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Determination of the limit of detection of penicillin G residues in poultry meat using a low cost microbiological method

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Abstract: Broiler chicken is often grown actively with antibiotics to attain maximum weight within a short period of time. The uncontrolled and unlimited use of these antibiotics may however lead to the accumulation of undesirable residues in the animals treated and their products. In the Kenyan poultry industry there are no inexpensive and easy to perform antibiotic residues screening methods with the capability for a high sample throughput, which can be used to rapidly sift large numbers of samples for suspect or potential non-compliant results. The aim of this study was to determine the limits of detection (LODs) of penicillin G (PEN G) in chicken tissues using a low cost microbiological method. Microbiological detection was achieved by agar well diffusion using *Bacillus cereus* and *Bacillus subtilis*. PEN G was detected below the maximum residue limits (MRLs) of 50 ng/g in both liver and kidney with the LODs being 2 times below the MRLs on these plates. It was concluded that both *B. cereus* plate at pH 7 and *B. subtilis* plate at pH 7 could be effectively used for routine screening for PEN G residues.

Keywords: Limit of detection, *Bacillus subtilis, Bacillus cereus*, penicillin G, chicken

Introduction

A wide range of antibiotics are used in poultry not only to treat disease but also to maintain health, promote growth and enhance feed efficiency (Gaudin *et al*., 2004). Antibiotic usage has facilitated the efficient production of poultry, allowing the consumer to purchase, at a reasonable cost, high quality meat and eggs (Donoghue, 2003). In particular, broiler chicken are often grown actively with antibiotics to attain maximum weight within a short period of time (Nonga *et al*., 2009).

In Kenya, penicillins are among the most widely used group of antibiotics (Mitema *et al.*, 2001). The uncontrolled and unlimited use of these antibiotics may however lead to the accumulation of undesirable residues in the animals treated and their products. Drug residues in the edible portions of the animal usually occur because the withdrawal period has not been observed. Benzylpenicillin (penicillin G) is widely used in Kenya to treat specific infections and also as a prophylactic. It is administered as one or more of a variety of salts which are used to prolong the activity of the drug. These can be the soluble sodium or potassium salts or the longer acting procaine and benzathine salts. Concern has been expressed over the possible presence of residues of this drug in foods of animal origin due to the occurrence of penicillin hypersensitivity in humans and development and transfer of antibiotic resistance between animals and man (Lee *et al*., 2001; Mitema *et al*., 2001; Mccracken *et al*., 2005).

To protect the public against possible health risks caused by such hazards, regulations regarding veterinary use of drugs including withholding periods after antibiotics therapy and tolerance levels have been formulated (WHO/FAO, 1998; WHO/FAO, 1999) and followed in developed countries (Lee *et al*., 2001; Donoghue, 2003). However, such regulations are not usually adhered to in countries where routine monitoring of drug residues in food is not done (Shitandi and Sternesjo, 2001).

In the Kenyan poultry industry there is lack of inexpensive and easy to perform antibiotic residues screening methods with the capability for a high sample throughput and that can be used to rapidly sift large numbers of samples for suspect or potential non-compliant results. This study evaluated the performance of *Bacillus cereus* and *Bacillus subtilis* in the detection of penicillin G in poultry meat and established a screening test, materials and conditions most suited for use in testing this antibiotic.

Materials and Methods

Bacterial suspensions

Bacillus cereus (Difco Laboratories, Detroit, Michigan, USA) and *Bacillus subtilis* BGA (E. Merck, Darmstadt, Federal Republic of Germany) were used as spore suspension. Three batches of

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Mueller Hinton agar at pH 6, 7 and 7.3 were prepared for use. Mueller Hinton agar was prepared as per the manufacturer's instruction and the pH adjusted appropriately using 0.1M HCl or 0.1M NaOH. After autoclaving at a pressure of pressure of 15 psi at a temperature of 121°C for 15 minutes, it was cooled to 45-55°C. The spore suspension of *B. cereus* and *B. subtilis* were inoculated into the molten agar and mixed thoroughly to ensure uniform distribution. Approximately 10^5 spores per ml were determined by a spectrophotometer and inoculated. Sterile Petri plates (diameter 90 mm) were filled with 15 ml of the inoculated media and incubated at 30°C for 18-24 hours to determine the effect of pH of bacterial growth.

