

# Establishment of a Single Plate Assay for the Detection of Antimicrobial Residues in Feed

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**Abstract**—Antimicrobial screening technique for animal feed will be a valuable tool to ensure quality of feed given to livestock or in aquaculture. The facility for screening animal feed for antimicrobials had been a felt need among animal feed industries, as well as by the regulatory authorities. Further, in situations where food commodities were positively detected for antimicrobial residues it is important to trace-back the origin of such antimicrobial residues. A single plate assay for the detection of antimicrobial residues was established. The assay was validated under the present laboratory conditions using spiked feed sample, with serial dilutions of different antimicrobials. The test was sensitive to all the subjected antimicrobials except for flavomycin. Of the validated antimicrobials erythromycin gave the highest sensitivity whereas sulphadiazine and ciprofloxacin were less sensitive.

**Keywords**— Antimicrobials, feed, bioassay, screening.

## I. INTRODUCTION

The antimicrobials are added into animal feed for prevention and treatment of diseases and to promote growth. For growth promotion, therapeutic antimicrobials cannot be used because it has both promoted and maintained the incidence of multiple drug resistant organisms in animals which are raised for human consumption. However there are non-therapeutic antimicrobials permitted to use for this purpose (1, 14).

The current global food safety efforts are mainly producer-oriented thereby needs to control feeding, management and treatment practices in food-producing animals. There are legislatives addressing food safety of animal origin covering diverse aspects (15). In spite of all these efforts it is well known that producers are very much inclined to use low levels of therapeutic antimicrobial agents with the belief of controlling or preventing diseases although this is not an approved farming practice (16). In the event of that such a

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malpractice is adopted; the easiest and economical method of performing this is by adding into animal feed. The major reason for this malpractice is that the veterinary drugs can be easily purchased 'over the counter' from pharmacies in most countries in spite of prescription restrictions (2).

The unauthorized use of therapeutic antimicrobials in animal feed may eventually lead to unacceptable levels of such drugs in food commodities of animal origin. Indiscriminate use of therapeutic antimicrobial agents in animal feed had been frequently implicated in acquiring resistance among bacteria (3,4,5).

### Non-therapeutic antimicrobials

Several non-therapeutic antimicrobials are permitted to incorporate in the animal feed as feed additives. Mainly the in-feed antimicrobials and anticoccidials are used as growth promoters and to prevent coccidiosis (17). Non-therapeutic antimicrobials in the poultry feed increase the live weight of birds predominantly by influencing the intestinal micro flora. Another positive effect of this in feed antimicrobials is that it increase amino acid levels in the gut thereby improve nitrogen balance (6).

Virginiamycin, zinc bacitracin, avoparcin, spiramycin and tylosin were previously licenced as zootechnical (non-prescription) feed additives, which have been suspended by the European Union (EU) marketing authorizations (7).

Currently avilamycin is allowed for growth promotion in pigs and poultry. It also has a prophylactic effect on necrotic enteritis in poultry when added at growth promoting dosages. Bambermycin marketed as flavomycin also known as flavophospholipol is a member of the glycolipid group of antibiotics. Zinc bacitracin was used in calves, piglets, lambs and poultry for growth promotion and to improve the egg production of chickens. It was banned for growth promotion in the EU since 1<sup>st</sup> July 1999 but will remain licensed for therapeutic use in member states where used (18).

Currently only four substances left in the EU as feed additives namely monensin, avilamycin, flavomycin and salinomycin (1). All the other antimicrobial growth promoters are banned due to public health concerns. As a solution for the situation the researchers are trying to introduce Direct Fed Microbes (DFM) as growth enhancers. The constituents of DFM include rapidly proliferating and lactic acid producing organisms. All these organisms in the product survive steam pelleting. The DFM given broilers have shown better growth rates and low mortality rates (8).

### Coccidiostats

The most widely used coccidiostats in feed are Carboxylic acid ionophores (9). This group includes salinomycin, lasalocid, naracin and monensin. In UK, monensin is licensed for use in broilers at dietary concentrations of 100-120mg/Kg. The important factor is that no MRL has been established for any of these compounds (10). They are added to medicated animal feeds as the sodium salt to the finished feed. They can be used until three days before slaughter.

Several reports have indicated some side effects of the ionophores. Halofuginone is prophylactically used as anticoccidial in the poultry industry. A study has revealed, that the residues of halofuginone remain in broiler birds until four days of medication (9). The use of in-feed antimicrobials has been a major concern because of the occurrence of antibiotic residues and their adverse effects on human health.

