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Receptor-Based Screening Assays for the Detection of Antibiotics Residues – A Review

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Abstract

Consumer and regulatory agencies have a high concern to antibiotic residues in food producing animals, so appropriate screening assays of fast, sensitive, low cost, and easy sample preparation for the identification of these residues are essential for the food-safety insurance. Great efforts in the development of a high-throughput antibiotic screening assay have been made in recent years. Concerning the screening of antibiotic residue, this review elaborate an overview on the availability, advancement and applicability of antibiotic receptor based screening assays for the safety assessment of antibiotics usage (i.e. radio receptor assay, enzyme labeling assays, colloidal gold receptor assay, enzyme colorimetry assay and biosensor assay). This manuscript also tries to shed a light on the selection, preparation and future perspective of receptor protein for antibiotic residue detection. These assays have been introduced for the screening of numerous food samples. Receptor based screening technology for antibiotic detection has high accuracy. It has been concluded that at the same time, it can detect a class of drugs for certain receptor, and realize the multi-residue detection. These assays offer fast, easy and precise detection of antibiotics.

Abbreviations: AMP (ampicillin); APH (3') -IIa (Aminoglycoside 3'-O phosphoryl transferase II a); ATP (adenosine triphosphate);AOAC (Association of Official Analytical Chemists); CE (capillary electrophoresis); DHPPP (6-hydroxymethyl-7,8-dihydropterin pyrophosphate); DHPS (dihydropteroate synthase); DIG (digoxigenin); EU (European union); ELRA (Enzyme-labeling receptor assay); ELISA (enzyme linked immuno-sorbent assay); FDA (food and drug administration)); GICA (gold immuno-chromatographic assay); HPLC (high performance liquid chromatography); HRP (horse radish per-oxidase); HMM (high molecular mass); LC-ESI-MS/MS (liquid chromatography-electrospray-tandem mass spectrometry analysis); LMM (low molecular mass); LOD (limit of detection); MS (mass spectrometry); MIP (molecularly imprinted polymer); Mg (magnesium); MRL (maximum residue limit); PABA (para-aminobenzoic acid); PBP (penicillin binding protein); SPR (surface plasmon resonance); Tc (tetracycline); TIM (triose phosphate isomerase); TAR (HIV-1 Tat-responsive element); UTI (urinary tract infection).

Keywords: antibiotics; labeling technique; residue; receptor; screening assays

1. Introduction

Antibiotics are drugs, which are originated from synthetic, semisynthetic and by a natural way. They have been widely used in the treatment of infectious diseases in animals and humans. Moreover, antibiotics also act as growth promoting factor in veterinary field [1]. Antibiotic residues in food products become problematic by many aspects such as bacterial resistance against the antibiotic, emergence of hypersensitivity reaction, aplastic anemia in several sensitive humans and can also hinder the growth of lactic acid bacteria [2, 3]. Regarding these outcomes, numerous laws and regulation have been established, such as the development of antibiotic residue detection method [4]. Methods for the detection of antibiotic residues are mainly categorized into two groups, screening and confirmatory. Confirmatory assays are typically based on quantifying the concentration of the analyte [1]. However, application of these methods is restricted due to time consuming, expensiveness, skilled personals and difficult sample preparation [1, 5]. Screening methods are to decide whether or not a sample comprises the antibiotic residue. In case of positive outcomes, it is essential to use an appropriate method for verification, recognition and quantification of residue [6]. Screening methods identifies an analyte or a family at the level of concern, and commonly delivers semi-quantitative results. The model features of a screening assay are fewer ratios of false-positive samples, high throughput, simplicity, time-saving, low-cost and better selectivity [1].

In recent years the receptor assay technology has become a hotspot for the screening of antibiotic residues. Since receptors can only bind to active substances therefore, the receptor technology has higher accuracy in the detection of antibiotics [5, 7, 8]. Screening methods based on receptor proteins, have been established for the detection of antibiotics residues such as β-lactam, sulfonamide, tetracycline, for example, radio receptor assay, colloidal gold receptor assay, enzyme colorimetry, enzyme labeling assay and biosensor assay [1, 5, 7, 8]. There is still a solid desire to develop and implement a simple economical and time saving antibiotics screening assay. Several methods were considered to be applied for the screening of antibiotics, which are specific but expensive and time consuming.

This review is planning to deliver an overview of newly established receptor based screening methods for antibiotic residue detection in various matrices. It will also benefit us to recognize the limitations in a direction to permit a stable choice among receptor based screening and conventional methods, to be operational in drug residue monitoring program. These assays hold boundless potentials for much broader application in the future. Many of the receptors based screening assays are immunoassays and more currently, biosensors assays. In general, these assays are easy, fast, accurate, high throughput and time saving.

2. Methods used for antibiotic residue detections

Numerous methods have been used for the screening of antibiotics residue, they are mainly categorized into three groups: chromatographic, microbiological and immunoassay. Chromatographic method used for the qualitative or quantitative antibiotics residue analysis. This assay mainly includes, such as high performance liquid chromatography (HPLC) [9, 10], capillary electrophoresis (CE) [11, 12], mass spectrometry (MS) and so on. Continuous development of CE, hyphenated technology has become the focus of chromatographic method, such as liquid chromatography–electrospray–tandem mass spectrometry analysis (LC–ESI-MS/MS) which has been used for the detection of macrolides and lincosamides in milk and muscle [13]. Another method used for the detection of antibiotic residue is microbiological screening assay, a specific recognition reaction among antibiotics and sensitive organism present in the sample takes place [1]. Microbiological assays are based on the microbial growth inhibition by measured antibiotic concentration [14]. Compared with spectrophotometry and reversed phase high performance liquid chromatography, microbiological method is an acceptable alternative method for the routine quality control of linezolid in injectable solution [15]. This mothed is inexpensive and simply operated. However, it is time-consuming, poor stability and has more interference factors.

A precise reaction between antibody and antigen is the basic principle for immunoassay [16]. Immunoassay is sensitive, precise and easy to use. However, due to its high specificity of antibody, this method can detect different drugs simultaneously [1, 17]. Another interesting approach for the detection of antibiotic residue is biosensor method. Biosensor assay is based on

recognition element such as protein, cell, enzyme, nucleic acid, tissues…. which combined with an element for transducing the signals [1, 18].

3. Antibiotics and their interaction with the corresponding binding proteins

The interaction of antibiotics with their targets can result in either killing the bacteria or inhibiting the bacterial cell growth. For example, β-lactams work as cell wall synthesis inhibitor. All the antibiotic groups and their interacting proteins reviewed here are listed in Table 1 [1, 19].

3.1 Interaction of β-lactams and their binding proteins

β-lactams which consist of β-lactam ring, including penicillin, cephalosporin, play an important role within the field of veterinary and human medicine. This group of antibiotics bind to their receptor protein termed as penicillin-binding proteins (PBPs), thus inhibiting the bacteria cell wall synthesis [20-23]. Regarding to their size of molecular mass, PBPs can be divided into two categories, high-molecular-mass PBPs (HMM PBPs) (≈50-100 kDa) and low-molecular-mass PBPs (LMM PBPs) (≈30-40 kDa). Whereas according to their function HMM PBPs are divided into Class A and Class B. In Class A HMM PBPs, the N-terminal domain has the transglycosylase activity, and the C-terminal domain has transpeptidase activity. Class B HMM PBPs only have the activity of the transglycosylase. LMM PBPs have D, D-carboxypeptidase activity, which is generally used for the enzyme colorimetry for detection of β-lactams [24].