Test plates

Six different plates were used for antibiotic detection: plate I and VI, Mueller Hinton agar (MHA) pH 6, plate II and V, MHA pH 7 and plate III and VI MHA pH 7.3. Plates I-III were seeded with *B. subtilis* while plates VI-VI were seeded with *B. cereus*. The test plates were coded such that the first two letters represented the test organism, the third letter the antibiotic, the fourth letter poultry organ to be used while the fifth number represented the pH, e.g. BCPL7 representing plate II with PEN G spiked chicken liver supernatants. The sterile Petri dishes (diameter 90 mm) were filled with 15 ml of the prepared and seeded media. After solidification, the media was used immediately or stored at 2-5°C for a maximum of 5 days.

Seven wells with diameter of 10 mm were punched into the agar layer and filled with 100 µl of the artificially spiked kidney or liver supernatants at different concentration of the antibiotic, an antibiotic-free negative control and a positive reference standard fortified with the established MRL for PEN G. The wells were at a distance of at least 30 mm from each other. After a pre-diffusion period of about 1 hour, at room temperature, the plates were incubated at 30°C for 13-18 hours. The samples and the standards were run in triplicates. The diameters of the zones of inhibition, from the edges of the wells, were measured and an inhibition zone of ≥2 mm was considered as positive. These diameters were measured minus the diameter of the punch well which was 10 mm (Omija *et al*., 1994; Aila *et al*., 2009; Nonga *et al*., 2009).

Stock antibiotic solutions

Stock solution of 0.05 g/l of PEN G was prepared in distilled water. *In vitro* sensitivity was carried out by serial dilutions of the stock solution. Dilutions were made in distilled water and/or kidney/liver supernatants. PEN G was obtained from Sigma Chemical Company (St. Louis, MO, USA).

Control samples

Fifty µg/l of PEN G, which is MRL concentration for liver and kidney, of PEN G were used as positive control in the search for the limits of detection of each drug in the two tissues. Liver or kidney tissues that were free of any antimicrobial drugs were used as the negative control.

Preparation of fortified liver and kidney

One hundred frozen samples consisting of 50 livers and 50 kidneys from chicken that had not been treated with antibiotics were homogenized with distilled water at a ratio of 1:2 (tissue: distilled water) and the homogenates centrifuged for 5 min at 3000 g to eliminate tissue debris. Supernatants from these extracts were used to dilute stock solutions to produce working solutions standards of 0.0125 - 0.083 µg/ml of PEN G. These are dilutions of the established MRLs of PEN G for the two tissues (Aila *et al*., 2009).

Determination of limits of detection

To verify the detection limits, the spiked supernatants were added to the holes on the agar such that each well containing a replicate of each drug concentration received 100 µl of test solution. Each concentration was analyzed in three replicates. The plates were allowed to stand for 1 h to allow the supernatant to diffuse into the media. They were then incubated at 30°C for 13-18 h. The zones of inhibition were then measured using a caliper. A regression line of log concentration (µg/ml/well) vs inhibition zone diameters was used to calculate the LOD (Koenen *et al*., 1995).

Data analysis

Experimental treatments were arranged in a randomized complete block design. The data was analyzed using GraphPad Prism 5 statistical software (GraphPad Software, Inc. 2009). Two way analysis of variance (ANOVA) was applied to test for the significant differences in mean inhibition zones among the pH and concentrations combinations. Bonferroni posttests were carried out to compare replicate means. Comparisons were considered significantly different at p values <0.001. Regression analysis of concentration and the inhibition zone diameters was used to determine the LODs.

Results

Poultry liver and kidney samples with known concentrations of PEN G were analysed and the influence of the type of organ and pH on the sensitivity of the organism was also examined. Concentrations of PEN G of 0.0125, 0.0167, 0.025, 0.05, 0.0625 and 0.083 µg/ml in liver and kidney were tested. Positive results showed inhibition activities around the well while negative results showed no inhibition. Negative controls showed no inhibition of test organism. The zones of inhibition are presented as mean ±SE in tables 1 and 2.

