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TOTAL ANTIBIOTICS — A NEW POSSIBLE ALTERNATIVE FOR THE SCREENING OF COCCIDIOSTAT RESIDUES IN POULTRY MEAT

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ABSTRACT

The Total Antibiotics test is a microbial inhibition test which has been recently introduced for the detection of antibiotics in meat. The aim of this study was to determine whether it would be suitable for the detection of coccidiostats in poultry meat. A comparison with the Premi®Test was assessed also for the suitability of the detection of coccidiostats in poultry meat. A selection of poultry meat samples of different organ parts were assessed with 14 samples from Slovakian farms that had previously been tested for coccidiostats by the Veterinary and Food Institute in Košice. In addition, another 8 samples from varied Slovakian supermarkets such as Lidl, Billa and Tesco with samples of chicken or duck meat, were tested. Each prepared sample was added to the Total Antibiotics kit tubes and incubated. The samples from all sources showed a mixture of positive and negative results for the detection of coccidiostats.

For the Premi®Test, the samples used the same extraction procedure as the Total Antibiotics, placed in Premi®Test kit tubes and incubated. The Premi®Test demonstrated a mixture of positive and negative results, as similar to the Total Antibiotics for coccidiostats in the poultry farm samples. However, the Premi Test revealed many more negative results for the supermarket sources compared to the Total Antibiotics. Therefore, based on the total number of positive results, we concluded that Total Antibiotics is more sensitive for the detection of coccidiostats in poultry meat, but depending on the source of the samples, both Total Antibiotics and Premi®Test had either similar or opposite results for the detection of coccidiostats.

Key words: coccidiostats; detection; poultry; residues

INTRODUCTION

The human consumption of poultry meat has increased over the years due to an increased population and demands for poultry meat. Also, the availability of poultry meat has become more available for consumers to purchase. However, the requirements for large production of poultry

within a short time frame cannot, sometimes, meet the demand. This is because poultry flocks have to meet welfare and hygienic demands so they can be stated fit to eat. Many veterinary drugs and prophylactic treatments are given to prevent the spread of poultry diseases [6].

Coccidiosis is a serious disease of poultry that can cause huge economic losses particularly where birds are reared intensively. To avoid the disease, coccidiostats are routinely given in poultry feed prophylactically and there is concern that this may lead to residues in poultry meat and eggs and present a risk for consumers. The farmer does prevent the parasitic disease with preventive measures of administrating coccidiostats into routine care, however the withdrawal period of coccidiostats before slaughter needs to be determined to prevent the accumulation of coccidiostat residues [5].

The European Union (EU) has many regulations governing food and feed safety. Regulation (EC) No. 1831/2003 [8] is regarding additives for use in animal nutrition; only additives that have been through a specific authorisation procedure may be placed on the market. The Regulation (EC) No. 1831/2003 followed food and feed safety requirements outlined in Regulation (EC) No. 178/2002 [7] by validating a 10 year authorisations of coccidiostats which are included in the additives regulated by this legislation. The regulation also set up EU Reference Labs for the testing of feed additives. Currently the EU has granted 28 authorisations for 11 coccidiostats for different species and uses. The authorisation contains information such as: the characteristic of the coccidiostat, maximum, minimum and recommended dosages, labelling, withdrawal periods and maximum residue limits (MRLs) if required [2, 9].The safety of the use of coccidiostats has been extensively assessed by the European Food Safety Authority (EFSA).

EU member states are legally obliged to control the residues of veterinary medicinal products including coccidiostats in food and feed under the Council Directive 96/23/EC [3]. As with the screening of food of animal origin for human consumption, it is important that the methods used for the screening must be: rapid, allow a high throughput of samples, and relatively simple sample preparations. The current residue control strategy is based on an initial qualitative screening test followed by a quantitative confirmatory test [1].

