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Til Fødevarestyrelsen

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**Levering af bestillingen "Forslag til fremtidige lovgivningsmæssige krav til resistensundersøgelser i forbindelse med godkendelse af Coccidiostatika"**

Fødevarestyrelsen har i en bestilling sendt den 8. juli 2020, bedt DCA – Nationalt Center for Fødevarer og Jordbrug – om at "*komme med forslag til hvordan kravene i EU-forordning 429/2008 til post-market monitorering kan gøres mere præcise på en sådan måde, at resistensproblematik og -udvikling kortlægges på en grundig, ensartet og brugbar måde for coccidiostatika som fodertilsætningsstof*".

Besvarelsen på denne bestilling består dels af nedenstående rapport og dels af et Annex 1, hvori der med track changes og kommentarfelter er givet input til "*GENERAL REQUIREMENTS TO BE SATISFIED BY THE DOSSIER PROVIDED FOR IN ARTICLE 3 - GENERAL ASPECTS*". Sidstnævnte leveres i Word-format og forventes ikke at blive offentliggjort elektronisk, men kan fremsendes i fald det efterspørges. Selve rapporten vil blive lagt i AU's offentligt tilgængelige database "PURE" i PDF-format. Besvarelsen er udarbejdet af Lektor Ricarda M. Engberg, Institut for Husdyrvidenskab, Aarhus Universitet. Adjunkt Ida Thøfner, Institut for Veterinær- og Husdyrvidenskab, Københavns Universitet, har været fagfællebedømmer, og rapporten er revideret i lyset af hendes kommentarer.

Besvarelsen er udarbejdet som led i "Rammeaftale om forskningsbaseret myndighedsbetjening mellem Miljø- og Fødevareministeriet og Aarhus Universitet" med relation til "Ydelsesaftale Husdyrproduktion 2020-2023".

Venlig hilsen

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# Suggestion for future legislation in relation to the evaluation of coccidial resistance connected to the approval of new coccidiostats

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## Bacground and task

Coccidiostatica are drugs that are added to poultry feed to minimize disease and production losses related to the infection with coccidia *(Eimeria* spp.). Currently, these substances are considered feed additives and are therefore regulated by Regulation (EC) No 1831/2003 on additives for use in animal nutrition. Article 11 of this regulation lays down that the Commission shall submit a report to the European Parliament and the Council regarding the use of coccidiostats and histomonostats as feed additives with a view to a decision on the phasing out of the use of these substances as feed additives by 31 December 2012. The report of the commission dated 5.5 2008 concluded that the use of coccidiostats as a preventive measure for the control of coccidiosis in modern poultry production is essential, that alternatives currently do not offer the same advantages as the use of coccidiostats as feed additives and that the regulatory framework (Regulation (EC) No 1831/2003) can be considered as working properly.

In a recent position paper, the Federation of Veterinarians of Europe (2016), recommends that coccidiostats should be under veterinary prescription following clinical examination and diagnosis. This would allow for better surveillance and the veterinarian to diagnose and choose the best strategy to extend the useful life of coccidiostats, such as through 'shuttle use' or 'rotational use' or the use of vaccines. Additionally it would allow more frequent reporting of any adverse reactions seen, including lack of efficiency, ensure withdrawal periods are respected and could allow monitoring through the the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) monitoring system.

Concerning the authorization of feed additives, Commission Regulation (EC) No 429/2008 of 25 April 2008 provides more detailed rules for the implementation of Regulation (EC) No 1831/2003 as regards the preparation and the presentation of applications, the assessment and the authorisation of feed additives. To assist aplicants in the preparation and the presentation of dossiers for approval of new coccidiostats, the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) has published a *Guidance for the preparation of dossiers for coccidiostats and histomonostats* (EFSA FEEDAP Panel, 2011). Further, more detailed assisting information is given in a guidance document concerning *Identity, characterization and conditions of use of feed additives* (EFSA FEEDAP Panel, 2017) and *Technical guidance for microbial studies* (EFSA FEEDAP Panel, 2008).

Commission Regulation (EC) No 429/2008 of 25 April 2008 is currently under revision and in this relation, The Danish Veterinary and Food Administration has asked Aarhus University to provide suggestions that contribute to clarification and if possible simplification of the current legislation with special attention to sensitivity tests needed for the documentation of the efficacy of anticoccidial feed additives.

