POST SCREENING METHODS FOR THE DETECTION OF BETA-LACTAM RESIDUES IN PIGS.

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Abstract

Microbial growth inhibition assays are used to detect antimicrobial residues in meat. Post-screening methods may facilitate the selection of an appropriate chemical confirmatory method. Two such methods, the Charm II radioreceptor assay and High Voltage Electrophoresis (HVE), were evaluated for the detection of incurred β-lactam residues in pigmeat. Pig liver, kidney and muscle containing penicillin G, ampicillin, amoxycillin, cephalexin or ceftiofur was produced. In kidney, Charm detected residues of all 5 compounds in all of the replicates tested (90/90). HVE detected all of the amoxycillin and ampicillin replicates (18/18), 17/18 for Pen G, 14/18 for cephalexin and 7/18 for ceftiofur. In liver, Charm detected all of the cephalexin and ceftiofur replicates but only detected 3/9 pen G replicates and none of the amoxycillin or ampicillin replicates. HVE detected amoxycillin and ampicillin in 2/9 replicates, each of the cephalosporins in 1/9 replicates and did not detect pen G. In muscle, Charm detected 0, 9, 9, 3 and 5 replicates out of 27 for each of pen G, ampicillin, amoxycillin, cephalexin and ceftiofur. The equivalent detection for HVE was 4, 13, 15, 4 and 1. It is concluded that Charm is superior to HVE for cephalosporin detection. Both assays performed better with kidney than with either liver or muscle.

Introduction

A microbiological growth inhibition assay the Four Plate Test (FPT) is used by this laboratory as a screening method for the detection of antimicrobial drug residues present in edible tissues.[1] For tissues which screen positive by FPT, precise identification and quantification of the inhibitor is necessary to determine if the concentration exceeds maximum residue limits (MRL).[2] Identification of the antimicrobial present and selection of the appropriate physiochemical confirmatory assay is based on the inhibitory zone pattern on all four plates. When more than one antimicrobial is present, identification can be difficult and secondary level screening methods such as high voltage electrophoresis (HVE)[3] or the Charm II microbial receptor assay[4] may be used. HVE enables electrophoretic separation of

antimicrobial compounds and identification by bioautography. A combination of migration distance and size and shape of the zone of inhibition allow different families of compounds, such as tetracyclines, aminoglycosides or β-lactams, to be identified. The Charm II microbial receptor assay was initially developed for milk but the method has since been modified to include tissue. Antimicrobials present in a sample extract compete for binding with a radiolabelled standard. Using a porcine model the present study compared the ability of HVE and Charm II to detect incurred residues of five of the most commonly used β-lactams in Northern Ireland agriculture.

Method and Materials

Animal management - Landrace pigs (n=18) (mean weight 68kg) were fed a drug-free pelleted 'finisher' ration for 7 days prior to treatment. The antibiotic free status of the feed was confirmed using a microbial growth inhibition assay[5] and HVE. Animals were allocated into 6 groups (n=3). Groups 1, 3 and 5 were injected intramuscularly (right gluteal) with a therapeutic dose of cephalexin (Ceporex, Schering - Plough), penicillin G (Norocillin, Norbrook laboratories) or received no treatment (control). Groups 2, 4 and 6 were treated with ceftiofur (Excenel, Pharmacia and Upjohn), amoxycillin (Clamoxyl, Pfizer) or ampicillin (Amfipen, Mycofarm). Animals were exsanguinated following captive bolt stunning 12 hours post injection and the diaphragm, gluteal (left and right), liver and kidney (both) were collected at post-mortem. Testing was completed within 11 days and before treatment on another group commenced. All tissues were analysed in triplicate, processed within 4 hours of collection and subjected to no more than one freeze-thaw cycle.

Charm II Test – (Charm Sciences Inc. Malden MA) A tissue samples (5g) was extracted, centrifuged and the duplicate supernatants pooled. Two 2 cm^3 sub-samples were split from the pooled extract, incubated and scintillation counted. Positive control points (A) were established as per kit instructions for each tissue by spiking the supplied multi-antimicrobial standard (containing pen G) into 6 drug-free tissue replicates at 0.05 μg g^{-1} (mean distintegrations per minute (dpm) + 20%). Control points (B) were determined for amoxycillin, ampicillin, cephalexin, penicillin G (all sigma) and ceftiofur (Pharmacia) by spiking drug-free kidney and diaphragm at a concentration of 0.05 μg g^{-1} (mean dpm + 20%).

The control points (C) were recalculated after changes made to the manufacturer's protocol, by subtracting 30% from the negative control average.

