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A SIX PLATE MICROBIOLOGICAL ASSAY FOR SCREENING OF ANTIMICROBIAL RESIDUES IN FOOD OF ANIMAL ORIGIN

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ABSTRACT

Occurrence of antimicrobial residues in livestock and aquatic products is a current public health concern. The monitoring of food commodities for antimicrobial residues will ensure the consumer safety and promote trade. Therefore, the objective of this study was to establish a screening test to detect antimicrobial residues in food of animal origin. A bioassay consists of six plates was established, which enables screening of six groups of antimicrobials, namely; penicillins, sulphonamides, streptomycin, erythromycin, tetracyclines and fluroquinolones. The Six Plate Test (SPT) is a bioassay technique, which is carried out using Bacillus subtilis BGA (Merck), Bacillus cereus (ATCC 11778), Micrococcus luteus (ATCC 9341) and Escherichia coli (ATCC 11303) as indicator organisms. The Mueller Hinton Agar (MHA) was used as the test medium at optimum pH for all indicator organisms except B.cereus, for which the Media No.8 was used. The Minimum Detectable Concentrations (MDC) of SPT were determined using serial dilutions of antibiotics. All the MDC values obtained on SPT for the validated antimicrobials were below the recommended MRL of each antimicrobial. SPT can be developed to a quick, easy to perform, robust and low cost screening test to detect antimicrobial residues in food of animal origin.

1. INTRODUCTION

Antimicrobials are used in food producing animal for treatment. The antimicrobial residues could occur in animal-derived food due to their misuses, failure to observe Withholding Period (WP) or as a result of inadvertent contamination of animal feed with antimicrobials during feed milling [1], [2].

These antimicrobial residues are a concern due to direct harmful effects to humans such as allergies, idiosyncratic reactions and indirect effects as development of antibiotic resistance and long term effects such as mutagenic or teratogenic effects [3], [4], [5]. Therefore, antimicrobial residue monitoring has become an integral part in food safety issues, which promotes local sales and international trade.

Bioassay techniques have been used for several years in order to screen food commodities for antimicrobial residues. Microbial Inhibition Tests (MIT), which apply microorganisms as indicators are considered as multiresidue screening tests for detection of antimicrobial residues [6], [7], [8], [9]. Initially a single plate assay was done using an indicator organism. Later it was developed to two or three plates, which contained different indicator organisms at different pH. Currently modified European Union (EU) Four- Plate Test (FPT) is being used in different countries.

[7], [9], [10]The FPT being used as preliminary screening test, to detect the presence of a wide range of antimicrobial substances, which are inhibitory at the level of residues on growth of microorganisms [11], [12].

Modification to bioassay can be done by changing the indicator organism, type of medium and the pH of the medium. Accordingly, each laboratory within or between countries has adopted their own method to suit the existing laboratory setup. The EU-FPT is based on microbial growth inhibition of Bacillus subtilis on agar medium at pH 6.0, 7.2, and 8.0 while Micrococcus luteus at pH 8.0 media. This is a less time consuming, robust, and an economical technique. It is sensitive to penicillins, tetracyclines, quinolones, macrolides and lincosamides and insensitive for anticoccidials, chroramphenicol and nitrofurans [12], [13].

Since quinolones and aminoglycosides are frequently used in treating animal diseases, it was felt highly necessary to acquire capability of detecting residues of these antimicrobials. Under these circumstances, the modified Six-Plate assay system was developed and validated with the objective of establishing a routine screening assay in Sri Lanka. This assay system has another advantage it can be validated to screen a variety of food commodities including meat, eggs, milk and fish.

2. MATERIALS AND METHODS

2.1. MICROORGANISMS USED

Bacillus Subtilis BGA (Merck)
Bacillus cereus ATCC 11778
Micrococcus luteus ATCC 9341
Escherichia coli ATCC 11303

2.2. PREPARATION AND MAINTENANCE OF MICROORGANISMS

2.2.1. PREPARATION OF SPORE SUSPENSIONS

Spores of *Bacillus subtilis* BGA and Bacillus cereus ATCC 11778 were used in the assay system and the spore suspensions were prepared.