### Therapeutic Antimicrobials in feed

Other than non-therapeutic antimicrobials, therapeutic antimicrobials are allowed to use in treatment of diseases in food producing animals. The best and easiest method of administration is by mixing in feed. When there are disease outbreaks, recommended levels of therapeutic antimicrobials are added to batches of animal feed. As an example Oxytetracycline (OTC) is widely used in shrimp farming to treat diseases such as Vibriosis, septicaemia and necrotizing hepatopancreatitis (11).

There is also a possibility that animal feed gets unintentionally contaminated at the level of milling, if a common mixing drum is used alternatively for medicated and non medicated feed (12). It is now believed that antimicrobial agents from medicated feed may adhere to the walls of the mixing drum, which needs about seven flushes to completely clear these adhered particles. If this flushing practice is not regularly adopted the cross contamination of unmedicated feed with therapeutic antimicrobial agents is a possibility (12).

Under these circumstances it is felt that an antimicrobial screening technique for animal feed will be a valuable tool to ensure quality of feed given to livestock or in aquaculture. The facility for screening animal feed for antimicrobials had been a felt need among animal feed industries, as well as by the regulatory authorities. Further, in situations where food commodities were positively detected for antimicrobial residues it is important to trace-back the origin of such antimicrobial residues. In such situations testing feed given for the identified batches of animals may provide the required information. As Sri Lanka did not have tests to screen antimicrobial residues in animal feed, the single plate assay was established, which could be used as a preliminary screening test.

## II. MATERIALS AND METHOD

The screening for antimicrobial agents in feed was performed using a bioassay technique in which the indicator organism was *Bacillus stearothermophilus* var *calidolactis* Strain C 953.

### A. Preparation and maintenance of the microorganism

*Bacillus stearothermophilus* was subcultured and maintained on Nutrient Agar (NA) slants and stored at 4°C. The organism was subcultured at monthly intervals.

Every time testing was carried out the test culture was prepared in Tryptone Dextrose Yeast Extract (TDYE) broth. Two loopfulls of *B.stearothermophilus* was taken from freshly prepared NA slants and inoculated into 20 ml of TDYE broth. Then it was incubated for 24 hours at 55°C. The test culture was tested for purity using the Loeffler's polychrome M.B. stain which specifically stains bacterial spores. This test culture can be stored in 4°C up to 1 week.

### B. Preparation of test plates

The routine assay was performed in Mueller Hinton Agar (MHA). The autoclaved MHA was kept at 55°C water bath for 30 minutes. Three milliliters of TDYE broth containing the indicator organism was inoculated into 15 ml of MHA and poured into 9mm diameter Petridishes. Allowed to solidify and stored upto 1 week at 4°C until used.

### C. Validation of the test

The assay was validated under the present laboratory conditions using spiked feed sample, with serial dilutions of different antimicrobials. A known negative feed sample for antimicrobials was obtained from Veterinary Research Institute (Gannoruwa, Peradeniya, Sri Lanka) and it was spiked with the antimicrobials within 2 days of submission.

The antimicrobials used for validation are zinc bacitracin, chlortetracycline, streptomycin, furazolidone, sulphadiazine, ciprofloxacin and erythromycin. Each antimicrobial was diluted in acetone phosphate buffer to obtain 100, 50, 25, 10, 1, 0.1 and 0.01 µg/ml solutions. Using these serial dilutions, 50g of spiked feed samples for each dilution were prepared having final antimicrobial concentrations of 1, 2.5, 5, 10, 25, and 50 mg/Kg. Four replicates from each concentration were tested on separate assay plates. Antimicrobial free feed sample was prepared and assayed in the same manner as the negative control. The spiked feed samples were held for 24 hours in room temperature to permit any possible interactions. Then they were extracted using the acetone-phosphate buffer.

Other than the serial dilutions several feed ingredients including maize, rice polish, coconut poonac, fish meal, and chlorchordide were tested individually in order to detect any antimicrobial effect originating from the base samples.

### D. Extraction of feed for assay procedure

The feed was extracted in acetone buffer. Two hundred milliliters (200 ml) of extraction buffer was added to 50 g of feed. Samples were shaken for 1 hour in a rocker. Then the samples were allowed to stand for 2 hours at room temperature. The supernatant was taken for the assay using a pasture pipette.

