The complex nature of studies contributing to the reputation of Krka's veterinary medicines

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Meta-analysis, in vivo studies, bioequivalence, residues, withdrawal period, tolerance, flubendazole, fipronil, toltrazuril, enrofloxacin, pigs, dogs, cats, fleas, ticks, lice, calves

Abstract
Krka's Animal Health Programme offers modern, effective and safe products that are the result of our own know-how and years of experience in the area of veterinary care. Many in vivo studies in the research and development of veterinary medicines are performed to fulfil the regulatory requirements for providing evidence of their safety and efficacy. All in vivo studies are performed in compliance with animal welfare regulations, good laboratory and clinical practices and study-specific guidance.
From the safety point of view, the following two general requirements have to be adequately addressed by in vivo studies: human (consumer) safety if the medicinal product is intended for use in food-producing animal species (residue studies), and the safety of the animal species intended to be treated (local and systemic tolerability studies). From the efficacy point of view, all claims have to be based on the findings from in vivo efficacy studies. However, Krka also employs several techniques to justifiably substitute in vivo studies for in vitro studies, or scientifically based justifications.
The present overview of in vivo studies will demonstrate the great variety of study designs employed by Krka and shed some light on Krka's approaches to them. With this article we would like to present some of the recently performed in vivo studies that were approved by the regulatory authorities and are the foundation of the claims in the Summary of Product Characteristics (SmPC) for Krka's products.
Krka's experts also perform meta-analyses to review and combine the results of the studies. They provide an upgrade option for discovering and exploring data in the existing scientific literature and can yield plausible explanations or even result in the discovery of new knowledge. They can also give practical answers to controversial clinical issues and save costs of additional clinical experiments.

Introduction

Krka is a specific company since all the knowledge gained in the human medicines development can be incorporated in the veterinary medicines development. The aim of *in vivo* studies is to adequately confirm the safety and efficacy profile of Krka's veterinary products. These studies have to be performed in accordance with regulatory requirements and guidelines, good laboratory/

clinical practice and animal welfare regulations. Each study is a big project on its own, with several complex procedures involved from designing to performing and quality control/assurance. Some of these procedures have to be specifically developed for a study, e.g. bioanalytics, statistical analysis and challenge models. The projects thrive on interdisciplinary approach employed by Krka where experts from several different fields of expertise work closely together and share knowledge gained on a daily basis. This overview of *in vivo* studies will demonstrate some of the study designs employed by Krka and present the use of meta-analysis as a powerful tool for combinatorial analysis of *in vivo* studies with potential for new discoveries.

Recent *in vivo* studies performed in Krka to support marketing authorisation

Residue depletion studies

In order to protect human health a withdrawal period needs to be established for all the animal species that are intended as source of food for human consumption and are to be treated with the proposed veterinary medicinal product. A withdrawal period specifies how long after any particular treatment the food products derived from the treated animals may be safely used for human consumption. For this purpose the company responsible for the veterinary medicines has to perform residue depletion studies. These studies determine how rapidly the marker residue for the particular active substance is depleted from edible tissues and edible products, and how quickly the levels of the marker residue fall below the predetermined maximum residue limits (MRLs). Since Krka is a generic company, it can use the already available data in order to reduce the number of animals in experiments (3R's approach) and still ensure no risk to consumer health. In a recent marketing authorisation procedure Krka successfully implemented a residue depletion study using reduced numbers of animals. This so-called confirmatory study design is currently not described in any EU guideline and is an alternative approach that was proposed by Krka's professionals.

Confirmation of the withdrawal period for Flimabend in pigs[1]

The active substance in Flimabend* is flubendazole, a broad-spectrum benzimidazole methylcarbamate anthelmintic which is orally administered to pigs, chickens, turkeys and game birds. The presence of flubendazole in edible tissues, resulting from the medication of pigs, is regulated and controlled by the European Commission. The Committee for Medicinal Products for Veterinary Use of the European Medicines Agency has defined the MRLs for this anthelmintic agent in pig tissues. Flubendazole is included in Commission regulation (EU) No 37/2010 of 22 December 2009. The specified limits that ensure consumer safety are listed in Table 1.

A confirmatory residue depletion study was carried out in pigs to establish the withdrawal period for Flimabend for the posology of 2.5 mg of flubendazole per kilogram of body weight for two consecutive days. Six clinically healthy castrated male and female domestic pigs with a body weight ranging from 69.2 to 89.2 kg were assigned to this study.

		Marker residue concentration (µg/kg)			
Withdrawal time (days)	Parameter	Muscle	Liver	Kidney	Skin and Fat
4	Range	< LOD–< LLOQ	< LLOQ	< LLOQ	< LOD–< LLOQ
LLOQ (µg/kg)		25	200	150	25
MRL (µg/kg)		50	400	300	50

MRL – maximum residue limit (Commission regulation (EU) No 37/2010); LOD – limit of detection; LLOQ – lower limit of quantification

Table 1. Concentration range of flubendazole marker residue in target tissues

* The product is marketed under different brand names in different countries (Flimabend, Flimabo).