Zone diameters decreased significantly ($p<0.05$) when *B. subtilis* and *B. cereus* were used to detect PEN G at decreasing pH from 7.3, 7.0 to 6. However *B. cereus* plate at pH 7 produced largest zones (Table 1 and 2). Bonferroni posttests showed that there was significant ($p<0.05$) difference in the inhibition zones measured in BSPL6 and BSPL7, BSPL6 and BSPL7.3, BSPL7 and BSPL7.3, BCPL6 and BCPL7, BCPL6 and BCPL7.3, BCPL7 and BCPL7.3, BSPK6 and BSPK7, BSPK6 and BSPK7.3, BSPK7 and BSPK7.3, BCPK6 and BCPK7, BCPK6 and BCPK7.3 and BCPK7 and BCPK7.3. There was no significant ($p>0.05$) difference in mean inhibition zones in the plates compared at 0 µg/ml concentration.

Table 1. Average inhibition zones (mean±SE) obtained from the analysis of chicken kidney spiked with different levels of PEN G onto Mueller Hinton agar at different pH

PEN G concentration (µg/ml)	Mean zones of inhibition (mm)					
	B. subtilis			*B. cereus*		
	pH 6.0	pH 7.0	pH 7.3	pH 6.0	pH 7.0	pH 7.3
0	0	0	0	0	0	0
0.0125	0	12.9±0.1	14.2±0.1	0	4.6±0.1	2.5±0.1
0.0167	0	13.8±0.1	15.3±0.1	0	5.8±0.1	3.7±0.1
0.025	0.6±0.1	14.8±0.1	16.8±0.1	0	6.8±0.1	5.4±0.1
0.05[a]	3.1±0.1	15.5±0.1	17.6±0.1	0	7.7±0.1	6.1±0.2
0.0625	4.7±0.1	16.7±0.1	18.6±0.1	0.7±0.0	8.2±0.1	7.3±0.1
0.083	6.3±0.1	17.6±0.1	19.8±0.1	0.7±0.0	8.9±0.1	9.0±0.1

[a]MRL concentration for PEN G in chicken kidney; SE-standard error

There was significant difference in zone diameters produced by spiked kidney and liver supernatants on *B. cereus* and *B. subtilis* plates. Mean inhibition zones differed significantly ($p<0.05$) between BSPK7 and BCPK7, BSPK7.3 and BCPK7.3, BSPL7 and BCPL7 and BSPL7.3 and BCPL7.3. In BSPK6 and BCPK6 and BSPL6 and BCPL6, there were no significant ($p>0.05$) differences in measured zones at 0.0125 µg/ml and 0.0167 µg/ml although zones differed significantly ($p<0.05$) at other concentrations. There was no significant ($p>0.05$) difference in mean inhibition zones in the plates compared at 0 µg/ml concentration. These are results of Bonferroni posttests (Table 1 and 2).

When *B. subtilis* was used, there was no significant ($p>0.05$) difference in the detection of PEN G in both kidney and liver samples at the different pH values. However, Bonferroni posttests showed 0.025 µg/ml, 0.05 µg/ml, 0.0625 µg/ml and 0.083 µg/ml produced inhibition zones that were significantly ($p<0.05$) different when BSPL6 and BSPK6 were compared. When *B. cereus* was used, there was no significant ($p>0.05$) difference in mean zone diameters produced by spiked kidney and liver supernatants although at 0.0625 µg/ml and 0.083 µg/ml zones diameters were significantly different between BCPL6 and BCPK6 and between BCPL7 and BCPK7 ($p<0.05$). At 0.025 µg/ml and 0.05 µg/ml, the zones were slightly different ($p<0.05$) between BCPL7.3 and BCPK7.3 and between BCPL7 and BCPK7 respectively. The 0.0125 µg/ml concentration produced inhibition zones that differed significantly ($p<0.05$) when BCPL7 and BCPK7 were compared.

Table 2. Average inhibition zones (mean±SE) obtained from the analysis of chicken liver spiked with different levels of PEN G onto Mueller Hinton agar at different pH

PEN G concentration (µg/ml)	Mean zones of inhibition (mm)					
	B. subtilis			*B. cereus*		
	pH 6.0	pH 7.0	pH 7.3	pH 6.0	pH 7.0	pH 7.3
0	0	0	0	0	0	0
0.0125	0	12.9±0.1	14.1±0.1	0	5.2±0.1	2.4±0.1
0.0167	0	13.6±0.1	15.4±0.1	0	6.1±0.1	3.5±0.1
0.025	1.6±0.1	14.7±0.1	16.8±0.1	0	7.1±0.1	5.1±0.2
0.05[a]	4.6±0.1	15.6±0.1	17.6±0.1	0	8.0±0.1	5.9±0.2
0.0625	6.6±0.2	16.5±0.1	18.5±0.1	1.2±0.0	9.1±0.1	7.3±0.2
0.083	8.7±0.1	17.5±0.1	19.7±0.1	1.5±0.0	10.4±0.1	8.8±0.2