BlaR is a kind of PBP, which is involved in the induction of β-lactamase in Gram-positive bacteria, such as *Bacillus licheniformis*. BlaR shows similarities in primary structures of the class of β-lactamases [23]. Both of the BlaR from *B. licheniformis* and DD-peptidase/PBP from *Actinomadura R39* belong to the receptor of β-lactams [25-27]. In summary, the number, type and function of PBPs are different in different bacterial species, e.g., *Bacillus subtilis* has a dozen kinds of PBPs. Since different PBPs show different affinity and sensitivity to the same kinds of β-lactam drugs, the selection of PBPs are important for the establishment of methods for detection of β-lactams [28].

3.2 Interaction of sulfonamides and their binding proteins

Sulfonamide drugs were the first operative antimicrobial agents synthetically being established and are still extensively used in the field of veterinary [7]. The existence of sulfonamide residues in animal derived food have a toxicological and regulatory alarm because they can be carcinogenic, cause hypersensitivity reaction and therapeutic incompetence in human medicine [29]. To decrease the risk of sulfonamides residue, the maximum residue limit (MRL) was set as shown in Table 1 [7].

DHPS (dihydropteroate synthase); is the receptor for sulfonamides, which act as competitive inhibitors with regard to PABA (para-amino benzoic acid). In *Streptococcus pneumonia*, the matching subunit of DHPS encoded by the gene SulA is 34 kDa. There is a fixed order of substrate binding: DHPPP (6-hydroxymethyl-7,8-dihydropterin pyrophosphate) binds first, tailed by the second substrate PABA. Binding of sodium pyrophosphate decahydrate also permits the enzyme to identify PABA or sulfonamides, which performs PABA analogues [7, 30]. In *E. coli* and *S. aureus* DHPS crystal structure has been stated: that the protein accepts a TIM-barrel type fold (triosephosphateisomerase (most common enzyme fold)), with the active site situated at the end of the central b-barrel [31]. The identification of the pterin moiety of the substrate DHPPP seems to be well conserved between the two enzymes, containing amino acids that are highly conserved through all the species. The interaction of the drug with Arg63 and Lys221 has been displayed by the exposure of sulfonamide to the *E. coli.* The common features of substrate and sulfonamide recognition by the enzyme are highly conserved through different species. A natural implication from these results is that the DHPS:DHPPP binary complex is the target for sulfonamides in *pneumococci*, rather than the apoprotein form. The equilibrium binding studies have permitted us to associate the binding affinities of sulfonamides for DHPS straightly with the affinity for PABA [7]. In summary the type of DHPS varies regarding species with the same function. Sulfonamides bind with higher affinity to the DHPS:DHPPP as compared to DHPS:pyrophosphate complex used in the equilibrium bindings studies. The selection of DHPS for using in the development of novel micro-plate assay for the screening of sulfonamide is very important.

3.3 Interaction of tetracycline's and their binding protein

Tetracycline's are among the record usually used broad-spectrum antibiotics [32]. The initial binding site of tetracycline is positioned inside the decoding center of the small subunit and

overlays in the position with the anticodon loop of an A-site bound tRNA [33]. Resistance to tetracycline has been developed by four key mechanisms. From the attained resistance mechanisms, the utmost widespread tetracycline resistance mechanism is an efflux pump; so far, 28 different classes of efflux pumps have been recognized. Resulting diligently is the so-called ribosome protection proteins, and these proteins bind to the ribosome and eliminate the drug from its binding site. Another resistance mechanism which is thought to be less prevalent contain two different genes, it modifies tetracycline and encourages their degradation, which is encoded by mono-oxygenases, and mutations within the 16S rRNA that decrease the binding ability of the drug for the ribosome. Moreover, a unique tetracycline resistance factor, tetU, encoded on the plasmid pKq10 in *E. faecium* has been stated to consult some tetracycline resistance [33]. The ultimate common mechanism includes a TetA, a protein associated with membrane that transfers out antibiotic from the cell afore it stops the polypeptide elongation. Together to TetA and divergently concerned is TetR, and their gene product strongly switches the expression of each TetR and TetA. The intergenic region amid the TetA and TetR genes encloses two matching operators. TetR interact to operators and therefore stops transcription from each promoter. The operator sequences overlay with promoters for TetR and TetA, thus inhibiting the expression of each of the genes. Once tetracycline enters it interacts with Mg^{2+} then it will bind to TetR, and a conformational modification occurs that track the TetR to make it incapable of binding to DNA. As a result, TetA and TetR are expressed as shown in Fig. 1 [8, 32, 34].

3.3.1 TetR structure

The TetR homodimer is organized by two matching monomers that fold into 10-helices with connecting turns and loops. Hydrophobic helix-to-helix contacts essentially stabilize the three dimensional structure of TetR monomer. The overall arrangement of the TetR homodimer can be separated into two DNA-binding domains located at the N-terminal end of every monomer, and a regulatory core domain involved in dimerization and ligand binding. The regulatory domain is responsible for dimerization and encloses, for every monomer, a binding pocket that kept tetracycline in the existence of a divalent cation. In both monomers the binding pocket of tetracycline is identical. After the entering of [TcMg] complex into the tunnel, so it's A ring creates links with loop 4-5, and the contact with the effector generates a cascade of conformational modifications. The 6-7 loop is also pushed near the inducer, so that Arg104 and Pro105 interact with tetracycline. Translation of 6 forces 4 to move in the same direction due to

van der Waals contacts. His 64 of 4, anchored to 5 and to tetracycline, acts as a pivot point, and 4 moves like a pendulum. As a consequence of the rotation of 4 and 4, recognition helices 3 and 3 moves more apart and the contacts of DNA are disturbed [32, 35, 36].

In conclusion the number, type and function of TetR protein are different in different species of bacteria. TetR protein displays a direct binding with tetracycline, and it seems feasible that TetR proteins play an essential role in the identification of tetracycline by using different method.

3.4 Interaction of fluroquinolones and their binding proteins

Over a period of a few spans, quinolones have been renovated from a slight and insignificant family of drugs used primarily for the treatment of urinary tract infections (UTI) to be certain of the most usually prescribed antibacterial worldwide [37]. Quinolones interact in a non-covalent way at the enzyme-DNA crossing point in the cleavage−ligation active site. Drugs interact with the protein and introduce into the DNA at both cleaved scissile bonds. It was decided that gyrase plays the primary target role and topoisomerase IV is a secondary target for quinolone antibiotic. Dependable with this decision, quinolones are more effective against *E. coli* gyrase than topoisomerase IV, and encourage upper levels of gyrase−DNA cleavage complexes in bacterial cells. Remarkably on the base of genetic study that rather than gyrase, topoisomerase IV have been found as the primary target for ciprofloxacin in *Streptococcus pneumonia* [37].

