JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

Jabbar et al. 2013 : 2 (5) 2349-2354

MICROBIOLOGICAL EVALUATION OF ANTIBIOTIC RESIDUES IN MEAT, MILK AND EGGS

*Abdul Jabbar *, Sajjad-Ur-Rehman*

Address(es): Abdul Jabbar
Institute of Microbiology, University of Agriculture, Faisalabad- 38040, Punjab, Pakistan

*Corresponding author: dr.jabbar@live.com

ARTICLE INFO

Received 20. 12. 2012
Revised 24. 3. 2013
Accepted 25. 3. 2013
Published 1. 4. 2013

Regular article

ABSTRACT

Microbiological tests are widely used to detect antibiotic residues in the meat, milk and eggs for better care of the quality and health safety. In the present study microbiological inhibition test i.e. Swab Test on Animal Food (STAF) was developed indigenously for screening of animal foods for presence of antibiotic residues. In this test local isolated culture of *Bacillus subtilis* was used as a test microorganism due to its high sensitivity to detect a wide range of antibiotics commonly used in animal disorders. The concentration of spore suspension of *Bacillus subtilis* JS2004 used in the formation of STAF plate was optimized at $2x10^7$ spores/ ml. At this concentration, inhibition zone around Neomycin control disc was 10-16 mm. Nutrient agar was used as a medium in spore suspension and 0.4% dextrose was added as a constituent of medium. Zones of inhibition around swab samples and Neomycin control disc were observed and the diameter was measured. All swab samples showing a minimum of 2 mm wide inhibition zone around them were considered as positive for presence of antibiotic residues. The swab samples showing no zone of inhibition or a zone measuring less than 2 mm were considered as negative. Results of application of STAF test was on animal food samples revealed the high incidence of antibiotic residues.

Keywords: Meat, milk, egg, *Bacillus subtilis* JS2004, antibiotic residues

INTRODUCTION

Human beings require energy, for their growth, development and normal functioning of the body. The carbohydrate, lipids and proteins are the essential dietary ingredients. The proteins are the principal materials of skin, muscles, tendons and nerves etc. These are required for the normal wear and tear of cells. Free market economy, competition in the domestic and international market, growing requirements of consumers, force the manufacturers of dairy and meat products to take a better care of the quality and health safety of milk and meat.

Drugs or their metabolites left over in the body after their administration for longer time are termed as residues. After the treatment of infected animals with drugs, the residues of drugs are present at some level in edible products like milk, eggs and meat of treated animals. Drug residue concentration vary considerably from tissue to tissue and are generally observed to higher in the tissues of storage such as body fat, or in organs that actively metabolize and excrete them e.g. residues may be highest in body fat , liver and kidneys **(Booth, 1973).**

These residues are sometimes present in such a sufficient amount in edible products that on consumption may cause potential human health hazards. So it becomes worthwhile to take preventive measures against the residues by establishing their withdrawal period from edible products.

Exposure to low levels of antibacterial causes the development of pathogenic microorganisms. Some drug residues may destroy useful micro flora present in gastro-intestinal tract especially of children, leading to indigestion. Drug residues cause the drug resistance. Drug resistance is the bacteria's tolerance to drugs, which make drugs ineffective in killing bacteria. Drug resistant bacteria pose a threat to public health because in case of a human bacterial infection there would be less or no drug effective for the treatment. Residues may involve immuno-pathological mechanisms which leads to human health hazards amongst immuno-pathological, mostly immediate hypersensitivity has been linked to the presence of drug residues in edible products of food producing animal, however, other types of hypersensitivity redactions cannot be excluded (**FAO/WHO, 1984**).

Some drugs and or their metabolites, which possess proven or suspected carcinogenic potentials, are of major concern. For example sulphamethazin residues containing meat, preserved with sodium nitrite may develop a Trizine complex, which has a considerable carcinogenic potential (**Mitchell, 1984**). Some other compounds like Nitrofurans, Nitromidiazole, Quinoxalin-N, N-dioxide, Griseofulvin and some Stibene derivatives, also have carcinogenic properties (**FAO/WHO, 1984**).

