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Evaluation of a Microbiological Growth Inhibition Assay as a Screening Test for the Presence of Antibiotic Residues in Poultry Meat

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Abstract: The present study was conducted to monitor the presence of antibiotic residues in poultry meat. Swab Test on Animal Food (STAF) employing *Bacillus subtilis* as test organism on nutrient agar with 0.4% dextrose at pH 7, was used. A spore suspension of 2×10^7 spores mL⁻¹ was used in 100 mL of nutrient agar to make STAF plates. A total of 100 tissue samples (33 liver, 33 kidney and 34 muscles) were collected from local market of Rawalpindi and Islamabad. Thirteen (39.4%) liver, 9 (27.3%) kidney and 7 (20.6%) muscle samples were detected positive for antibiotic residues. Antibiotic sensitivity and Minimum Inhibitory Concentration (MIC) of Oxytetracycline, Gentamicin, Neomycin, Enrofloxacin, Chloramphenicol, Amoxicillin, Benzyl Penicillin, Streptomycin and Tylosin against *Bacillus subtilis* was determined to be 0.6100, 0.0549, 0.1525, 0.1525, 0.0023, 0.0143, 0.0095, 0.0380 and 0.0023 µg, respectively. It is concluded from the study that STAF test can be used for the preliminary detection of antimicrobial residues in poultry tissues.

Key words: Antibiotic residues, STAF test, *Bacillus subtilis*, MIC

INTRODUCTION

Poultry meat has emerged as good substitute of beef and mutton. Its importance can be judged from the fact that almost every family in rural areas and every fifth family in urban areas is associated with poultry production activities in one way or the other. It contributes about 11.9% of the total GDP (Economic Survey of Pakistan, 2004-2005).

Antibiotics are widely used as therapeutic, prophylactic and growth promoting agents in livestock and poultry production (Donoghue, 2003). Various antibiotics take different time periods to be excreted from the body. This time period is known as withdrawal period for that particular antibiotic and has to be observed before taking eggs from the birds, slaughtering the animals and before taking milk from lactating animals. Improper dosages, as well as neglecting withdrawal period, results in antibiotics residues in meat, milk and eggs. It becomes a potential hazard to human health (Kozarova *et al.*, 2001).

Farmers and supporting groups (veterinarians and livestock dealers) are devoted to producing safe as well as nutritious dairy and poultry products. In developed countries, government agencies ensure the supply of safe and wholesome products for human consumption. Consumers wish that their food supply be free from residues of pesticides, drugs, or antibiotics. Approximately 10% of the human population is considered to be hypersensitive to a number of substances including penicillin and other antibiotics and suffers allergic reactions like skin rashes, hives, asthma and anaphylactic shock (Booth, 1988).

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Bacterial inhibition tests used to screen milk and tissues for antimicrobial veterinary drug residues must be high volume, quick, rugged, inexpensive and sensitive. Bacterial inhibition tests-such as the Swab Test on Premises (STOP), the Calf Antibiotic and Sulfa Test (CAST), the Fast Antibiotic Screen Test (FAST), the Charm Farm Test (CFT), the Antimicrobial Inhibition Monitor 96 (AIM-96) assay, the German Three Plate Test, the European Union Four Plate Test and the New Dutch Kidney Test-have been used to screen tissues for antimicrobial activity. There are certain limitations in the use of these screening tests for regulatory control and avoidance of veterinary drug residues in meat. The ideal bacterial inhibition test for screening antimicrobial residues in slaughtered animals does not exist. Each of the current and potential tests has limitations (Korsrud *et al.*, 1998).

In the present study, a microbiological assay called Swab test on animal food (Rehman and Jabbar, 2006) using locally isolated *Bacillus subtilis* as test organism was used for the screening of poultry meat for the presence of antibiotic residues.

MATERIALS AND METHODS

Isolation of *Bacillus subtilis*

Plate of nutrient agar (0.4% dextrose) was inoculated (by spread plate method) with 0.1 mL of a heated (80°C for 15 min) dilution of soil and incubated aerobically at 37°C for 24 h. Cultural and morphological characteristics were studied by inoculating on Nutrient agar and by Gram's staining, spore staining methods, respectively. Biochemical and sugar fermentation tests were performed and hemolytic properties were studied on blood agar having 5% sheep blood as described by Cruickshank *et al.* (1975) and Cheesbrough (1989) for identification of pure *Bacillus subtilis*.