DNA gyrase protein has a tetramer structure, which is consisting of each of two A and B subunit. This protein binds and wraps DNA into a positive supercoil. Gyrase eliminates negative supercoils from DNA during the lack of ATP (adenosine triphosphate). Supercoiling is depressed by inhibitors of gyrase and decrease-of-function alleles that rise in reaction to an insufficiency of topoisomerase I. This enzyme stops excess supercoiling from accumulating. These declines in supercoiling rise up the expression of gyrase, which indicates that a homeostatic mechanism present for the controlling of supercoiling. The drive of replication and transcription complex is also facilitated by gyrase over DNA by the addition of negative supercoils. The supercoiling action of purified gyrase has been inhibited by nalidixic and oxolinic acid when it is extracted from wild-type cell, while it doesn't effect on mutant strain, which contains resistant gene gyrA (nalA).

Alike gyrase, topoisomerase IV is also consisted of subunits, two of par C and two of par E. Gyrase and topoisomerase IV both use a double-strand passage action of mechanism. Though these enzymes diverge in a central way, topoisomerase IV doesn't wrap the DNA while gyrase wraps DNA around itself. In difference as like topoisomerase II of eukaryote topoisomerase IV is probable to identify DNA crossovers. It has been observed that topoisomerase IV has the ability for the decatenation of DNA earlier to the replication of cycle completion. It has been observed that 15 to 50 times quinolone is required for the inhibition of topoisomerase IV decatenating activity in *E. coli* as compared to dose required for inhibition of gyrase supercoiling activity. In recent years topoisomerase IV has been identified as second target of action [38]. In order to summarize gyrase and topoisomerase consist of two subunits. These subunits are involved in the interaction with fluroquinolone. Regarding to the feasibility of using Gyrase and Topoisomerase for the screening of fluroquinolone seems not to be suitable.

3.5 Interaction of chloramphenicol and their binding protein

Chloramphenicol group of antibiotic have a broad spectrum of antimicrobial activity and is used for the treatment of both animal and humans [39]. Chloramphenicol is firstly, isolated from *Streptomyces venezuelae* [40]. Chloramphenicol inhibits protein chain elongation by targeting peptidyl-transferase center [41]. It has been observed that chloramphenicol stops the peptidyl-transferase activity by hindering the transfer RNA binding to A-site [42]. However, notably, they are safe in domestic animals, but they express numerous effects to human health for example gastrointestinal disorder, hypersensitivity reaction and depression of bone marrow [39]. Regardless of legal banning, evidence proposed the use chloramphenicol in poultry farm in some countries [39]. An evidence had been displayed by the early observation that chloramphenicol interact to the two site for 50S subunit [43]. Furthermore, a suggestion is made by the evidence that binding sites of chloramphenicol incompletely overlap with that of macrolide and erythromycin [41]. By the observation, it has been reported that L16 protein plays a significant role in binding with chloramphenicol antibiotic. This suggestion is based upon the examination which displays the covalently association of L16 protein and iodoamphenicol residue [44]. It has also been examined that L16 protein plays an essential role in the reconstruction of peptidyl transferase activity [45]. On the base of X-ray crystallography in a complex structure of antibiotic and ribosomal subunit, some other binding sites for chloramphenicol have been

observed. According to one of the complex with *Haloarcula marismortui* large subunit of ribosome chloramphenicol interact to the hydrophobic crevice. While in another complex, the binding of chloramphenicol with the A-site of *Deinococcus radiodurans* 50S subunit has been noticed [41].

The discovery of a dynamic efflux pump for chloramphenicol is stretched the identified mechanistic range of many bacteria to chloramphenicol resistance. The efflux system has a high specificity and have the ability to put up a wide range of structurally dissimilar agents for example detergents, dyes, antibiotics, solvents, etc. [46]. Numbers and range are varied from organism to organism. Gene such as cmr of *E. coli* and TtgR of *P. putida* displays the phenotypic features of active efflux for chloramphenicol [47]. TtgR belong to TetR family, which is basically a transcriptional repressor proteins and is also shown to be responsible for the expression of drug transporter gene in bacteria [46]. Classically, these repressor proteins consist of two functional domains a C-terminal domain which is less conserved and take part in binding and dimerization and N-terminal domain which is highly conserved and involved in DNA binding [46]. Regarding to the summary of chloramphenicol binding protein L16, their feasibility has been observed in the detection of iodoamphenicol residues, and the resistance protein TtgR also shows a clear binding with chloramphenicol. The choice of selection L16 and TtgR protein is very important in the method development of chloramphenicol screening.

3.6 Interaction of aminoglycosides and binding protein

Aminoglycosides is a class of a broad spectrum antibiotic with aminocylitol and amino-sugar in their structure. The main target for aminoglycoside antibiotic is located on the 30S ribosomal subunit. The combination of tRNA and 30S ribosomal subunit plays a significant role in the synthesis of protein. Aminoglycoside directly binds to the A-site of 30S ribosomal RNA, which affect the entire process and interfere with protein translation and lead to inaccuracy in protein synthesis [48, 49]. In addition to the ribosome, the function and structure of various RNA constructs, e.g. TAR (HIV-1 Tat-responsive element), ribozyme, has been moderated by binding with aminoglycoside [50]. Ribosomal protein S12 located on 30S subunit encoded by RpsL gene. RpsL gene thought to be involved in the interaction of streptomycin with 16S rRNA. It has been reported that amino acid mutation in S12 protein can cause resistance to streptomycin [51]. Streptomycin binds to 16S RNA through hydrogen bond and salt bridge. Furthermore,

streptomycin has an also direct effect on S12 protein [52]. Aminoglycoside 3'-O phosphoryl transferase II a (APH (3') -IIa) has the function of the phosphorylation of kanamycin, and APH (3')-IIa can specifically bind and recognize kanamycin molecule through the mutation [53]. Aminoglycosides modifying enzyme and 16S rRNA methylation enzyme showed an important role in prevalence of resistance against aminoglycosides. These enzymes efficiently block the target site of aminoglycoside and lead to lose the antibacterial activity [54]. Some of the researcher have also been evidenced that aminoglycosides modifying enzyme such as AAC and the aminoglycoside phospho-transferase play an important role in the prevalence of resistance against aminoglycosides [55, 56]. In summary; the selection of A-site of 30S ribosomal RNA plays an important role in the method development for the screening of aminoglycoside antibiotics.