Rapid microbiological tests are used in slaughter establishments to detect the presence of antimicrobial residues in food animal tissues. The Swab test on Premises (STOP) is used for all red meats species except bob veal calves, where then Calf Antibiotic and Sulpha Test (CAST) is used. Pre- Harvesting Antibiotic Screening test (PHAST), an in-plant screen test, was developed in 1989 to improve the capability of the antibiotic detection program. PHAST has greater sensitivity and can detect wide range of antibiotics (**USDA/FSIS, 1998**). These microbial inhibition tests are simple to perform, cost effective and allow routine testing and release of large number of food animal carcasses in the shortest possible time.

It is advisable to use kidney rather than muscle samples as the test material for microbiological identification of antimicrobial residues in a carcass. (**Myllyniemi *et al*., 1999**).

Bacillus. subtilis is often referred to as a 'soil dweller'. *B. subtilis* actually grow in soil or in a place where spores accumulate until they encounter conditions suitable for their germination and proliferation. The use of fluorescent antibodies to distinguish vegetative and spore forms of *Bacillus subtilis* in diverse soil samples revealed that the organism was most often in its vegetative form when associated with decaying organic material (**Norris *et al*.,1961**).

Bacillus subtilis grow at optimum temperature 37°C, aerobic meaning that they require oxygen to grow and abundant growth occurs on the ordinary culture medium and 5% sheep blood agar. Large, flat, dull, with ground-glass appearance, somewhat membranous growth; may be pigmented (white, yellow, orange or brown) may be beta-hemolytic (**Cruickshank *et al*., 1975**).

Bacillus subtilis are Gram-positive, large rods, spore forming and motile. The vegetative cell width was less than 1 µm. Spores were central, shorter, and thinner with rounded ends and did not swell the cell **(Forbes *et al*., 2002**).

Bacillus subtilis is a model organism for studying endospore formation in bacteria. Endospores in *Bacillus subtilis* bacteria are mostly formed in the tips of protuberances extending downward from liquid surface pellicles (**Schaechter *et al*., 2006**).

Screening of milk and other animal food samples for the presence of antibiotic residues is usually performed with the help of microbial inhibition assays. Their sensitivity to different drugs depends on the indicator micro-organism used and the concept of the test. Microbiological assays for the detection of antibiotic residues utilize the genus *Bacillus,* because of its high sensitivity to the majority of antibiotics (**Jevinova *et al*., 2003**).

The lowest concentrations (in microgram/ml) of 27 selected antimicrobial drugs with a semi-standardized inoculum of *Bacillus subtilis* strain ATCC 6633 were determined on Mueller-Hinton agar plates (***Traub et al*., 1982**).

Objectives:

Present study was planned with the following objectives:

1. Detection of residues of commonly used antibiotics in meat, milk and eggs.
2. Use of local *Bacillus subtilis strain* as a test microorganism in antibiotic screening test.
3. Antibiotic sensitivity testing of local *Bacillus subtilis* strain
4. Standardization of antibiotic screening test on animal foods in Pakistan.

MATERIAL AND METHODS

Collection of soil samples

Ten soil samples from fertile land were collected and transported to Microbiology Laboratory University of Agriculture Faisalabad for further studies.

Isolation and identification of microorganisms

All the soil samples were dusted directly over the surface of sterilized nutrient agar (0.4%dextrose) plates and incubated for 24 hours at 37° C then growth was inoculated on 5% sheep blood agar and incubated for24 hours at 37° C **(Carter *et al*., 1991)**. Colonies of various morphological structures were separately examined for their morphological, cultural, biochemical and sugar fermented tests (**Cruickshank *et al.*, 1975).**

Preparation of spore suspension of pure isolate

Single colony of pure isolate was streaked out on the surface of nutrient agar plate and incubated for 18 hours at 37° C. The growth was confirmed through Gram's staining method for its purity and growth was harvested in 5 ml sterilized nutrient broth. Freshly prepared 125ml nutrient agar Roux flask was inoculated with 5 ml of pure growth isolate and spreaded homogeneously over the surface of Roux flask. Then Roux flak was kept at room temperature for the remainder of week (6days). The growth was harvested from Roux flask by adding 20 sterile glass beads and approximately 25ml of normal saline per flask. Gently agitate flask to dislodge bacterial growth. The bacterial suspension was aseptically transferred into sterile centrifuge tubes and was heated in boiling water (100°C) for 10 minutes. Wash the heated suspension three times with sterile distilled water by centrifugation at 5°C for 20 minutes @ 20,000 X G and decanting the supernatant and resuspending the pellet in distilled water each time. Pooled all spore suspension into sterile bottle. Stock suspension was stored at 4°C.