The offered amount of medicated water was completely consumed by all animals during the 4-hour treatment period on each treatment day. Tissues were sampled 96 hours after treatment in accordance with the approved withdrawal period of the reference product. The samples were analysed with the bioanalytical procedure for determining marker residues (i.e. the sum of flubendazole and (2-amino-1H-benzimidazol-5-yl)(4-fluorophenyl)-methanone) using ultra performance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS). The results are presented in Table 1.

The flubendazole marker residue was well below the MRL in all tissue samples 96 hours after the end of the treatment. Close examination of the obtained results showed that flubendazole marker residue was below the LLOQ in all tissue samples 96 hours after the end of the treatment. Flubendazole concentrations were in the great majority of samples below the LOD, with two samples out of 24 below the LLOQ. Flubendazole metabolite concentrations were quantified in two samples out of 24, with the concentrations close to the LLOQ.

This confirmatory residue depletion study demonstrated the adequacy of the 4-day withdrawal period for Flimabend administered to pigs for two consecutive days at the dose 2.5 mg of flubendazole per kilogram of body weight, as established for the reference product.

Safety studies

Safety studies (e.g. the margin of safety and local tolerance) must be conducted in target animal species (in the most susceptible category of the target animal species to be exposed to the product, e.g. young animals). Such studies should be designed to allow valid determination of safety (even at the overdose) and used as requested by regulatory guidance. Careful consideration should be given to parameters evaluated and tests/examination performed in the study as well as to the statistical evaluation. Hence, all available (toxicological) data for the (active) ingredients of the product have to be considered before designing such studies. Another challenge with these studies can be the physiological properties of the study animals (e.g. rapidly changing physiological values in very young animals during the intensive growth period and/or their susceptibility to any kind of excessive stress or to diseases). Due to the complexity of these studies, underlined by highly sensitive subjects, they have to be well designed, monitored and evaluated in order to preserve an unbiased assessment of a formulation's safety profile.

Target animal safety study of Krka's fipronil spot-on solution in dogs (puppies)[2]

Fipronil is used to control external parasite infestations on dogs and cats. This study was a parallel group, randomised, blinded, controlled target animal safety study. Beagles of both sexes (16 female puppies and 16 male puppies), approximately 8 weeks old at the first treatment were subject to topical administration of the product for three times at 28-day intervals. Female and male dogs were ranked according to their body weight within each sex and randomly allocated into test groups: control, once, three times and five times the recommended dose in compliance with the guideline requirements. The observed clinical alterations in some animals during the study were properly recognised, diagnosed and treated. Concurrent treatments and vaccinations did not interfere with the study, but they were considered as necessary for the study animals due to their age and origin.

For local tolerance evaluation the skin of animals was monitored on a regular basis for any signs of inflammation or differences in skin appearance. Only minor cosmetic changes were noticed in individual animals; predominantly in animals given five times the recommended dose. In the group administered the recommended dose, scurfs were observed in one animal only after the first treatment. For systemic tolerance (safety margin) evaluation, several parameters were monitored on a regular basis and statistically evaluated. The body weights of study animals increased during the study period. An increase in the mean weekly feed consumption per animal was similar between most test groups, and lower feed consumption was observed in females of one of the groups during the first five weeks; it normalised after week 6.

Some variations in hematology and biochemistry were observed, as can be anticipated in this subcategory of animals (growing puppies):

- platelet and basophil counts were higher in one of the groups (irrespective of the day);
- white blood cell and neutrophil counts were higher in one of the groups (irrespective of the day).

However, no statistical differences were observed between the test groups and the control group.
The weights of selected organs (tissues) were different between sexes as were the body weights. No treatment-related macroscopic findings were observed in any of the organs and/or tissues.
Evaluation of the data analysis for the systemic tolerance assay revealed no clinically significant differences between the groups.

Thus, the following general conclusion can be drawn from the study:
The product was safe when administered to puppies as young as 8 weeks, even at 5-times the recommended dose.

Efficacy studies

Efficacy studies are required to establish and confirm the proper use of medicines in each of the indications claimed in the product literature. In the generic industry, the first choice is to use a bioequivalence study; however, for some products this is not an option. For products used to control external parasite infestations, dose-confirmation studies are required for the confirmation of the products' efficacy against external parasites in target animal species (e.g. dogs and cats). In the absence of properly validated *in vitro* methods, these studies provide the necessary data to determine the efficacy of the products in target animals. These studies should be controlled and blinded to allow reliable determination of efficacy. If induced infestation is used (for this type of studies it may be preferred over natural infestation), parasites (e.g. fleas and ticks) free of vector-borne diseases should be used to eliminate the possibility of transferring vector-borne diseases to experimental animals. The colonies of the parasites are maintained in the laboratories for several generations and they are regularly refreshed with field strains to mirror the current field situation. These studies are extremely challenging due to the combination and interaction of two study objects (target animal and parasite). Efficacy can only be confirmed if the threshold of 90% for ticks or 95% for fleas is exceeded (reduction of infestation in comparison with untreated control animals). For a valid and robust evaluation, a sufficient number of parasites should be present on control animals at any timepoint (i.e. 50% of fleas and 25–50% of ticks used for infestation). Immediate efficacy is determined up to 48 hours after the administration of the product (on animals previously infested with parasites). For the determination of persistent efficacy, animals are infested (challenged) with parasites once weekly and after 48 hours any remaining parasites are removed from them and counted.
Studies with a novel formulation of fipronil (recommended for once monthly administration) are presented as an example of efficacy studies in companion animals. Studies in cats are even more challenging due to the difficulties related to cats as experimental animals (e.g. their intensive grooming behaviour).