4. Preparation of antibiotic binding protein and principle of receptor-based screening methods

The important feature for the screening of antibiotics based on receptor based assay is the preparation of receptor protein. Preparation of receptor is mainly based on the following three methods [57]. First; the whole bacterial cells are directly used as receptors, and these cells contain specific binding targets that can bind to a variety of antibiotics. It can be used to establish the method such as CHARM kit [58-60]. Second; the receptor protein can be obtained by extracting a particular receptor protein such as the separation of subcellular fractions and the protein purification. Third; receptor protein was expressed by heterologous recombinant. For example, for the N-terminal hydrophobic trans-membrane region of PBP2x was truncated, and over-expressed in *E. coli,* which can increase the soluble of the recombinant receptor protein, but does not affect the activity [61]. Receptor protein for the quinolone antibiotics had been prepared by many examiners, such as GyrA, gyrB, parC and pare E [62-67]. Bacterial DHPS protein is used as the basic target for the interaction with sulfonamide antibiotics. A receptor based micro-plate assay has been developed by using DHPS as binding protein [7]. However, the target resistance protein interaction of tetracycline is belonged to TetR family proteins [35, 46]. At present, the majority of technologies being developed for detecting the receptors of antibiotics and mainly of them are following receptor protein purification method to obtain receptors.

The receptor-based screening method is an analytical method based on the specific recognition between antibiotics and receptors. The receptor-based screening methods for antibiotics are divided into two major categories. One is the receptor adsorption method, which is based upon the combination of antibiotics and their binding protein, as similar in binding of antigen and antibodies in immunoassay [17]. Receptor adsorption assay commonly used binding protein as a receptor, such as PBP2x, BlaR for β-Lactam as shown in Fig. 2, DHPS for sulfonamide while TetO is used for tetracycline. There will be intact bacterial cells or cell components containing receptor, such as CHARM method. Receptor adsorption assay can be combined with a variety of labeling techniques, which can improve the sensitivity, such as isotopic labeling, enzyme labeling, and colloidal gold labeling, which can also label the receptor as well as the drug antigen. The other is the enzyme colorimetry which has been discussed [68]. The principle is to determine the inactivation degree of antibiotics class, but it has low sensitivity to antibiotics. Both receptor adsorption method and enzymatic colorimetry can be combined with biological sensor. A variety of kits for the detection of antibiotics shown in Table 2 [69, 70].

5. Receptor based screening assays

Receptor is a macromolecular substance that can identify and selectively bind to ligand. Antibiotics have the corresponding target position in the bacteria, which are known as the receptors of the antibiotics. The role of receptors and ligands to meet three main characteristics: specificity, saturation and high affinity. Antibiotics combine its specific receptor to hinder the normal function of the receptor, which plays an antibacterial role. The main receptor screening assays of antibiotics are discussed in this chapter.

5.1 Radio receptor assay

Radio receptor analysis method recognized by the Association of Official Analytical Chemists (AOAC), and was identified as the standard testing tool. This method has been widely used in various countries, laboratories; ministry of agriculture and other agencies, such as China has made the relevant national standards for the radio receptor analysis of antibiotics in animal derived foods. CHARM I and II tests employ radio-receptor assay. As shown in principle A (Fig. 2), radioisotope (^{14}C or ^{3}H) labeled penicillin competes the receptors with free drug. The rate of

binding is measured through a scintillation counter and it can analyze the antibiotic content in the sample.

CHARM I is the method for the detection of β-lactams residues in milk, it was the first rapid research method that admit by the AOAC. CHARM II test can detect β-lactams, tetracyclines, sulfonamides, aminoglycosides, chloramphenicols, novobiocin, and macrolides, the detection range of sample include milk, meat, seafood, honey and so on. Salter used CHARM II test for the detection of tetracycline residues in pork tissue, the LOD is 10 $\mu g \cdot kg^{-1}$ [71]. It can effectively detect the tetracycline residues in pork with more than 10 $\mu g \cdot kg^{-1}$ in compliance with Russian import regulations. CHARM MRL BLTET test used Rapid One-Step assay lateral flow technology for the detection of β-lactams and tetracycline's residues in ewe and goat milk, the limit of detection (LOD) of amoxicillin, ampicillin, benzylpenicillin, dicloxacillin, oxacillin, cefacetrile, cefalonium, cefapirin, desacetylcefapirin, cefazolin, cefoperazone, cefquinome, ceftiofur, desfuroylceftiofur, cephalexin, chlortetracycline, oxytetracycline and tetracycline are lower than MRLs which has been regulated by EU [60].

5.2 Enzyme-labeling receptor assay

Enzyme-labeling receptor assay (ELRA) is based on the different combinations of package material, enzyme species, and competitive methods. This method is based on the specific interaction of receptor, ligand and enzyme labeling technique, and shows similar principle to ELISA. This is the most widely used method in receptor assays; shown in Table 3. Structural modification of the receptor protein will change its affinity for antibiotics, which can be used to detect different antibiotics. For example, PBP2* can be obtained by intercepted the N-terminal hydrophobic transmembrane region of PBP and expressed in *E.coli*. It has been observed that PBP and PBP2* have different affinities for different drugs, and it is also used as a receptor for detection the antibiotic residues [72, 73].

The carboxy-terminal of BlaR from *Bacillus licheniformis* was used to establish ELISA which is mainly based on receptor protein for the detection of β-lactam antibiotics. This assay was grounded on directly competitive inhibition of binding of horseradish peroxidase (HRP)-labeled ampicillin to the immobilized BlaR-CTD [5]. It can simultaneously detect 15 β-lactams, the detection limits of 11 β-lactams are lower than MRLs which are regulated by EU. By incorporating a 6 His-tag at the N-terminus to DHPS of *S. pneumonia* was purified in a high

amount for the detection of sulfonamide residues. The sensitivity of this assays were improved by optimizing some chemical and physical conditions, And below the 100 $\mu g \cdot kg^{-1}$ level of PABA and 9 sulfonamides could be detected under optimized condition [7]. There are numerous kits that used receptor analysis techniques for the detection of antibiotic residues. Three SNAP^{TM} kits with LOD for antibiotic residues at or below the US Food and Drug Administration (FDA) tolerance levels were used for the initial screening of antimicrobial residues in milk samples. The New SNAP β-lactam Test Kit was used to screen the following β-lactam drugs: penicillin, amoxicillin, cephapirin, and ceftiofur residues in raw milk. In addition, the SNAP Test Kit can also screen the tetracycline, sulfonamide and gentamicin residues in milk [74].

5.3 Colloidal gold receptor assay

This method used the colloidal gold colored particles to label the receptor protein for the detection of antibiotic residues. Degelaen used the BlaR-CTD as the receptor, lined with biotin and used colloidal gold to mark the protein, which can be used to detect β-lactams in 5 min, the LOD is lower than MRLs which regulated by EU [75]. Beta Star and Twins Sensor BT produced by Belgium, and Charm Rosa MRL produced by US are using the colloidal gold-labeling receptor assay (Table 4). After adding the sample to the colloidal gold test strips, there will be 2 red bands, if the test band is weaker than the control, the result is positive, otherwise negative. It is simple and rapid, and it can be detected only in a few minutes.

Gaudin established a colloidal gold receptor assay for the detection of sulfonamides and tetracycline residues in hone, [76, 77] while Chen has developed a novel gold immune-chromatographic assay (GICA) based on anti-β-lactam receptors by replacing with the antibodies which are used traditionally, It has been successfully permits a rapid and simultaneous detection of 15 β-lactams in milk samples in 5-10 min, and it satisfied the MRLs which are set by the EU [78].

