.6 ng ml^{-1}) beginning from 20–40 min after CRI start and through the whole study. The steady state in dog A was of 3.0 ng ml^{-1}, it was maintained for 6 h and then decreased (1.5–2.0 ng ml^{-1}) during the last 4 h. Dogs B and C had a steady state of 1.0–2.0 ng ml^{-1} dur-

ing all the study. No adverse behavioral effects were observed during or after CRI. The loading dose of BUP is needed to facilitate BUP transfer to SNC and so the onset of analgesia; studies on a single IV bolus may consider CRI plasma concentration reported in the present study in order to find the best combination for effective analgesia. CRI of BUP may be a useful treatment to provide analgesia in dogs, but this requires further investigation.

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19.3.

Measuring the excitation of canine dorsal root ganglia to pruritogens

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INTRODUCTION

Sensory nerve fibers that innervate the skin have their cell bodies in the dorsal root ganglia (DRG). The activation of DRG leads to open voltage-dependent calcium channels and an influx of calcium ions resulting in the excitation of the neuron and initiating the sensory process. Therefore, measurement of calcium influx is a direct correlation to the excitation and function of that neuron. The excitation of DRG to possible pruritogens is a common procedure in mice. However, no published data are available for dog DRG. Canine DRG express several receptors associated with pruritus (mRNA level) [1], but as there is a disconnect between the administration of pruritogens (e.g. histamine and substance P) inducing itch behavior, it is important to determine the effects of the pruritogens directly on the DRG.

MATERIAL AND METHODS

The purpose of this study is the development of a canine DRG cell culture for intracellular calcium measurements. DRG from dogs recently euthanized, for reasons unrelated to this study, were collected through a dorsal approach. DRG were enzymatically digested with collagenase and dispase. Single cell suspensions were obtained by trituration of enzymatically softened ganglia by passages through the tips of fire-polished Pasteur pipettes. Cells were seeded onto poly-l-lysin- and laminin-coated glass slides. Within 18–24 h single cell calcium measurements were performed using Fura-2 AM and UV imaging at excitation 340 nm and 380 nm, with the software calculating the ratio 340/380 nm. To begin, we used the classical pruritogens, histamine and protease-activated receptor 2 (PAR2) agonists, as stimulations to identify excitable neurons.

RESULTS AND DISCUSSION

We obtained pilot data from the DRG of three dogs. From 174 excitable neurons 50 (28%) responded with a specific calcium influx to histamine (10–100 μmol l^{-1}). Only one dog showed a positive response to the canine protease-activated receptor 2 agonist (SLIGKT-NH_2). Nineteen out of 47 neurons (40.4%) reacted to SLIGKT-NH_2 but not the scrambled protein (TKGILS-NH_2). There was a significant variation, among the dogs sampled, of responding cells. Thus a further increase of the number of dogs is necessary to compare canine data to mouse and human DRG data. Taken together this method will allow for further development of the interaction of any stimulant with DRG in the canine system.

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19.4.

The spontaneous hypertensive rat, a model to study hypertension as a risk factor of neurodegenerative disorders

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INTRODUCTION

In Humans, hypertension is one of the most important risk factors for the development of Alzheimer's disease (AD) (Carnevale *et al.*, 2012), but the mechanisms by which hypertension may affect memory processes require further exploration. We chose the model of spontaneously hypertensive rats (SHRs) that show a spontaneous and stable hypertension without developing comorbidity, a major caveat often observed in other rodent models of hypertension.

OBJECTIVE

To examine to what extent hypertension that developed over time in SHRs could be a triggering factor of some of the deleterious features associated with AD, namely, memory deficits, we examine the associative olfactory memory (in SHR rats treated chronically against hypertension with the anti-hypertensive drug Losartan (He *et al.*, 2014). The drug was added to the drinking water of SHR rats from 2 to 7 months of age but absent in the control group.

MATERIALS AND METHODS

Blood pressure, clinical and behavioral parameters were monitored regularly in SHR rats during the 2 to 7 month period.

The posology of Losartan was adjusted each week between 13–18 mg kg^{-1} day^{-1} to ensure maintenance of a blood pressure close to physiological values (observed in the Wistar Kyoto strain).

RESULTS

The losartan treatment was effective in reducing blood pressure in SHR rats and in maintaining it close to physiological values. During the 5 month treatment period both strains did not show differences in term of anxiety/stress-related behavior, indicating that Losartan administered chronically did not induce obvious side-effects that could represent confounding factor. Testing of memory performance is currently under investigation ... Losartan reduces the blood pressure as expected, without affecting others behavioral features. These last results indicate that the treatment could be tested on memory.