Microbial inhibition tests (MITs) are used as part of the qualitative screening and they are based on the principle that the growth of a strain of bacteria present in the agar can be inhibited by the presence of an antimicrobial (inhibitory) substance. These tests can be plate tests or tube tests. The tube tests are very popular commercially and produce broad spectrum screening tests. They are designed to detect most of the relevant antimicrobial compounds, such as antibiotics or chemotherapeutics in food of animal origin at or below their MRLs within 3–4 hours. A positive result is presented by the absence of colour change from purple to yellow of the indicator (bromocresol purple) in the agar medium. The major bacterial strain used in the tube tests is Bacillus stearothermophilus var. calidolactis.

Premi®Test and Total Antibiotics are examples of this type of tests. The Premi®Test was developed by DSM Food Specialties (Delft, the Netherlands) and it is by far the most widely used test for the rapid screening of antibiotic residues in meat samples. The Total Antibiotics is one of the newest tube tests on the market developed by the Eurо-Clone S. p. A. (Pero, MI, Italy) for the same purpose. Both tests use Bacillus stearothermophilus var. calidolactis as the bacterial strain. In order to gain more insight in the detection capacity of the test Total Antibiotics, the aim of our study was to evaluate this test for the possible screening of coccidiostat residues in poultry meat. The Premi®Test was also assessed and compared with the Total Antibiotics for its suitability in the detection of coccidiostat residues in poultry meat.

MATERIALS AND METHODS

Poultry meat samples

To determine whether the Total Antibiotics and the Premi®Test would be suitable for the detection of coccidiostats in poultry meat, a selection of poultry meat samples from Slovakia was made. A total of 22 samples of poultry tissue were collected during the time period from November 2015 to February 2016 as follows: 14 samples were from Slovakian farms that had previously been tested for coccidiostats by the Veterinary and Food Institute in Košice, 4 samples were from Lidl, 3 samples were from Billa and 1 sample was from Tesco. For the control, a negative breast chicken sample was used. A variety of poultry tissues [chicken breast (6), duck fat and skin (1), duck breast (1), chicken thigh (4), chicken fat and skin (2), chicken spleen (2), chicken gizzard (2), chicken kidney (1), chicken liver (2), and chicken heart (1)] were stored in a freezer at –20 °C until the analysis.

Preparation of samples

All of the samples for both MIT tests used the same preparation method as for the Total Antibiotics. Poultry meat samples were thawed and 2.5 g of meat was weighed in a tube and minced with a sterile lancet. The extraction buffer provided from Total Antibiotics was diluted 1 : 10 with demineralised water. Ten ml of freshly prepared extraction buffer was poured into the test tube with the minced poultry tissue and foil tops were placed on top. The test tubes were vortexed for half a minute and incubated for 2 hours at 37 °C. The clean supernatant was collected and used in the respective tests.

Total Antibiotics

Product detail: A commercial antibiotic broad spectrum test kit supplied by EuroClone S. p. A. (Italy) containing 96 ready-to-use capped test tubes with Bacillus stearothermophilus var. calidolactis in a solid agar medium and an extraction buffer concentrate were used for the preparation of the samples for the residue analysis. The Total Antibiotics test tubes and extraction buffer must be stored in the fridge at 4 °C before use.

Test procedure: 200 µl of clear supernatant taken from the surface of the previously prepared sample was pipetted with a single disposable pipette to each of the Total Antibiotic kit test tubes. The test tubes were tightly closed with a yellow cap and incubated in a pre-heated thermostatic dry bath at 65 °C for 3 hours. The sample incubation time can be extended until the negative control has changed colour.

The interpretation of results: Results were read by analysing the agar medium present in the Total Antibiotic test tubes. The incubation period has to be completed and stopped if the agar medium in the negative control has changed to a clear yellow colour. If the colour of the agar medium remains unchanged (purple), the sample is labelled positive. All shades of purple are also read as positive samples. Complete change in medium colour from purple to yellow is labelled negative. Photos of samples can be made directly after reading the samples to ensure that no changes had taken place.

Premi®Test

Product detail: A commercial antibiotic broad spectrum screening test kit supplied by R-Biopharm AG (Germany) containing 25 ready-to-use ampoules with Bacillus stearothermophilus var. calidolactis in a solid agar medium was also used to test the same samples.