5.4 Enzyme colorimetry

Enzyme colorimetry was usually used to detect β-lactam antibiotics in milk. The principle of enzyme colorimetry is the detection antibiotic residues by measuring the degree of inactivation of the DD-carboxy peptidase for β-lactams. The β-lactams specifically bind with the enzyme and inactivate it, thus interfering with the bacterial cell wall formation. Under the action of this

enzyme, the peptide substrate releases D-alanine. Hydrogen peroxide can be produced when the D-alanine is oxidized into pyruvic acid, it can be presented by redox indicator.

Methods and kits for the detection of antibiotic residues using the DD-carboxy peptidase from *Actinomadura R39* have been discussed. This method can detect the β-lactams in milk, the LOD of which is 0.017 IU•ml^{-1}. In the Penzym test, if the colour of redox indicator is yellow, the sample contained the β-lactams, and if it is peach-coloured, the sample did not contain the β-lactam, while if the colour is between the yellow and peach, the content of β-lactams was between 0~0.017 IU•ml^{-1} in 15 min [79].

5.5 Receptor analysis combined with biosensor

As a new type information technology, the biological sensor played the important role in the field of the detection for antibiotics residues because of its highly automated and miniaturization. The most widely used which combed with the receptor assay are the surface plasmon resonance (SPR) and electrochemical sensor. A novel approach was established for the development of an indirect tetracycline screening assay based on SPR. Tetracycline resistance mechanism of gram negative bacterial species is the basic principle for this assay. A luciferase operon has been added to downstream of the repressor protein. Though after tetracycline entering into the sensor cell it will binds to the repressor protein, so the complex will released and it permits the luciferase gene expression which will lead to a luminescence signal shown in Fig. 3. Seven of the most commonly used tetracycline was screening on the base of this assays [8, 36, 80].

After adding the known amount of PBP2x* to the samples, only a sample containing PBP2x* would covalently bind to β-lactam antibiotics. After the incubation of DIG-AMP (digoxigenin-ampicillin), it was supplemented to non-complex PBP2x*. For the determination of tagged complex the solution was then introduced to biosensor. All molecule of PBP2x* forms a complex with DIG-AMP in the absence of β-lactams in the sample. While the complex PBP2x*/DIG-AMP is caught by the antibody against digoxigenin which have been immobilized on the surface of the sensor. The signal has been changed by the response of the sensor chip surface, the size of the signal changes and the content of antibiotics in the sample were inversely proportional. The detection limit of this method for penicillin G is 4 μg •kg^{-1} [81]. Gamella established the method that PBP2x was immobilized onto His-Tag-Isolation-modified magnetic beads, and enzyme labeled with HRP as a tracer for the determination of antibiotics. The

amperometric response was used as the transduction signal for the specific detection and quantification of β-lactam antibiotic residues in milk. This methodology showed very low detection limits, and it took only 30 min [82]. Others receptor analysis method combined with biosensor are shown in Table 5.

5.6 Advantages and disadvantages of receptor based screening assays

Different receptor analysis assays have different advantages, but they also have some disadvantages. As show in Table 6, the radio-receptor assay has high sensitivity, specificity and less sampling amount, and this technology is relatively mature, but radioisotopes is harmful to health, environmental pollution, and this mothed need special inspection equipment and high cost [83]. Enzyme-labeling receptor assay has less environmental pollution, easy sample pretreatment, and the enzyme markers are stable and less costly, which has a broad application prospect, but the protein stability is poor [5]. Colloidal gold-labeling receptor assay is simple and fast, but it is only suitable for single-determination, and can't be used for the quantitative detection [84]. Enzyme colorimetry is fast and convenient, but the result is easy to be interfered [85]. Biological sensor has the characteristics of high throughput, real-time monitoring, low reagent consumption, but due to the expensive equipment limits its application in the field of detection, also need to be improved on the portable device in the future [86].

6. Conclusion and future perspective

Antibiotics studied in this reviewed literature are β-lactams, sulfonamides, tetracycline's, fluoroquinolone's, chloramphenicol's and aminoglycosides. By this review we attempt to draw a diverse figure of awareness into a unified vision on receptor based screening of antibiotics. Receptor-based screening methods can be combined with a variety of labeling techniques to attain higher sensitivity. Quantum dots as a kind of semiconductor nano-crystals, the features of its multi-color mark can be detected simultaneously in several different compounds. It has been demonstrated that the quantum dots can be used to label the corresponding antibodies for the multi-residue detection of antibiotics [87, 88]. The combination of receptor analysis and quantum dot labeling will further expand the scope of antibiotic multi-residue detection. In addition, molecular imprinted polymers as a marker for the detection of antibiotics residues can make their

application wider [89]. Some other markers can also be used for the screening of antibiotics, such as SPR combined with molecularly imprinted polymer (MIP), thin film can be used to identify ciprofloxacin and azithromycin [90]. Ultrasensitive electrochemical aptasensor which is based on metal ions doped metal organic frame materials can be used for the simultaneous detection of oxytetracycline and kanamycin [91]. Although it is not currently used for receptor-based screening methods, it is believed that with the development of technology, these markers will be gradually applied to this mothed.

In order to summarize, the receptor-based screening methods of antibiotic residue can be improved from the labeling technique, in order to obtain higher sensitivity and expand the types of test substance. In addition, due to the various types of antibiotics binding protein such as PBPs, DHPS, Tet, gyrase and topoisomerase which filter out the high affinity receptor for antibiotics and standardize output to rely on advances in biotechnology. In addition the AAC(6')-1b-cr protein of fluoroquinolone and nuclear protein L4 and L22 of macrolides had more extensive research in recent years, they also can be used as a receptor for the detection of antibiotic residues in receptor-based screening. Currently a great interest has been arising in the development of receptor-based screening of antibiotics residue, because these assays can easily operate, fast, high throughput and high sensitivity. Meanwhile, it has been expected that the sensitivity of micro-plate assay might be enhanced by the receptor reconstruction and choosing a more optimal combination of corresponding HRP-labeled hapten and receptor. Moreover a trend has also been increasing for the antibiotic screening on the base of miniaturization such as micro-array, chip, and micro-titer plate. With the development of science and technology, we believe there will be identified more new antibiotics receptor assays.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Novelty statement

Our novelty resides in a unified approach that deals with all the receptor based screening assays for antibiotic residues detection in various matrices. This review will offer a knowledgeable assistance for the recognition and preparation of specific receptor proteins. By this overview we believe that application objectives of receptor based screening assays will essentially benefit.

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Figures legends.

Fig. 1. Schematic overview of tetracycline resistance mechanism. In the presence of tetracycline in resistant bacteria it will bind to repressor protein TetR as magnesium complex. This complex leads to conformational change and release TetR protein from operator 01 and 02. Efflux protein TetA and TetR is produced and take out tetracycline from the cell.