[a]MRL concentration for PEN G in chicken liver; SE-standard error

Bacillus cereus test organism was able to detect PEN G at the legally acceptable levels at pH 7 and 7.3. However the LODs were above the MRLs at pH 6. The LODs were less than twice below the MRLs on a *B. subtilis* plate (Table 3). Figures 1 and 2 show regression curves for PEN G in plates I-IV that were used to calculate respective LODs.

Table 3. Limits of detection for PEN G at different pH

Tissue	Antibiotic	[a]MRL (ng/g)	Limits of detection (ng/ml)					
			B. cereus			*B. subtilis*		
			pH 6	pH 7	pH 7.3	pH 6	pH 7	pH 7.3
Liver	PEN G	50	126.90[b]	3.7	11.2	24.0	0.1	0.1
Kidney	PEN G	50	662.2[b]	3.2	10.6	29.3	0.1	0.1

[a]Chicken tissues MRLs, [b]LODs above MRLs

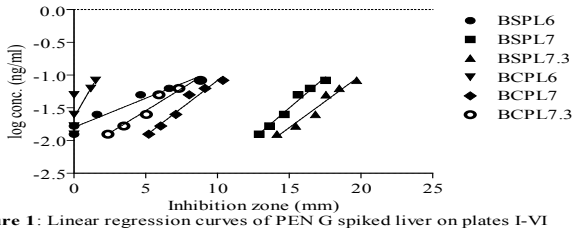

Figure 1: Linear regression curves of PEN G spiked liver on plates I-VI

R^2: BSPL6=0.9548, $p<0.0008$; BSPL7=0.9774, $p<0.0002$; BSPL7.3=0.9579, $p<0.0007$, BCPL6=0.6284, $p<0.0600$; BCPL7=0.9654, $p<0.0005$; BCPL7.3=0.9592, $p<0.0006$. The curves were used to calculate LOD of PEN G in chicken liver on plates I-VI.

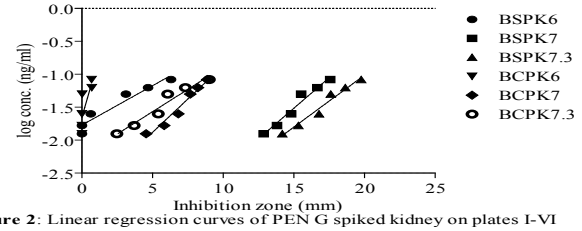

Figure 2: Linear regression curves of PEN G spiked kidney on plates I-VI

R^2: BSPK6=0.9275, $p<0.0020$; BSPK7=0.9632, $p<0.0005$; BSPK7.3=0.9685, $p<0.0004$; BCPK6=0.6292, $p<0.0597$, BCPK7=0.9647, $p<0.0005$; BCPK7.3=0.9462, $p<0.0011$. The curves were used to calculate LOD of PEN G in chicken kidney on plates I-VI.

Discussion

Microbiological inhibition tests are used for preliminary screening of foods for antibiotics residues so that samples that probably contain one or more analytes and should be investigated further with more sophisticated immunochemical and/ or chromatographic methods could be selected. Screening tests should be simple, cheap and fast (Okerman *et al*., 1998). A plate test consists of a layer of inoculated agar, with samples applied on top of the layer or in wells in the agar (Okerman *et al.*, 1998; Pikkemaat *et al*., 2009). Screening methods are based on the use of sensitive bacteria and *B. cereus* and *B. subtilis* are normally incorporated (Koenen *et al*., 1995; Pikkemaat *et al*., 2008). Bacterial growth turns the agar into an opaque layer, which yields a clear growth-inhibited area around the sample if it contains antimicrobial substances. The presence of antimicrobial residues in foods is of particular concern in countries where legislation regarding MRLs for marketed products is lacking and violation of withdrawal periods frequently occurs (Shitandi and Sternesjo, 2001).