Test procedure: 100 µl of clear supernatant taken from the surface of the previously prepared sample was pipetted with a 100 µl pipette to each of the Premi®Test kit test tubes. The test tubes were covered with sealing plastic foil and incubated in pre-heated thermostatic dry bath at 65 °C for 3 hours. The sample incubation time can be extended until the negative control has changed colour.

Interpretation of results: Results are read by analysing the agar medium present in the Premi®Test test tubes. The incubation period has to be completed and stopped if the agar medium in the negative control has changed to a clear yellow colour. If the colour of the agar medium remains unchanged (purple), the sample is labelled positive. All shades of purple with no clear colour change are considered dubious. All dubious results are also suspected samples but the concentration of coccidiostats or antibiotics is at the level of detection limit of the test (not so high but still detectable according to the presence of the violet colour). If the colour of the agar medium has a clear change to the yellow colour, the sample is consider negative. Photos of samples can be made directly after reading the sample to ensure no changes take place.

RESULTS

The first set of results was done with samples from the Slovakian farms. The results of the screening for the presence of coccidiostat residues in poultry meat by MIT Total Antibiotics and Premi®Test are presented in Table 1.

The samples showed that both the Total Antibiotics and the Premi®Test had both negative and positive results. All two MIT kits were functional in detecting the presence of coccidiostats in the chicken tissues. Samples 1, 2, 9, and 10 representing the breast muscle and thigh muscle were negative for the presence of coccidiostats by both MITs. Samples 3, 4, 5, 11, 12 and 14 representing the chicken fat and skin, spleen and gizzard were positive for the presence of coccidiostats by both MITs. Sample 7 and 13 representing the chicken liver were positive for the Total Antibiotics and dubious for the Premi®Test. Sample 6 representing chicken kidneys was negative for the Total Antibiotics and dubious for the Premi®Test. Sample 8 representing the chicken heart was positive for the Total Antibiotics and negative for the Premi®Test. All samples with dubious results were suspected samples for the presence of coccidiostat residues.

Table 1. Results of screening for coccidiostats with MIT of first batch of samples

	Sample (number/matrix)	Total Antibiotics	Premi®Test
1	CHBM	–	–
2	CHTM	–	–
3	CHF&S	+	+
4	CHS	+	+
5	CHG	+	+
6	CHK	–	±
7	CHL	+	±
8	CHH	+	–
9	CHBM	–	–
10	CHTM	–	–
11	CHF&S	+	+
12	CHG	+	+
13	CHL	+	±
14	CHS	+	+

+ — positive sample; ± — dubious sample; – — negative sample
CHBM — chicken breast muscle; CHTM — chicken thigh muscle; CHF&S — chicken fat and skin; CHS — chicken spleen; CHG — chicken gizzard; CHK — chicken kidney; CHL — chicken liver; CHH — chicken heart

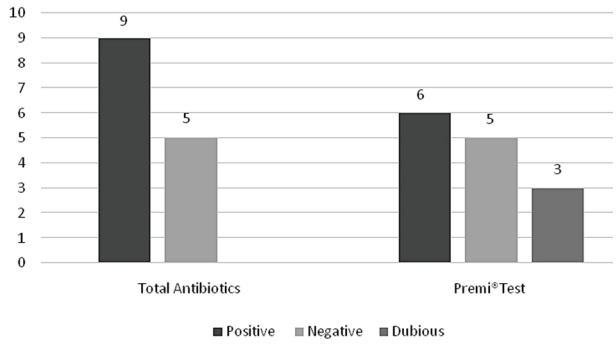

Fig. 1. Bar chart showing the differences of positive, negative and dubious results from the first batch of samples

Table 2. Results of screening for coccidiostats with MIT of second batch of samples

	Sample (number/matrix)	Total Antibiotics	Premi®Test
1	CHTM	–	–
2	CHTM	+	–
3	CHBM	–	–
4	CHBM	–	–
5	CHBM	+	–
6	CHBM	+	–
7	DBM	+	–
8	DF&S	+	+

+ — positive sample; – — negative sample
CHBM — chicken breast muscle; CHTM — chicken thigh muscle; DBM — duck breast muscle; DF&S — duck fat and skin