Preparation of working spore suspension

Working spore suspension as processed through Breed's smears method for the spore counting using Malachite green staining method **(Awan and Rahman, 2002).**

Breed's smear method (Total count)

Breed count named after its originator, is an accurate and useful technique for counting bacterial populations in semi pure and pure cultures. Breed slides has an area of 1cm^2 marked on it.

A. Smear was prepared into a square and placed 0.01ml fluid to be examined with a commercially available micro- syringe or loop.

B. Dip in KOH to remove the fat layer, allowed it to dry and stain with Gram's staining method.

C. Examine with an oil immersion lens. Few microscopic fields were selected randomly and average number of bacteria was calculated.

D. The microscopic factor **(MF)** was determined as follows:

With the help of stage micrometer, the diameter of one microscopic field (area visible under microscope, Nikon, Japan) at 100X objective lens was measured in microns (μ):

$a = r^2 \prod$

$a = (D/2)^2 \prod$

One small division of stage micrometer is equivalent to 10 μ in size.

Number of divisions of stage micrometer = 32

32 x 10 = 320

r = 320/2 = 160

$a = (160)^2 \times 3.14 = 80384$

As the area of scaled microscope slide (A) = 1cm^2 =10^8 μ

MF = A/a Microscopic Factor (MF) may be defined as total number of microscopic fields present in 1cm^2 prescribed area of microscope glass slide.

Microscopic Factor of Microscope (Nikon, Japan)

MF= A/a = 10^8/80384

= 1244

E. Finally, total spores were calculated as:

Spores/ml = Average number of Spores x MF x dilution factor

Average number of spores = 82,160,240

Dilution factor = 100 (as volume was taken 0.01ml)

Spores/ml = 160 x1244 x 100

=20000000

= 2 x 10^7 spores/ml

Antibiotic sensitivity and Minimum Inhibitory Concentration (MIC) of native isolated strain against various antibiotics: Disc diffusion method - Kirby-Bauer test (Tortora *et al.*, 2001).

Nutrient agar medium was inoculated (seeded) uniformly over its entire surface with a standard amount (2x 10^7 spores /ml) of a pure isolate strain. Two fold serial dilutions were made of different antibiotics to perform disc diffusion method. Next, filter paper discs (6 mm diameter) impregnated with known concentrations of different groups of antibiotics were placed on the solidified agar surface. During incubation, the chemotherapeutic agents diffused from the disk into the agar. A zone of inhibition formed after16-hours at 37° C. The diameter of the zone was measured.

Swab test on animal food (STAF)

Medium Preparation

Nutrient agar (0.4 % dextrose) was used as testing medium.

Table 1 Composition of Nutrient agar

Ingredient	Quantity
Beef extract	3.0g
Peptone	5.0g
NaCl	5.0g
Agar	15.0g
Dextrose	4.0g
Distilled water	1000ml
pH	7.0

Autoclave at 121C ° for 15 minutes at 15 lbs.

Table 2 No. of samples of meat (kidney, liver, muscle) milk and eggs collected from different locations of district Faisalabad

Species	Type of sample	Total No. of samples collected
Cattle/ Buffalo	Milk	24
-	Kidney	20
-	Liver	20
-	Muscle	20
Sheep/Goat	Kidney	20
-	Liver	20
-	Muscle	20
Poultry	Eggs A. Albumen B. yolk	24 each
-	Kidney	20
-	Liver	20
-	Muscle	20

Collection of muscle, kidney, liver and milk and egg samples

Samples of muscle, kidney, liver and milk were collected aseptically from cattle/buffalo and sheep/goat from the slaughterhouses and milk establishments of district Faisalabad. Similarly, samples of muscle, kidney, liver and eggs of chicken were collected from slaughtered birds and marketed eggs.

Preparation of STAF Plate

Two different methods of STAF plate were applied for the processing of *Bacillus subtilis* JS2004 spores in the STAF test.

Pour plate method

Aseptically add 1 ml of 2 X 10^7 spores/ml *Bacillus subtilus* JS2004 spore suspension per 100 ml of the agar. Mixed thoroughly.20ml of the agar was poured into each 6 x 6 inch plate and tilt plates to ensure even distribution. Allow the plates to harden on a flat, level surface. Refrigerate plates sealed in double plastic bags to prevent moisture and evaporation. These plates can be used for a period of 10 working days.