Collection of Meat Samples

A total number of 100 poultry meat samples including 33 liver, 33 kidney and 34 muscles were collected randomly from slaughtered birds, commercially available in the local market of Rawalpindi and Islamabad, Pakistan and were transported to National Veterinary Laboratories, Islamabad under cold conditions, where all the experiments were conducted during 2006. The samples were kept at -20°C until the time of analyses.

Antibiotic Sensitivity and Minimum Inhibitory Concentration of *Bacillus subtilis*

Antibiotic sensitivity and Minimum Inhibitory Concentration (MIC) of *Bacillus subtilis* against various commonly used antibiotics were determined using Kirby-Bauer disc diffusion method as described by Tortora *et al.* (2001). A single well-isolated colony of *Bacillus subtilis* was inoculated in 5 mL normal saline and turbidity was adjusted to 0.5 McFarland opacity standards. A homogenous bacterial lawn was prepared on nutrient agar (Oxoid, CM3) with this standardized bacterial suspension. Two fold serial dilutions were made in microtitration plates from the standard solutions of Oxytetracycline, Gentamicin, Neomycin, Enrofloxacin, Chloramphenicol, Amoxicillin, Benzyl Penicillin, Streptomycin and Tylosin by adding 50 µL of each antibiotic solution in 1st well upto the 24th well in distilled water. Then filter paper discs (6 mm i.d. 2 mm thickness, 25 µ disc⁻¹ absorbing capacity) were impregnated in each dilution from 1st well to 24th well for each antibiotic used. Antibiotic discs were placed on the solidified agar surface and incubated at 37°C for 18 h. The highest dilution of antibiotic that inhibited the growth was the MIC of that antibiotic (Table 1).

Swab Test on Animal Food (STAF)

Freshly prepared 125 mL nutrient agar Roux flask was inoculated with 5 mL of pure broth culture and spreaded homogenously over the surface of Roux flask. The Roux flask was incubated for remainder of week (6 days) at 37°C. The growth was harvested from Roux flask by adding 20 sterile

glass beads and approximately 25 mL of sterilized normal saline per flask. Gently agitated the flask to dislodge the bacterial growth. The bacterial suspension was aseptically transferred into sterile centrifuge tubes and was heated at 80°C for ten minutes. Washed the heated suspension three times with sterile distilled water by centrifugation at 5°C for 20 min @ 20,000 x g (Sigma 3K30, Rotor No. 12150-H, Germany) and decanting the supernatant and resuspending the pellet in distilled water each time. Pooled all spore suspension into sterile glass bottle. Stock suspension was stored at 4°C until further use.

A working spore suspension of *Bacillus subtilis* having 2×10^7 spores per mL in normal saline was prepared through Breed's smear method for the spore counting using Gram's staining method (Awan and Rehman, 2002). Aseptically added 1 mL of this spore suspension to 100 mL of nutrient agar (0.4% dextrose, pH 7) at 55°C. Using pour plate method, 20 mL of nutrient agar was added in 6×6 inch petriplates to make STAF plates and refrigerated at 4°C until use.

Frozen samples collected from market were allowed to thaw completely placing at room temperature. Sterile cotton swabs were immersed aseptically in liver, kidney and muscles samples for 5 min. Muscle samples were first agitated to dislodge muscle fibers and to release tissue fluid. Swab samples were separately placed over STAF plates having Neomycin 5 µg antibiotic filter paper disc as a positive control. Above plates were incubated at 37°C for 18 h. An inhibition zone greater than 2 mm i.d. around swab samples was considered as positive; while that less than 2 mm i.d. was taken as negative for antibiotic residues.

RESULTS AND DISCUSSION

Morphological and Cultural Characteristics

Bacillus subtilis was isolated and identified as Gram positive, small rods occurred singly or in short chains, spore forming and motile. Spores were central with rounded ends and did not swell the cell. The optimum temperature for growth was 37°C, aerobes and abundant growth occurred on Nutrient agar and 5% sheep blood agar. The colonies were large (3 mm) undulated, rhizoidal, flat and dull, with ground glass appearance. In broth showed flaky deposits with clear supernatant. On blood agar plates, growth was pigmented (orange), glistening and showed beta hemolytic characteristics, adherent somewhat membranous growths that tend to spread. Similar morphological and cultural characteristics of *Bacillus subtilis* have been reported by Cruickshank *et al.* (1975).

Biochemical Reactions

The organism was positive for catalase, citrate utilization test, Voges Proskaur test and negative for indole production, methyl red test, urease and lecithinase test. It fermented glucose and manitol with the production of acid and no gas.

