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Review

Residues of veterinary medicinal products and coccidiostats in eggs – causes, control and results of surveillance program in Poland

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Abstract

The use of veterinary medicinal products in food producing animals for a variety of purposes causes that their residues may be presented in edible tissues. As a result, in concern of public health, European Union Countries establish each year monitoring plans and they control the levels of harmful substances in food of animal origin. This paper presents survey of residues of veterinary medicinal products and coccidiostats in eggs for Poland and European Union in years 2007-2010. Despite the decrease in reported non-compliant results for coccidiostats, the numbers were still higher than those for veterinary medicines. The most often determined coccidiostats were: nicarbazin, dinitrocarbanilide, salinomycin and lasalocid, and the most often reported non-compliant results for veterinary medicines were: antimicrobials, enrofloxacin and doxycycline.

Key words: antimicrobials, coccidiostats, eggs, residues, veterinary medicinal products

Introduction

Eggs are important nutriment component because they are inexpensive, highly nutritious, and commonly available. They can be called "perfect food"as they contain everything what is needed to support life. They can be consumed in the primary form or as intermediate products (like: dried eggs, egg powder, blend of bakery or protein powder) used to manufacture goods.

The problem is that nowadays there is no animal food production without using veterinary medicinal products and feed additives. Contemporary intensive methods of production may cause faster spreading of diseases among the animals, and thus modern commercial food production, oriented on high efficiency, has to face animal health issues. The outbreaks of a disease are more often and the illnesses sometimes get out of control. Currently the most common poultry diseases are: Newcastle Disease, Gumboro Disease, Marek's Disease, coccidiosis, E. coli infections, Salmonellosis (Mazanowski 2011). Among others, coccidiosis is one of the biggest problems in poultry production (Tewari and Maharana 2011). It is caused by protozoa of the genus *Eimeria* and causes mortalities, poor weight gain and feed conversion ratio and decreases egg production (Mazurkiewicz 2005). To prevent coccidiosis of poultry many substances called coccidiostats are widely used. For many reasons, these compounds are mainly registered as feed additives and are not authorised for laying hens within the European Union (Olejnik et al. 2011). Also antimic-

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robials are not only used for treating but also for preventing numerous bacterial infections, which is more effective than treating them. Furthermore, they may be also illegally used for fattening purposes because they act as growth-promoters when are administered to healthy animals in lower doses than those used in the treatment of infections (McEwen and Fedorka-Cray 2002).

Using veterinary medicinal products and feed additives in animal food production may results in the presence of their residues in edible tissues and food of animal origin, like milk and eggs, which enter the human food chain. The residues are small quantity levels (expressed in μg/kg or mg/kg) of substances in food obtained from animals after some kind of administration. Because of the potential negative influence of antimicrobial related residues on animals and humans' health, like: cancerogenic, genotoxic, immunotoxic, potential antibiotic resistance (Voogd 1981, Sørum et al. 2002, Michalova et al. 2004, Lu et al. 2010) the European Union countries make efforts to protect public health by monitoring residual level of harmful substances in animal products and to keep them on toxicologically acceptable levels. As a result properly planned monitoring programs are established each year in every European Union country.

Sources and arising of residues in eggs

Veterinary medicinal products can be administrated to animals in a variety of ways e.g. in feed, water, orally or injected. The residues in eggs destined for consumption may occur when laying hens are mistakenly or intentionally treated with prohibited substances, when they are off-label clinically used, the withdrawal period is not restricted or the animals are given feed contaminated in mill (during the mixing) or during the storage or transport.

A hen lays an egg every 24 hour, but it takes couple of days to develop an egg *in vivo*. Sometimes residues may be presented in eggs up to few weeks after administration because some of egg components are formed months before the egg is fully developed and laid. That is the reason why sometimes several week time period is required after the treatment to obtain eggs free of residues.