Overlay Method:

Aseptically added 1 ml of 2 x10^7 spores/ml *Bacillus subtilus* JS2004 spore suspension per 100 ml of the agar and mixed thoroughly. 10ml of the simple nutrient agar was poured into each 6 x 6 inch plate and tilted the plates to ensure even distribution. After this 10 ml of agar containing spore suspension (2 x10^7 spores/ml) was spread on the plate already containing simple nutrient agar. Allowed the plates to harden on a flat, level surface. Refrigerated and sealed plates in double plastic bags to prevent moisture and evaporation.

Use of Neomycin (5µg) Control disc on STAF plate

Neomycin **(**5µg**)** control disc was placed at a distance of one inch from sample swab on the plate.

Standard Test Procedure

Samples were placed at 4°C or below. Allowed frozen samples to thaw completely at room temperature for a sufficient period of time such that ice crystals are no longer present within the sample.

1.Opened a sterile cotton swab pack (sterilized under UV light at 265°A), removed one swab, and inserted the sharp end of the swab shaft about 1/2" to 3/4" into each kidney and liver sample.

2. For muscle samples, moved the swab shaft back and forth several times to macerate the tissue, disrupting tissue cells and releasing tissue fluid.
3. kept the swab in the tissue samples (kidney, liver and muscle) for minimum of five minutes.

4. For milk and egg samples, swab was dipped in fresh milk and albumen, yolk of egg for 30 seconds.

5. Allowed refrigerated STAF plates to warm to room temperature for about 10 minutes. Each plate was checked for absence of contamination, cracking of agar or dryness.

6. Placed a Neomycin 5µg disc on the agar surface on a STAF plate.

7. Removed the swab from the sample; break the shaft approximately two inches from the swab end.

8. Gently placed the sterile sample swab on the surface of the STAF plate with the broken end of the shaft (1inch) from the Neomycin (5µg) disc making sure not to break the agar surface. Made sure the sample swab had a uniform contact with the agar.

9. Incubated the plate upright at 30°C for 16-18 hours.

Observation and Measurement of inhibition zones

a. Incubated plates were removed from incubator.

b. Measured the ZI by the N5 disc with an mm ruler or with an antibiotic zone reader. The zone should be 10-16 mm wide. If the zone is not 10-16 mm in width, the test is inconclusive and should be repeated.

c. Observed the plates for inhibition of *Bacillus subtilis* JS2004 growth surrounding the swabs.

i. If a zone of inhibition is observed, the test was considered positive. Measured the width of the zone and recorded the results.
ii. If no zone of inhibition is observed, the test is negative.

RESULTS

Isolation and identification of pure isolates from soil samples

Out of 10 samples, 8 samples showed successful positive growth on nutrient agar plates after 24 hours of incubation at 37°C. Following cultural, morphological and biochemical characteristics were commonly observed.

Table 3 Cultural, Morphological and Biochemical Characteristics of *Bacillus subtilis* JS2004 isolated from soil samples

Characteristics	Results
Gram's staining	Positive
Shape/Size/Arrangement	Large rods (1.5-5 x 0.5-0.8 um), convoluted threads of Bacilli in chain
Spore Staining	Spores central, non-bulging with rounded ends, greenish in color
Nutrient agar	Yellowish brown granular discs (3 mm), smooth margin, and white curly growth after 24hr at 37 °C
Blood Agar	Beta-hemolytic, white glistening, adherent somewhat membranous growth
Glucose	Positive with absence of gas
Manitol	Positive with absence of gas
Catalase	Positive on nutrient agar
V.P.	Positive
Indole	Negative
MR	Negative

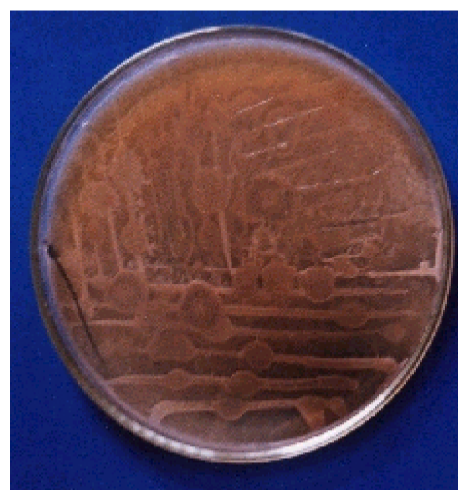

Figure 1 Colonies of *Bacillus subtilis* strain JS2004 on nutrient agar (0.4% dextrose)

