



Methods for the detection of antibiotics and its residues in edible animal products

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ABSTRACT

In recent years, AMR (Antimicrobial resistance) is a very fervent issue and having great concern for different regulatory agencies and consumers not only in India but also in worldwide. So, reliable and consistent screening methods for detection of antibiotics and its residues are necessary in different food commodities of animal protein origin for food safety point of view. Analytical methods are mainly divided into two groups i.e. screening methods and confirmatory methods. Screening methods are qualitative or semi quantitative, whereas confirmatory methods are quantitative, sensitive and accurate. Selection of the suitable method will depend on the intended degree of accuracy, convenience, urgency, availability of resources, availability of technical expertise and cost.

INDEX TERMS – AMR, SCREENING AND CONFIRMATORY METHOD, MICROBIOLOGICAL, ELISA, HPLC, UPLC, LC-MSs

INTRODUCTION

Antibiotics revolutionized medicines in 20th century. These are the very effective substances produced by some microorganisms to show antagonism effect. Antagonism effect is the kind of effect which can be seen when a microorganism inhibits or kills the other microorganism by producing some inhibitory substance. In 1921, Sir Alexander Fleming 1st observed this effect in his laboratory which leads to the discovery of antibiotic penicillin in 1928. After that discovery of penicillin, many scientists and researchers made several chemical alterations to obtain wide range of antibiotics for safe human and animal consumptions. Penicillin belongs to β -lactum family and few drugs which are the result of chemical alteration are, oxacillin, ampicillin, amoxicillin, etc.

Antibiotics are used for treating bacterial and few fungal infections in human as well as in animals. Some of their families are sulfonamide, β -lactum, fluoroquinolone, macrocyclics etc. Over last few decades, the use of antibiotics in human and animal husbandary practices has increased multifold due to the development of new generation antibiotics and also easy accessibility of antibiotics. However, it has been observed that their effectiveness and easy availability has led to their overuse, prompting bacteria to acquired resistance power. Acquired resistance is gained by bacteria from resistant bacterial strains via transformation, transduction or conjugation. Antimicrobial resistance is the ability of a microorganism to survive and multiply in the presence of an antimicrobial agent that would normally inhibit or kill this species of microorganism. Over use of antibiotics in animal as a growth promoter and weight booster became a trend, due to which excess antibiotics remains in the tissues or parts of edible animal products such as meat, milk, egg and enters into the food chain. This antibiotic resistance complicates the treatment of infection and associated with increased morbidity and mortality.

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animal protein origin for food safety point of view. It is small and general approach to review or evaluate different methods for detection of antibiotic residues in edible animal products.

Basically, analytical methods are mainly divided into two groups i.e. screening methods and confirmatory methods. Both the methods have its own advantages and disadvantages. Screening methods are qualitative or semi quantitative method. It is a rapid, selective, simple and specific method for detection of antibiotics and its residues whereas confirmatory methods are quantitative, sensitive and accurate. Selection of the suitable method will depend on the intended degree of accuracy, convenience, urgency, availability of resources, availability of technical expertise and cost.

SCREENING METHODS FOR THE DETECTIONS OF ANTIBIOTIC RESIDUES

In recent years, screening methods are very active methods for detection of antibiotic residues due to its nature of sample preparation, inexpensiveness, specificity and short result achievement time. There are different types of screening methods specific for wide range of products such as feed, meat, milk, and egg. Screening methods include microbiological screening, immunoassay screening and biosensor.

D) MICROBIOLOGICAL SCREENING METHODS

Microbiological inhibition assay is the earliest technique used for rapid screening for qualitative and semi quantitative detection of antibiotic residue and till now it is widely used in different research laboratories. Microbiological assay is divided into different types depending upon their mode of detection techniques i.e. zone of inhibition, UV/ Vis detection, conductimetry and luminescence [1].

In microbial zone of inhibition technique, the antibiotic residues in a sample are examined against the overgrown culture of test microorganism in a suitable media. Organisms mainly used for detection are *Escherichia.coli* at pH 6 and 7, *Staphylococcus.aureus* at pH 8 and 9 and *Bacillus.subtilis* at pH 6-8. It is an agar diffusion method based on inhibition – diameter measurement using vernier calliper. In this technique, the presence of antibiotic residue is detected by observing the zone of inhibition against the susceptible bacteria. Diffusion of an antibiotic residues or antimicrobial substance are shown by the formation of inhibition zone. The zone of inhibition should be ≥ 2 mm in diameter for the acceptance of results. This technique is mainly used for the detection in poultry meat, slaughter animal, milk.

In UV/VIS microbial screening method, the presence of antibiotic residue in the test sample is detected by changing color of the media due to the variation in pH. The principle behind this method is that if the test samples doesn't contain antibiotic residue which are incubated at 65°C for 3-4hrs with media containing bacterial spores (*Bacillus stearothermophilus*), it grows with the formation of acid which led to the change in pH of the media. The color changes to yellow due to the production of gas [2].

