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Drug Resistance Mechanism and Detection Method of *Salmonella*

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Abstract: As an important zoonotic food-borne pathogen, *Salmonella* is a concern for public health authorities. In particular, bacteria that are resistant to multiple antimicrobials can confuse the efficacy of treatment for infectious diseases. Drug-resistant bacteria have a variety of drug-resistant molecular and cellular mechanisms. These antimicrobial resistance mechanisms include antibiotic efflux, permeability changes in cell membranes, enzymatic drug inactivation, biofilm formation, drug target changes, and protection of antimicrobial targets. In this paper, the mechanisms of antimicrobial resistance in *salmonella* and the techniques of detecting antibiotic resistance by traditional and molecular methods are reviewed, with emphasis on their advantages and disadvantages, as well as the validity and reliability of the results.

Keywords: *Salmonella*; Anti-Microbial Resistance; Drug Resistance Mechanism; Microbiology Molecular Tools.

1. Introduction

Salmonella The causative agent of life-threatening infectious diseases belonging to Gram-negative bacteria that produce alarming numbers of morbidity and mortality[1]. Gram-negative bacteria have a thin peptidoglycan cell wall sandwiched between their inner and outer membranes, unlike Gram-positive bacteria, which have a thicker peptidoglycan cell wall[2]. Gram-negative bacteria are common in nature and can cause infection in many parts of the body, including the urinary tract, lower respiratory tract, biliary tract and blood. Due in large part to the selective pressure of antibiotic use, resistance in Gram-negative bacteria has increased significantly over the past two decades[3].

In terms of their structure and function, antimicrobials represent a highly diverse group of low molecular weight substances that interfere with bacterial growth, restrict growth (bacteriostasis) or kill bacteria (bactericidal action). Antibiotics have been used for more than 60 years to treat bacterial infections in humans, animals and plants[4]. At present, antibacterial drugs are one of the most commonly used therapeutic drugs in human and veterinary medicine[5].

Antimicrobial resistance was not considered a significant problem in the early days of antibiotic treatment due to the small number of resistant strains and the discovery of a large number of new and highly effective antimicrobial agents in different classes[6]. Since the 1950s, the widespread use of antimicrobials has increased selection pressures, which have clearly accelerated the development and spread of bacterial resistance to antimicrobials. In most cases, the first resistant target bacteria will appear three to five years after an antimicrobial agent enters the clinic. This is even more serious for broad-spectrum antimicrobials such as tetracycline, aminoglycosides, macrolides and beta-lacamides, which have been used for a variety of uses in human and veterinary medicine, horticulture and/or aquaculture[7]. In contrast, for narrow-spectrum drugs, such as glycopeptide drugs, the time span extends to more than 15 years because the dosage of these drugs is significantly lower. Multiple studies have also shown that resistance to fully synthetic antibacterial drugs such as sulfonamides, trimethoprim, fluoroquinolones and oxazolidones can develop rapidly. These observations highlight the tremendous flexibility that bacteria have to cope with adverse environmental conditions by constantly exploring new ways to survive in the presence of antimicrobial agents [8].

Salmonella have demonstrated a different set of mechanisms to degrade antibiotics, change the target of antibiotics, or regulate the flow of antibiotics into/out of bacterial cells, the production of biofilms, and the destruction of antibiotics by enzymes[9]. In addition, multidrug-resistant bacteria have developed mechanisms that confer the transfer of DNA as a genetic determinant of resistance to disease-causing species in clinical Settings, the food production industry, the human gut, and agriculture[10]. Understanding these resistance mechanisms is critical in epidemiology. In the broadest sense, understanding the mechanisms of antibiotic resistance helps to understand how resistance arises and how it spreads between bacteria and patients. The study of resistance mechanisms is also important in the pharmaceutical industry because multiple new drugs have emerged to circumvent known resistance[11].