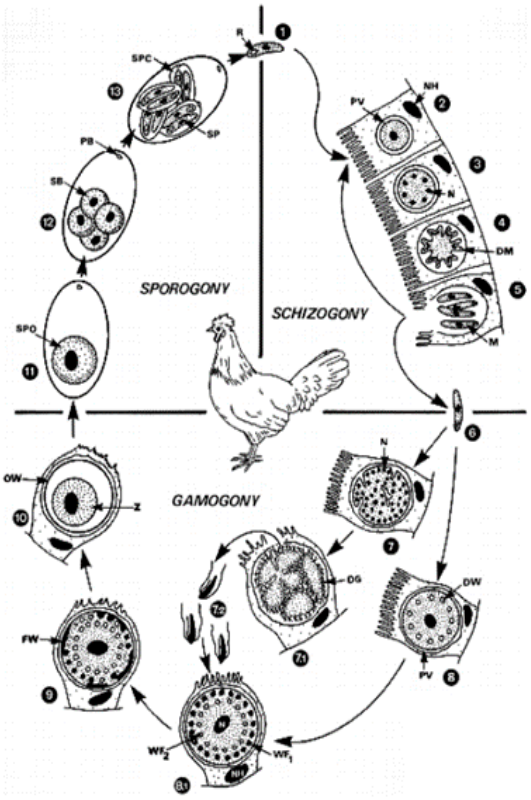
The aim of the present note is, to identify possibilities to clarify and simplify the present regulations considering evaluation and monitoring of coccidial resistance/sensitivity. Central technical terms related to the coccidial parasite, coccidiostats and resistance of coccidia and bacteria against coccidiostats are explained in the beginning of the document and a suggestion for the simplification of the current text of the Commission Regulation (EC) No 429/2008 of 25 April 2008, which may be

implemented in the Guidance for the preparation of dossiers for coccidiostats and histomonostats (EFSA, 2011) is provided as Annex1.

## Explanation of central terms related to coccidia and coccidiostats

***Coccidia:*** Coccidia are microscopic, spore-forming, single-celled eukaryote parasites of the subclass Coccidiasina. Unless otherwise noted in this note, the term "coccidia" is used to describe coccidia of the genus Eimeria which can infect poultry. Coccidia are obligate intracellular parasites with a quite complex lifecycle (Figure 1) involving asexual and sexual formation within the host intestinal cells. It is the intracellular stages that may lead to an intestinal injury, which may exceed beyond immunization of the infected chicken at high infection levels. The product of the sexual formation is the oocyst which is shed with the faeces and sporulates (sporogony) outside the host in the litter to become infective. *Eimeria* spp. have a narrow host spectrum, meaning that coccidial species that can infect chickens can not infect turkeys, and vise versa.

outside the host




inside the host
asexual formation

inside the host
sexual

**Figure 1**. *Life cycle of* Eimeria *spp. in chickens*
***1*** After oral uptake of sporulated oocysts the sporozoites hatch in the small intestine from the sporocysts. ***2–6*** After penetration, multinucleate schizonts are formed (**3**) inside a parasitophorous vacuole (*PV*). The schizonts produce motile merozoites (*DM, M*), which may initiate another generation of schizonts in other intestinal cells (**2–5**) or become gamonts of different sex (**7, 8**). *7* Formation of multinucleate microgamonts, which develop many flagellated microgametes (**7.1**-**7.2**). *8* Formation of uninucleate macrogamonts, which grow to be macrogametes (**8.1**) that are characterized by the occurrence of two types of wall-forming bodies ($WF_1$, $WF_2$). ***9*** After fertilization the young zygote forms the oocyst wall by consecutive fusion of both types of wall-forming bodies (*FW*). ***10*** Unsporulated oocysts are set free via faeces. ***11–13*** Sporulation (outside the host) is temperature dependent and leads to formation of four sporocysts, each containing two sporozoites (*SP*), which are released when the oocyst is ingested by the next host. Adopted from Peek (2010).