Now a day, different commercial microbial kits are manufactured by various companies which depend on the above principle for wide range of samples. It has been studied that commercial microbial kits are very effective, spontaneous, specific, low cost and high throughput with less time duration. Premi test is another type of test which contains viable spores of a strain of *Bacillus stearothermophilus* which is sensitive to antimicrobial residues, such as beta-lactams, tetracyclines, macrolides and sulphonamides. The growth of the strain is inhibited by the presence of antimicrobial residues in muscle tissue samples. It is mainly used for detection in poultry meat and slaughter animal. It is specific and mainly used for beta-lactams, tetracyclines, macrolides and sulphonamides. It has been seen in literature studies that the detection capabilities of Premi Test for amoxicillin, ceftiofur, tylosin and tetracycline were at the level of the respective maximum residue limits in muscle samples or even lower [2].

Delvotest is another standard method for antibiotic residue testing in the global dairy industry. It detects broad spectrum of antibiotic in milk. The test is made of an agar gel containing bacterial spores and a colour indicator. The milk sample is added onto the agar gel, and the test is incubated at 65°C for 3-4 hrs. The principle of the test is based on the diffusion of inhibitory substances that may be present in the milk sample into agar and inhibit the growth which can observe visually. In course of time various modifications have been done to develop the test more specific to particular group of antibiotic. In 1970s, the first version of Delvotest developed was the Delvotest P, which is designed to detect β -lactams group. Then Delvotest SP which is capable of detecting a broader spectrum of substances, notably sulphonamides, but also has increased sensitivity to tylosin, erythromycin, neomycin, gentamicin, trimethoprim and other antimicrobial inhibitory substance. Both Delvotest P and Delvotest SP are identical, the only difference in incubation time i.e. for Delvotest SP it is 2½ hrs and Delvotest P is of 3-4hrs. The Charm AIM-96 test is a micro-titre plate test, based on the principles of Delvotest and capable of detecting β -lactams, sulphonamides, tetracyclines, macrolides and aminoglycosides in 96 samples simultaneously. In the Charm test, liquid medium is used instead of agar

or gel. The Charm Farm Test-'Vial' and the Charm Farm Test-'Mini Vial' are two versions of the charm AIM_96 test but are designed for fewer samples [1,2].

II) IMMUNOASSAY METHOD

Immunoassay method is a semi quantitative method and used widely by different laboratories for its high sensitivity and specificity, simplicity and cost effectiveness. It is mainly based on the principle of antigen-antibody interaction [1].

ELISA techniques are capable of detecting low levels of residues in tissues, milk, egg, and feed. These assays are rapid method, require little sample clean-up and processing and impart themselves to routine testing of large numbers of samples. This detection method is used for qualitative screening or quantitative analysis and detection of specific drugs which include sulfonamides, chloramphenicol, β -lactams and aminoglycosides. It is used for conducting basic research to monitoring the presence of one or more antibiotic residues in a sample. ELISA technique is useful for analyzing antibiotic residues in milk, meat, fish, eggs, feed and honey.

The enzyme immunoassay technique for detection of antibiotic is based on the competition between the antibiotic to be assayed and the antibiotic conjugate, for binding to antibody directed against antibiotic coated onto micro wells. The sample containing the antibiotic, and antibiotic conjugate, when added to the microtiter wells, compete for binding to a limiting number of antibody sites. After incubation, each well is rinsed in order to remove non-bound components. The bound enzymatic activity is then measured by the addition of a chromogenic substrate. The intensity of the colour developed is inversely proportional to the concentration of antibiotic in the sample. The concentration is calculated on the basis of a standard curve. Now a day, ELISA kits are manufactured by several companies and are widely available in worldwide.

III) ENZYMATIC COLOURIMETRIC ASSAYS

It is another technique for detection of antibiotic which is an enzymatic test widely used for the detection of beta-lactam antibiotic residues in milk. It is a specific method with good sensitivity to this group of antibiotics and enables results to be obtained within a short time. Penzym test is one of the qualitative enzymatic colorimetric methods for a rapid determination of β -lactam antibiotics in milk. The test principle is based on establishing the level of inactivation of the DD-carboxypeptidase enzyme by β -lactam antibiotics. These residues bind specifically with the enzyme and inactivate it, thus interfering with the bacterial cell wall formation. The degree of inactivation of the enzyme depends on the amount of antibiotics present in the sample [3].

IV) BIOSENSOR

In these biosensors, specific receptors or enzymes are utilized to generate a bio-recognition reaction, whose signal is then detected with a suitable transducer. It is latest generation screening method and are widely accepted due to fully automatical working procedure. Receptor/enzyme-based biosensors usually employ optical or electrochemical signal detection principles. For optical detection, Surface Plasmon Resonance (SPR) is most commonly used. The application of SPR technology secures low detection limits, even below the established MRL values. The main drawbacks of SPR biosensors are their high cost; nonspecific binding of compounds of sample matrix to the sensor surface; and assay time (including chip preparation, incubation of receptors, detection, and system regeneration), which could take even a couple of day. There are different types of biosensor i.e. microbial biosensors, immunosensors, Molecularly Imprinted Polymer (MIP) sensors, receptor and enzyme-based biosensor and aptasensor [4].

CONFIRMATORY METHODS FOR ANTIBIOTIC RESIDUES DETECTION.

