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Microbial inhibition assay for detection of antibiotic residues in chicken meat using vegetative form of *Geobacillus stearothermophilus*

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Abstract
Food safety is a prime requirement for consumers. One of the key challenges to ensure food safety is the availability of fast, sensitive and reliable methods to identify specific hazards like antibiotic residues. A microbial inhibition assay (MIA) was optimized using vegetative form of *Geobacillus stearothermophilus* as indicator organism to screen for the presence of antibiotic residues in chicken meat. The spectrum of sensitivity to antibiotics was wide with *Geobacillus stearothermophilus* and *B. subtilis* while *E. coli* was resistant to 3/16 antibiotics screened. The limit of detection (LOD) was 1 ppb for enrofloxacin and 0.5 ppb for ciprofloxacin and oxytetracycline. A total of 360 tissue samples collected from enrofloxacin or oxytetracycline -fed experimental broilers were screened with the developed MIA. Highest percentage of positive samples was detected in skin followed by breast muscle in either oxytetracycline or enrofloxacin-fed chickens. This test can be used as a preliminary screening assay to detect the presence of any antibiotic residue qualitatively.

Keywords: Screening, antibiotic residues, microbial inhibition assay, chicken, meat

1. Introduction
Food safety is a prime requirement for consumers. One of the incriminating factors against the growing antibiotic resistance in humans is the indiscriminate veterinary applications of antibiotics. In animal production and protection, antibiotics are used for preventing and treating several diseases as well as for promoting growth in food producing animals (Carson *et al.*, 1994; Di Corcia and Nazzari, 2002; Donoghue, 2003) [1, 6, 5]. Lack of stringent control on the withdrawal period of antibiotics further facilitates antibiotic resistance in human. Meat or milk with antibiotic residues becomes potential hazard for human health (Kozarova *et al.*, 2001; Hind *et al.*, 2014) [12, 10]. The availability of rapid and feasible tests for detection of such residues in food would not only curtail their indiscriminate use but deter the poultry and livestock farmers to sell their produce before the recommended withdrawal periods.
Currently available antibiotic-residue detection tests are of two types: the screening methods that includes microbiological tests (Disc assay, modified Premi and Delvotest) (Hind *et al.*, 2012) [9] and the confirmatory methods using more complex and advanced techniques such as enzyme linked immunosorbant assay (ELISA) and high performance liquid chromatography (HPLC) (Tajik *et al.*, 1998) [18]. The microbial inhibition assays were the earliest methods used and are preferred as a preliminary screening assay by virtue of their convenience, ease of performance, low cost and ability to detect wide spectrum antibiotics. However, these tests are less specific and don't give information on the quantity and the type of antibiotic present. Such screening methods would help us to identify suspect samples for further confirmation of positivity by using more sensitive chromatographic techniques.
Commercial microbiological tests have been manufactured by several companies under different trade names. Some of them include the Nouws antibiotic test (NAT), the Premi test (DSM, The Netherlands), the Brilliant Black Reduction test, the Copan microbial inhibitor test, Delvotest (Gist- brocades BV, The Netherlands), Swab Test on Premises (STOP), the Calf Antibiotic and Sulfa Test (CAST), the Fast Antibiotic Screen Test (FAST), the Charm Farm Test (CFT) and the Antimicrobial Inhibition Monitor 96 (AIM-96) assay (Pikkemaat, 2009; Gaudin *et al.*, 2004; Chafer- Pericas *et al.*, 2010) [16, 8, 3]. The ideal bacterial inhibition test for screening antibiotic residues in slaughtered animals does not exist. Each of the tests has limitations (Korsrud *et al.*, 1998) [11].