Fig. 2. Principles of the receptor-based screening methods for the detection of antibiotics. Principle A: Using enzyme labeled drug, the compound was is detected with the properties of labeled substance; Principle B: After the antibody recognized the compound, adding the 2nd

antibody which marked by enzyme, the result was showed by the chromogen substrate; Principle C: Using biotin labeled drugs, the results was showed by the identification of biotin and avidin; Principle D: Using enzyme labeled receptor, the results was showed by the chromogen substrate; Principle E: Using enzyme labeled drug, the results was showed by the chromogen substrate; Principle F: Using enzyme labeled receptor, the compound was detected with the properties of labeled substance.

Fig. 3. Schematic overview of tetracycline biosensor assay. On downstream of TetR binding site a luciferase operon has been placed. Luciferase gene expression has been stopped in the absence of tetracycline (situation A), while in the presence of tetracycline it will bind to repressor protein and permit the luciferase gene expression, and luminescence signals has been detected (situation B).

Table 1 Antibiotic groups and their interacting proteins.

Antibiotics	MRL (μg •kg^{-1})	Interacting proteins	Role of binding protein	References
β-lactam	20-100	PBPs	Involved in final stages of bacterial peptidoglycan synthesis	[5, 92]
		BlaR	Involved in the induction of β-lactamase in Gram-negative bacteria	[93]
Sulfonamide	10-100	DHPS (SulA)	Catalyzes the condensation DHPPP with pABA to form dihydropteroate and pyrophosphate.	[7, 92, 94]
Tetracycline	100-600	TetR family	Family of transcriptional regulators that is well represented and widely distributed among bacteria with an HTH DNA-binding motif	[32, 92]
		TtgR	Family of transcriptional regulation in bacterial gene expression, multidrug binding repressor	[46]
Fluroquinolone	30-100	Gyrase (GyrA, GyrB), topoisomerase IV (ParC and ParE)	Breakage and reunion of DNA, ATPase activity	[38, 92, 94, 95]
Chloramphenicol	Not applicable	L16	Reconstruction of peptidyl-transferase activity, transcriptional regulation	[45]
		TtgR	Family of transcriptional regulation in bacterial gene expression, multidrug binding repressor	[46]
Aminoglycoside	100-20000	S12, APH(3')- IIa	Inhibition of protein synthesis or the loss of catalytic activity of aminoglycoside phosphine acyltransferase	[52, 53]

Table 2 Limit of detection (LOD) of commercial based receptor test kits for the detection of antibiotics [92].

Test kit	Time (min)	LOD(μg•kg^{-1})											Principle
		Amoxicillin	Ampicillin	Penicillin G	Oxacillin	Cloxacillin	Dicloxacillin	Cephalexin	Cefazolin	Ceftiofur	Cefoperazone	Tetracycline	
Delvo-X-Press βL	<10	-	-	4	-	-	-	-	-	-	-	100	Enzyme-labeling receptor assay
Delvo-X-Press βL-□	<7	4-8	4-8	2-4	25-50	30-60	25-50	25-50	-	4-8	-	-	
SNAP	<10	6	4-5	2-4	35-40	30	30	-	25-27.5	50	-	-	
New SNAP	14	-	-	2-3	-	-	-	-	-	-	-	-	
Parallux	4	3.6	2.9	3.2	10	7.4	20	-	-	33.7	-	-	
BetaStar	5	2-4	2-5	2-4	5-10	5-10	5-10	-	-	75-150	-	-	Colloidal gold-labeling receptor assay
TwinSensor	-	3-5	3-5	2-3	-	4-8	-	-	20-25	10-15	-	80-100	
Rosa MRL3	3	4-5	3-5	2-3	-	20-30	15-25	15-30	12-20	10-20	5-9	-	
Rosa SL3	3	7.8	8.7	4.2	-	-	-	-	-	51	-	-	
Rosa SL-6	8	7.1	9.6	4.2	-	8.3	-	-	-	72	-	-	
CHARM □	8-15	9	8	3.5	75	50	50	30	30	10	-	-	Radio-receptor assay
Penzym	15	4-6	4-7	4-6	30-50	60-100	-	-	-	40-70	-	-	Enzyme colorimetry

Table 3 Enzyme labeling receptor assay for detection of antibiotics.

Principle (as show in Fig. 2)	Protein or kit	Detection results				Reference
		Drugs	Sample	LOD(μg•kg^{-1})	Time(min)	
Principle A	1)DHPS from *S. pneumoniae*	9 sulfonamides and *p*-aminobenzoic acid	-	<100	-	[7]
	2)Parallux	6 β-lactams	milk	-	-	[96, 97]
Principle B	1)PBP2a from *Bacillus subtilis*	penicillin and cephalexin	-	-	-	[72]
	2)PBP2x from *Streptococcus pneumoniae*	ceftiofur	milk	20	-	[98]
	3)PBP2x* from *Streptococcus pneumoniae*	6 β-lactams	milk, meat juice, egg	2	-	[17]
Principle C	PBP2x from *Streptococcus pneumoniae*	penicillin G	milk	2	-	[72, 99]
Principle D	1) Delvo X-Press βL test	ampicillin and cloxacillin	milk	<MRL	10	[100]
	2)BlaR-CTD from *Bacillus licheniformis*	6 β-lactams (solid support: magnetic beads—cephalosporin)	milk	-	-	[75]
		3 β-lactams (solid support: microplate-cephalosporin)	milk	-	-	
		6 β-lactams (solid support: tube coated)	milk	-	-	
	3)IDEXX-SNAP kit	β-lactams	milk	-	10	[79]
	4)SNAP test	penicillin, amoxicillin, cephapirin, and ceftiofur	milk	-	-	[74]
Principle E	1)APH(3')- IIa	kanamycin, neomycin	-	0.06-27.45	-	[101]
	2)PBP2x* from *Streptococcus pneumoniae*	15 β-lactams	milk		45	[61]
	3)PBP3* from *Streptococcus pneumoniae*	27 β-lactams	milk	0.26-109.46	45	[102]
	4)BlaR-CTD from *Bacillus licheniformis*	15 β-lactams	milk, edible	<MRL(11 β-lactams)	-	[5]

Table 4 Colloidal labeling receptor assay for antibiotic screening.

Protein or kit	Detection results				Reference
	Type of drug	Sample	LOD(μg•kg^{-1})	Time(min)	
1)BlaR-CTD from *Bacillus licheniformis*	11 β-lactams	-	<MRL	5	[75]
2)BetaStar	amoxicillin, ampicillin, cephapirin, cloxacillin, and penicillin G	milk	below the FDA tolerance/safe levels	-	[103]
3)Beta-s.t.a.r. 1+1 protocol	β-lactams	milk	-	2	[104]
4)BetaStar plus kit	penicillins and cephalosporins	milk	-	5	[105]
5)Betastar Combo 3.0	penicillin G	sheep	<MRL	-	[106]
	amoxicillin and oxytetracycline	milk	slightly higher than the MRL		
6)Sulfasensor kit	sulfamethazine, sulfamethyldiazine, sulfathiazole, sulfapyridine	honey	25	40 samples can be detect in 90	[76]
	sulfadiazine and sulfadimethoxine		25~50		
	sulfaquinoxalinum		150		
	compound sulfamethoxazole, sulfadiazine		1500		
7)Tetrasensor kit	chlortetracycline, oxytetracycline, tetracycline and doxycycline	honey	5~15	-	[77]
8)Rosa Charm test	penicillins and cephalosporins	milk	2	3	[107]
9)TwinsSenseor kit	β-lactams and tetracyclines (simultaneous)	milk	-	6	[84]
10)Anti-β-lactam receptors	15 β-lactams	milk	<MRL	5~10	[78]

Table 5 Receptor analysis combined with biosensor for the detection of antibiotics.