Confirmation of the efficacy of Krka's fipronil spot-on for the treatment and control of fleas, ticks and chewing lice on dogs[3]
The immediate and persistent effect of a novel spot-on formulation containing fipronil was evaluated in several laboratory studies to confirm its efficacy against fleas (*Ctenocephalides felis*), ticks (*Rhipicephalus sanguineus* and *Dermacentor reticulatus*) and chewing lice (*Trichodectes canis*) on dogs and the duration of its effect of at least one month (Table 2). All study treatments were applied at the recommended dose rate (at least 6.7 mg of fipronil/kg of body weight) and using the recommended route of administration (at one or two spots between the shoulder blades) on day 0. No adverse health effects related to the product were observed in any of the experimental animals.

	Study A	Study B	Study C
Experimental groups	Control (n = 8), IVP (n = 8), CVP (n = 8)		
Parasite species (number used for each infestation; origin)	*Ctenocephalides felis* (100; S. Africa and Europe) *Dermacentor reticulatus* (50; Europe) *Rhipicephalus sanguineus* (50; Europe)	*Ctenocephalides felis* (100; Europe)	*Trichodectes canis* (natural infestation, ≥ 10 live adult lice or ≥ 2 live lice + viable eggs at the pre-treatment evaluation; Europe)
Animal data	24 dogs (both sexes) Age: ≥ 4 months Body weight: 11.0–18.6 kg	24 dogs (both sexes) Age: 8–84 months Body weight: 10.3–18.4 kg	24 dogs (both sexes) Age: adult Body weight: 8.6–18.1 kg
Infestation (days)	–2, 7, 14, 21, 28	–2, 7, 14, 21, 28, 35, 42, 49, 56	Natural infestation
Counts (days)	2, 9, 16, 23, 30	2, 9, 16, 23, 30, 37, 44, 51, 56	2, 7, 14, 21, 28, 35

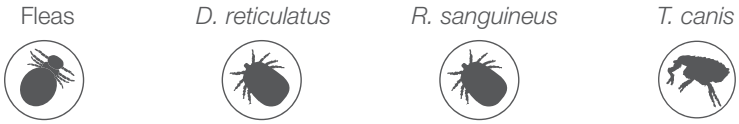
IVP – investigational veterinary product; CVP – comparator veterinary product

Table 2. Design of studies to confirm the efficacy of Krka's fipronil formulation against fleas, ticks and lice on dogs

Parasite infestations and counts were made on each animal to determine the adulticidal activity of the investigational and the comparator product compared to the control group. Mean parasite counts were calculated as geometric means. The percent efficacy relative to the untreated control group was calculated using the mean according to Abbott's formula:

$$Efficacy\ (\%) = \frac{mean\ control - mean\ treated}{mean\ control}$$

Mean control: the mean number of live parasites in the untreated control group
Mean treated: the mean number of live parasites in the treated group; in tick studies, ticks of the following categories were considered: live free ticks, attached ticks of the categories: live, unengorged; live, engorged; dead, engorged



	Fleas		*D. reticulatus*		*R. sanguineus*		*T. canis*	
	Control	IVP	Control	IVP	Control	IVP	Control	IVP
Day 2	77	0	27	2	25	2	37	5
Week 1	70	0	31	0	24	0	35	0
Week 2	67	0	30	0	22	0	32	0
Week 3	72	0	25	0	27	0	28	0
Week 4	58	0	36	1	34	3	27	0
Week 5	73	0	/	/	/	/	27	0
Week 6	82	0	/	/	/	/	/	/
Week 7	72	0	/	/	/	/	/	/
Week 8	77	2	/	/	/	/	/	/

IVP – investigational veterinary product

Table 3. The efficacy (presented as geometric mean of parasite count) of Krka's fipronil spot-on against fleas, ticks (*D. reticulatus* and *R. sanguineus*) and lice (*T. canis*) on dogs

These studies confirmed that treatment with Krka's fipronil spot-on at the recommended dose rate rapidly reduces infestations with fleas, ticks and chewing lice on dogs. The treatment provided control of re-infesting fleas for up to eight weeks, an up to four-week control of ticks and control of chewing lice.

