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Validation of Commercial Test Kit for Microbiological Screening of Antimicrobials in Chicken Eggs

[1]I.O. Fagbamila, [1]S.S. Ngulukun, [1]S.S. Ardzard, [2]N. Sati, [1]O.T. Ajayi, [3]P.I. Ankeli, [1]Y. Akalusi, [1]L. Okeke, [1]L. Ikpa, [1]M.O. Odugbo and [1]M. Muhammad
[1]Bacterial Research Division, [2]Poultry Division, [3]Bacterial Vaccine Production Division, National Veterinary Research Institute, Vom, Nigeria

Corresponding Author: I.O. Fagbamila, Bacterial Research Division, National Veterinary Research Institute, Vom, Nigeria Tel: +234-803-366-9580, +234-709-626-3441

ABSTRACT

The present study was conducted to evaluate a commercial test kit for the qualitative screening of eggs submitted to our laboratory as a first step for the testing of drug residues. Forty hens at the point of lay were kept for 4 weeks without administering any antibiotic. Eggs were then collected and tested for the absence of drug residues. Antibiotic-free birds were then divided into two groups. One group was administered tetracycline for three days and the other group left as control. Eggs were collected daily for two weeks from both groups and tested for tetracycline residues using the disc diffusion method and a commercial test kit. Both methods detected the presence of drug residues in test eggs with the commercial test kit able to detect residues over a longer period up to day 10. The study indicated that the commercial test kit could be used for the detection of drug residues particularly when the aim is to screen large numbers of samples rapidly. However, it is not sensitive enough to detect drug residues at lower concentrations and may not be suitable for confirmatory testing.

Key words: Residue, qualitative screening, tetracycline

INTRODUCTION

The indiscriminate use of antimicrobials either as preservative or at subtherapeutic levels for the control of animal diseases has been a problem in many countries (Salehzadeh *et al*., 2006, 2007; Al-Nazawi and Homeida, 2005; Erdogrul and Cakiroglu, 2002). There are ethical and professional concerns as to the safety of the public from drug-residue related diseases and the development of antibiotic resistant pathogenic microorganisms (Khaniki, 2007; Al-Nazawi, 2006; Hassan *et al*., 2007). The occurrence of antimicrobial residues is a serious poultry farm problem in Nigeria due to non-compliance with guidelines and recommendations on antimicrobial use and the lack of a drug monitoring programme leading to the build up of antimicrobial residues in eggs. Confirming the presence of drug residues in animals and animal products is expensive, time consuming and laborious. Microbiological screening methods are seen as a suitable and simple for the detection of drug residues when the aim is to screen large numbers of samples rapidly and at relatively low cost (Pikkemaat *et al*., 2007).

Qualitative tests are capable of detecting a broad range of antimicrobials and reducing the number of samples for confirmatory analysis (Shahid *et al*., 2007; Biswas *et al*., 2010). Screening tests are used to select samples that require further quantitative analysis with methods such as immuno-enzyme-assays, high voltage electrophoresis, Charm II receptor assay and/or chromatography (Jackman, 1993; Smither and Vaughan, 1978; Charm and Chi, 1988). Based on bacterial inhibition, several methods can be used for screening (Korsrud *et al*., 1998). These include the Calf Antibiotic Sulfa Test (CAST), two Microbial Inhibition Tests; one using swab stick, Swab Tests on Premises (STOP) and the other using filter paper discs as simple applicator (Korsrud and MacNeil, 1987; Korsrud *et al*., 1998). Others include Combined Plate Microbial Assay (CPMA), Eclipse '100ov'® method, Plate Count Agar, Difco and the Delvotest SP method (Ferrini *et al*., 2006; Yamaki *et al*., 2006; Pikkemaat *et al*., 2007; AOAC, 2004). The limit of detection of a microbiological test for a given antimicrobial depends primarily on the innate sensitivity of the test bacterium. The concentration of the test bacterium needs to be standardized as this relates to the size of the zone of inhibition of the growth of the test organism (TerHune and Upson, 1989). A moderate concentration of the test bacteria improves the sensitivity of the test drug producing a well-defined zone of inhibition (Renard *et al*., 1992). Temperature, duration of incubation and the content of culture media are also an important factor. Other factors such as centrifugation and the use of more than one disc to inoculate each sample have also been reported as affecting the size of the zone of inhibition of the growth of the test organism (Brady and Katz, 1987, 1989). The Premi®Test kit has been used to screen for drug residues in tissues (Stead *et al*., 2007) but not in poultry eggs. The disc diffusion method has been applied for antimicrobial screening in eggs (Kabir *et al*., 2004). This study was conducted to validate the Premi®Test kit in the screening of commercial eggs for drug residues and to compare its sensitivity to the disc diffusion method.