In India there is no routine screening followed for antibiotic residue detection in meat samples. One of the possible reasons could be the lack of the availability of in-house developed microbial screening assay in an easy-to-use kit formulation. The approximate cost of commercial kits like PREMI test is Rs. 350/- per test which may be prohibitive for routine use.
Thus the aim of the present study was to develop a microbial screening method to determine antibiotic residue in poultry meat and formulate the test as a kit for field use. In addition this method was used to detect the presence of residues in selected tissues of broiler chicken experimentally fed with antibiotics, oxytetracycline or enrofloxacin.

2. Materials and Methods

2.1 Bacterial strains

Geobacillus stearothermophilus (ATCC 7953), *Escherichia coli* (MTCC 443) and *Bacillus subtilis* (MTCC 441) were used.

2.2 Antibiotic standards

Standard Oxytetracycline (HIMEDIA CMS653) and Enrofloxacin was procured from Indian Pharmacopoeia Commission **(**IPC- Cat No.E-015, Lot No. IPRS/42/13**)**

2.3 Spectrum of antibiotic sensitivity of the three indicator bacterial strains

The conventional Kirby- Bauer disc diffusion method was used for antibiotic susceptibility testing of the indicator organisms. Briefly, Muller-Hinton Agar (MHA; Hi-Media) plates were streaked with 100 µl of the reconstituted freeze dried cultures of *G.stearothermophilus, Bacillus subtilis* or *Escherichia coli* in different plates and commercially procured antibiotic discs (HiMedia) were placed on the plate and incubated at 37°C. After 24 h, the diameters of zone of inhibition were measured by using scale/calipers. In accordance to Performance Standards for Atimicrobial Disk Susceptibility Tests, Clinical and Laboratory Standards Institute (CLSI), the organism was classified as being resistant or sensitive.

2.4 Microbial Inhibition Assay using *G.stearothermophilus*

a. Bacterial culture

Geobacillus stearothermophilus was inoculated into nutrient broth and incubated at 37 °C. The purity of test organism was determined by quadrant streaking on nutrient agar plates and Gram staining methods. The pure culture of *Geobacillus stearothermophilus* were freeze dried using nutrient broth with cryoprotectant (10% w/v skim milk; Cody *et al.*, 2008) [2] until further use.

b. Preparation of freeze dried assay medium

The assay medium consisted of nutrient broth with 5% w/v sucrose, 2.5% w/v bovine serum albumin and 2% v/v bromocresol purple indicator (ATCC, 2012).The test organism concentration was adjusted to 0.5 MacFarland standards which are equal to 10^6 cells and added to the assay medium. Each 2 ml cryovials added with 900 µl of assay medium with test organism were subjected to freezing drying as per the following conditions: Freezing for 14 hours at -40 °C. Primary drying was done for 16 hours at -30 °C, followed by secondary drying at 37 °C for 2 hours. Vials were sealed under vacuum and then further sealed with aluminium caps. The vials were stored at 4 °C until use.

c. Enumeration of viable cells

To reveal the effect of freeze drying on viability of the test organism, plate count was carried out. The assay medium before and after freeze drying was serially diluted up to 10^{-4} and 100 µl from each dilution of assay medium was spread on to nutrient agar plates. The plates were incubated at 37 °C and cfu per ml was calculated.

d. Sample preparation

Approximately 2 G of the tissue samples (liver, kidney, breast and thigh muscles and skin) were taken for extraction of fluid. Using meat press the fluid from breast and thigh muscles was collected. The other samples were homogenised, centrifuged and filtered using 0.45 µ syringe filters and stored at -20 °C until use.