Bacillus subtilis or *Bacillus cereus* spores were sub-cultured in Tryptone Soya Agar (TSA) slants and incubated at 30^0C for 18 hours. The culture was then suspended into peptone salt solution and transferred into Finley and Fields sporulation medium. These inoculated flasks kept at 300C for 5 days for sporulation. The spores were tested for purity, and were harvested using peptone salt solution. Then the spores were suspended with sterile saline, heated at 70^0C for 35 minutes and diluted with saline to obtain a 25% light transmission at 620 nm wavelength using the spectrophotometer. These spore suspensions were dispensed into 1ml aliquots, which stored at -70^0C. The spore suspension was diluted with sterile distilled water for use in the bioassay.

2.2.2. MAINTENANCE OF VEGETATIVE ORGANISMS

Micrococcus luteus ATCC 9341 and *Escherichia coli* ATCC 11303

The vegetative form of these organisms is used in the assay system; therefore, a common method is described for both organisms with the differences in incubation time.

M. luteus and *E.coli* was subcultured in Nutrient Agar (NA) slants and incubated at 37^0C for 48 hours and 37^0C for 24 hours. Following incubation, cultures were checked for purity. Then the cultures were suspended into peptone salt solution.

Prepared bacterial suspensions were stored at 4^0C and used in bioassay within one month. The working bacterial suspensions in peptone salt solution were held at 4^0C whilst the subcultures on slants maintained at 4^0C, which needs sub-culturing at monthly intervals.

2.3. PREPARATION OF ASSAY MEDIA

Mueller Hinton Agar (MHA)

For B.subtilis, M.luteus and E.coli the assay medium used was Mueller Hinton Agar. Bacillus subtilis was used in three assay plates at pH 6.0, pH7.2 and pH 8.0. Inorder to enhance the sensitivity for detection of sulphonamide, 75µl trimethoprim (10µg/ml) solution was added to 15ml of media at pH 7.2.

Mueller Hinton Agar (Oxoid CM 337) (38g) was suspended in 1 litre of distilled water, boiled to dissolve, cooled down to 55^0C and adjusted the pH using solutions of 0.1N HCL and 0.1N NaOH. This media were dispensed to 15ml aliquots in universal bottles for autoclaving.

Medium N0 .8 for Bacillus cereus

Since B.cereus did not show satisfactory growth on MHA; medium No.8 [14] was used as the assay medium for this organism. Media No.8 was prepared adding Bacto Peptone 6g, Yeast Extract 3g, Lab lemco powder 1.5g and Agar 15g in 1 litre of distilled water. Then it was boiled to dissolve and cooled down to 550C in order to adjust the pH. Adjusted the pH 0.2 above the required pH, dispensed to 15ml aliquots in McCartny bottles and autoclaved at 121^0C for 15 minutes.

2.4. PREPARATION OF ASSAY PLATES

The sterilized aliquots of Mueller Hinton medium and medium No.8 were held in a 55^0C waterbath for 30 minutes to stabilize the temperature. Each 15 ml aliquots of the media were inoculated with the appropriate innoculum of microorganism as shown in table 1. The petry dishes having 9cm diameter were labeled properly and warmed at 55^0C incubator for 10 minutes before pouring media. The petrydishes were then poured with inoculated media (5ml of media/plate) while rocking on the palm in order to get an even spread. Then the plates were kept at room temperature to solidify. These plates can be stored at 4^0C for one week.

Validation of the bioassay technique was done using serial dilutions of antibiotics. Technical grade antibiotics were purchased from Sigma (Table 2). Each antimicrobial was dissolved in the appropriate solvent (Table 2), correcting for potency to obtain a concentration equivalent to 1mg/ml of the active antibiotic. The serial dilutions were prepared using sterile distilled water to obtain dilutions ranging from 100 µg/ml to 0.025 µg/ml for all the antimicrobials.

2.5. ASSAY PROCEDURE

The prepared assay plates, held at 4^0C, were dried at 30^0C for 30 minutes in the incubator just before use.

Paper discs (5mm diameter) were prepared using filter papers (Whatman 42 Cat. No. 1442 055). The plates were numbered and the blank paper discs were kept on the agar using a sterile forcep. Using an adjustable pipette 10 µl from each concentration of the antibiotic was added on to each disc. On the same plate with the serial dilutions of antibiotic, the commercially available antibiotic disc (Mast Group Ltd., Merseyside, UK) containing the same antibiotic was placed. The plates were incubated at 30^0C for 18 hours.

This procedure was repeated 5 times for each antimicrobial.