MATERIALS AND METHODS

The experiment was conducted within a four month period (October, 2009 to February, 2010) at the Bacterial Research Laboratory, National Veterinary Research Institute, Vom, Nigeria. Forty layers at the point of lay and with a history of not having been administered any antibiotic in the previous 6 weeks were purchased from a commercial farm in Jos, north central Nigeria. The birds were then kept for another 4 weeks without administering any antibiotic. Eggs were then collected after one month to confirm the absence of tetracycline residue using the High Performance Liquid Chromatography (HPLC) method similar to that of (Senyuva *et al*., 2000). Briefly, standard oxytetracycline hydrochloride (Sigma-Aldrich, Germany) was used for the analysis. Isocratic separation was achieved using a hypersil BDS C_{18} (5 mm, 250×4 mm) column. The mobile phase consisting of distilled water (pH = 2.1 with H_2SO_4). Acetonitrile, 85:15 (v/v), was pumped at a flow rate of 1.5 mL min^{-1}. The extraction was carried out by adding 0.1 g of citric acid to 2 mL of homogenized egg. To the mixture, 1 mL of nitric acid (30%), 4 mL of methanol and 1 mL deionized water were added, respectively. This was then mixed thoroughly using a vortex mixer and kept in an ultrasonic bath for 15 min and then centrifuged for 10 min at 5300 rpm. It was then filter through a 0.45 μm nylon filter and 20 μL of the solution was injected into HPLC for analysis. The chromatography was performed at 24°C and the analyses detected at 360 nm using a setting of 0.01 Absorbance Units Full Scale (AUFS). Having certified the eggs free of oxytetracycline residues these were then used for the experiment.

The antibiotic-free birds were divided into two groups; the first group was administered oxytetracycline hydrochloride (Pfizer, Nigeria) for three days at the recommended dose of

137.5 μg L^{-1} while the control group was left untreated. Eggs were collected daily from both groups for two weeks and examined for tetracycline residues using both disc diffusion and the Premi®Test kit. The samples were also tested with HPLC method for confirmation.

Test for tetracycline residues using disc diffusion method: An 18 h culture of *Bacillus cereus* in 10 mL nutrient broth (Oxoid, UK) was inoculated onto Mueller Hinton (MH) agar plates. This was achieved by dipping sterile cotton swab sticks into the suspension of the test organism until it was saturated with the organism. The plates were then gently and thoroughly seeded to achieve a lawn of confluent growth. Inoculated plates were then allowed to dry for 5-10 min. With the use of sterile forceps, a paper disc (0.6 cm in diameter and 0.2 cm thick) was dipped into the homogenized egg and allowed to soak. Excess egg was drained off before gently placing the disc on the surface of the seeded MH plate. The disc was firmly but gently pressed onto the surface of the agar to allow for proper diffusion around the disc. The plates were then labelled accordingly and incubated at 37°C for 18-24 h. After incubation, plates were viewed for the presence or absence of zones of inhibition of the test organism. With the use of a tape rule, the diameters of the zones of inhibition were measured in millimeters from one edge of the zone to the other across the disc. The difference between the diameter of the zone of inhibition and that of the disc was calculated. Any disc with a difference of 1 mm or more was considered positive for the presence of antimicrobial substance.