***Coccidiostats*.** Coccidiostats are drugs added to poultry feed that serve to retard the life cycle or reduce the population of pathogenic coccidia to the point that disease is minimized and the host develops immunity. According to the register of approved feed additives, the following 11 types of coccidiostats are currently approved within the EU (European Union Register of Feed Additives pursuant to Regulation (EC) No 1831/2003 Annex I: List of additives). These are: Narasin, lasalocid sodium, monensin sodium, salinomycin sodium, maduramycin ammonium, semduamicin sodium, robenidine hydrochloride, diclazuril, decoquinate, halofuginon and nicarbacin. Basically three categories of anticoccidial products are currently used in poultry production (Peek and Landman, 2011).

a. *Synthetic compounds*. These compounds are produced by chemical synthesis and are often referred to as 'chemicals'. Synthetic drugs usually have a specific mode of action against the parasite metabolism. There are products affecting cofactor synthesis, e.g. the folate pathway, or affecting mitochondrial function of the parasite. However, the precise mode of action of some synthetic coccidiostats, e.g. diclazuril, halofuginone, nicarbaczin and robenidine, is still not yet completely clarified (Noack et al., 2019).

b. *Polyether antibiotics or ionophores*. These products are produced by the fermentation of Streptomyces spp. or Actinomadura spp. and destroy coccidia by interfering with the balance of important ions like sodium and potassium. Polyether antibiotics influence the transport of mono- or divalent cations ($Na^+$, $K^+$, $Ca^{++}$) across cell membranes inducing osmotic damage. The following groups of ionophores exist: Monovalent ionophores (monensin, narasin and salinomycin). Monovalent glycosidic ionophores (maduramicin and semduramycin). Divalent ionophores (lasalocid).

c. *Mixed products.* These products consist of two components, e.g. a synthetic compound and ionophore (nicarbazin/narasin).

It is important to note that in contrast to synthetic coccidiostats, most ionophores inhibit growth of Gram-positive bacteria in the digestive tract of poultry (Table1). In particular, lactic acid bacteria and the potential intestinal pathogen *Clostridium perfringens* causing necrotic enteritis are targeted by ionophores (Engberg et al., 2000; VKM, 2015).

## Resistance of microorganisms against antimicrobial drugs

***Acquired resistance.*** This is resistance of a microorganism (in this case of coccidia or bacteria) to a particular antimicrobial agent, to which the microorganism previously was susceptible. The change is the result of genetic alteration in a microorganism due to mutation(s), the acquisition of foreign mobile genetic material or a combination of both mechanisms.

***Cross-resistance and Co-selection.*** Cross-resistance occurs when the same or similar mechanism(s) of resistance apply to different antimicrobials. As an example in relation to coccidiostats, cross-resistance between different monovalent ionophores has been reported (Chapman and Shirley, 1989; Marien et al., 2007). Beside cross-resistance, different antimicrobial drugs may induce co-selection of resistance. This is the case, when separate resistance mutations or genes are linked. A selection of resistant bacteria with one drug will in this way lead to the selection of bacteria resistant to another drug (Figure 2).

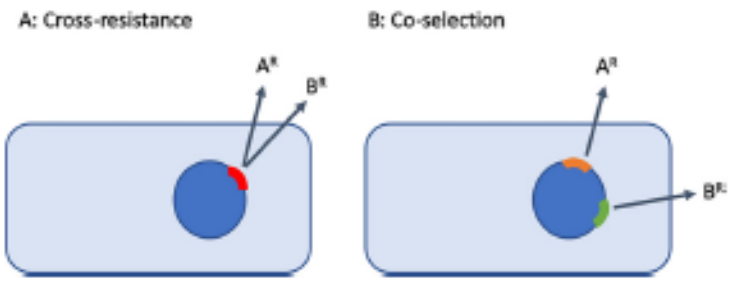



**Fig. 2. (**A) A single mutation or gene may confer cross-resistance to two or more drugs "A" and "B" as hypothetical examples ). (B) Alternatively, if separate resistance mutations or genes are linked, selection with one drug will co-select for resistance to a second drug. (Adopted from Wong (2019)).

**Table 1. Antibacterial propertes of coccidiostats. (adopted from VKM report, 2015)**

| Compound | Active substance | Antibacterial activity |
|---|---|---|
| **Ionophores** | Narasin | Mainly active against Gram-positive bacteria |
| | Lasalocid sodium | Active against Gram-positive bacteria, but not against Gram-negative bacteria. |
| | Monensin sodium | Mainly active against Gram-positive bacteria. |
| | Salinomycin sodium | Active against Gram-positive bacteria, but not against Gram-negative bacteria. |
| | Maduramicin ammonium | Active against Gram-positive bacteria, but not against Gram-negative bacteria. |
| | Semduramicin sodium | Has limited antibacterial activity against Gram-negative microorganisms tested, and a minimal activity against selected Gram-positive control organisms |
| **Non ionophores** | Robenidine hydrochloride | No known antibacterial effect |
| | Diclazuril | No substantial antibacterial activity |
| | Decoquinate | Most tested strains of bacteria appear resistant to the effects of decoquinate at concentrations of > 64 mg /-1, substantially higher than the concentration of decoquinate expected in the digestive tract |
| | Halofuginon | No known antibacterial effect |
| | Nicarbazin | No known antibacterial effect |