2. Mechanisms and Epidemiology of Antibiotic Resistance in Salmonella

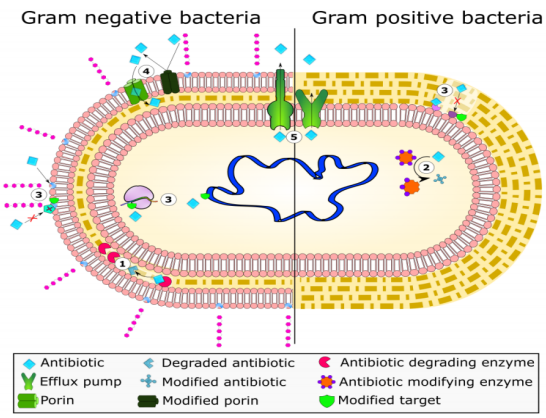

Figure 1. Mechanisms of *salmonella* resistance to antimicrobial agents. Common antibiotic resistance mechanisms in *salmonella* include: (1) enzymatic hydrolysis; (2) Enzyme modification of antibiotics through group transfer and REDOX processes; (3) Modification of antibiotic targets; (4) Reducing antibiotic permeability by modifying porin; (5) membrane efflux pump

Microbial resistance to antibiotics is usually caused by several mechanisms: (1) enzymatic inactivation of antibiotics: inactivation of antibiotics by enzymatic modification through phosphorylation, adenylation, or acetylation; (2) Modification of antibiotic targets; (3) Antibiotic efflux pump: antibiotics are released from cells through various transporters; (4) decreased membrane permeability [9]. The diagram of four common resistance mechanisms in *salmonella* is shown in Figure 1.

2.1. Enzymatic Drug Inactivation

The most common resistance mechanisms in *Salmonella* are hydrolases, enzymes produced by the bacteria that destroy antibiotics and render them useless, thus destroying or disabling the drugs before they can reach bacterial cells, there are three main types of drug inactivated enzymes: hydrolase (mainly β-lactamase), passivase (aminoglycoside inactivation enzyme, chloramphenicol acetyltransferase, erythromycin esterase, etc.) and modification enzyme (aminoglycoside modification enzyme). Enzymatic mechanisms of antibiotic resistance include hydrolysis group transfer and REDOX processes[12]. Beta-lactamase is the oldest and most diverse antibiotic degrading enzyme known, which can cut the beta-lactamase ring of penicillin-like antibiotics and break the amide bond, inactivating the antibacterial activity of the drug. Multiple mechanisms of β-lactam resistance have been reported including ESBLs, AmpC, and carbapenase. Including $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}, bla_{OXA}, bla_{CMY}, bla_{FOX}, bla_{MOX}, bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP}, etc [13].

Beta-lactam is the largest class of antibiotics in clinical use and includes penicillins, cephalosporins and carbapenems, all of which are characterized by beta-lactam rings containing 3 carbon 1 nitrogen. *Salmonella* has a thin peptidoglycan layer, followed by an outer membrane[14]. Synthetic antibiotics that target cell walls, such as naturally occurring beta-lactam (penicillin), often fail to penetrate the outer membrane. However, semi-synthetic beta-lacamides such as ampicillin and naturally occurring carbapenems can inhibit their growth by passing porin across the outer membrane[15]. β-lactam antibiotics are structural analogizes of penicillin-binding proteins (PBPs) substrates, which play a key role in peptide crosslinking during peptidoglycan cell wall biosynthesis. The structural imitation of the terminal fragment of the cross-linked peptide d-Ala-d-Ala by β-lactam promotes competitive inhibition of PBPs, which prevents cell wall synthesis and leads to bacterial cell lysis and death. However, bacteria gain resistance to lactam antibiotics by altering their PBPs, which are no longer susceptible to antibiotic binding. Alternatively, bacteria produce powerful lactamases that hydrolyze beta-lactam rings before the antibiotic binds to PBPs, inactivating beta-lactam antibiotics[16]. Since their discovery in the early 1940s, the beta-lactamase family has grown and grown, with more than 300 enzymes discovered worldwide.

The genes responsible for encoding beta-lactamase encode on chromosomes (e.g., AmpC beta-lactamase) or are present in transferable plasmids. A point mutation of TEM and SHV lactamases led to the development of broad-spectrum β-lactamases (ESBLs) that hydrolyze multiple cephalosporins. ESBLs hydrolyzes a broad spectrum of cephalosporins, including the first, second and third generation cephalosporins, but does not hydrolyze cephalosporins and carbapenems, and is inhibited by clavulinic acid[17]. *Salmonella* acquired ESBL genes (e.g., $bla_{\text{CTX-M-1}}$) through transferable plasmids such as IncC and Incl1. Carbapenem resistance, on the other hand, can be achieved through the acquisition of carbapenase (carbapenem hydrolase) or through porin loss and overexpression of beta-lactamases that have a mild affinity with these carbapenems. $bla_{\text{KPC-2}}$ is the first carbapenase gene reported in Streptococcus intestinalis [18].