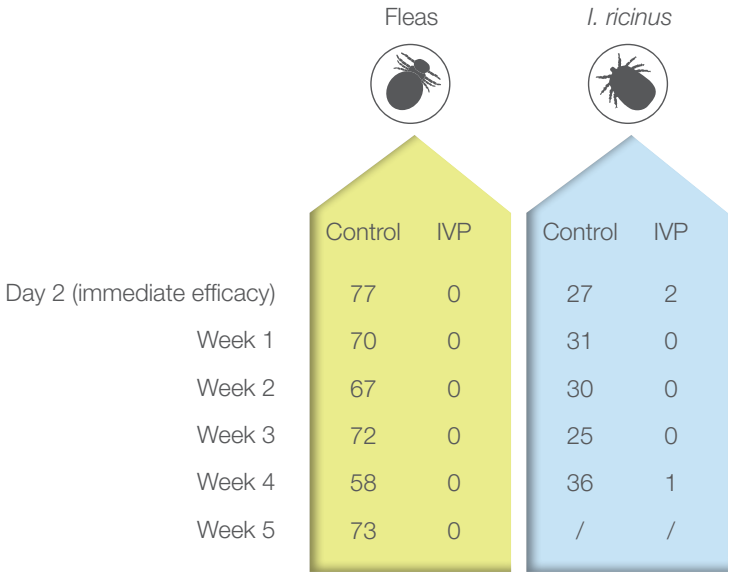
The efficacy of Krka's fipronil spot-on for the treatment and control of induced infestations with adult cat fleas (Ctenocephalides felis) and castor bean ticks (Ixodes ricinus) on cats[4]

The product's efficacy was evaluated against cat fleas (*Ctenocephalides felis*) and ticks (*Ixodes ricinus)* in experimentally infested cats in two clinical trials (Table 4). Cats were treated once topically with the recommended dose rate (unit label dose: 50 mg fipronil per cat (10.6–23.8 mg/kg); route of administration: two spots, one at the base of the skull and a second 2–3 cm further back). The product was well tolerated in all treated animals and no treatment-related adverse reactions were observed in any of the studies. Only transient changes in appearance (such as greasiness and/or clumping of the hair coat) were observed at application sites, but they resolved within 48 hours in the majority of animals.

	Study 1	Study 2
Experimental groups	Control (n = 8), IVP (n = 8)	
Parasite species (number used for each infestation; origin)	*Ctenocephalides felis* (100; Europe)	*Ixodes ricinus* (60; Europe)
Animal data	16 cats (both sexes) Age: ≥ 4 months Body weight: 2.0–3.5 kg	16 cats (both sexes) Age: > 8 months Body weight: 2.2–5.3 kg
Infestation (days)	–2, 7, 14, 21, 28, 35	–2, 7, 14, 21, 28, 35
Counts (days)	2, 9, 16, 23, 30, 37	2, 9, 16, 23, 30, 37

IVP – investigational veterinary product

Table 4. The design of studies to confirm the efficacy of Krka's fipronil formulation against fleas and ticks on cats



IVP – investigational veterinary product

Table 5. The efficacy (presented as geometric mean of parasite count) of Krka's fipronil spot-on against fleas and tick (*I. ricinus*) on cats

Appropriate infestation rates were provided in control animals, despite recognised difficulties related to cats as experimental animals. These studies confirmed that treatment with Krka's fipronil spot-on at the recommended dose rate rapidly reduces the existing infestations with fleas and ticks on cats and prevents infestation with fleas for up to five weeks and with ticks for up to four weeks.

Bioequivalence studies

In the development of generic veterinary medicines, similarity of their plasma profiles with those of the reference products have to be demonstrated in all animal species for which a product is intended. The performance of such studies in animals is quite challenging since it requires high standardisation of study conditions while respecting animal welfare. Krka uses all available data to calculate the minimal number of animals required to ensure a reliable result. An additional challenge arises when a medicinal product is intended for a target population that differs significantly in the anatomy and physiology from adult animals; in such cases the study must be performed on non-adult animals.

***Single-dose, parallel bioequivalence study of Toltarox use in calves*[5]**

The active substance in Toltarox* is toltrazuril, a coccidiocidal triazinon derivative that acts against the coccidia of the genera *Isospora* and *Eimeria*, which is orally administered to piglets, calves and lambs.

An *in vivo* bioequivalence study was conducted in the target animal population – preruminant calves (8–32 days of age, 38.5–74 kg of body weight) in order to demonstrate bioequivalence between Toltarox and the reference product. Due to the long elimination half-life of the active ingredient, the study had to be conducted according to parallel design. The study medications were administered once in doses of 15 mg of toltrazuril per kg of body weight. For the administration of the medicine the suckling reflex of the calves was induced by inserting a finger into the animals' mouths. During the study, milk was served as recommended, corn starter feed and water were available *ad-libitum* except during the standardised period related to the dosing of the study medication. Due to the fact that prandial state is a high source of variability, the following standardisation was introduced on dosing days: animals were fasted overnight prior treatment (at least 10 hours) and fed 1 hour post treatment. Fluid was restricted 1 hour before each treatment and allowed as desired 1 hour post-dose. Due to the complexity of the study (i.e. the availability of the calves dependent on parturition frequency and the long elimination half-life of the active ingredient), the clinical phase of the study lasted for approximately 4 months.