***Resistance of Eimeria spp. against coccidiostats.*** The World Health Organization (WHO) defines drug resistance in antimalarial chemotherapy, which can also be applied to coccidiology, as 'the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug in doses equal to or higher than those usually recommended but within the limits of tolerance of the subject' (WHO, 1965). Generally, drug resistance in coccidia can be complete, in which case increasing doses up the maximum tolerated by the host is ineffective (i.e. diclazuril and nicarbazin). In contrast, relative resistance to anticoccidial drugs is characterized by the fact that increasing doses tolerated by the host still will show efficacy (i.e. ionophores) (Peek and Landman, 2011).

Resistance of *Eimeria* spp. against coccidiostats is a common phenomenon (Chapman et al., 1984). In relation to the development of coccidial resistance, it has been shown that resistance against ionophores generally occurs slower than that against synthetic coccidiostats. In order to avoid resistance, the use of so called "shuttle programs" (shift from one coccidiostat to another coccidiostat within the same rotation), the use of combinations of ionophore and synthetic coccidiostat, or a so called "switch" (the change of coccidiostat from one rotation to the next rotation) are commonly applied in practice (DANMAP, 2015).

***Resistance of bacteria against antimicrobial compounds***. There are a number of definitions of bacterial resistance which are summarized by Davison et al. (2000): 1. Antimicrobial resistance is the property of bacteria that confers the capacity to inactivate or exclude antibiotics, or a mechanism that blocks the inhibitory or killing effects of antibiotics. 2. Antimicrobial resistance is the ability of a microorganism to withstand an antibiotic. 3. Antimicrobial resistance is a relative term which provides an interpretation of the clinical significance of concentrations of an antimicrobial that inhibit the growth of an organism or kill it in laboratory systems (*in vitro*). 4. Either microbiological resistance, where resistant organisms are those that possess any kind of resistance mechanism or resistance gene, or clinical resistance, where a bacterium is classified as susceptible or resistant depending on whether an infection with that bacterium responds to therapy or not.

As mentioned above, most ionophore coccidiostats inhibit growth of primarily Gram-positive bacteria in the digestive tract of poultry (Table 1). The continuous use of the same ionophore in the control of coccidiosis does therefore not only result in resistance development of *Eimeria* spp. but may also induce resistance in certain intestinal bacteria to these compounds. The Panel on Animal Feed of the Norwegian Scientific Committee for Food Safety (VKM, 2015) concluded that enterococci (*Enterococcucus faecium* and *Enterococcus faecalis*) isolated from poultry fed with narasin, monensin and salinomycin may become resistant to these drugs. Blood infections in humans related to these types of enterococci occure quite often. However, whether or not the extensive use of ionophores contributes to resistance problems is not very well established. Recent evidence suggests that ionophore use in some cases may co-select for resistance to vancomycin (Wong, 2019), which is considered a critical antibiotic used in human medicine (WHO, 2019). Wong (2019) concludes that there is an urgent need to systematically investigate the contribution of ionophores to the burdon of antimicrobial resistance.

In line with this, the Federation of Veterinarians of Europe (2016), advise the inclusion of coccidiostats in the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) ESVAC monitoring system.

**Assessment of sensitivity/resistance of *Eimeria* spp. against coccidiostats**

Drug sensitivity is the converse of drug resistance and, therefore, the criteria used to evaluate efficacy are also used to measure resistance (Chapman, 1998). Due to their complex life cycle (resulting in a lack/the absence of valid *in vitro* assays where full life cycles are possible), the assessment of sensitivity/resistance of *Eimeria* spp. to coccidiostats is generally quite laborious and requires *in vivo* passage/infection in the host/target animal (i.e. poultry) when compared to the assessment of bacterial sensitivity/resistance, which is done under standardized conditions *in vitro*. According to the current regulation (Regulation (EC) No 1831/2003; Commission Regulation (EC) No 429/2008 of 25 April 2008 and to the Guidance for the preparation of dossiers for coccidiostats and histomonostats (EFSA 2011), the efficacy of coccidiostats has to be demonstrated in the target animal, which requires the conduction of challenge experiments. In order to minimize animal experiments, *in vitro* assays that could be used for sensitivity assessment, e.g. in the initial phase of drug development have been descibed (COST 89/820, 1995; Habibi et al., 2016) but their application is currently limited. In the future, efforts should be made to develop reliable and valid *in vitro* assays for efficacy/sensitivity/resistance assessment.