CONCLUSIONS

Our results suggest that long-lasting hypertension could be regulated by anti-hypertensive drugs during months. This result is promising to use SHR strain to study if hypertension is risk factor of Alzheimer's disease.

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19.5.

Determination of Ketamine and Norketamine enantiomers in dog plasma by chiral LC-MS/MS: preliminary results

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INTRODUCTION

Ketamine has been extensively used in clinical medicine for more than fifty years. The recent introduction on the market of pure S-ketamine, which has been proved to have a four times higher affinity to the NMDA receptor compared to the R-isomer eith faster elimination and recovery from anesthesia compared to the racemic mixture, has led to new interest in this drug. The identification of optical isomers requires chiral separation, which is known to be a challenging analytical technique. Methods are available that are capable of measuring the enantiomers of ketamine and of its active metabolite norketamine in plasma includes LC-MS/MS (Moaddel *et al.*, 2010; Rosas *et al.*, 2003). In the light of the above, a rapid, selective and sensitive technique wasdeveloped for the simultaneous determination of ketamine and norketamine enantiomers in canine plasma by LC-MS/MS, within a project aimed at evaluating differences in their pharmacokinetic profiles.

MATERIALS AND METHODS

Sample extraction was performed following the procedure proposed by Sergi *et al.* (2009), with slight modifications. Briefly, 150 μl of plasma added of the internal standards underwent a liquid/liquid extraction with methanol and, after centrifugation, the supernatant was filtered and injected in the UPLC-MS/MS system (Waters, Milford, MA). Chromatographic separation was achieved through a Phenomenex Lux 3u Cellulose-3 (150 × 2.00 mm, 3.0 μm) HPLC column, using a gradient of acetonitrile and water containing ammonium acetate and ammonium formate. The analytes were quantified by selected reaction monitoring (SRM) in positive electrospray ionization (ESI+) mode. Two transitions were observed for each analyte and each internal standard: ketamine 238 > 125, 238 > 179 *m/z*, ketamine-d4 242 > 129, 242 > 183 *m/z*, norketamine 224 > 207, 224 > 125 *m/z*, norketamine-d4 228 > 211, 228 > 129 *m/z*.

RESULTS AND CONCLUSIONS

The proposed method allows to efficiently separate and quantify the optic isomers of ketamine and its main metabolite in canine plasma. Linearity was assessed over the range 17–16.700 ng ml^{-1} for ketamine and 17–5.333 ng ml^{-1} for norketamine. The lower limit of quantification (LLOQ) was 17 ng ml^{-1} for the enantiomers of both compounds. The method is being fully validated according to current European guidelines and its performances, combined with the small amount of plasma required and the quick sample preparation, make it a perfect tool to be employed in the PK study it was developed for.

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Session 20: Non Conventional Drugs & Generics

20.1.

The effect of different vehicles on transdermal permeation and skin reservoir of thiamazole (methimazole) in cats

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INTRODUCTION/OBJECTIVE

Thiamazole (methimazole) effectively treats hyperthyroidism in cats by inhibition of the production of thyroid hormones. Since some cats are difficult to pill, the transdermal application of thiamazole is of special interest. Therefore, the present study deals with the effect of different vehicles on skin permeability of thiamazole through feline skin.

MATERIALS AND METHODS

The examinations were performed *in vitro* in Franz-type diffusion cells with feline split skin (500 μm). Five different thiamazole containing formulations (50 mg/g) were prepared: 1) phosphate buffered saline (PBS; pH 7.4); 2) petrolatum; 3) ½ propylene glycol (PG) ½ PBS; 4) 1/3 dimethylsulfoxide (DMSO) 2/3 PBS; 5) ½ ethanol (EtOH) ½ PBS. Thiamazole permeation was studied over 28 h and thiamazole storage in the skin was studies after 28 h as well (whole skin and stratum corneum samples). The amount of thiamazole in the acceptor medium and the skin extraction media was determined by HPLC.

RESULTS

After 6 h the highest thiamazole permeation was found for DMSO, followed by pure PBS and EtOH. Interestingly after 28 h the highest thiamazole permeation was found for pure PBS, followed by DMSO and EtOH. PG showed lower thiamazole permeability, which in turn was fallen below by petrolatum. This permeability order is also reflected by the Papp-values. A significant higher Papp-value was observed for PBS and ethanol in comparison to petrolatum. PG and DMSO showed a higher Papp-value than petrolatum, which was notwithstanding non-significantly higher.

The thiamazole residues in the *stratum corneum* are nearly similar for all vehicles (15–22 μg cm^{-2}). The whole skin biopsy shows different results. Petrolatum exhibits the worst thiamazole reservoir within the skin (50 μg cm^{-2}), while all other formulations exhibit thiamazole concentrations of approximately 200 μg cm^{-2}.