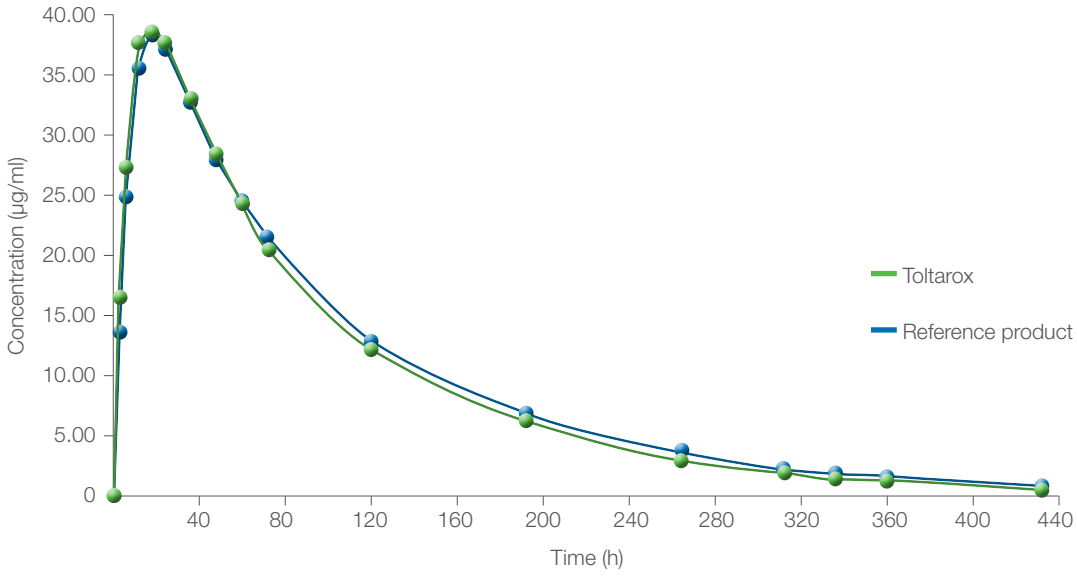


Figure 1. Comparison of mean plasma profiles (linear) of Toltarox determined after administration of Krka's and the reference product to calves

* The product is marketed under different brand names in different countries (Toltarox, Tolzesya, Tolracol).

The obtained plasma samples were analysed using HPLC (high-performance liquid chromatography) with a fluorimetric detector. The result of the study is based on 55 animals randomly assigned to treatment with Krka's or the reference product. The 90% confidence interval of the ratio (test/reference) of least-squares means from the ANOVA of the ln-transformed AUCt and C_{max} parameters were within 80–125% and therefore bioequivalence between the two formulations was demonstrated. Similarity of the plasma profiles of toltrazuril from the two compared formulations was confirmed and is presented in the diagram (Figure 1).

Meta-analysis and systematic review

Introduction

A meta-analysis is the process of using statistical methods to review and combine the results of different, independent clinical studies.[6] This analytical method is of particular importance in the assessment of therapeutic efficacy when individual studies do not provide reliability. As their samples are too small, individual studies do not allow for quantitative evaluation of the effect of treatment, nor can they test the null hypothesis. A quantitative systematic review or meta-analysis is a systematic review that uses statistical methods to combine the results of two or more studies.

A brief summary of all meta-analysis procedures would comprise the following rational definitions:

1. definition of the problem and the criteria for inclusion of studies;
2. positioning of studies, classification and coding of characteristics of individual studies and quantitative measurement of the studies' characteristics (scale);
3. integration of results and comparison with the studies' characteristics (analysis and explication of results);
4. reporting of results.[7–10]

Feature	Narrative review	Systematic review
Question	Often broad in scope	Systematic: often a focused clinical question
Sources and search	Usually not specified, potentially biased	Comprehensive sources and explicit search strategy
Selection	Usually not specified, potentially biased	Criterion-based selection, uniformly applied
Appraisal	Variable	Rigorous critical appraisal
Synthesis	Qualitative summary	Qualitative or quantitative (meta-analysis)
Inferences	Sometimes evidence-based	Usually evidence-based

Table 6. Differences between narrative reviews and systematic reviews[10]

In the past two decades, meta-analysis has been increasingly used in all fields of science. This is particularly evident in the medical science, where two other methods are used as well – the systematic review and evidence-based medicine; especially in medicine, the decision analysis and cost-effectiveness analysis have emerged. All methods are connected, and the latter two are the upgrade of the first two.[8–13] Systematic reviews are exact summaries of the best evidence related to exactly specified clinical dilemmas. Special centres like the Cochrane Collaboration have been organised in different places around the world where systematic reviews of scientific literature and their own findings are published in databases collecting data on most appropriate therapies for individual illnesses. These reviews support the synthesis of the best evidence for treatment or establishment of the best medical practice.[14]

Systematic review and meta-analysis of the efficacy of enrofloxacin in the treatment of domestic animals[15]

In our research we reviewed literature describing the use of enrofloxacin in domestic animals. In addition to a meta-analysis, a systematic review was performed and in the end the general findings were evaluated. The applicability and efficacy of enrofloxacin in the health care of pigs, poultry and cattle were reviewed and evaluated.
About 28 years ago, fluoroquinolones were introduced to the clinical veterinary medicine and they promised a new era in the development of antimicrobial agents. Due to their broad spectrum of action, excellent clinically significant pharmacokinetic properties and low toxicity – these being great advantages over other groups of antibiotics – they were considered as almost ideal antimicrobial agents.