Physicochemical properties of compounds determine whether they deposit in the egg yolk or white. The distribution of residues depends, among other things, on the compound's ability to bind with tissue proteins, pass through different tissue types and mainly water solubility. Because of the large amount of lipids the egg yolk is an apolar while the egg white is a polar medium. Due to that we can observe differences between depositions of e.g. antimicrobials between these two compartments (Aerts et al. 1995). While the egg is developing first yolk lipoproteins are depositing in concentric layers, and liphophilic substances which result as residues in the yolk accumulate during this time. Further water soluble proteins of the egg white are depositing around the yolk and the shell membranes are formed. The egg shell is formed at the end, when white proteins are diluted with water (Simkiss K and Taylor TG 1971). Goetting et al. (2011) have described literature concerning different antibiotic classes' residues and their deposition into eggs which concludes that chemical properties cannot always be the base to foresee antibiotic's pharmacokinetic properties. The presence of residues varies across individual differences such as rate of metabolism, age, gender, etc.

Donoghue and Hairston (2000) reported that residues of veterinary medicines first appear in the egg white when a medicament is distributed through this compartment. If plasma shows constant levels of the medicament, the egg white reflects plasma levels of residues and reaches a constant level after 2-3 days. The residues in the egg yolk reflect medicament level of plasma only during the time of exponential growth and they require an 8-10 day exposure to obtain the constant level. However, single exposure to a medicament can be detected in the egg depending on the method used and compound physicochemical properties. Kan (2003) has revealed that the disappearance of veterinary medicinal residues from the egg depends mostly on plasma levels of the medicament. When the medicament disappears quickly from the body the residues usually disappear within 2-3 days from the egg white, while disappearing from the yolk usually takes 10 days.

Limits of residues

Some of the substances whose residues are found in eggs are banned and others are allowed for the use under strict conditions and acceptable on established limits. For those whose residual levels are acceptable in eggs for human consumption the European Union provides maximum residue limits (MRL's) and maximum levels (ML's). Residue limits of pharmacologically active substances in foodstuffs of animal origin are harmonised at the Community level by the Regulation No. 470/2009 (2009). The veterinary medicinal products and coccidiostats with established limits in eggs for the European Union countries are listed in Table 1 (Regulation No. 37/2010, 84/2012) and Table 2 (Regulation No. 124/2009).

Table 1. Veterinary medicinal products with established maximum residue limit in eggs for the EU.

Substance	Class of medicine	MRL* in eggs (μg/kg)
Chlortetracycline	antibiotic	200
Colistin	antibiotic	300
Erytromycine	antibiotic	150
Flubendazole	antiparasitic agent	400
Lincomycine	antibiotic	50
Neomycin	antibiotic	500
Oxytetracycline	antibiotic	200
Penicillin V	antibiotic	25
Phoxim	antiparasitic agent	60
Piperazyne	antiparasitic agent	2 000
Tetracycline	antibiotic	200
Tiamuline	antibiotic	1 000
Tylosine	antibiotic	200

MRL* – Maximum Residue Limit

Table 2. Limits for coccidiostats authorised for use in chickens destined for laying hens within the EU, expressed in μg/kg.

Coccidiostat	MRL*	ML**
Decoquinate	–	20
Diclazuril	–	2
Halofuginone	–	6
Lasalocid	150	–
Maduramicin	–	2
Monensin	–	2
Narasin	–	2
Nicarbazin	–	100
Robenidine	–	25
Salinomycin	–	3
Semduramycin	–	2

MRL* – Maximum Residue Limit
ML** – Maximum Level

The European Commission set maximum levels for eleven coccidiostats in eggs and maximum residue limit only for lasalocid. These substances are forbidden for use in egg laying hens (they can be administrated only to chickens destined for laying hens up to 12-16 weeks old). To avoid the presence of coccidiostats in non-target feed the European Commission set maximum levels of unavoidable carry-over of these substances in animal feed to obligate manufacturers to make some efforts to avoid cross-contamination during the production, storage and transport (Directive 2009/8/EC, 2009). As Olejnik et al. (2009) reported, this regulation entered into force in July 2009 and was included as permitted levels of coccidiostats content in non-targeted feed in Poland.

There are very few medicinal products registered for use in chickens destined for laying hens (e.g. with tylosine) or to treat laying hens during the laying period (e.g. with colistin, flubendazole). The withdrawal period in this kind of products must be short, and it is set e.g. as 0 days for tiamulin or 3 days for oxytetracycline or neomycin.