There are certain quantitative methods for the detection of antibiotic and its residues from the samples. It is used to detect antibiotic residue with different sample preparation techniques. Most extraction procedures employed in reference methods are time consuming and costly, and required large volume of sample and solvent. Confirmatory method involves liquid chromatography-mass spectroscopy (LC-MS), UPLC and HPLC and Capillary zone electrophoretic (CZE) method [1].

A) LIQUID CHROMATOGRAPHY – MASS SPECTROSCOPY (LC-MS)

Liquid chromatography-mass spectroscopy (LC-MS) is a combination of two highly equipped instruments. Both of them works synergistically and enhance the result to more specific and sensitive. In this methodology, liquid chromatography is used for separating the individual components in antibiotics present in the sample and mass spectroscopy is used to detect the structural identity and concentration value of each component. The method is used for the detection of wide spectrum of antibiotics in poultry muscle tissue. It is based on the matrix solid-phase dispersion technique with heated water as the extractant followed by liquid

chromatography (LC)–tandem mass spectrometry (MS). Targeted compounds are extracted from tissues by heat treatment at 70°C. After several processes of acidification and filtration, extracted liquids are injected into the LC column. MS data acquisition was performed in the multi-reaction monitoring mode, selecting two precursor ions to product ions transitions for each target compound. It has been found that the absolute recovery data ranged between 70% and 78%. The accuracy of the method is determined at three spike levels in muscle tissues. The limitation associated with this method is laborious sample preparation, time consuming and the cost [5].

B) ULTRA-PERFORMANCE LIQUID CHROMATOGRAPHY (UPLC) AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

HPLC and UPLC are highly equipped methods used for quantification of antibiotic residues. It is based on the principle of chromatography in revised ways. These are the confirmatory technique for detection of antibiotic but it is time consuming, laborious sample preparation, costly and highly equipped laboratory with skilled personnel. Chromatography method is used to separate mixtures of substances into their components on the basis of their molecular structure and composition. This involves a stationary phase (a solid, or a liquid supported on a solid) and a mobile phase (a liquid or a gas). The mobile phase flows through the stationary phase and carries the components of the mixture with it. Sample components that display stronger interactions with the stationary phase will move more slowly through the column than components with weaker interactions. In both the process, solvent is drip from the column under high pressure up to 400 atmospheres rather than gravity. Both are same in their working principle but there are few differences which are listed below [6, 7].

Table-1: Comparison Between UPLC and HPLC

Characteristics	HPLC	UPLC
Particle size	3 to 5µm	Less than 2µm
Maximum backpressure	300-400 bars	1000 bars
Analytical column	C18	UPLC BEH C18
Column dimensions	150 X 3.2 mm	50 X 2.1 mm
Injection volume	5µL	2µL
Column temperature	30 ° C	65 °C
Total run time	10 min.	1.5 min
USP resolution	3.2	3.4
Plate count	2000	7500
Flow rate	3.0 ml/min	0.6ml/min

C) CAPILLARY ZONE ELECTROPHORETIC METHOD

It is an alternative method of liquid chromatography used for the quantitative studies of antibiotics and its residues. Capillary Zone Electrophoresis (CZE), also known as free solution capillary electrophoresis. In this technique, antibiotics present in the sample are extracted by extraction procedure and run in the capillary. The separation is based on the differences in electrophoretic mobility, which is directed proportional to the charge on the molecule, and inversely proportional to the viscosity of the solvent and radius of the atom. The velocity at which the ion moves is directly proportional to the electrophoretic mobility and the magnitude of the electric field. It is mainly used for the separation and quantification of a mixture of eight cephalosporins – cefadroxil (CFL), cefixime (CIX), cefuroxime sodium (CFR), ceftriaxone sodium (CTR), ceftizoxime (CFT), cefaclor (CFC), cefradine (CFD), and cefotaxime (CTA). It is then observed by UV absorbance at 214nm. Limits of detection are in the range 0.5–5 µg mL⁻¹ [8, 9].

CONCLUSION

This manuscript provides a brief overview of the screening and confirmatory methods for detection of antibiotic residues in different edible animal products. Microbial screening methods are rapid, sensitive and specific with less sample preparation time. Results can be detected by using simple techniques such as UV/VIS, zone of inhibition by vernier caliper, indicators etc. It does not require any highly-equipped laboratory and skilled technician. It is semi quantitative and qualitative method for detection. There are many commercial kits available in the market to make the screening more precise and easier. Other methods such as ELISA technique, enzymatic colorimetric assay, biosensor is also used for the screening of antibiotic residues in animal-based food commodities.

In contrast, confirmatory methods i.e. LC-MS, UPLC, HPLC, CZE are quantitative method for detection of antibiotic residues. These methods are highly selective, specific, and detection level is more accurate and consistent than screening methods. Both the methods have their own advantages and disadvantages, so the application methods are depending on urgency, detection level, accuracy, convenience, availability of resources, availability of technical expertise and cost.

Now a day, there is an on-process development on the screening methods for their rapidness, specificity, low cost, convenience and high throughput. Nano-technological studies are conducted for the development on screening method for better prospects.

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