2.5 Limit of detection of Enrofloxacin and Oxytetracycline by MIA

Stock solutions of antibiotics Oxytetracycline and Enrofloxacin at 1000 ppm were prepared using methanol as solvent and stored at -20 °C until use. Working solutions at 100 ppm concentration were prepared from the stock with water and was used for MIA plate method.
Rehydrated assay medium in the cryovials was added with 100µl of 500ppb to 50 ppb concentration of Enrofloxacin or Oxytetracycline. The tubes were incubated at 37 °C overnight. The colour change due to growth of test organism in assay medium was observed and compared with control (without antibiotic addition). When the blue colour of the indicator dye changed from blue to colourless, it is indicative of absence of antibiotic residues thereby enabling bacterial growth. When the blue colour remained without change as in control vial, it is indicative of presence of antibiotic residue which has prevented bacterial growth. Any intermediate colour change is treated as suspicious.

a. Detection of antibiotics in spiked samples

Chicken meat extracts were spiked with various concentrations ranging from 200 ppb to 0.25ppb (at and below the MRL) of Enrofloxacin and Oxytetracycline. The antibiotic spiked samples (100 µl) were added to the rehydrated (900µL) assay medium of *G. stearothermophilus*. The vials were incubated at 37°C overnight. The microbial growth inhibition by the antibiotics was observed as the change in colour of the medium.

2.6 Experimentally fed broiler chicken with Oxytetracycline or Enrofloxacin

Three weeks old broiler chickens (Strain- B_3 Nandanam) were randomly divided into control (one) and treatment groups (two groups). One group was treated with Enrofloxacin at recommended therapeutic dose of 10 mg / kg body weight via drinking water (freshly prepared daily medication) for five days consecutively from 25th to 29th day of age (Sureshkumar *et al.*, 2013) [17]. Six birds each were sacrificed ethically on 1, 3, 5, 7 and 9 days post treatment along with two control birds on each occasion.
The other group was treated with Oxytetracycline at a therapeutic dose of 40 mg / kg body weight via drinking water (freshly prepared daily medication) for 7 consecutive days (Odore *et al.*, 2015; Cornejo *et al.*, 2016) [15, 4] from 23rd to 29th day of age. Six birds each were sacrificed ethically on 1, 3, 5, 7, 9, 11 and 14 days post treatment along with three control birds on each occasion.

Samples such as liver, kidney, muscle (breast and thigh) and skin were collected immediately after sacrificing the birds from both treated groups along with control and were stored in deep freezer for further analysis. The samples were processed for fluid extraction and stored frozen after filtering using 0.45µ syringe filter. 100 µl of the meat extract was subjected to antibiotic residue detection using MIA.

3. Results and Discussion

The analysis on zone of inhibition of the three indicator organisms by Kirby-Bauer MHA plate method using antibiotic discs showed that both *G. stearothermophilus and B. subtilis* showed sensitivity to all the 16 antibiotics tested. However, *E. coli* strain was found resistant to three antibiotics, erythromycin, amoyclav and neomycin (Table 1).

The MIA developed using freeze dried preparation of the vegetative form of *G. stearothermophilus* as the indicator organism could detect the antibiotics, enrofloxacin, ciprofloxacin and oxytetracycline at and below the MRL level prescribed by the European Council Regulation 2377/90 with respect to meat (Table 2). The lyophilisation protocol did not affect the viability of the test organism. The number of viable cells, before freeze drying was 2 x 10^6 cfu/ml. After 72 hours freeze drying the count was 1.4 x 10^6 cfu/ml.

The developed MIA was formulated as a kit using 10 vials of freeze dried preparation of *G. stearothermophilus* for testing 10 samples along with controls. A portable incubator earlier designed for the ABT Choice kit (developed at TRPVB, TANUVAS) was also applicable for this assay for incubation of the reconstituted vials (Figure 1).

Oxytetracycline residues were predominantly detected in the skin, breast muscle, thigh muscle, liver and kidney in that order (Table 3). By day 11 post-feeding of oxytetracycline, only 13% of samples tested were positive for antibiotic residues. On day 14 post-feeding, no tissue tested showed the presence of antibiotic residues.

Enrofloxacin residues were predominantly detected in the skin, breast muscle, kidneys, thigh muscle and liver in that order (Table 4). By day 9 post-feeding of oxytetracycline, only 13% of samples tested were positive for antibiotic residues. Of the positive samples on day 9 post-feeding, 75% were from skin and 25% from breast muscle.