Biosensor	Receptor	Detection results				Reference
		Type of drug	Sample	LOD(μg•kg^{-1})	Time(min)	
SPR	1)PBP2x*	6 β-lactams	milk	4	-	[81]
	2)D, D-carboxypeptidase from *Streptomyces R39*	penicillin G	milk	2.6	-	[108]
		penicillin G	milk	1.2	-	[18]
		penicillin G	milk	1.5	-	[109]
	3)TetO	tetracycline	milk	<MRL	30	[8]
Electrochemical sensor	1)PBP	penicillin G	milk	5	3-6	[110]
	2)PBP2x from *Streptococcus pneumoniae R6*	penicillin G, amoxicillin, ampicillin, cloxacillin, oxacillin, cefapirin	milk	<MRL	30	[82]
	3)Protein G	sulfonamides and tetracyclines (simultaneous)	milk	<MRL	30	[111]
	4)PBP	cephalosporins, sulfonamides and tetracyclines	milk	-	5	[112]

Table 6 Advantages and disadvantages of receptor-based screening methods.

Methods	Advantage	Disadvantage	Reference
Radio-receptor assay	high sensitivity and high specificity, less sampling amount	harmful, special equipment and high cost	[83]
Enzyme-labeling receptor assay	simple sampling preparation and low cost	poor protein stability	[5]
Colloidal gold receptor assay	simple and fast	only suitable for single-determination	[84]
Enzyme colorimetry	fast and convenient	susceptible to interference	[85]
Receptor analysis combined with biosensor	high throughput, real-time monitoring, low reagent consumption	expensive equipment	[86]