High Voltage Electrophoresis (HVE) - Each tissue was analysed on 1% (w/v) agarose type 1 (Sigma) and 1% (w/v) agar purified (Oxoid) at pH 6.0 and pH 8.0, poured to give a uniform media depth of 2 mm. Tissue cubes (2 cm^3) were placed directly onto the agar surface and allowed to diffuse for 1 hr. Standard disks (penicillin G 0.03 iu/disk and oxytetracycline 0.5 μg/disk - Mast Diagnostics), were included on each assay 15 min before electrophoresis commenced. Tissue was removed after diffusion and a constant voltage of 1500 v was applied for 1.5 hrs using the appropriate electrolyte buffer. The support gel was overlayed with 150 cm^3 antibiotic medium No. 1 (Difco) pH 6.6 inoculated with *Bacillus subtilis* (Difco) 10^6 organisms cm^3. After incubation overnight at 30°C, zones of inhibition were measured from the start line to the end of the zone farthest from the line.

Results

All replicate tissues from the control animals were negative by both methods. The results for medicated animals are shown in table 1.

Table 1. Comparison of Charm and HVE methods: number positive %.

Drug	Liver		Kidney		Muscle	
	Charm	HVE	Charm	HVE	Charm	HVE
Penicillin G	3 (33.05)	0	18 (100%)	17 (94.4%)	3 (11.1%)	4 (14.8%)
Ampicillin	0	2 (22.2%)	18 (100%)	18 (100%)	9 (33.0%)	13 (48.1%)
Amoxycillin	0	2 (22.2%)	18 (100%)	18 (100%)	9 (33.0%)	15 (55.6%)
Cephalexin	9 (100%)	1 (11.1%)	18 (100%)	14 (77.8%)	3 (11.1%)	4 (14.8%)
Ceftiofur	9 (100%)	1 (11.1%)	18 (100%)	7 (38.7%)	5 (18.5%)	1 (3.7%)
Total Rep	45	45	90	90	135	135
Total Pos	21 (46.6%)	6 (13.3%)	90 (100%)	74 (82.2%)	29 (21.5%)	37 (27.4%)

The Charm assay detected β-lactam residues in all kidneys tested (90/90: 100%). Of these 82.2% were detected by HVE. Amoxycillin and ampicillin were not detected by Charm in the liver samples. HVE had a detection rate of 22% for both drugs. Charm detected cephalexin

and ceftiofur in all liver replicates. Cephalexin and penicillin G were not detected in any muscle sample by Charm but were detected in 6/54 samples by HVE. Ceftiofur residues were detected in 34/54 tissue replicates by Charm whereas HVE detected 8/54 ($p<0.001$). The control points (A) determined using the supplied standard varied between the different tissue types. Variation was also obtained when the control points (B) were calculated using the individual β-lactams spiked at 0.05 μg g^{-1} in both kidney and diaphragm (Table 2).

Table 2. Positive control points expressed as a percentage of the negative control counts.

Control Point	Standard (Spiked in drug-free tissue)	Kidney	Liver	Diaphragm	Gluteal
A	Multi-standard +20%	33.3%	45.8%	24.7%	29.6%
B	Penicillin G +20%	39.9%	-	24.7%	-
	Amoxycillin +20%	77.6%	-	56.3%	-
	Ampicillin +20%	70.4%	-	47.8%	-
	Cephalhexin +20%	72.4%	-	74.0%	-
	Ceftiofur +20%	83.1%	-	77.7%	-
C*	Negative control - 30%	68.4%	67.8%	72.4%	66.5%

* Determination in kit buffer, no tissue added.

Determination of the control point using the negative control minus 30% resulted in an increase (from 8% to 76%) in the number of muscle samples above the positive threshold.

Discussion

Determination of the positive control point for the Charm assay will affect the number of positive samples obtained. Using the negative control counts minus 30% eliminated matrix interference but increased the number of positive muscle samples. In regulatory laboratories this increase in samples requiring chemical confirmation could prove unacceptable. Control points obtained using the supplied multi-antimicrobial standard were compared with individual spiked analytical standards. Ceftiofur and cephalexin demonstrated a lower reduction in the counts required for a positive result. Similar findings on the sensitivity of the assay for the individual β-lactams have been previously reported[6]. Calculation of the control point from the negative control raises the positive threshold to that obtained by spiking ceftiofur or cephalexin at 0.05μg cm^{-3} thereby increasing the number of other β-lactams,

particularly pen G, amoxycillin and ampicillin which would be referred for chemical confirmation. The control point set by spiking with pen G only would result in residues of ceftiofur and cephalexin in excess of the MRL remaining below the positive threshold of the assay.

Despite the differing sensitivities for the detection of the cephalosporins the Charm assay performed well for the detection of cephalexin and ceftiofur residues. Structural differences of the cephalosporins compared to penicillins confer resistance to the bacterial enzyme β-lactamase resulting in increased therapeutic effectiveness against gram-negative bacteria but decreased activity against gram-positive organisms. This factor may be considered in selecting a suitable microbial inhibition assay for β-lactams. It is concluded that Charm is superior to HVE for cephalosporin detection. Both assays performed better with kidney than with either liver or muscle.

References

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