Table 1: Detailed layout of six plates in the assay system

Plate No.	Medium	pH	Microorganism	Innoculum Size Per 15 ml of media
1	Mueller Hinton	6.0	*B.subtilis*	50 µl
2	Mueller Hinton (Trimethoprim added)	7.2	*B.subtilis*	20 µl
3	Mueller Hinton	8.0	*B.subtilis*	20 µl
4	Mueller Hinton	8.0	*M.luteus*	100 µl
5	Media No.8	6.0	*B. cereus*	20 µl
6	Mueller Hinton	8.0	*E.coli*	100 µl

2.6. INTERPRETATION OF RESULTS

A complete inhibition of growth of the microorganism around the antibiotic disc was considered as a positive result. The diameters of the inhibitory zones were measured (Fig. 1) to the nearest millimeter using the Vernier caliper. The mean zone size was calculated for each antimicrobial concentration in each plate. The smallest observable zone, for a series of antimicrobial dilution was considered as the minimum inhibitory zone for that antibiotic. The antimicrobial concentration, which gave the minimum observable inhibitory zone, was considered as the Minimum Detectable Concentration (MDC) of the antimicrobial for the relevant plate.

Table 2: Diluents used in preparing antimicrobial stock solutions

Antimicrobial	Product code	Diluent
Penicillin G	P8721	Distilled water
Amoxycillin	A 8523	N HCl
Tetracycline	T 3258	N HCl
Doxycycline	D 9891	N HCl
Erythromycin	E 6376	Methanol
Ciprofloxacin	17850	N HCl
Streptomycin Sulphate	S 6501	Distilled water
Sulphadiazine	S 6256	30% HCl

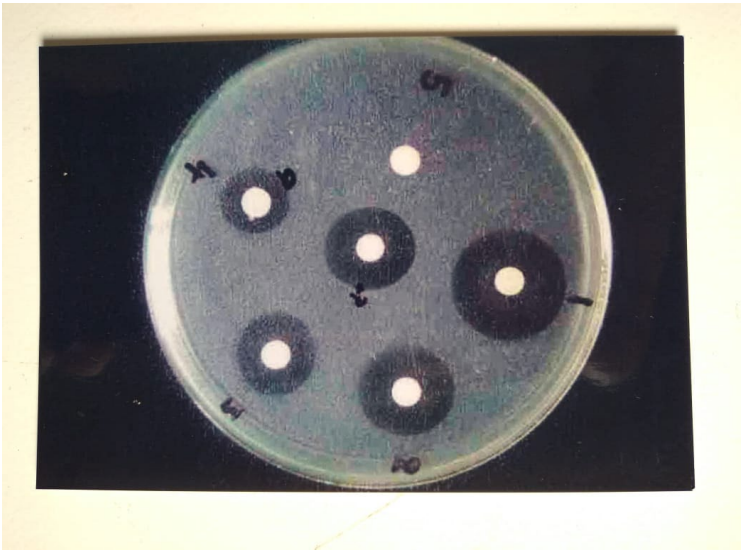

Figure 1: Assay plate showing the inhibitory zones around the discs containing serial dilutions of antimicrobials. a) Minimum Detectable Zone

3. RESULTS AND DISCUSSIONS

Minimum inhibitory zones were measured with respect to the antimicrobial concentration of the disc and Minimum Detectable Concentrations (MDC) are presented in table 3.

For penicillin the MDC was 0.00001 IU for plate 1,2,3 and 4, but when compared the zone sizes given for the MDC, the largest inhibitory zone size was shown in plate1.

Amoxycillin gave highest sensitivity to plate 1 in which the MDC was 0.00025µg/disc. Tetracycline gave MDC of 0.00025µg/disc for three plates, that is plate 1, 2 and 5 but the largest minimum inhibitory zone was given in plate 5. The doxycycline gave highest sensitivity to plate 5 in which the MDC was 0.00025µg/disc. The MDC for streptomycin and erythromycin was 0.0025µg/disc and they were highly sensitive to plate 3 and 4 respectively. Ciprofloxacin gave best sensitivity on plate 6 in which the MDC was 0.00025µg/disc. The MDC for sulphadiazine was 0.00025µg/disc on plate 2, which showed the best sensitivity for that antibiotic.