Antibiotic Sensitivity & MIC of local strain *Bacillus subtilis*JS2004 against different Antibiotic Groups on STAF Plate

Antibiotic sensitivity of local *Bacillus subtilis* strain JS2004 was observed by using Disc Diffusion method. This test is simple and inexpensive and is most often used. It was used to estimate the Minimal Inhibitory Concentration (MIC), the lowest antibiotic concentration that prevented visible bacterial growth. The MIC determined for commonly used antibiotics is given in (Table 4). The values showed that *Bacillus subtilis* strain JS2004 was sensitive to detect very minute amounts of antibiotics e.g. 0.0625 ug of Gentamycin while Maximum Residue Limit (MRL) approved by US/FDA for Gentamycin is 0.1 ppm in animal foods which can be very clearly detected by *Bacillus subtilis* JS2004.Maximum Residue Limit (MRL) for Enrofloxacin is 0.1-0.3ppm, Amoxicillin 0.01ppm, Tylosin 0.2ppm and Procain Pencillin-G 0.05-0.01ppm. The MRL values of commonly used antibiotics are much higher than the Minimum Inhibitory Concentration (MIC) of respective antibiotics for which *Bacillus subtilis* strain JS2004 is sensitive.

Table 4 Antibiotic Sensitivity & MIC of local strain *Bacillus subtilis* JS2004

Antibiotic	Concentration	Dilution	MIC
Gentamicin	40 mg/ml	15	0.0625 ug
Sulphadiazin/ Trimethoprim	400 mg/ml 80 mg/ml	19	0.0378 ug 0.015 ug
Enrofloxacin	100 mg/ml	22	0.0011 ug
Chloramphenicol	200 mg/ml	23	0.001 ug
Oxytetracycline	50 mg/ml	24	0.00149 ug

Amoxicillin	150 mg/ml	29	0.000013 ug
Tyrosine	200 mg/ml	32	0.000002 ug
Benzyl Penicillin/ Procaine Penicillin	100,000 i.u 150,000 i.u.	34	0.000000002 ug 0.000000004 ug
Streptomycin	200mg/ml	34	0.0000005ug

Table 5 Results of STAF test on meat, milk and egg samples

Species	Type of sample	No. of samples	Antibiotic positive*	Antibiotic negative**	Percentage positive
Buffalo / cattle	Milk	24	10	14	41.6
-	Kidney	20	7	13	35
-	Liver	20	6	14	30
-	Muscle	20	6	14	30
Sheep / Goat	Kidney	20	9	11	45
-	Liver	20	8	12	40
-	Muscle	20	9	11	45
Poultry	Eggs a.Albumen b.Yolk	24 each	16 9	8 15	66 37.5
-	Kidney	20	14	6	70
-	Liver	20	12	8	60
-	Muscle	20	10	10	50

* Clear zone around swab & N-5 disc between 10-16 mm.
**Opaque bacterial growth right up to the swab& clear zone around N-5 disc between 10-16 mm.

Interpretation of results

Antibiotic positive

Inhibition of *Bacillus subtilis* JS2004 growth was observed in plates surrounding the sample swab and Inhibition zone around Neomycin disc was measured 10-16 mm wide.

Antibiotic negative

Opaque bacterial growth right up to the swab was observed surrounding the sample swab and clear zone around N-5 disc between 10-16 mm was measured.

Test inconclusive

Opaque bacterial growth right up to the sample swab was observed and clear zone around N-5 disc was less than 10-16 mm was measured. Rerun test.