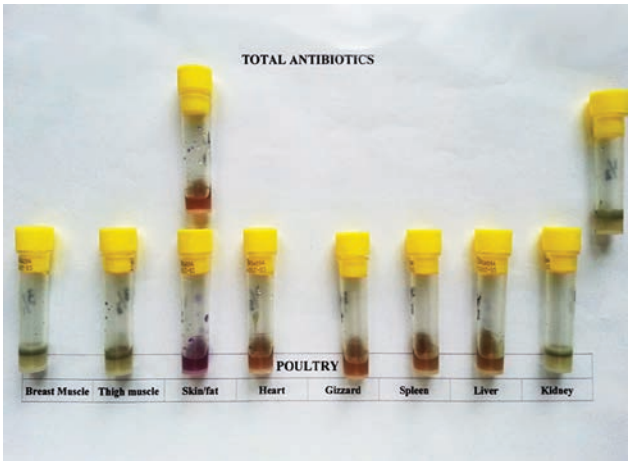

Fig. 2. Results from first batch of samples testing with Total Antibiotics

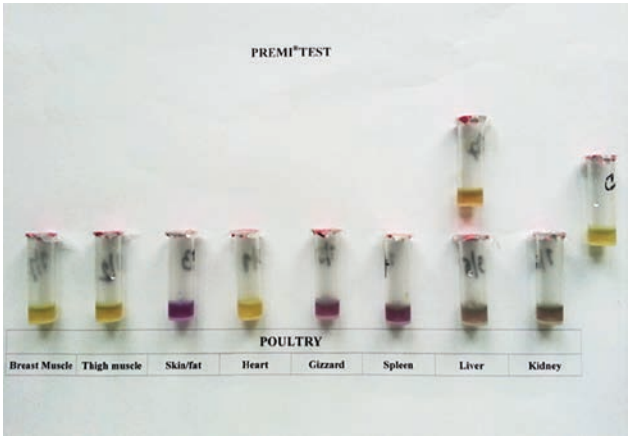

Fig. 3. Results from first batch of samples testing with Premi®Test

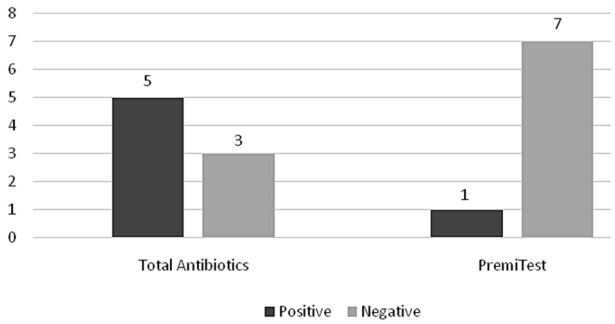

Fig. 4. Bar chart showing the differences of positive and negative results from second batch of samples