The current practical procedure involves the following steps: a. Collection of samples of litter, droppings or intestinal content in the field (recent samples), b. Isolation of single or mixed oocysts from these samples, c. Identification of isolated *Eimeria* spp., d. Counting of oocysts and titration for the preparation of the infection inoculum. Challenge experiment with naïve medicated and non-medicated birds (fed the respective coccidiostat). Oocysts can be stored in the laboratory in potassium dichromate for months, however storage time is still limited. In order to ensure infectivity of oocysts, coccidial strains or mixtures are passaged (juvenilized) via infection of naïve birds followed by sampling of intestinal content, isolation of oocysts and preparation for the challenge experiment.

Holdsworth et al. (2004) published detailed guidelines for evaluating the efficacy of newly developed anticoccidial drugs in chickens and turkeys. The authors suggest the necessicity of 3 types of animal studies for efficacy assessment: a. Dose determination studies, which are battery cage studies of limited duration, b. Dose confirmation studies that involve the housing of birds in floor pens in standard grow out facilities, and c. Field effectiveness studies, which are carried out under commercial production conditions in he field.

a. Dose determination studies are used to evaluate the ability of e.g, a newly developed drug to treat or control coccidiosis, or measure the effect of different concentrations of drugs, study the efficacy af a drug against different field strains, and determine the stage of the life cycle affected by ananticoccidial drug under carefully controlled conditions.
b. Dose confirmation studies provide an opportunity to test the new drug against a wide selection of recent field isolates either as single species infections or mixtures to support claims against the most important coccidial species, which are *E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, and *E. tenella* in chickens and *E. meleagrimitis*, *E. gallopavonis and E. adenoeides* in turkeys. Dose confirmation tests carried out in floor pens allow to test drug efficacy under environmental conditions that pertain to commercial practice. Criteria used by various investigators are weight gain, feed conversion, mortality, production costs and lesion scores, dropping scores, oocyst counts, skin pigmentation scores, packed erythrocyte volumes blood xanthophyll or caretonoids and immunity levels (Holdworth et al., 2004).
c. Field effectiveness studies allow to study the efficacy of the coccidiostat under practical farming conditions. Compared to studies involving artificial infection of birds with recent field isolates under housing conditions simulating practice (floor pens), field effectiveness studies have significant disadvantages. In particular, the lack of appropriate control groups, i.e. an unmedicated

control group (negative control) or a group medicated with another already authorised coccidiostat (positive control), is a drawback. Holdsworth et al. (2004) points out that in relation to field trials a number of precausions have to be taken to avoid errors. Usually many people participate in field trials, which may increase the risk of errors, e.g. in connection with feed preparation, as well as sample and data collection. Further appropriate control groups are often lacking. Usually the outcome variables of these trials are production results (final body weight, feed consumption, feed conversion, production costs and total mortality), all of which can be affected by other infections common in commercial poultry flocks.

In accordance with Holdsworth et al. (2004), the Guidance for the preparation of dossiers for coccidiostats and histomonostats (EFSA 2011, Section 4) currently stipulates that data concerning coccidiostat efficacy should derive from three types of target animal experiments: a) screening for response using artificial single and mixed infections, b) natural/artificial infection to simulate use conditions (e.g., floor pen studies with poultry) and c) actual use conditions in field trials (or sensitivity trials). However, due to their inherent weaknesses, the value of field trials in the assessment of coccidial sensitivity is questioned by EFSA (2011, Section 4), suggesting that the conduction of sensitivity trials is preferred. These sensitivity tests are expected to involve artificial infections using field strains of *Eimeria*, recently confirmed as pathogenic/resistant by a sensitivity test or recognized problems in the poultry operation (confirmed by veterinary certificate).

In section 5 of the Guidance (EFSA 2011), it is stated that the post-market monitoring plan for coccidiostats shoud involve field monitoring in the form of a sensitivity study as described under Section 4.