Determination of residues in eggs in the European Union

In compliance with the Commission Staff Working Paper (2004), the methods for determination of residues can be divided into three groups: microbiological, immunochemical and physico-chemical. The microbiological methods are fast screening methods used to avoid false compliant results. They can only detect the presence of substance and supply preliminary information about its concentration. A further confirmatory method is required to provide precise identification of the substance and to confirm whether the MRL was exceeded. The immunochemical methods are widely used in residue analysis; they are rapid, sensitive and selective. The physico-chemical methods give better identification and quantification results. The criteria for the validation and interpretation of results for screening and confirmatory methods are specified in the Decision 2002/657/EC (2002) and since it entered into force analytical results exceeding the permitted limits are termed as "non-compliant" (previously "positives"), where: "Non compliant: is a sample that has been analysed for the presence of one or more substances and failed to comply with the legal provisions for at least one substance. Thus, a sample can be non-compliant for one or more results".

As Serratosa et al. (2006) reported, the biggest analytical challenge in residue of veterinary medicinal products analysis are multi-residue methods which could simultaneously determine residues of different chemical groups due to the use of mixtures consisting of various prohibited substances in a low concentration. This makes the approval of illegal use of banned substances much more complicated. Olejnik et al. (2011) reported that after a multi-residue method for coccidiostats was implemented in the control program in Poland, the number of non-compliant samples significantly increased. So far most of the methods allow for the determination over a dozen analytes (examples are given in Table 3), what additionally enhances the

Table 3. Examples of methods used for the analysis of veterinary pharmacologically active substances in eggs.

Analytes	Detection system	Number of analytes	CCα* [μg/kg]	References
Coccidiostats	LC-MS/MS**	11	2.2-174	Galarini R. et al., 2011
Macrolides	LC-MS/MS	3	5.5-230	Bogialli S. et al., 2009
Nitroimidazoles	LC-MS/MS	4	0.05-0.44	Mitrowska K et al., 2010
Sulphonamides	LC-MS/MS	10	16.1-20.5	Forti AF, Scortichini G, 2009
Chloramphenicol	LC-MS/MS	1	0.09-0.192	Rrnning HT et al, 2006
Nitrofuran metabolites	LC-MS/MS	4	0.16-0.24	Śniegocki T. et al., 2008
(Fluoro)quinolones	HPLC-FLD*** LC-MS/MS	9	15-37 3-7	Gajda A. et al, 2012
Tetracyclines and their metabolites	HPLC-UV**** LC-MS/MS	17	nd*****	Zurhelle G. et al, 2000
Benzoimidazoles	HPLC-UV	10	nd	Bistoletti M. et al, 2011
Multi-residue: Coccidiostats Macrolides Lincosamides	LC-MS/MS	10	0.87-229.7	Spisso BF et al, 2010
Multi-residue: Sulfonamides Diaminopyridine derivates Quinolones Tetracyclines Macrolides Penicylines Lincosamides	LC-MS/MS	41	0.3-232.3	Jimenez V. et al, 2011
Multi-residue: Penicylines Macrolides Tetracyclines Lincosamides Sulfonamides Quinolones	LC-MS/MS	46	25-235	Błądek et al., 2012

* CCα – decision limit, means the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant (2002/657/EC, 2002); ** LC-MS/MS – Liquid chromatography tandem mass spectrometry; *** HPLC-FLD – High-pressure liquid chromatography with fluorescence detector; **** HPLC-UV – High-pressure liquid chromatography with UV detector; ***** nd – no data.

need of work under the multi-residue methods, which may be divided into two categories: qualitative or quantitative screening methods and quantitative confirmatory methods. Cháfer-Pericás et al. (2010) have reported that according to the Web of Knowledge database actually high pressure liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is the most common detection system used in the antibiotic residue analysis in food samples; it is applied in 38% of published methods.

Commission Decision 98/179/EC (1998) requires that official samples analyses should be implemented in accredited laboratories which apply validated methods to guarantee the quality of results. Additionally those laboratories must confirm their competency by the regular participation in proficiency testing schemes organized by the national or Community reference laboratories. However, due to the differences between limits of detection in methods applied in national control laboratories there is still a possibility that some laboratories may categorize samples as compliant while in other country they might be found as non-compliant (Serratosa et al. 2006) (within the European Union and according to EU legislation). These differences causes that EFSA (European Food Safety Authority, established by European Parliament in 2002) is not fully satisfied with data received.