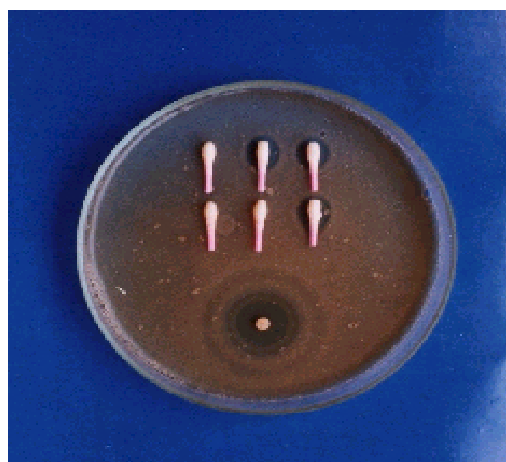

Figure 2 STAF test with overlay method on meat milk, and Egg samples

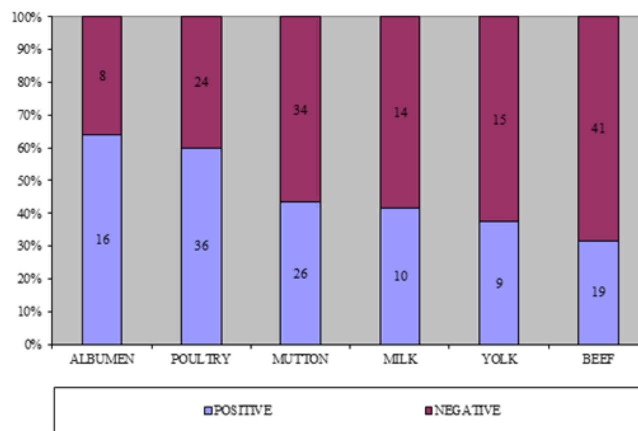

Figure 3 Overall % age of antibiotic residues in various tissues / secretions using STAF

DISCUSSION

Results of application of STAF test was on animal food samples revealed the high incidence of antibiotic residues. All samples showing a clear inhibition zone around swab was considered positive. Antibiotic residues may be a result of extra-label use of drugs which includes using subcutaneous (SQ) when labels indicate intravenous (IV) or intramuscular (IM) and increases the dose above the label dose as there is no strong veterinary infra -structure in Pakistan and mainly quacks/ Veterinary assistants are treating the animals. Another main cause of high antibiotic residues is no consideration of withdrawal time of drugs. The use of antimicrobials for growth promotion in animals should be terminated if it is used in human therapeutics and known to select for cross resistance to antimicrobials used in human medicines. In Pakistan there is no system for detection of antibiotic residues in animal foods. There are some studies on detection of antibiotic residues in poultry meat, eggs and milk by Spectrophotometry. But this procedure is lengthy, laborious and costly as compared to microbiological inhibition assay. So therefore present study was carried out to develop an antibiotic residue detection test by using local *Bacillus subtilis* strain JS2004 as a test organism, for this purpose all necessary conditions were optimized for STAF test.

All samples showing a clear inhibition zone around swab was considered positive. The result was recorded as positive when the width of inhibition zone was equal to 2 mm or more. **(Myllyniemi *et al.*, 1999)**.

Screening of milk samples for the presence of antibiotic residues is usually performed with the help of microbial inhibition assays. The assays with *Bacillus stereothermophilus* as the test microorganism are routinely used in the milk industry worldwide. In a study, it was concluded that *Bacillus stereothermophilus* is not sufficiently sensitive to the residues of oxytetracycline in milk. **(Jevinova *et al.*, 2003)**.

In STAF (Swab Test on Animal Food), there were three spore concentration of local *Bacillus subtilis* strain JS2004 used to get an optimum inhibition zone (10-16mm).At concentration of spore suspension 1×10^{7} spores per ml, the width of inhibition zone around control disc of neomycin was observed 20-26mm as was described in STOP **(USDA /FSIS,1998)**. This concentration of spore suspension presented some problems like large width of inhibition zone, limited space on plate to apply maximum no. of samples. At concentration of spore suspension 3×10^{7} spores per ml, inhibition zone of 5- 7mm was observed. This spore concentration did not give optimum inhibition zones.

So, to resolve these problems and to get optimum inhibition zones, 1mlof 2×10^{7} spores per ml concentration of spore suspension in 100 ml of nutrient agar with 0.4 % dextrose was used. This spore concentration was resulted in clear growth of *Bacillus subtilis* JS2004 on STAF plate producing optimum inhibition zone measuring 10 -16 mm and provision of application of more no. of samples in same plate at same time.