Minimum Inhibitory Concentration (MIC) of *Bacillus subtilis* Against Different Antibiotics

MIC of most of the antibiotics against *Bacillus subtilis* was below the Maximum Residue Limits (MRLs) recommended by EU, FAO/WHO and FDA of USA, except oxytetracycline, for which detection sensitivity was 0.6100 µg while its MRL is 0.2, 0.6 and 1.2 µg g⁻¹ for meat, liver and kidney, respectively (Table 1).

In present study, the MIC of Oxytetracycline was higher than other antibiotics used. This result is consistent with the study of Hrdlicka (1990), who found that *Bacillus subtilis* ATCC 6633 was least sensitive to tetracyclines when compared to other antibiotics. However, DeWacsh *et al.* (1998) who reported that an inhibition test with a medium at pH 6 and *B. subtilis* as test organism was well suited to screen pork and chicken muscle tissue for residues of tetracycline antibiotics. The MIC for Enrofloxacin was 0.1525 µg, which is similar to the sensitivity, determined by Choi *et al.* (1999) using

Table 1: Minimum inhibitory concentration (MIC) of different antibiotics against *Bacillus subtilis*

Antibiotics	Standard solutions of antibiotics used (mg mL ⁻¹)	Dilution at which MIC was observed	MIC (µg)	*MRL (µg g ⁻¹)
Oxytetracycline	200	13	0.6100	0.2(M), 0.6(L), 1.2(K)
Gentamicin	40	14	0.0549	0.1(M)
Neomycin	50	13	0.1525	1.2(M), 3.6(L), 7.2(K)
Enrofloxacin	100	14	0.1525	0.3(M), 0.1(L), 2(K)
Chloramphenicol	200	21	0.0023	-
Amoxicillin	150	18	0.0143	0.01(M)
Benzyl penicillin	200,000 ^a	19	0.0095 ^a	0.1(K)
Streptomycin	200	17	0.0380	0.5(M), 0.5(L), 2(K)
Tylosin	200	21	0.0023	0.2(M), 0.2(L)

^a = IU, M = Muscle, L = Liver, K = Kidney, *MRL = Maximum Residues Limit (values of MRL are adopted from FDA, 1996)

Table 2: No. of samples of different inhibiting zones in STAF test

Inhibiting zones	Type of tissue samples		
	Liver	Kidney	Muscle
≥ 2 mm	13*	9*	7*
<2-1 mm	5	3	2
<1 mm	2	0	0

*Samples with zone of inhibition ≥ 2 mm were considered STAF positive

Table 3: Results of STAF test on poultry meat samples

Type of samples	Total No. of samples	Antibiotic positive*	Antibiotic negative**	Percentage positive
Liver	33	13	20	39.4
Kidney	33	9	24	27.3
Muscle	34	7	27	20.6

*Clear zone around swab ≥ 2 mm and Neomycin (5 µg) disc between 10-16 mm, **Opaque bacterial growth right up to the swab and clear zone around Neomycin (5 µg) disc between 10-16 mm

Bacillus subtilis ATCC (3491). They also reported that *B. subtilis* was sensitive to only enrofloxacin in the fluoroquinolones groups and detection sensitivity was 0.250 µg g⁻¹. MIC values of penicillin, amoxicillin recorded in present study are in line with those reported by Popelka *et al.* (2005). Carlisle and Falkinham (1989) studied the enzymatic activity and antibiotic susceptibility of *Bacillus subtilis* colonial variants VT30M and VT30NM. They reported that MIC of Chloramphenicol for both variants was 0.002 µg which is in line with present study.

Swab Test on Animal Food

Out of 100 samples processed by STAF test, 29 samples had zone of inhibition ≥ 2 mm and were considered STAF positive, while 12 showing zone <2 mm were considered STAF negative (Table 2). The highest number (39.4%) of antibiotic residues was observed in liver samples. While 27.3% kidney samples showed positive results. In case of muscle samples 20.6% were detected as positive (Table 3). These results agreed well with Myllyniemi *et al.* (2000) who used kidney samples rather than muscle samples as the test material for microbiological identification of antibiotic residues in a carcass. Jabbar (2004) reported that incidence of antibiotic residues in kidney samples was 70%, in liver it was 60%, while for muscle it was 50%.

CONCLUSION

From the results of the present study, it can be concluded that STAF test using *Bacillus subtilis* can be used as a screening method in monitoring of antibiotic residues in poultry meat. Due to unspecific nature of the microbial inhibition assays, the presence of residues presented in the

potentially positive samples must be further confirmed by using more specific physico-chemical methods like High performance liquid chromatography. It would also be interesting to develop the microbial methods, which combine the residue detection and identification stages.

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