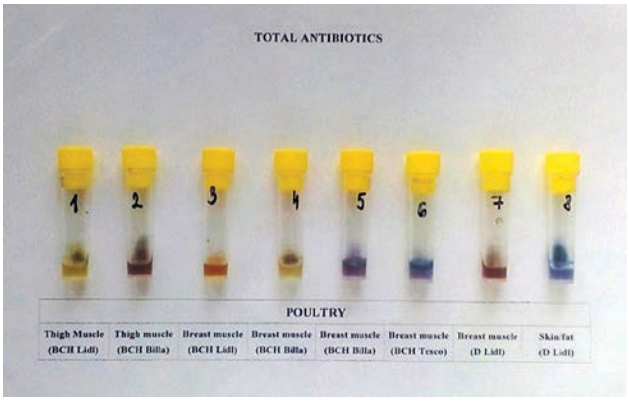

Fig. 5. Results from second batch of samples testing with Total Antibiotics

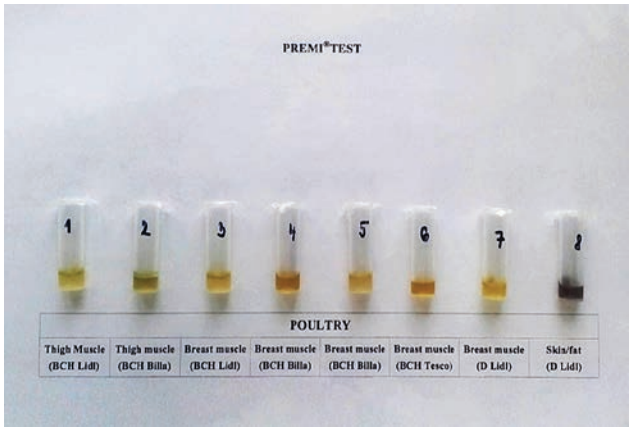

Fig. 6. Results from second batch of samples testing with Premi®Test

Figure 2 shows the resulting colour change in the agar medium for the Total Antibiotics. Figure 3 shows the resulting colour change to the agar medium for the Premi®Test.

The bar chart below in Figure 1 shows the differences of positive, negative and dubious results shown in Table 1.

The second set of results was done with samples from different Slovakian supermarkets (Lidl, Billa, Tesco). The results of the screening for the presence of coccidiostat residues in poultry meat by MIT Total Antibiotics and Premi®Test are presented in Table 2.

Figure 5 shows the resulting colour change in the agar medium for the Total Antibiotics. Figure 6 shows the resulting colour change to the agar medium for the Premi®Test.

The samples also showed that both the Total Antibiotics and the Premi®Test had both negative and positive results. All two MIT kits were functional in detecting the presence of coccidiostats in the chicken and duck tissues. Samples 1, 3, and 4 representing the chicken thigh muscle and breast muscle were negative for the presence of coccidiostats by both MITs. Sample 8, representing the duck fat and skin, was positive for the presence of coccidiostats by both MITs. The other four samples (2, 5, 6, 7) representing the chicken thigh and breast muscles and duck breast muscle were positive only for the Total Antibiotics. No dubious results were detected after screening.

The bar chart below in Figure 4 shows the differences of positive, negative and dubious results shown in Table 2.

DISCUSSION

In this study of establishing the performance of the newly developed MIT, the Total Antibiotics and a comparison with the commonly used MIT, Premi®Test was made. The comparison was done to evaluate the efficiency and sensitivity of the Total Antibiotics by using the same samples of poultry meat.

The sample preparation for both MIT was the same. The extraction buffer from the Total Antibiotics kit was used to extract the contents of the tissue and convert it into a clear supernatant solution so it could be used in both MIT. The extraction buffer must be correctly diluted with demineralized water (9 parts water and 1 part extraction buffer). If normal tap water or bottled water was used, this could contaminate the extraction buffer and serve as a "carry over" with the samples once mixed. Samples must be minced into smaller pieces so the surface area of the poultry meat is high. Ampoules/test tubes used in the kit must be handled with care and stored in the fridge at 4 °C and if the medium

agar had changed colour inside the well before use then the ampoule must be discarded. To ensure the extraction buffer and the ampoules used for both the Premi®Test and the Total Antibiotics were suitable to be used in residue detection, the kits must be kept at 4–6 °C. Manipulation of the kits were handled with care.

Each test run in the study had one negative control. The negative control was the same sample in all series of screening. This was done to ensure fair and consistent results and recognition once the colour had changed. The negative control was a breast muscle sample from a chicken confirmed free of bacteria at the Veterinary and Food Institute in Kosice. The first incubation at 37 °C of the extraction of poultry meat was put into an incubator which was stable at this set temperature. The incubator had a sealed door to ensure no escape of heat could exit and no external air draughts could enter. In addition, no preheat treatment was required with the incubator. During the second incubation at 65 °C, only 2 °C below and above was allowed to change in the thermostatic dry bath. All series of MITs performed were kept within the margins of 65 °C and therefore were constant because the thermostatic dry bath was electronically automated. Before each run of the test tubes, the thermostatic dry bath was preheated before the three hour time set. If the negative control had not changed to a clear shade of yellow then the incubation time was prolonged. In this study, the prolongation time extended for another 30 minutes for the second batch of samples. Ensuring stable temperatures were run in accordance to the kit manual so the efficiency of the MIT could be analyzed successfully and recognized as a good performance of the assay.