Survey of residues in eggs

Council Directive 96/23/EC (1996) requires the Member States to implement the national residue

Table 4. The list of substances included in the National Residue Control Program for eggs.

Group	Analytes	Method	Number of samples/year
A 6	Nitrofuran metabolites: 4 analites	LC-MS/MS*	~ 45
	Nitroimidazoles and its metabolites: 7 analites	LC-MS/MS	~ 10
	Chloramphenicol	ELISA**/LC-MS/MS	~ 45
Summarize group A			~ 100
B 1	Antimicrobials	4-i 5- plate test/LC-MS/MS	~ 145
	Sulfonamides: 6 analites	HPLC-UV*** HPLC-DAD****/ HPLC-FLD*****	~ 30
	Tetracyclines: 4 analites	HPLC-UV/LC-MS/MS	~ 25
	Fluorochinolones: 2 analites	HPLC-FLD	~ 25
Summarize group B 1			~ 225
B 2a	Anthelmintics Benzoimidazoles and its metabolites: 19 analytes	LC-MS/MS	~ 15
B 2b	Coccidiostats: 12 analytes	LC-MS/MS	~ 100
Summarize group B 2			~ 115
Summarize group A and B			~ 440

* LC-MS/MS – Liquid chromatography tandem mass spectrometry; ** ELISA – Enzyme-linked immunosorbent assay; *** HPLC-UV – High-pressure liquid chromatography with UV detector; **** HPLC-DAD – High-pressure liquid chromatography with diode array detector; ***** HPLC-FLD – High-pressure liquid chromatography with fluorescence detector.

monitoring plan for groups of residues detailed in Annex I of the Directive and according to the rules for the analytical methods and levels detailed in Annex IV. The Member States annually submit the results of their monitoring to the Commission, including occurrence data. Following the guidelines of this Directive, samples are targeted with the aim to detect illegal treatment or control compliance with maximum levels laid by legislation. Suspect samples are taken as a consequence of non-compliant results of the monitoring plan, possession or presence of forbidden substances through the food production and suspicion or evidence of illegal treatment or not restriction of withdrawal period of prohibited veterinary medicinal products.

Every year the concept of the National Residue Control Program in Poland is prepared in the National Veterinary Research Institute, National Reference Laboratory, in Puławy (Żmudzki et al. 2005). It gives the guidelines for which substances are monitored and it also provides methods and their performance criteria for different types of analytes. Substances are divided into two groups: A – with anabolic effect and banned substances, and B – medicinal products and other substances which may be used for veterinary purposes. Group B includes also environmental contaminants such as: organochlorine pesticides, polychlorinated biphenyls and toxic metals (~ 200 samples). The list of substances, methods and approximate number of collected samples for eggs are given in Table 4 (Polish National Control Program 2010).

In case of eggs, within the European Union, a requirement for the national residue control program is to collect minimum one sample for every 1000 t of yearly egg production every year with a bare minimum of 200 samples (Decision 97/747/EC 1997). Total amount of egg production for Poland and for the European Union, and overall number of targeted samples during the 2007-2010 period are given in Table 5 (SANCO 2008, EFSA 2009-2011). Generally, the European Union production maintains the same level, while in Poland it increases every year. As a consequence the number of annually collected samples in Poland also increases. Moreover, Poland fulfils the European requirements for eggs with surplus every year what subsequently has an influence on percentage partitioning of reported non-compliant results for the entire EU. The numbers of targeted samples collected for eggs by every EU country are presented in Fig. 1. In 2010 Poland was the 7th country in Europe minding this requirement.

Table 5. Production of eggs and number of targeted samples over 2007-2010 period.