The pH of the test medium is an important factor influencing the detection limits of most antibiotics. The pH influences the permeability of bacteria to antibiotics, the stability and activity of enzymes which inactivate antibiotics and the stability and kinetics of certain antibiotics whereas changes in ionization of the antibiotic may be the crucial factor (Corkill *et al*., 1994; Lakaye *et al*., 2002). The activity of PEN G decreased on a *B. subtilis* plate when pH was decreased. The results were not consistent for PEN G on *B. cereus* seeded plates where the zones of inhibition were larger in plate V than at both plates IV and VI. This may be attributed to the fact that both *B. cereus* and *B. subtilis* have penicillinase whose activity is enhanced at low pH. It has also been reported that the mechanisms of the effect of pH on antimicrobial activity are inconsistent from drug to drug and also based on the sensitivity or resistance of the test microorganisms to various antibiotics (Amsterdam, 1996; Karraouan *et al*., 2009). PEN G was detected below the MRLs on plates I, II, III, V and VI. The LODs were lowest on plates II, III, and V in both kidney and liver. The LOD was above the MRL on plate IV in both liver and kidney being highest in kidney fluid. Plate III gave the largest zones of inhibition for PEN G for both kidney and liver and the LODs were also optimal, meaning that this plate was the most sensitive to residues of PEN G. However these results were at variance with those reported by Popelka *et al*., 2005). This difference can be attributed to different sample preparation and matrix effects.

Incurred samples obtained from routine monitoring programs have been used to evaluate the performance of a method (Myllyniemi *et al*., 2001; Okerman *et al*., 2004). However, such an approach is very much limited by the availability of these samples. It is also impossible to produce incurred samples from different animal species with a specified concentration of specific residue. This has resulted in validation of most microbiological methods using antibiotic standard solutions and hence potential matrix effects are neglected. It is generally expected that the presence of matrix components has a negative effect on the sensitivity of an assay (Okerman *et al.*, 2004; Pikkemaat *et al*., 2007). However Myllyniemi *et al*. (2001) showed that incurred kidney samples containing penicillin G (PEN G) or oxytetracycline (OTC) at their MRLs caused wider inhibition zones compared to standard solutions of corresponding concentration. Fortifying extracted matrix fluid with the analyte or analytes at the required concentration may give more realistic results although tissue binding is not taken into account (Cantwell and O'Keeffe, 2006; Pikkemaat *et al*., 2009). In this study chicken liver and kidney were spiked with known concentrations of PEN G.

Kidney and liver are commonly used for screening slaughter animals for the presence of antibiotic residues (Hassan *et al*., 2007; Pikkemaat *et al*., 2008; Pikkemaat *et al*., 2009). Penicillins are rapidly absorbed from the gastrointestinal tract of chicken due to their high tissue penetrating ability (Alhendi *et al*., 2000) and the maximum mean concentrations are found in the kidney followed by the liver. This is due to the fact that these organs are involved in storage, metabolism and the elimination of the drug (Anadon *et al*., 1994; Alhendi *et al*., 2000). Although chicken liver and kidney have equal MRLs for PEN G, inhibition zones produced by kidney and liver spiked with PEN G were at variance. However, the LODs were generally comparable in both liver and kidney. The presence of naturally occurring growth inhibiting compounds in kidney causes non-specific inhibition (Pikkemaat *et al.*, 2008). This may have led to the variability in sizes of inhibition zones produced by PEN G spiked kidney and liver fluids. However, some high molecular weight naturally occurring growth inhibiting compounds in kidney, such as lysozymes, were removed by the centrifugation step.

Screening tests are designed to be easy, inexpensive and time efficient. The results from this study reveal that PEN G residues can be detected microbiologically, in poultry tissues by both *B. subtilis*

and *B. cereus*, inexpensively and with ease. The materials used in this method are readily available in most microbiology laboratories while the procedure is simple and understandable. The method is also fast as the residues can be detected within 13 hours. Plate II, III and V are efficient for assay of PEN G residues in chicken liver or kidney. These plates gave optimal inhibition zones and LODs for this antibiotic.

Conclusion

Penicillin G can reliably be detected below the MRLs by *B. cereus* and *B. subtilis* although detection is influenced by pH of the growth media, concentration of the antibiotic and type of organ. Analysis of chicken liver using one of the plates is sufficient for the detection of antibiotic residues in chicken meat. This approach offers the advantage of using unspecialized microbiology facilities in order to perform the analysis.

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