From various sources we obtained 919 articles describing the efficacy of enrofloxacin. With further selection of articles that described clinical studies and enabled numerical combinations, we included in the meta-analysis 110 studies on health care in pigs, 67 in ruminants and 60 in poultry, i.e. a total of 237 studies.
A special meta-analysis of the treatment of specific infections with enrofloxacin in individual animal species was carried out. We prepared 19 meta-analyses of data on different uses of enrofloxacin in various diseases in pigs, poultry and domestic ruminants (mainly cattle), while in 7 cases we also calculated the individual effect size (odds ratio) for a specific parameter.
By following a systematic way of reviewing, we ensured repeatability of our meta-analyses. The homogeneity of the studies was graphically evaluated with funnel plots (Figure 2). In heterogeneous meta-analyses, we additionally calculated the total size of the effect according to a random calculation model for total effect size.
The Comprehensive Meta-Analysis (Borenstein, 2000) computer programme was used for the statistical analysis.

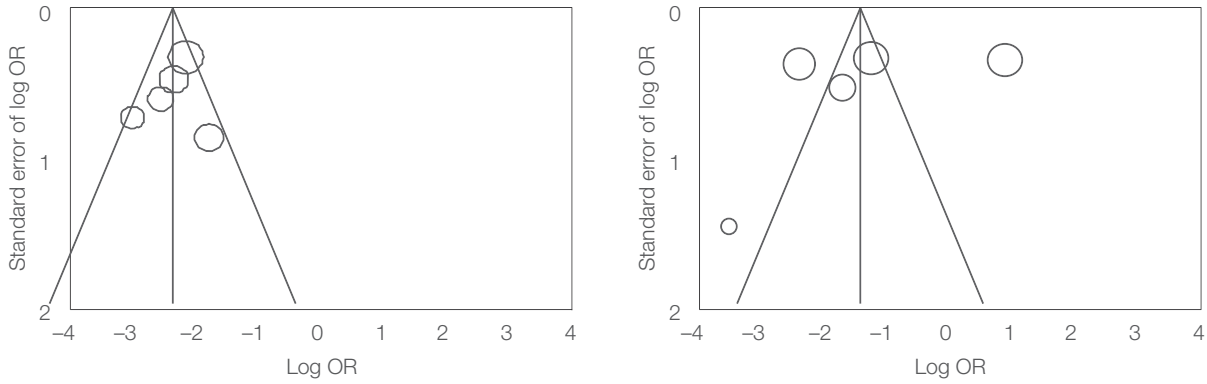


Figure 2. Funnel plots – an example of homogeneity (left) and heterogeneity (right); OR – odds ratio

In addition to clinical studies, we reviewed data on bacterial *in vitro* susceptibility to enrofloxacin. These results were also considered in the final opinion about the individual meta-analysis of the efficacy of enrofloxacin. In a systematic review, the efficacy of enrofloxacin and that of other antimicrobial agents was compared.[15]

Results

Enrofloxacin was very effective in the treatment of all coli and salmonella infections in pigs and poultry ($p < 0.001$).
The results also showed that additional studies about coli-bacillosis and salmonellosis in cattle would be necessary. In cattle, *in vitro* resistance to enrofloxacin was established in 11.8% of *E. coli*

strains (n = 195), 1.8% of *E. coli* strains isolated in the udder (n = 1,695) and in 8.4% of salmonella strains (n = 1,211).
It is evident from the results that enrofloxacin is potently effective in the treatment of respiratory infections in all domestic animals ($p < 0.01$). After taking into account all the findings (*in vivo* and *in vitro*), it was revealed that enrofloxacin is effective in the treatment of mycoplasma infections in poultry and pigs, while additional studies would be necessary in cattle. A meta-analysis in poultry revealed that administration of enrofloxacin is effective in pasteurellosis in turkeys ($p < 0.001$) and in infectious coryza, staphylococcosis and *R. anatipestifer* infection in ducks ($p < 0.001$). These results were confirmed by the findings showing high *in vitro* susceptibility to enrofloxacin of these pathogens.
In pigs, treatment with enrofloxacin was significantly more effective in the trial group than in the control group in the mastitis metritis agalactia (MMA) syndrome ($p = 0.002$), urinary tract infections ($p < 0.05$) and streptococcal infections (fewer deaths, $p = 0.045$). These results were confirmed by the findings of high *in vitro* susceptibility of this pathogen to enrofloxacin. In Glässer's disease the difference in comparison with the control group was not significant ($p = 0.25$); however, the pathogen (*H. parasuis*, n = 124) was 100% susceptible to enrofloxacin. In greasy pig disease there was a high *in vitro* susceptibility of *S. hyicus* to enrofloxacin (98.3%, n = 744).