Fig. 1

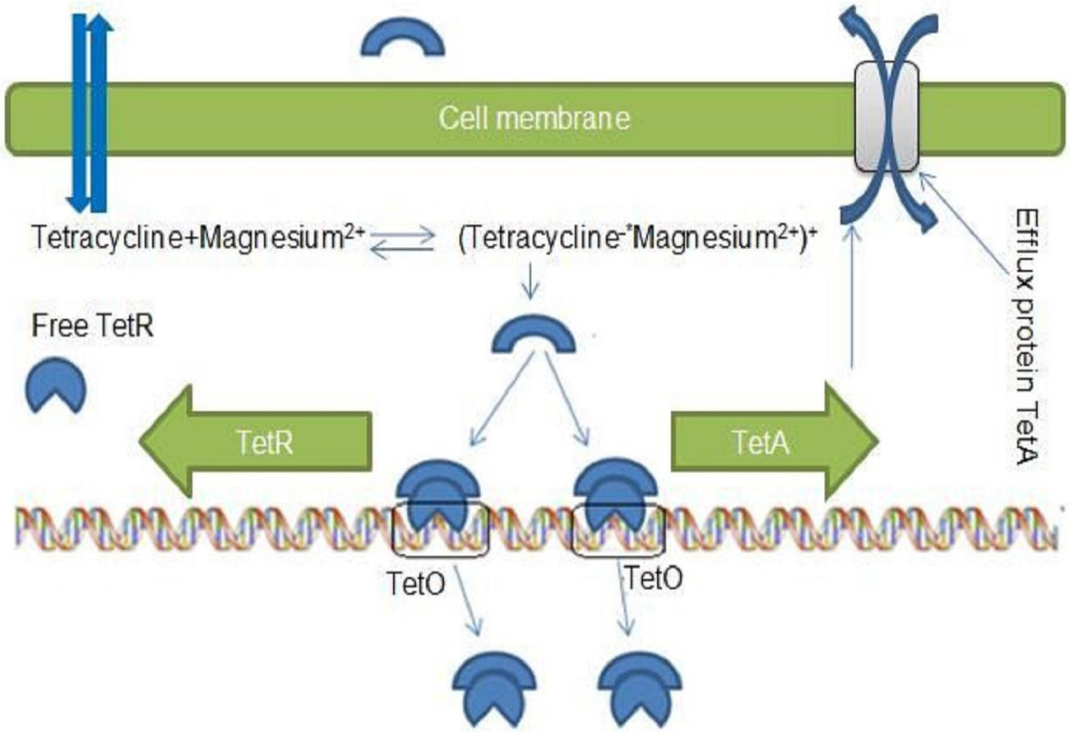


Fig. 2

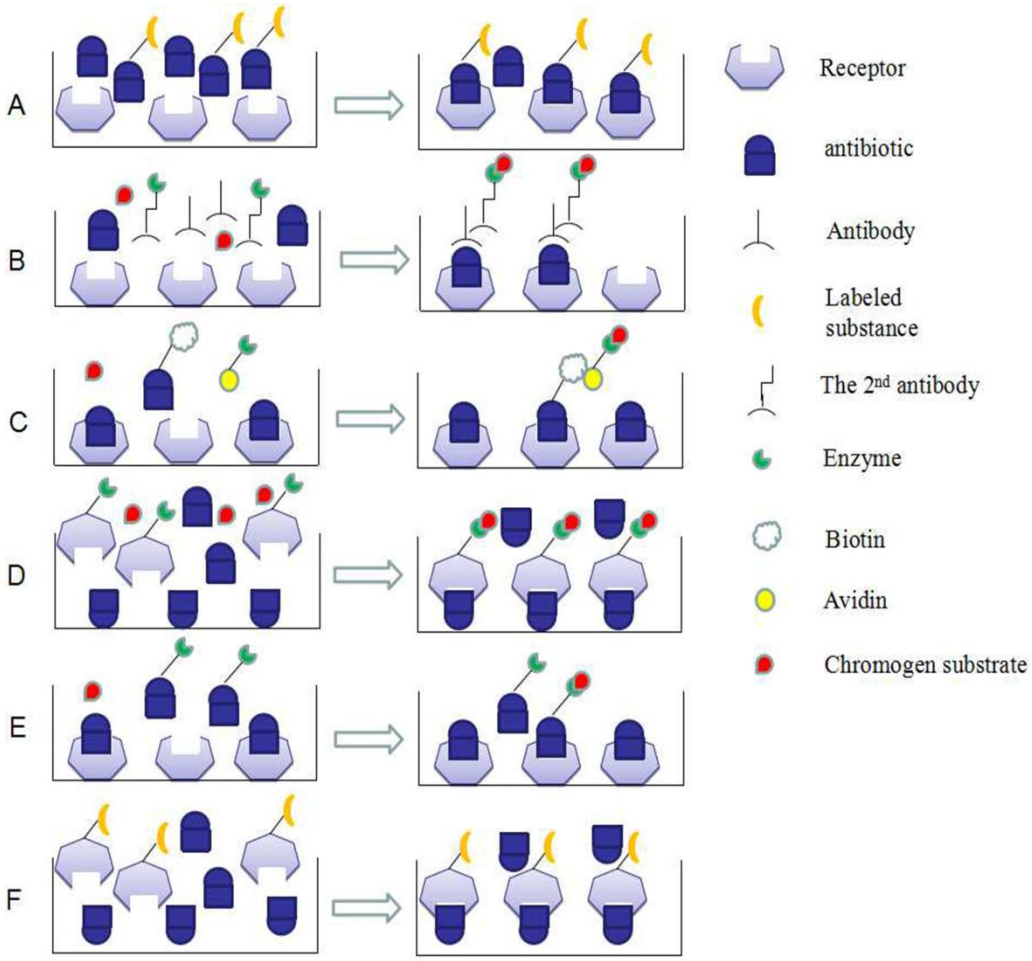
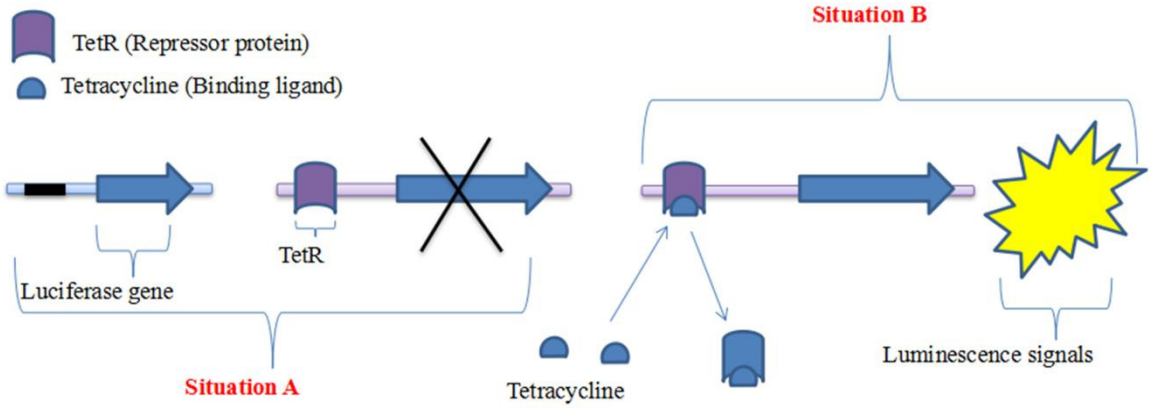
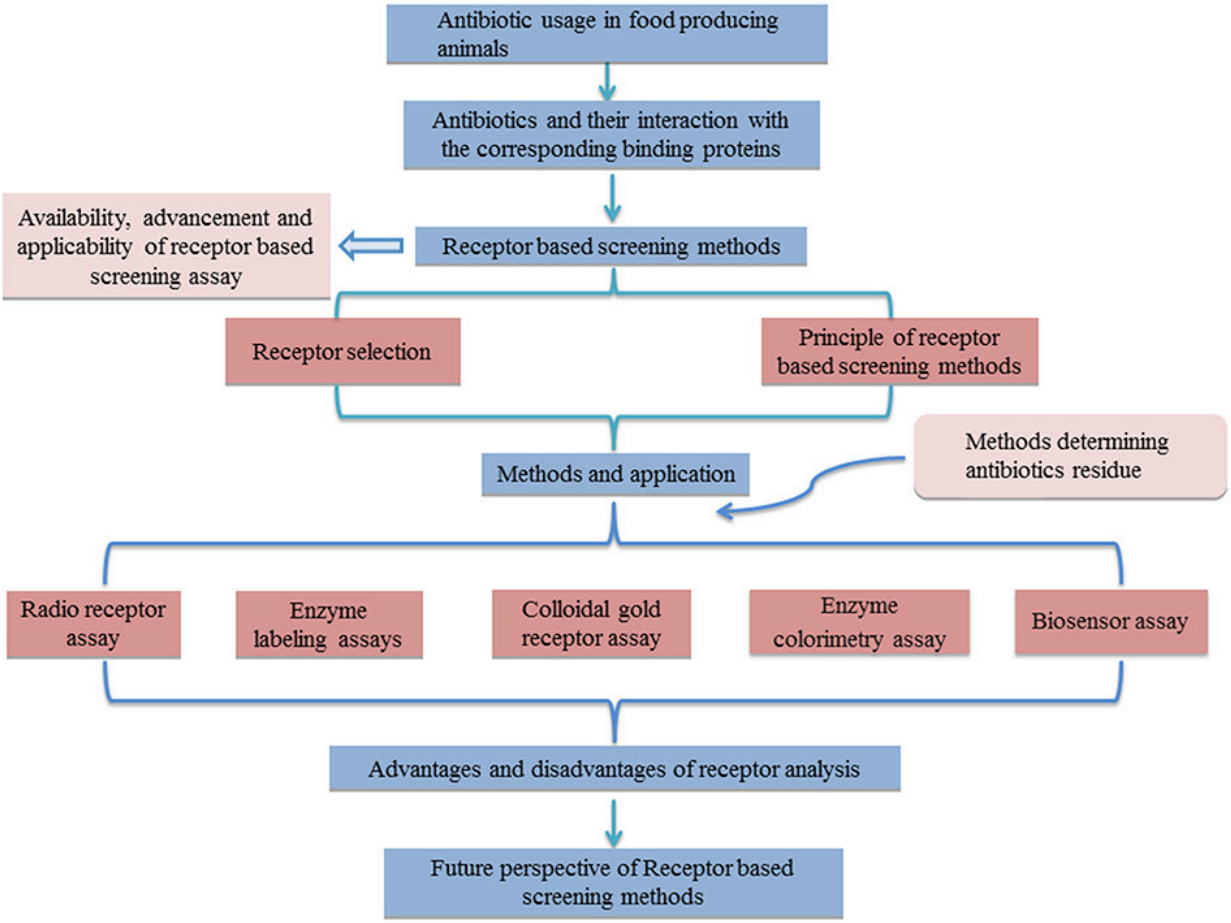

A
B
C
D
E
F
Receptor
antibiotic
Antibody
Labeled substance
The 2nd antibody
Enzyme
Biotin
Avidin
Chromogen substrate

Fig. 3


TetR (Repressor protein)
Tetracycline (Binding ligand)
Situation B
TetR
Luciferase gene
Situation A
Tetracycline
Luminescence signals

Highlights:

- Overviews of receptor based screening assays for the detection of antibiotic residues
- Selection and preparation of receptor protein for receptor based assays
- Permits the choice of stable selection among receptor based and other screening assays


Antibiotic usage in food producing animals
Antibiotics and their interaction with the corresponding binding proteins
Availability, advancement and applicability of receptor based screening assay
Receptor based screening methods
Receptor selection
Principle of receptor based screening methods
Methods and application
Methods determining antibiotics residue
Radio receptor assay
Enzyme labeling assays
Colloidal gold receptor assay
Enzyme colorimetry assay
Biosensor assay
Advantages and disadvantages of receptor analysis
Future perspective of Receptor based screening methods