### E. Assay procedure

The assay was based on the principal of agar-well-diffusion. The prepared assay plates, stored at 4°C were dried at 30°C in

an incubator for 15 minutes. The test sample (100µl) was added into wells of 8mm diameter cut on agar using a metal cutter. Duplicate from each sample was added on to the assay plate. Every assay plate carried a commercial disc of penicillin antibiotic (Mast group Ltd., Merseyside, UK) at the center as the reference standard. Known negative feed samples were extracted and placed on assay system to exclude false positive results. The assay plates were incubated at 55°C for 4 hours.

#### F. Interpretation of results

A complete inhibition of growth of the microorganism around the feed sample was considered as a positive result. The antimicrobial concentration of the feed sample, which showed the lowest inhibitory zone, was considered as the Minimum Detectable Concentration (MDC) for that antimicrobial in feed.

#### G. Statistical Analysis.

Statistical analysis of data was performed using the SAS General Linear Model (GLM) procedure of SAS software (SAS Institute Inc., Cary, NC). Duncan's multiple range test was used to find significant differences ( $p < 0.05$ ) between treatments at a particular time. For each growth condition, the test was repeated four times.

### III. RESULTS

The observed MDC for sulphadiazine and ciprofloxacin was 0.25 mg/kg, whilst Zinc bacitracin, chlortetracycline, streptomycin, furazolidone showed MDC of 0.025 mg/kg. The best sensitivity (0.0025 mg/kg) was given with erythromycin while flavomycin was not detectable as shown in Table 1.

### IV. DISCUSSION

It is important to develop screening facility for antimicrobial residues in animal feed for several reasons. If a batch of feed is suspected to be contaminated, samples from such a batch can be tested prior to feeding during finishing stages of productive animals. Further, if residues were unexpectedly detected in a food commodity, in the event of trace-back of its origin, a feed testing facility may provide confirmatory evidence, if feed had been the origin of the contaminant antimicrobial. The Single Plate Assay does not differentiate between antimicrobial compounds but it is important as a primary screening method to screen the bulk of feed prior to send for confirmatory testing. This test is simple, economical and results could be given within one working day.

The MHA was used as the assay medium, which is cheap and easily available in Sri Lanka. A series of pilot trials were done in order to decide the best agar thickness, inoculum size of the microorganism, the incubation time, which gives the best detectable inhibitory zones. The selection of antimicrobials for test validation was dependent on their frequency of use in animal production.

The test was sensitive to all the subjected antimicrobials except for flavomycin. Of the validated antimicrobials

erythromycin gave the highest sensitivity whereas sulphadiazine and ciprofloxacin were less sensitive (Table 1)

All the feed ingredients including maize, rice polish, coconut poonac, fish meal, and chlorchloride did not show zones of inhibition, excluding possibility of false positive results. As the validation is in progress the assay is being validated to all the other possible antimicrobials in feed.

TABLE 1 MINIMUM DETECTABLE CONCENTRATIONS (MDC) OF ANTIBIOTICS SHOWN ON THE SINGLE PLATE ASSAY FOR SCREENING ANIMAL FEED.

Antibiotic	MDC
Zinc bacitracin	0.025 g/kg
Chlotetracycline	0.025 g/kg
Streptomycin	0.025 g/kg
Furazolidone	0.025 g/kg
Sulphadiazine	0.25 g/kg
Ciprofloxacin	0.25 g/kg
Erythromycine	0.0025 g/kg

### V. CONCLUSION

This single plate assay procedure is suitable for detecting the presence of inhibitory substances in animal feed and provides with a simple and rapid, multi-analytic screening test. The assay system is non-specific and unable to identify or to quantify the inhibitory antimicrobial agent. The development of a secondary screening procedure such as Thin Layer Chromatography (TLC) method is suggested which could be used to identify and quantify the inhibitory antimicrobial agents in the event of detecting positive samples on routine screening.

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University of Peradeniya to continue her career in research and teaching in food safety.



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Madhavi joined North Carolina A&T State University in 2009 to pursue a PhD in Energy and Environmental Systems interdisciplinary program under the thrust of Environmental Food Science. She received several scholarly awards at North Carolina A & T State University. She was given the award for scholarly accomplishment and academic excellence by the International Students and Scholars Office (ISSO) in 2009–2012. She is a recipient of the Wadaran L. Kennedy 4.0 Scholars award from the School of Graduate Studies from 2009–2011.

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