Table 1: Spectrum of antibiotic susceptibility of *G. stearothermophilus, E. coli and B. subtilis* based on zone of inhibition measurement

Antibiotic / disc content (µg)	*G. stearothermophilus*		*B. subtilis*		*E. coli*	
	Zone of inhibition (mm)	Inter-pretation	Zone of inhibition (mm)	Inter-pretation	Zone of inhibition (mm)	Inter-pretation
Choramphenicol C^{30}	25	Sensitive	36	Sensitive	32	Sensitive
Erythromycin E^{15}	35	Sensitive	35	Sensitive	12	**Resistant**
Oxytetracycline O^{30}	45	Sensitive	32	Sensitive	28	Sensitive
Cefepime CPM^{30}	15	Sensitive	18	Sensitive	26	Sensitive
Cefotaxime CTX^{10}	25	Sensitive	32	Sensitive	36	Sensitive
Co-Trimazole COT^{25}	35/40	Sensitive	36	Sensitive	32	Sensitive
Cefexime CFM^{5}	22	Sensitive	26	Sensitive	26	Sensitive
Amoyclav AMC^{30}	23	Sensitive	22	Sensitive	10	**Resistant**
Kanamycin K^{30}	25	Sensitive	26	Sensitive	32	Sensitive
Streptomycin S^{10}	25	Sensitive	34	Sensitive	16	Sensitive
Gentamicin GEN^{10}	22	Sensitive	26	Sensitive	22	Sensitive
Neomycin N^{30}	20	Sensitive	18	Sensitive	12	**Resistant**
Enrofloxacin EX^{10}	42	Sensitive	36	Sensitive	40	Sensitive
Norfloxacin NX^{10}	38	Sensitive	44	Sensitive	40	Sensitive
Ofloxacin OF^{5}	33	Sensitive	38	Sensitive	40	Sensitive
Ciprofloxacin CIP^{30}	41	Sensitive	36	Sensitive	42	Sensitive

Table 2: Limit of detection (LOD) of selected antibiotics in poultry meat as detected by the MIA using *G. stearothermophilus*

Antibiotic	Poultry meat	
	MRL (ppb) (EU)	LOD (ppb) in spiked meat fluid
Enrofloxacin	100	1.0
Ciprofloxacin	100	0.5
Oxyteyracycline	100	0.5

Fig 1: Schematic representation of the MIA named ABT Detect kit along with portable incubator

Table 3: Detection of antibiotic residue by MIA in selected tissue samples collected at different time intervals from oxytetracycline fed broiler chickens

Day of sample collection post feeding of antibiotic	Liver	Kidney	Breast muscle	Thigh muscle	Skin	Total (percent positivity)
1	4*	3	3	3	5	18 (60%)
3	2	2	4	1	4	13 (43%)
5	2	2	3	2	4	13 (43%)
7	-	1	3	2	3	9 (30%)
9	-	-	2	1	3	6 (20%)
11	-	-	1	1	2	4 (13%)
14	-	-	-	-	-	0
Total (percent positivity)	8 (19%)	8 (19%)	16 (38%)	10 (23%)	21 (50%)	

* indicates no. of samples positive in MIA of 6 samples tested on each occasion

Table 4: Detection of antibiotic residue by MIA in selected tissue samples collected at different time intervals from enrofloxacin fed broiler chickens

Days of sample collection post feeding of antibiotic	Liver	Kidney	Breast muscle	Thigh muscle	Skin	Total (percent positivity)
1	3*	5	6	4	6	24 (80%)
3	3	2	4	1	6	16 (53%)
5	1	2	3	2	6	14 (46%
7	-	1	1	0	4	6 (20%)
9	-	_	1	-	3	4 (13%)
Total (percent positivity)	7 (23%)	10 (33%)	15 (50%)	7 (23%)	25 (83%)	

* indicates no. of samples positive in MIA of 6 samples tested on each occasion

Availability of farmer-friendly pen-side screening assay for the presences of the antibiotic residues in poultry meat is an essential prerequisite to ensure food safety and also to gain consumer confidence. Some of the commercial poultry marketing units do claim that their chicken meat is antibiotic residue-free. However it was found that such claims were made by testing representative samples at one time point. This does not ensure that all the meat sold by such marketing units would be antibiotic residue-free on all occasions.