CONCLUSION

The present study demonstrates the best permeability of thiamazole through feline skin out of hydrophilic vehicles, while the lipophilic petrolatum is no suitable vehicle for transdermal thiamazole application.

20.2.

Comparison of antibacterial activity of cultured and naturally growing lyophilized extracts of marjoram (*Origanum majorana* L.) hydrosols

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INTRODUCTION

Natural products are still major sources of new compounds to be processed as antimicrobial agents. Discovery of new antimicrobial agents is not an easy task and the situation is further complicated by a variety of commercial factors. These factors have determined a poor financial return for pharmaceutical companies on developing new products. Screening for new antimicrobials has been left to small and relatively poorly resourced start-up companies. Hydrosols, also known as floral water, distillate water or aromatic water, are the co-products or the byproducts of hydro- and steam distillation of plant material. Hydrosols are quite complex mixtures containing traces of the essential oil and, of course, several water-soluble components. They have practically been used as beverages for a long time in Turkey. Thyme and oregano have commonly been used in foods mainly for their flavour, aromas and preservation, herbal tea, alternative medicines and natural therapies. Residual hydrosols after distillation of essential oils from plant materials can be used as economical sources of antimicrobial components. Marjoram (*Origanum majorana* L.), is a well-known species for its antimicrobial activity against food borne bacteria and mycotoxigenic fungi.

MATERIAL AND METHODS

In this context, lyophilized marjoram hydrosols which were obtained from cultured and naturally growing samples, were investigated for their antibacterial activity *in vitro* by broth micro dilution and bioautography methods against *Staphylococcus aureus*, *Escherichia coli* and *Enterococcus faecalis*.

RESULTS

Minimal Inhibitory Concentration (MIC50) values ranged between 11.58 and 79.87 μg ml^{-1} for *E. coli* and 137.61 and 44.52 μg ml^{-1} for *E. faecalis* in natural and cultured species, respectively. MIC50 value could not be calculated for *S. aureus* since no concentration dependency was observed. MIC50 values were then confirmed by the autobiography test using tetrazolium violet dye where white spots against purple background

on the TLC plates were found for *E. coli* and *E. faecalis*. These results suggest that marjoram could be a potentially useful source for the herbal treatment of infections caused by *E. coli* and *E. faecalis*, hence justified the ethnopharmacological use in folkloric medicine.

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20.3.
Improving immunocompetence in foals: future prospects for oligosaccharide supplements

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INTRODUCTION

In humans and experimental animal species, oral supplementation with oligosaccharides, derived from natural products such as colostrum, milk or plants, has been shown to be beneficial for health and immunity. Next to indirect immunomodulation by oligosaccharides through prebiotic effects, which are well documented, the focus of current research is drawn to direct immunomodulatory effects of oligosaccharides as well. As of yet, very little is known about the effects of such oligosaccharide fractions in horses. Improving immunocompetence in young foals by means of preventive medicine, hence lowering the susceptibility to infections and improving health and well-being, would be a very valuable addition to the current management and therapeutic strategies in the equine sector.

MATERIALS AND METHODS

The presented research project includes studies into immunomodulatory effects of several specific oligosaccharide fractions in *ex vivo* models using peripheral blood mononuclear cells (PBMCs) of adult horses and neonatal foals, and a pilot study into the effects of an orally supplemented defined oligosaccharide fraction to foals during the first weeks of life. In the latter study, clinical and immunological blood parameters were investigated, as well as PBMC responsiveness to a standardized lipopolysaccharide (LPS) challenge.

RESULTS AND CONCLUSION

In *ex vivo* cultured equine PBMCs, we found distinct immunomodulating effects of the investigated carbohydrate fractions, which either stimulated or suppressed the LPS-induced inflammatory response. In our first *in vivo* pilot study, oral supplementation with galacto-oligosacchardes (GOS) appeared to reduce pro-inflammatory responses in PBMCs following an *ex vivo* LPS challenge (on a transcriptional level). With this knowledge, a longer follow up of GOS-treated foals may reveal long-term beneficial effects on the incidence and severity of immune-mediated inflammatory diseases, in line with published studies in other mammalian species. Moreover, both stimulation and suppression of LPS-induced inflammatory responses by oligosaccharides require additional investigation, to elucidate underlying modulatory mechanisms, and to translate this knowledge into the clinical application of oligosaccharide supplements in foals and other neonates. In conclusion, these results are a valuable starting point for further research and for the future development of strategies to improve immunocompetence in young foals.

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