	Production (t)		Targeted samples		Minimum 96/23/EC
Year	European Union	Poland	European Union	Poland	
2007	6 114 369	448 000	13 685	630	1/1000 t
2008	6 021 476	459 500	10 859	651	
2009	6 137 732	497 100	13 031	660	
2010	6 101 039	510 000	12 687	700	

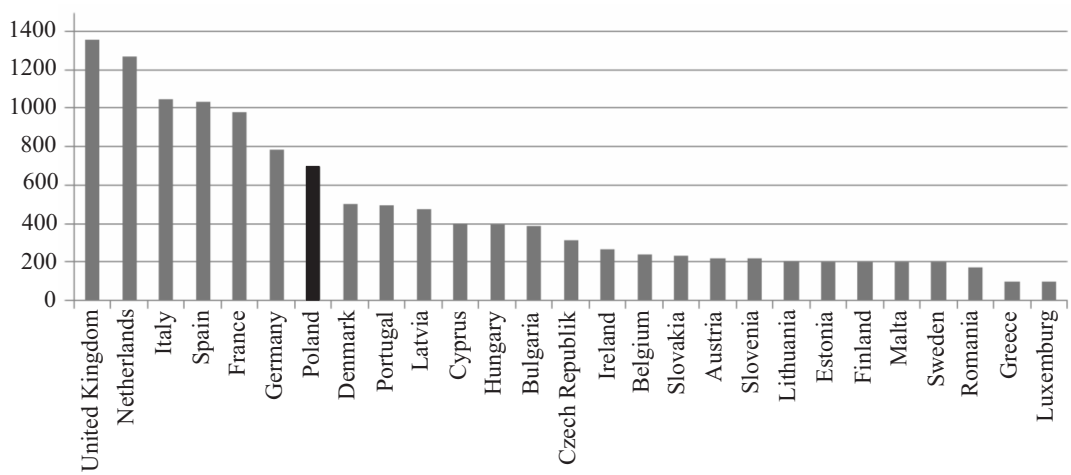

Fig. 1. The numbers of targeted samples for eggs collected by EU countries in 2010.

The percentage of non-compliant results in the EU in reference to the number of collected egg samples over the years 2007-2010 is presented in Fig. 2. Despite the fact it decreased significantly during this years, from ~ 0,8% to ~ 0,2%, it is still two times higher than that found for all analysed food samples of animal origin, which equals ~ 0,1%.

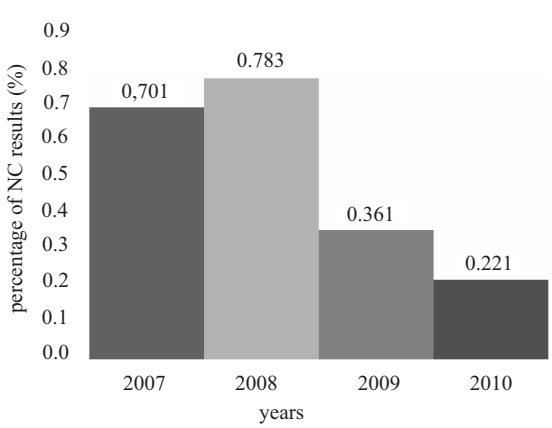

Fig. 2. Percentage of non-compliant results for eggs over 2007-2010 period for EU countries.

The number of non-compliant results for targeted and suspected egg samples aimed for veterinary medicines and coccidistats for the European Union and for Poland during the last four years are presented in Table 6. The table contains the data summarised by the European Commission and EFSA (SANCO 2008, EFSA 2009-2011). The results are given for targeted and suspected samples; they do not include those received from other samplings (e.g. import). In some cases the number of non-compliant results can be higher than the number of non-compliant samples because when multiresidue method is applied one sample can be analysed for more substances in one group.

Graphical percentages of NC results are presented in Fig. 3. Only one non-compliant result for metronidazole (group A6) was reported in 2008 in France and for this reason it is not included in the diagram. The total amount of non-compliant results for veterinary medicines maintains at a constant level, while the number of reported non-compliant results for coccidiostats in Europe decreases every year. It can be indirectly related to the new regulations laid by Directive 2009/8/EC. However, the comparison of re-

Table 6. Comparison of non-compliant results of the national control programs for the European Union and for Poland.

