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Research Note

Hog Charm II Tetracycline Test Screening Results Compared with a Liquid Chromatography Tandem Mass Spectrometry 10-μg/kg Method

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ABSTRACT

Pork tissue samples that tested positive and negative by the Charm II tetracycline test screening method in the slaughter plant laboratory were tested with the modified AOAC International liquid chromatography tandem mass spectrometry (LC-MS-MS) method 995.09 to determine the predictive value of the screening method at detecting total tetracyclines at 10 μg/kg of tissue, in compliance with Russian import regulations. There were 218 presumptive-positive tetracycline samples of 4,195 randomly tested hogs. Of these screening test positive samples, 83% (182) were positive, >10 μg/kg by LC-MS-MS; 12.8% (28) were false violative, greater than limit of detection (LOD) but <10 μg/kg; and 4.2% (8) were not detected at the LC-MS-MS LOD. The 36 false-violative and not-detected samples represent 1% of the total samples screened. Twenty-seven of 30 randomly selected tetracycline screening negative samples tested below the LC-MS-MS LOD, and 3 samples tested <3 μg/kg chlortetracycline. Results indicate that the Charm II tetracycline test is effective at predicting hogs containing >10 μg/kg total tetracyclines in compliance with Russian import regulations.

Russian import regulations require tetracycline-free tissue <10 μg/kg, which is significantly more stringent than the Codex Alimentarius maximum residue limit of 200 μg/kg in muscle tissue (600 μg/kg in liver and 1,200 μg/kg in kidney) *(2, 7)*. AOAC International method modifications that substitute MS-MS detection for UV allow detection of all tetracycline antibiotics and their 4-epimers at LOD levels of 2 to 10 μg/kg *(1)*. Enforcement of the Russian level has stimulated import screening and has resulted in import bans. The pork industry has implemented muscle tissue screening in the slaughter environment by using the Charm II and RIDASCREEN immunoassays to predict positive and negative hog pens for import *(6)*.

The Charm II method is a simple, 30-min screening test that uses polyclonal antibodies to tetracycline, oxytetracycline, and chlortetracycline *(3, 4)*. It is also cross-reactive to the drugs' 4-epimer metabolites. The method uses dilution to predict pork tissues exceeding regulatory levels, with the most sensitive 1:2 dilution confirmation allowing detection of spiked parent drug into samples at 25 to 50 μg/kg, and the more common 1:4 dilution allowing detection of spiked parent drug at 50 to 100 μg/kg. However, because the method of detection is specific to cumulative levels of all tetracycline drugs and their epimer metabolites, incurred samples are detected well below the method spiked drug detection claims and the European Union maximum residue limit of 100 μg/kg total tetracyclines *(3, 5)*.

The true sensitivity of the method needs to be determined by analysis of incurred positive and negative screened tissue. This study reports liquid chromatography tandem mass spectrometry (LC-MS-MS) analysis results of screening positive and negative muscle to determine its effectiveness at predicting tissue containing more or less than 10 μg of total tetracyclines per kg of tissue.

MATERIALS AND METHODS

The Charm II tetracycline test screening method using the prescribed operator's manual procedure of a 1:4 dilution was implemented at the Tyson Fresh Meats pork slaughter facilities. There were 4,195 samples tested, with 218 tetracycline presumptive-positive samples sent to the Eurofins Central Analytical Laboratories (Metairie, LA) for LC-MS-MS analysis. The laboratory used an AOAC International modified method that substituted MS-MS analysis (AB SCIEX API4000 triple quadrupoles) for UV detection, and hydrophilic lipophilic balanced solid-phase extraction cartridge (Oasis HLB, part 186000115, Waters Corp., Milford, MA) methanol elution cleanup (instead of C-18 methanol oxalic acid) with LC columns (50 by 2.0 mm; Synergi 4 u Polar-RP, 80A, Phenomenex, Torrance, CA) and mobile-phase gradient MP A (0.5% formic acid and 10 mM ammonium acetate in water) and MP B (0.1% formic acid in acetonitrile) to achieve limits of detection of 10 μg/kg 4-epichlortetracycline, 10 μg/kg 4-epioxytetracycline, 10 μg/kg 4-epitetracycline, 2 μg/kg chlortetracycline, 2 μg/kg oxytetracycline, 5 μg/kg doxycycline, and 2 μg/kg

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TABLE 1. *Fourfold table analysis of LC-MS-MS samples tested*

		Charm II Test		
		Positive	Negative	Total
LC-MS-MS	Positive	210	0	210
	Negative	8	30	38
	Total	218	30	248

Index	Estimate
Sensitivity	1.0000
Specificity	0.7895
Efficiency (correct classification rate)	0.9677
Predictive value of positive test	0.9633
Predictive value of negative test	1.0000
False-positive rate	0.2105
False-negative rate	0.0000
Prevalence	0.8468
Cohen's kappa	0.8640
Positive agreement	0.9813
Negative agreement	0.8824

TABLE 2. *Fourfold table analysis of all Charm II samples tested*

		Charm II Test		
		Positive	Negative	Total
LC-MS-MS	Positive	210	0	210
	Negative	8	3,977	3,985
	Total	218	3,977	4,195

Index	Estimate
Sensitivity	1.0000
Specificity	0.9980
Efficiency (correct classification rate)	0.9981
Predictive value of positive test	0.9633
Predictive value of negative test	1.0000
False-positive rate	0.0020
False-negative rate	0.0000
Misclassification rate	0.0019
Prevalence	0.0501
Cohen's kappa	0.9803
Observed agreement	0.9981
Positive agreement	0.9813
Negative agreement	0.9990

tetracycline by using demeclocycline as a internal standard *(1)*. Thirty negative (not found) screening test samples from randomly selected hogs were also sent for analysis.