Different methods of STAF plate were applied for the processing of *Bacillus subtilis* JS2004 spores in the STAF test. But overlay method was considered best method. The inhibition zone around neomycin control disc was optimized by using the different concentrations of spore suspension. The maximum zone measuring 20-26 mm observed on a concentration of 1×10^{7} spores/ml and minimum zone measuring 5-7mm on a concentration of 3×10^{7}.But when concentration of spore suspension was 2×10^{7} per ml, the optimum zone was observed 10-16mm. As this was the final concentration of spore suspension used in the test, zone around neomycin disc was optimized.

Both STOP (Swab Test On Premises) and PHAST(Pre-Harvesting Antibiotic Screening Test) are used for antibiotic residues detection in red meat only, but a modification is made so that it can also was able to detect drug residues in poultry meat, eggs and milk. Moreover both former tests were unable to detect sulphonamides because in these tests, Muller Hinton agar was used as a

testing medium. As in STAF test Nutrient agar with 0.4 % dextrose was used as a testing medium.

Spore concentration of *Bacillus subtilis* ATCC 6633 used in STOP was 1x10 ^{7} spores /ml while in PHAST spore concentration of *Bacillus megaterium* was1x 10 6 spores/ml but in STAF the concentration of local *Bacillus subtilis* strain JS2004 which was found optimum to get a inhibition zone (10-16) was 2x 10^{7} spores/ml of spore suspension in 100ml of nutrient agar with 0.4 % dextrose at PH 7. This spore concentration resulted in clear growth of *Bacillus subtilis* JS2004 on STAF plate producing inhibition zone measuring 10 -16 mm and provision of application of more no. of samples in one plate at same time.

One-plate screening method for the microbiological detection of antibiotic residues by growth inhibition of *Bacillus subtilis* in agar medium, at pH 7, which contains trimethoprim for better detection of Sulphonamides. Beta-Glucuronidase is added to the samples to enable the detection of chloramphenicol residues. The sensitivity was determined for 16 antimicrobial substances frequently used in animal husbandry (**Koenen-Dierick *et al.,* 1995**).

Modification of the EC Four Plate Method based on microbial growth inhibition of *Bacillus subtilis* on agar medium at pH 6.0, 7.2 and 8.0 and Micrococcus luteus at pH 8.0 developed to cope with large numbers of samples. The method's performance was evaluated by determining the Minimum Inhibitory Concentrations (MIC) of 66 commonly used drugs and determining the between-assay variation of antimicrobial control standards. The modified method proved particularly sensitive for beta-lactams, tetracyclines, quinolones, marcrolides and lincosamides and least sensitive for anticoccidials and nitrofurans. The pH 6.0 and 7.2 plates were more sensitive for 39 of the 66 antimicrobials (59%) whereas the two pH 8.0 plates (*B. subtilis*, M. luteus) were the most sensitive for 27 (41%). Muscle samples were taken from 1830 routine meat inspection investigations between 1994 and 1996. Of the 38 (2%) positive meat inspection carcasses, the following antimicrobials were confirmed above the MRL: penicillin G (10), oxytetracycline (16), sulphadimidine and sulphadiazine in combination (4) and chlortetracycline (1). The method as described is technically simple, cost effective, robust, and suitable for large sample throughput and for frozen, thawed or fresh tissues. When all four plates were used the pattern of inhibition reduced unnecessary confirmatory assays by indicating the antimicrobial group most likely to be present (**Currie *et al*., 1998**).

Results of STAF test on Beef samples

Total 60 samples of beef collected from different locations of district Faisalabad, Pakistan were analyzed for drug residues by STAF test. Results of STAF test concluded that out of 60 samples 19 samples were positive for antibiotic residues with a percentage of 31.6 %. These beef samples were collected from both buffaloes and cattle randomly and consist of tissues of kidney, liver, and muscle. Twenty samples each of kidney, liver, and muscle were used for antibiotic residues analysis. Among these three tissues the highest no. of positive samples were observed in kidneys having a percentage of 35 % as 7 samples were positive out of total 20 samples analyzed. But both in muscle and liver samples 30 % samples gave positive results as 6 samples out of total 20 were positive in each case. The results showed a relatively high incidence in kidney samples because kidney is main organ responsible for excretion of antibiotic residues. The overall prevalence of antibiotic residues (31.6 %) may indicate frequent use of antibiotics in cattle and buffalo to cure bacterial infections specially Mastitis and Hemorrhagic Septicemia which are main problems in these animals in Pakistan.