Year	Group	European Union			Poland	
		non-compliant results		MS reporting NC* samples	Non-compliant results	
		samples targeted	samples suspected		samples targeted	samples suspected
2007	B1	3	1	3	–	1
	B2b	71	21	9	4	1
2008	A6	1	–	1	–	–
	B1	7	6	3	3	–
	B2b	67	5	11	8	4
2009	B1	1	–	1	–	–
	B2b	43	3	13	3	3
2010	B1	11	8	7	2	6
	B2b	8	1	5	–	–

NC – non-compliant

Table 7. Details for substances reported by Member States in 2007-2010 period.

	2007		2008		2009		2010	
	EU	PL	EU	PL	EU	PL	EU	PL
Veterinary medicines:								
Metronidazole	–	–	1	–	–	–	–	–
Sulfadiazine	1	–	–	–	–	–	1	–
Ciprofloxacin	1	–	–	–	–	–	–	–
Enrofloxacin	1	–	–	–	–	–	5	7
Tetracycline	1	1	–	–	–	–	–	–
Sulfadimethoxine	–	–	–	–	1	–	1	–
Doxycycline	–	–	–	–	–	–	4	1
Antimicrobials	–	–	13	3	–	–	–	–
Coccidiostats:								
Salinomycin	15	2	5	1	6	1	1	–
Narasin	2	–	1	–	2	–	–	–
Nicarbazin	37	2	24	6	6	2	3	–
Lasalocid	4	–	10	2	13	1	2	–
Robenidine	8	–	3	–	1	–	–	–
Dicazuril	7	–	1	–	1	1	–	–
Dinitrocarbanilide	18	–	19	–	3	–	–	–
Maduramicin	1	–	4	1	12	–	3	–
Semduramicin	1	1	4	2	1	1	–	–
Monensin	–	–	11	–	–	–		

sults for Poland to those obtained in other European Union countries is not authoritative due to the different range of analytes included in control programs and set limits together with different amounts of collected egg samples mentioned earlier.

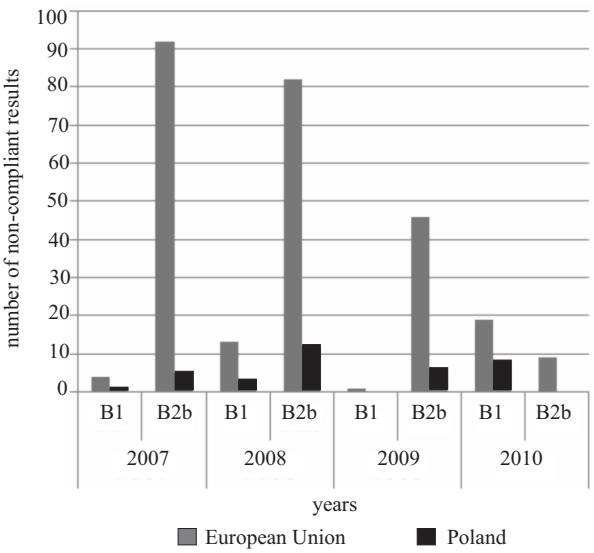

Fig. 3. Graphic comparison of reported non compliant results: European Union vs Poland.

The results for individual groups of substances determined within the EU and Poland are presented in Table 7. Within the 2007-2010 period more non-compliant results were reported for coccidiostats than for veterinary medicines. The most common detected coccidiostats were: nicarbazin, dinitrocarbanilide, salinomycin and lasalocid, while the most often reported NC results for veterinary medicines were: antimicrobials, enrofloxacin and doxycycline. In 2009 Poland reported the lack of non-compliant results within the group B1, and in 2010 the lack of non-compliant results for group B2b substances.

Conclusions

Egg production in Poland increases every year and laboratories receive more samples with large variety of residues to analyse as they are obligated to fulfil requirements for the national control program. Poultry diseases cause economical losses of animals and food of animal origin and due to that the amount of veterinary pharmacologically active substances used also increases. Despite the fact there are validated analytical methods for the determination of those substances in eggs, there is still a lack of multi-residue methods that could simultaneously determine residues of different analytical groups, combining veterinary medicinal products, coccidiostats and other chemical substances. Such methods are especially needed to improve effectiveness of detection of illegal use of banned substances, particularly minding a use of "coctails" which is now pretty difficult.

Decrease of non-compliant samples for coccidiostats is the result of improved quality of prepared feed and limits laid for carry-over of coccidiostats in non-target feed.

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