To be able to answer the complex questions about mastitis in cattle, one or more additional studies with enrofloxacin would be necessary, as our results indicate that enrofloxacin is not more effective than the drugs in the control groups (fixed model: odds ratio = 0.3; $p = 0.5$, random model: odds ratio = 1.19; $p = 0.79$). However, the *in vitro* results on susceptibility of mastitis pathogens to enrofloxacin are good. An additional study would also be necessary for the treatment of endometritis in cattle, since the difference between the trial and the control group was not statistically significant ($p = 0.9$), although the results were in favour of the treatment with enrofloxacin.[15]

Systematic review and meta-analysis of the efficacy of enrofloxacin in E. coli infections in poultry
Twenty studies were included in the survey, of which 8 were considered in the meta-analysis of their clinical data, while others were used to build a susceptibility profile of bacteria to enrofloxacin.
The total number of animals included in the systematic review was 21,948, of which 11,303 were from the enrofloxacin-treated test groups of different studies and 10,645 from their control or alternative treatment groups.[16–36]

It is evident from the results in the graph that enrofloxacin is effective in the treatment of *E.coli* infections in poultry ($p < 0.001$).

We also combined *in vitro* susceptibility of individual microbes to enrofloxacin and evaluated the MIC values with the clinical studies included in the meta-analysis and from this overwiew it is evident that enrofloxacin is very effective in the treatment of all coli infections in poultry ($p < 0.001$). (Table 7).

Category	MIC_{50}	MIC_{90}	MIC – Range	% of resistant strains (resistant/all)
Broilers	0.03–0.2	0.06–0.78	0.015–1.0	10.0% (1,820/18,238)
Turkeys	0.03	0.06–0.13	≤ 0.03–8	19.7% (285/1,445)
Layers	< 0.1	0.2	< 0.1–1.6	10.7% (2,105/19,683)

Table 7. *In vitro* susceptibility of *E. coli* (in poultry) – systematic review

Figure 3 shows compiled data on authors of individual studies, the year of study publication or performance, a numerical comparison between the treated and the control group and the mean values of effect size with 95% confidence intervals. The odds ratio (OR) was used as a scale of magnitude for the effect size. The characteristic of OR is that a value of 1.0 means that a certain therapy has no effect; values below 1.0 indicate that the investigational therapy (in our case the use of enrofloxacin) is better than the control therapy or the comparator therapy. Values above 1.0 indicate the advantage of the control or comparator therapy over the investigational therapy. The total effect size is always conditioned by the weights of individual studies; therefore, in a meta-analysis we speak about a weighted total value of the effect size which, the same as for individual studies, is presented by the mean value and confidence intervals. In the figure the weights for individual studies are shown as bigger or smaller green circles (●). This means that studies with smaller weights have smaller circles and those with larger weights have larger circles. The total effect size is shown as a green square (■).
It is evident from the results in Figure 3 that enrofloxacin is effective in the treatment of *E. coli* infections in poultry ($p < 0.001$).

We also reviewed *in vitro* susceptibility of *E.coli* to enrofloxacin and evaluated the minimal inhibitory concentrations (MICs) in the clinical studies included in the meta-analysis and in this overview, and performed a meta-analysis. It is evident that enrofloxacin is very effective in the treatment of all coli infections in poultry.