Results of STAF test on Mutton samples

Total 60 samples of mutton were tested and among them 26 samples were positive for drug residues with a 43.3 % may indicate frequent use of antibiotics in sheep and goat to treat bacterial infections specially Enterotoxaemia and Mycoplasmosis which are main problems in these animals in Pakistan. Mutton samples included 20 samples each of kidneys, liver, and muscle of sheep and goats collected from different locations randomly. In kidney and muscle samples 45 % were positive for residues as 9 samples of each tissue were positive out of total 20.But incidence of drug residues was slightly lower in liver samples with 40% positive samples as 8 samples out of total 20 showed positive results.

Results of STAF test on Milk samples

In this study total 24 milk samples taken from buffaloes and cattle from different locations were used for analysis, among these samples 10 samples were positive for antibiotic residues with a incidence of 41.6 %. The use of antibiotic therapy to treat and prevent udder infections in dairy animals is a key component of mastitis control in many countries specially in developing countries like Pakistan, that's why incidence of antibiotic residues in milk are very high.

Residues detection in milk by using high performance liquid chromatography (HPLC), 11 samples out of 100 were positive in district Faiasalabad for presence of sulphonamides and concentration ranged from 581 to 12980 µg/ L **(Munir, 2000)**.

The prevalence of oxytetracycline in milk, and concluded that all samples collected were positive in district Faisalabad for presence of oxytetracycline when analyzed by HPLC. The lowest concentration of tetracycline was 93 µg / ml and highest was 232 µg / ml.(**Afzal, 2000**)

Results of STAF test on Poultry meat and egg samples

For the treatment of various bacterial diseases in poultry and to increase the poultry products (chicken and eggs), various chemotherapeutic agents are used, among them antibiotics are mostly used. Poultry meat samples including 20 samples each of liver, muscle and kidney tissues were used for detection of antibiotic residues. Among these highest incidences of residues were observed in kidneys with a 70 % because 14 samples out of total 20 showed positive results. While incidence was 60 % in liver and 50 % in muscle samples as 12 and 10 samples each of 20 gave positive results respectively. Poultry eggs were also studied for residue detection. For this albumen and yolk of each egg sample was analyzed separately. Highest incidence of residues was observed in egg albumen as 16 samples out of 24 were positive with 66 % positivity. While incidence of antibiotic residues was lower in yolk which have 37.5 % positive results as 9 samples of yolk were positive out 24.

All samples of chicken meat collected from district Faisalabad were positive for tetracycline when analyzed by HPLC. The highest concentration of tetracycline was 81.3 and lowest was 21.32 µg /g. This result showed that incidence was 100 %. Similarly all egg samples were also positive showing a range from 74.41 µg / ml to 167.13 µg /ml. (**Iqbal, 2000**).

CONCLUSION

Microbiological inhibition test i.e. Swab Test on Animal Food (STAF) was developed indigenously for screening of animal foods for presence of antibiotic residues. All swab samples showing a minimum of 2 mm wide inhibition zone around them were considered as positive for presence of antibiotic residues. Antibiotic residues in meat, milk and eggs may be a result of extra-label use of drugs and no consideration of with drawl time of drugs.

RECMENDATIONS AND SUGESSIONS

Concentration of antibiotic residues can be measured by High Performance Liquid Chromatography (HPLC) assay and width of inhibition zones can be standardized according to the HPLC values. STAF kit can be developed to detect antibiotic residues for commercial producers of animal food.
Following are precautions which can be followed to avoid antibiotic residues in animal foods.

1. Using medication requiring no withdrawal time or follow drug withdrawal time.

2. Avoid extra-label use of drugs.

3. Mark and identify all treated animals.

4. Do not combine several antibiotics yourself.

5. The use of antimicrobials for growth promotion in animals should be terminated if it is used in human therapeutics and known to select for cross resistance to antimicrobials used in human
medicines.

Acknowledgments: Author acknowledges with deep heartiest gratitude to *Sajjad-Ur-Rehman*, professor, Institute of Microbiology, University of Agriculture Faisalabad for his dynamic and affectionate supervision made it very easy to undertake this project. Words are lacking to express my obligations to affectionate Father, *Zulifqar Ahmed* and *Mother*, in grateful appreciation of their pray, help, kindness, moral support and encouragement during the whole span of studies.

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