To avoid cross contamination of meat samples and the "carry over" of substances used to prepare samples, gloves were worn. The lancet was cleaned and disinfected with ethanol after mincing each sample. In addition, the cutting board was wiped clean in between sample preparation with ethanol.

Deposition of the supernatant was handled with care. The micropipette used was automatically set to the exact amount of supernatant needed to be collected and a disposable pipette was used in each one. This was to ensure the correct amount and prevent cross contamination of the supernatant. The supernatant was pipetted onto the top of the medium without touching the agar. Yellow tops for the Total Antibiotics were closed properly and for the Premi®Test sealing tape were applied to tightly seal the tubes. This was done to prevent any evaporation during the second incubation period.

The Total Antibiotics overall was found to be a more sensitive tests with both batches of the samples. Making comparisons with the more commonly used MIT, Premi®Test was a good indication to use as a comparison for the suitability of the Total Antibiotics.

With the first batch of samples, more than half of the samples were found to be positive in the Total Antibiotics in comparison with the Premi®Test. With the second batch of samples, more than three quarters of the samples were positive in the Total Antibiotics in comparison with the Premi®Test. The Premi®Test had most of the samples as negative, whereas the Total Antibiotics had more positive samples. This showed that the Total Antibiotics was able to detect the residues much more efficiently in comparison to the Premi®Test.

The Total Antibiotics is rapid, simple and multi-residually. The kit is suitable to screen multiple samples, and able to complete an assay run within a few hours. If consistent reading of the results was made, the Total Antibiotics overall showed a better detection to pick up the concentration of coccidiostat residues in all types of poultry tissues including different species. According to Gondova et al. [4], the Total Antibiotics has an increased sensitivity compared to other tube tests because it corresponds with the MRLs (however a confirmatory step afterwards is still essential) and there are a reduced number of false positive results as the sample preparation is simple and the extraction buffer is efficient in extracting the residue from the meat sample. With the positive samples used in our study, the second confirmatory test could be made so concentrations and individual identification of coccidiostats could be done.

CONCLUSIONS

The production of poultry meat is carried out to meet the consumer's demand for their nutritional health. The effects of coccidiosis on the poultry industry has detrimental effects on the number of poultry and the cost of rearing. The production of coccidiostats is widely used to control the spread of the protozoan parasites among the poultry flocks. However, the accumulation of coccidiostats in poultry is also toxic and if passed along the food chain for human consumption, then this can have negative impact in the quality of meat and increase health risks for consumers,

especially for those on a diet mainly of poultry meat. It is up to the Regulations set by the EU to make standards so they can be referred to during the manufacturing process.

In this study, the analysis of the presence of coccidiostats was determined because of the impact of the "carry over" of veterinary drug residues and specifically coccidiostat residues in poultry meat. The analysis of premixes, feeds and poultry meat can be done to determine the presence of coccidiostats and their residues in every sample to be tested. The analysis of the Total Antibiotics was made as it is a new and recently developed commercial MIT kit. Comparisons to the most commonly used MIT kit (Premi®Test) was made as it utilizes tube tests similar to the Total Antibiotics.

The presence or absence of coccidiostat residues in each sample involved a qualitative colour change of the agar medium. Making the analysis of the colour changes could be slightly different between different observers. Therefore, a colour scheme was devised. The results were recorded as either negatives or positive with the Total Antibiotics and positive, dubious or negative with the Premi®Test.

Poultry samples from the Slovakian farms (1st batch) and from the Slovakian supermarkets (2nd batch) provided a diverse source of samples to test. Poultry from Slovakian farms were tested by a confirmatory analysis before and many were found to be positive. However, poultry from Slovakian supermarkets should all be negative according to the EU Regulations because it is sold to the consumers.

Unfortunately, the Total Antibiotics was only able to detect the presence of a coccidiostat or of multiple coccidiostats in the sample; it was unable to determine the level of coccidiostats or if multiple coccidiostats were present in the sample. It is a rapid screening procedure which could be used, as it can still abide by the Regulations set by the EU. The test has the ability to detect the substances at their MRLs, however the confirmatory test afterwards need to be done by another laboratory test.

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