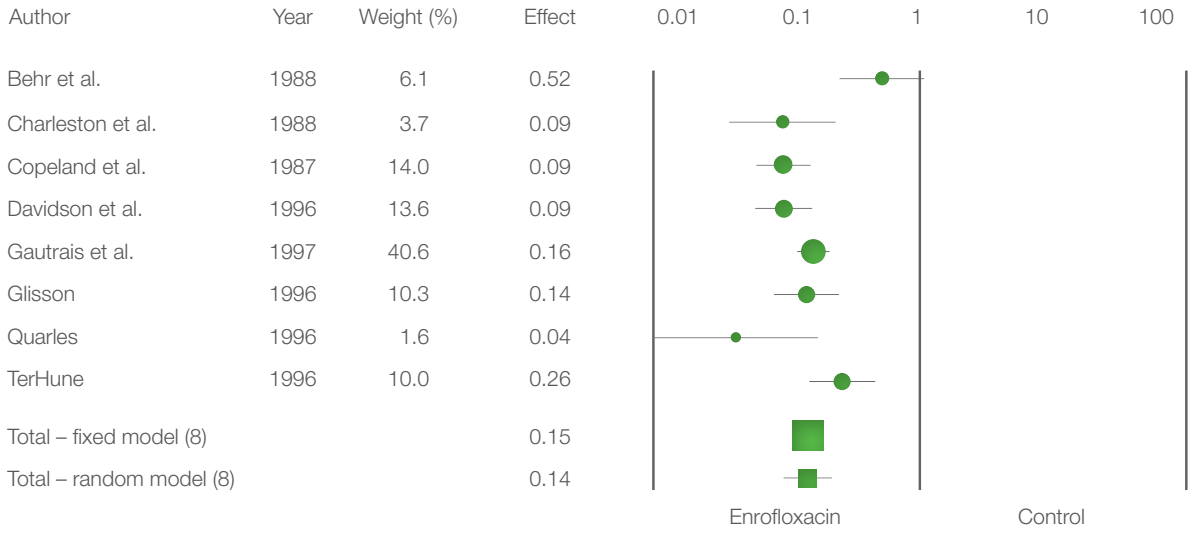
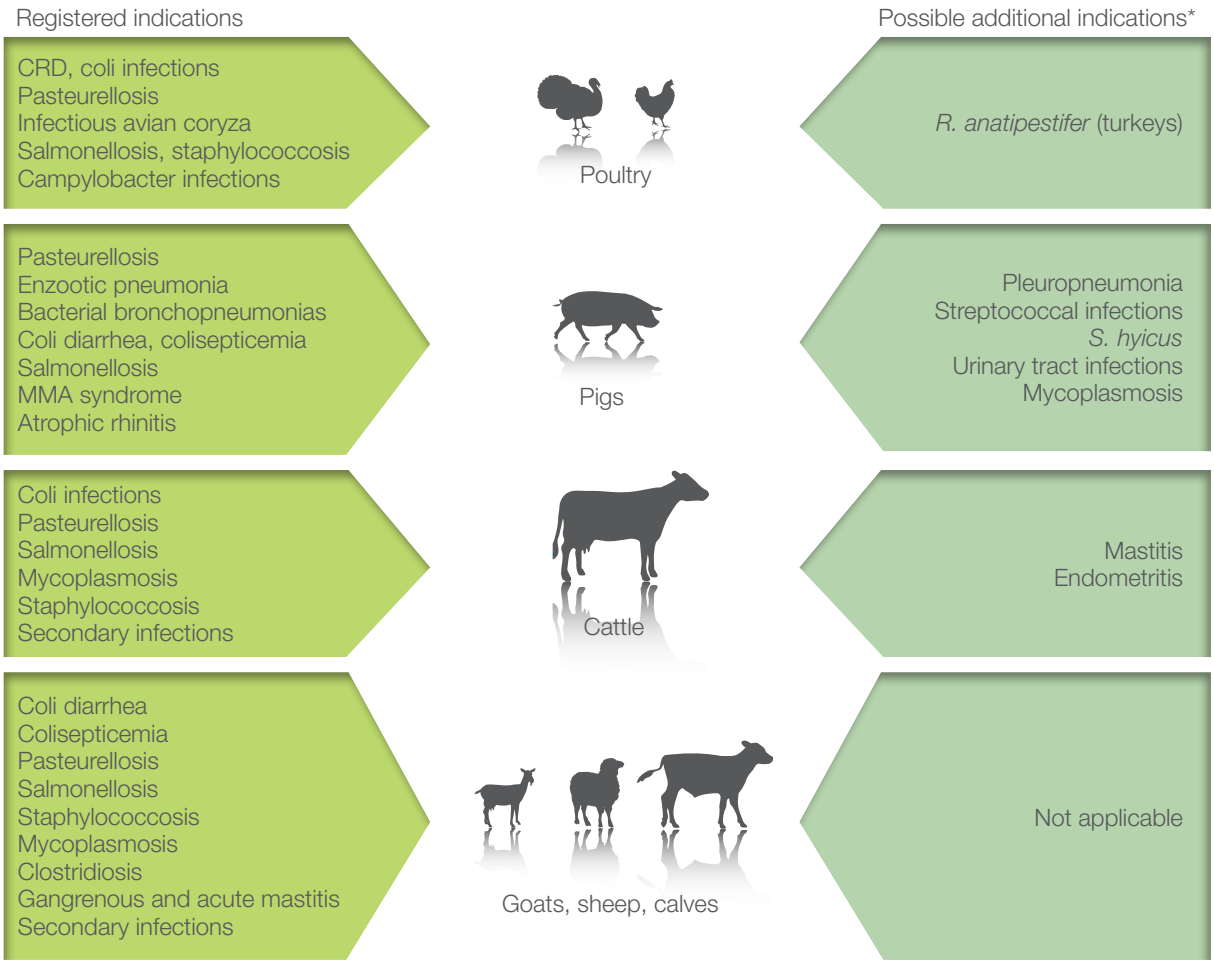


Figure 3. *E. Coli* infections in poultry (broilers)[37]

Applicability of the findings

Our findings can be considered useful for investigators, doctors of veterinary medicine in practice and for the breeders, as well as for the manufacturers of veterinary medicines and governmental authorities. Our work has a great economic impact too, since it offers an overall survey of the research field of interest and provides guidelines for further research.

If well performed, a meta-analysis can give practical answers to controversial clinical issues and save costs of additional clinical experiments and provide grounds for new indications of an existing active ingredient (Table 8).



* from meta-analysis and systematic review

Table 8. Comparison of the results of systematic review and official indications (2003)

Conclusions

As can be seen from this overview, *in vivo* studies performed to support veterinary medicine development are very diverse and quite challenging.

The field of *in vivo* studies is changing rapidly and all efforts are focused on substitution of *in vivo* by *in vitro* experiments. Hence, the company is implementing this substitution whenever possible and Krka's experts for *in vitro* test systems have already had great success in substituting *in vivo* studies by convincing results of *in vitro* studies, even though they are not regulated yet.

Krka uses a special system in product development, with the majority of the experts working in human and veterinary medicines development, which allows incorporation of knowledge gathered during the development of human medicines into the development of veterinary medicines. Even though animal health product sales represent 3.6% of total sales in the Krka Group, the development of veterinary medicines with its special pharmaceutical forms and species-specific biopharmaceutical properties represents a significant opportunity for the expansion of knowledge for all experts involved.

Krka is unique in performing a meta-analysis and a systematic review of enrofloxacin usage and its efficacy in domestic animals.

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