Test for tetracycline residues using Premi®Test kit method: The Premi®Test kit is a commercially available agar diffusion test based on the inhibition of growth of *Bacillus stearothermophilus*. The agar contains a standardized number of bacterial spores, selected nutrients and the pH indicator (Bromocresol purple). After adding 100 μL of homogenized egg directly onto the agar, it was then incubated first at 80°C for 10 min and later at 64°C for 3 h. During incubation, microbial metabolism will result in a change in pH and hence a change in color from purple to yellow. By contrast, if the sample contains sufficiently high concentrations of a drug residue, the colour remains purple.

RESULTS AND DISCUSSION

All test eggs were positive for tetracycline residue with the HPLC method. The mean recovery of the HPLC method was found to be between 6-100%. All control samples were negative. The disc diffusion method had a lower daily sensitivity than the Premi®Test kit. The Premi®Test kit had 50% sensitivity at day 6 while the disc diffusion method had 10% sensitivity at the same day. Both methods validated the screening test for drug residues with the commercial test kit able to detect residues for a longer period than the disc diffusion method. However, the detection of residue did not exceed 10 day post tetracycline administration in both methods (Table 1). The number of samples positive for drug residue increased up to the third day before gradually declining in both methods. The highest detection was on day 3 when the drug would have reached its peak blood concentration.

The microbiological method can be specific when targeting a particular class of drugs for analysis. For example *Bacillus megaterium* is most suitable for sulfonamide screening in animal tissues, urine, or milk (Gudding, 1976) while *Bacillus cereus* var. *mycoides* is ideal for tetracycline assay (Brady and Katz, 1989). Thus the target drug for analysis determines the best test organism. *Bacillus subtilis* produces bacitracin so it is unsuitable for tetracycline assay. *Bacillus cereus*

Table 1: Residues in eggs collected from hens administered oxytetracycline

		Positive/sensitivity					
		Premi test		Disc diffnsion test		HPLC (n = 10)	
Days	Total test eggs screened	No.	%	No.	%	Found (μg L^{-1})	Recovery (%)
1	10	5	50	3	30	121	88
2	10	7	70	5	50	137	100
3	10	9	90	7	70	137	100
4	10	8	80	5	50	136.5	99
5	10	7	70	3	30	135	98
6	10	5	50	1	10	132	96
7	10	3	30	0	0	128	93
8	10	2	20	0	0	113	75
9	10	2	20	0	0	104	66
10	10	1	10	0	0	99	61
11	10	0	0	0	0	79	57
12	10	0	0	0	0	37	27
13	10	0	0	0	0	19	14
14	10	0	0	0	0	8	6

produces penicillinase enzyme and thus will destroy the penicilhn in the sample (Read *et al*., 1971). *Bacillus stearothermophilus* has been widely used as the test organism for most antibiotics (Braham *et al*., 2001; Shitandi and Gathoni, 2005). This micro-organism is very sensitive to many antibiotics and sulphonamide residues. The problem with *Bacillus stearothermophilus* however, is its sensitivity to the inhibitory activity of lysozyme making the bacterium less suitable for drug residue detection in kidney tissue.

As a screening method, the microbiological method minimizes the number of test samples that would eventually be tested with a more sensitive test for confirmation. Although not always specific, these methods are recommended for screening as they can detect many classes of drug residues in animal products in a single test.

CONCLUSION

The validation of the commercial test kit has shown that it can be used to assay for drug residues in eggs. Although, it is not sensitive enough to detect drug residues at lower concentrations and is unsuitable for confirmatory testing but when the aim is to screen large numbers of samples rapidly and at relatively low cost, the commercial test kit may generally be applied.

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