RESULTS

The prevalence of presumptive-positive samples in the population ($n = 4,195$) of samples was 5.2%. A total of 218 samples tested presumptive positive with the Charm II Test. Eighty-three percent (182 of 218) of the presumptive-positive samples contained cumulative tetracycline levels greater than 10 μg/kg by LC-MS-MS; this represents 4.3% of the total samples tested. Of the presumptive-positive samples, 3.6% (8 of 218) had no detection greater than the LOD of the LC-MS-MS method; this represents a false-positive rate of 0.2% of all the samples tested. Of the presumptive-positive samples, 12.8% (28 of 218) had nonviolative levels of tetracyclines detected, <10 μg/kg; this represents 0.7% of the total samples tested. Of the 30 negative samples analyzed by LC-MS-MS, 27 had no detection by LC-MS-MS, while 3 samples had low-level chlortetracycline detected at 3, 3, and 2 μg/kg.

DISCUSSION

The positive and negative Charm II–screened samples that were analyzed by LC-MS-MS are compared in a fourfold analysis in Table 1, where ''positive'' and ''negative'' by LC-MS-MS are defined as having a cumulative level greater than the method LOD. Cohen's kappa (κ) of 0.864 indicates an almost perfect agreement with a positive agreement (0.981) and negative agreement (0.882). This negative agreement could be understated and the false-positive rate (0.21) overstated, because the total population of negative Charm II–screened samples are not considered in this analysis. The 30 negative samples selected for LC-MS-MS demonstrate a minimum selectivity of 90%, with 95% confidence and a low false-negative (0.00) rate that is in actuality is <3%, based on number of negatives analyzed. The prevalence (0.85) and specificity (0.78) are anomalous, because the total population of screened samples are not considered in the Table 1 analysis. Still, the predictive values of the positive and negative exceeding 0.95 indicate that the screening method has value in identifying samples likely to test positive or negative by LC-MS-MS, which is reflected in the overall efficiency (0.97). On the other hand, fourfold table and chi-square analysis using all samples tested (Table 2) indicates an even stronger κ of 0.980 between presumptive-positive Charm II and LC-MS-MS levels detected above the LOD, indicating an almost perfect agreement with the positive agreement of 0.981 and the negative agreement of 0.999. Table 2 analysis is probably a truer determination of the false-positive rate (0.2%), because the entire population of samples is considered, and all the positives were analyzed by LC-MS-MS. The parameter calculations of Table 2 analysis assumes no false negatives in the samples that were screened negative by Charm II but not analyzed by LC-MS-MS, and therefore could overstate the level of method agreement, selectivity (1.0), and specificity (0.998). It is likely that the true specificity of the screening method is between the two calculated values of 0.78 to 0.998. The predictive values and the efficiency are similar in both analyses, indicating excellent identification of samples likely to be positive and negative by LC-MS-MS analysis.

The false-negative rates in both sets of analysis are 0.000, and based on number of LC-MS-MS–negative samples analyses, give 95% confidence that the false-negative rate is less than 2%. The false-positive rate varies between 0.2 and 21% in the two analyses, depending on the number of negative samples used in the calculation. These do not consider the 12.8% false-violative samples in the Charm II presumptive-positive samples, because they are drugs detected less than the 10-μg/kg control threshold. If

false-violative samples are considered a false positive, the false-positive rate would be 0.9% (36 screening positive of 4,013 negative samples). The analysis indicates that the screening method might be oversensitive relative to the 10-μg/kg LC-MS-MS, but is very effective in identifying samples with detectable levels of drug above the LC-MS-MS LOD. In screening applications, where a single or several hogs might be selected as representative of an entire pen population, this oversensitivity could be a prudent attribute of the screening method, which is used to assure that product being exported into the more restrictively regulated environment will pass the determinative method analysis by using a different random sample.

LC-MS-MS analysis of Charm II tetracycline–positive muscle when using 1:4 dilution screening indicates that the Charm II method is effective at identifying hogs containing detectable levels of tetracyclines, with 83% of presumptive-positive samples containing more than 10 μg/kg, and only 1% of the total screened samples (16.4% of the presumptive-positive samples) containing nonviolative or nondetected levels of the drug. This method is useful in testing samples to be in compliance with Russian import regulations. Spiked-sample label claims do not indicate that the method is sensitive enough to detect the 10-μg/kg level; however, the cumulative effects of total tetracyclines, tetracycline impurities, and 4-epimer metabolites detected by the screening method do indicate that the method, when used in abattoir applications, is more sensitive than spiked-sample sensitivity determination.

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