**Suggestions for the revision of Commission Regulation (EC) No 429/2008 for further implementation in the Guidance for the preparation of dossiers for coccidiostats and histomonostats (EFSA 2011)**

It seems reasonable to suggest a clarification and simplification of the text in the current regulation (EC) No 429/2008 and consequently that of the guidance considering the assessment of the efficacy of coccidiostats. As the value of field trial outcomes has been questioned in the latest version of the guidance paper, the requirement for these kind of trials could be deleted and could be substituted by the requirement for so-called anticoccidial sensitivity tests (ASTs) in a revised version of the document. However, a general clarification of the text is needed to allow a better grasp of its contents.

*In the Guidance for Industry, Evaluating the effectiveness of anticoccidial drugs in food-producing animals,* the Food and Drug Administration (FDA) (2012) defines anticoccidial sensitivity tests (ASTs) as studies conducted with birds in battery cages to evaluate the efficacy of a variety of anticoccidial drugs against a particular field isolate. Because of their study design and objectives, the FDA proposes that ASTs do not provide substantial evidence of effectiveness and considers the conduction of commercial-scale field studies on an appropriate number of geographic locations necessary to confirm that the selected feeding level of a new drug is effective under commercial production conditions.

However, in acoordance with Holderworth et al. (2004) who describe the design and procedure of *dose confirmation studies (ASTs)*, sensitivity testing of coccidiostats can be carried out as fully controlled target animal trials involving artificial infection of poultry housed in floor pens under enviromental condition simulating practical conditions over the entire grow out period. The study design should comprise 4 groups: an untreated non-infected control, an untreated infected control, a

treated (coccidiostat in question) non-infected group and a treated infected group. Further, an appropriate number of replicates is required to ensure sufficient statistical power. The infection strains *Eimeria* spp. should be recent field isolates from different European countries EU. Sensitivity tests should be performed according to the principals established by Chapman (Chapman, 1998). Criteria could be taken from McDougald et al., 1986, 1987) and guidelines published by Holdsworth et al. (2004). The outcome variables measured should at least include production results (body weights, feed consumption, feed conversion ratio), mortality, scoring of gross lesions and oocyst shedding, e.g. Marien et al. (2007).

ASTs as described above should also be the studies of choice included in post marketing monitoring plans. Monitoring of coccidial sensitivity should be carried out towards the end of the approval period making up the documentation for a new approval of the product in question. For monitoring, the study design should also comprise 4 experimental groups as described above. As the consumption of coccidiostats may differ somewhat within the EU, recent field strains or strain mixtures from different locations (European countries) should be used as challenge material. Compared to the trials necessary for the documentation of the efficacy of new drugs, it could be discussd whether ASTs for monitoring purposes could have a shorter duration (e.g. infection at 16 days, oocyst counting and lesion scoring 20-24 days) as described by Marien et al. (2007) and Barrios et al. (2017). However, considering these tests as documentation for a re-newed approval of the product, it is suggested that tests for the purpose of monitoring also should be carried out over an entire production period.

***Microbial studies in relation to the approval of coccidiostats***

Similar to other feed additives, microbial studies for the determination of bacterial resistance should be carried out in relation to the first approval and as part of the post marketing monotoring. In the *Technical Guidance, Microbial Studies* (EFSA, 2008) a list of reference strains known to be succeptible to clinically relevant antibiotics, ionophores or biocides is given. As mentioned before, resistance of intestinal enterococci isolated from poultry against ionophores has previously been observed (VKM, 2015), which may indicate that both *Enterococcus faecalis* and *Enterococcus faecium* should be in the panel of microorganisms screened for resistance against ionophores. Further, *Clostridium perfringens* which causes necrotic enteritis in poultry and possible food poisoning in humans should likewise be included. Finally, besides staphylococci *(Staphylococcus aureus),* streptococci, in particular those causing disease in different hosts (i.e. *Streptococcus zooepidemicus*) would be reasonable to include in the panel of microorganisms. Although, resistance in Gram-negative bacteria has not been observed frequently in relation to the use of ionohores, *Escherichia coli* and a Salmonella type frequently causing disease in humans, i.e. *Salmonella* Typhimurium and/or *Salmonella* Enteritidis should be part of the panel of microorganisms. Standard methods for the determination of minimum inhibitory concentration (MICs) are provided by European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Dieases (ESCMID), 2000.

A suggestion for the clarification/simplification of the current text of the Commission Regulation (EC) No 429/2008 is provided as Annex 1.

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