In a residue-monitoring establishment the primary need is for screening techniques. Screening of food commodities can be done for variety of veterinary drugs, pesticides or few selected chemicals. In this study the priority was given to establish screening technique for antimicrobials due to their common use and the demand from producers. The antimicrobial screening techniques enable the qualitative identification of selected antimicrobial residues in livestock or aquatic products. For antimicrobial screening, bioassays had been used economically due to their rapidity, simplicity and large throughputs [15]. The Six Plate Test (SPT) was selected to establish in the laboratory because it is simple, easy to perform, less time consuming and due to its ability to detect different groups of antibiotics.

The Mueller Hinton Agar (MHA) and Media No.8 was used with necessary pH adjustments. The MHA was selected for B.subtilis, M.luteus and E.coli, as it is less expensive and easily available. Media No.8 is the recommended medium for B.cereus [14]. The 9mm diameter petry dishes were the assay plates used as they are reusable, hence economical and environmental friendly, when compared with disposable plates.

A series of pilot trials were undertaken in order to decide the best innoculum size of the microorganism for each plate, and thickness of the assay medium, which were known variables that can influence the minimum observable inhibitory zones.

The advantage of SPT having several assay plates at different pH over a single assay plate allows detecting a wide range of antimicrobials. Such a screening will help to decide the suitable confirmatory method if residues were detected [13].

The validation of the microbiological screening methods can be done by several methods. Most accurate method is to analyze samples, which are positive for residues in screening test with confirmatory techniques. However, validation had been done by testing serial dilutions of technical grade antimicrobials or spiked meat samples on bioassay plates [12]. For this study it was decided to use serial dilutions of technical grade antimicrobials in order to validate the assay system.

Table 3: Plate sensitivities against Minimum Detectable concentrations (MDC)

Antibiotic	Plate No.	MDC (µg/disc)	MRL ((µg/ 100mg)
Penicillin G	1	0.00025	0.005
Amoxycillin	1	0.00025	0.005
Tetracycline	5	0.00025	0.01
Doxycycline	5	0.00025	0.01
Erythromycin	4	0.0025	0.04
Ciprofloxacin	6	0.00025	0.01
Streptomycin sulphate	3	0.0025	0.05
Sulphadiazine	2	0.00025	0.01

When considered the overall results (Table 3), plate 1 is more sensitive to beta lactam antimicrobials, and plate 2, 3, 4, 5 and 6 are specifically sensitive to sulphonamides, streptomycin, erythromycin, tetracyclines and fluoroquinolones respectively. When consider the MDC for each antimicrobial all the antimicrobials were sensitive to Six Plate Test up to 0.00025 µg/disc except erythromycin and streptomycin. For these two antimicrobials the MDC was 0.0025µg/disc (Table 3). When compared the MDC values against recommended MRLs of each antimicrobial, all the MDC values are below the MRL level, which is a requirement of a good assay system. However, the given MDCs cannot be directly related to MRLs since MDCs are expressed as µg/disc but not as a concentration in tissues. When compared with the results of the previous studies done by Okerman [15] in 2001 and Currie in 1998 [12], this modified SPT is more sensitive for all the antimicrobials tested. The increased sensitivity may be due to the reduce thickness of the assay medium. All the MDC values detected are lower than the recommended MRL for each antimicrobial. Accordingly, SPT is sensitive enough to screen six groups of antimicrobials and invaluable for the routine use as a screening method in the laboratory.

4. CONCLUSIONS AND RECOMMENDATIONS

The Six Plate Test proved best sensitivity for all the antimicrobials for which it was validated and the Minimum Detectable Concentrations (MDC) are less than the recommended MRL for each antimicrobial. However, the given

MDCs cannot be directly related to MRLs since MDCs are expressed as µg/disc but not as a concentration in tissues. When compared with the sensitivity of the SPT with the previous studies, it is more sensitive for all the antimicrobials tested. Six Plate Test can be used as a routine screening method, because it is cheap, less time consuming and enable to screen different groups of antibiotics. Six groups of antimicrobials can be screened by the established modified SPT, namely Penicillin, sulphonamide, streptomycin, erythromycin chlortetracycline and fluoroquinolones. Future studies need to be carried out to evaluate the sensitivity of SPT for antimicrobials in different matrices. The SPT can be validated to screen different animal originated food commodities for antimicrobial residues.

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CONFLICT OF INTEREST

The author have declared that no competing interests exist.

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