In this context, if a simple MIA if formulated as a kit, this could be used by the poultry marketing units to repeatedly test all the batches of meat samples that are being used for human consumption.

Commercial kits are available for such assays such as Premi test, Delvo test, etc. These tests are expensive which prohibits routine use. Additionally the tests require heating the inoculated sample at 64 °C to ensure sporulation of the indicator organism used.

The main objective of our study was to develop an in-house MIA which is cost effective and overcomes the need for heating at 64°C. So instead of the spores of *G. stearothermophilus*, the vegetative form of the organism was used. To ensure stability and long shelf-life of the organisms and to convert the assay in to a kit, freeze drying was applied. The freeze dried bacteria reconstituted with the meat fluid samples could be incubated at 37°C. In many parts of our country, incubation can be done at room temperature during summer months. However for use in the cold region a portable incubator can be used for the assay.

The commonly used indicator organisms for MIA are *G. stearothermophilus, E. coli, B. subtilis, B. megatherium, etc*. Each of these organisms has a specificity to detect particular classes of antibiotic residues. For example, *E. coli* is preferred for quinolones, *B. subtilis* for tetracycline, penicillin, chloramphenicol, streptomycin *and G. stearothermophilus* for aminoglycosides, ß – lactams, tetracyclines, sulphonamides, macrolides (Chafer- Pericas *et al.*, 2010) [3].

In our study we found that *G. stearothermophilus* had a wide spectrum of sensitivity to 16 antibiotics tested, thereby, serving as an ideal indicator organism. The LOD of this organism for two antibiotics tested were hundred to two hundred folds lower than the MRLs permitted. Such high sensitivities have been reported in MIA using spores of *G. stearothermophilus* for specific detection of antibiotics (Pikkemaat, 2009) [16]. It is possible that the use of vegetative form of *G. stearothermophilus* could have also contributed to the increased sensitivity since germination of spores may not be 100 percent. Freeze drying is an ideal method for long term storage of bacteria, virus and other biologicals. In our study also freeze drying did not cause any major decrease in cfu of the bacteria.

Another requirement during antibiotic treatment is to know precisely when to market the treated chickens. Although withdrawal times are recommended for each antibiotic there is paucity of data in this area, especially with respect to MIA. Using five organs collected at different time points from OTC-fed broiler chickens in MIA revealed maximum positivity in the skin and on day one post-feeding. By 14th day, none of the organs showed any residue. The recommended withdrawal time for OTC is also 10 – 14 days (Odore *et al.*, 2015; Lee, *et al.*, 2000) [15]. It is recommended that withdrawal times are scrupulously followed to ensure absolute absence of antibiotic residues in the meat meant for human consumption.

Since skin has the highest positivity for presence of residues for both antibiotics, it can be recommended that skin of poultry should not be included in case the birds are sacrificed before the compliance of withdrawal period. This would largely reduce the presence of antibiotic residue and in turn development of resistance in humans. Similar results were also seen in Enrofloxacin fed birds in which 83% of skin samples were positive even on the 9th day of feeding.

The MIA was also formulated in form of a kit that can be used in poultry farms. It is hoped that the availability of MIA in kit format would play a pivotal role in ensuring food safety in relation to poultry meat consumption. This test can also be applied to meat, milk and eggs. Another recommendation through this study is to avoid use of poultry skin of birds that

have been fed with antibiotics.

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