Enzymatic modification of antibiotics by transferring functional groups, such as acyl, sugar, ribose, nucleotide, phosphoyl or sulfhydryl groups, can lead to resistance to a range of antibiotics [19]. Aminoglycoside antibiotics, for example, work by entering the ribosome A site and preventing aminoacyl-tRNA binding. N-acetyltransferase, O-nucleotide transferase and O-phosphotransferase are different kinds of aminoglycoside modifying enzymes. N-acetyltransferase encoded by *aac(3)-Ia* or *aacC1* genes in *Salmonella* typhimurium mediates acetyl group migration to aminoglycoside antibiotics[20]. The gene products of *ant (3")-Ia*, *aadA*, *aadA1*, and *aad(3")* have been shown to have O-nucleotide transferase activity and contribute to adenine transfer. O-phosphotransferase catalyzes the phosphorylation of these aminoglycosides. It has been reported that *aph(6)-Ic* and *str* genes have O-phosphotransferase activity in *Salmonella*.

2.2. Efflux Pump for Antibiotic Transport

Efflux pump, as the first defense barrier of antibacterial drugs, plays an important role in the output of antibiotics by cells. Pathogenic bacteria often utilize membrane proteins that use antimicrobial agents as transporters, which can actively export structurally unique antimicrobial agents from the cytoplasm where drug targets are located to the extracellular environment where molecular targets are lacking. Bacterial multidrug efflux pump systems are vigorously driven by ATP hydrolysis (primary active transport) and electrochemical ion gradients or ion dynamics (secondary active transport)[21].

Efflux pumps transport specific antibiotic compounds across cell membranes to the external environment, thereby reducing drug concentration and thereby mediating resistance. According to the secondary structure and the way of driving energy, effluents can be divided into five protein families, including: (1) Resistance Nodulation Division (RND superfamily); (2) ATP Binding Cassette (ABC) family, which uses ATP to drive the process and export; (3) Major Facilitator (MFS superfamily) (EmrAB, MdfA, and SmvA); (4) Multidrug and Toxic Compound Extrusion (MATE family); (5) Small Multidrug Resistance (SMR) family[22]. Figure 2 depicts five types of efflux pumps that mediate the transport of specific substrates across cell membranes. RND-type efflux pump plays an important role in antibiotic resistance of *salmonella*.

Resistance-Nodulation division (RND) superfamily. Which transports molecules through the cytoplasmic membrane, plays an important role in multidrug Resistance of *Salmonella*. RND tripartite multi-drug efflux pump system consists of three main domains, which constitute a tripartite structure[23]. The first third of the structure is the outer membrane-associated channel TolC, the middle part includes the ectoplasm-associated domain AcrA, and the third part is composed of AcrB, a widely studied member of the RND superfamily. *Salmonella* has five groups of proteins in this drug expulsion system: AcrA/B-TolC, AcrA/D-TolC, AcrE/F-TolC, MdsA/BeC, or MdsA/B-TolC and MdtAB-TolC. In the above efflux system, AcrA/B-TolC (AcrA: membrane fusion

protein, AcrB: RND transporter, and TolC: Outer membrane proteins) are the most common efflux system. AcrB proteins recognize many substrates in contrast to other transporters and are abundant in bacterial cells for the export of multiple drugs, such as quinolones, aminoglycosides, sulfonamides, chloramphenicol and tetracycline [24].

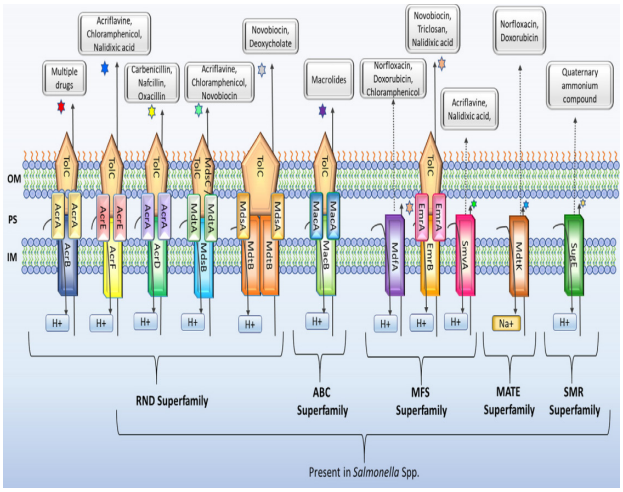

Figure 2. Schematic diagram of antibiotic transport mediated by *Salmonella* efflux pump in RND superfamily, ABC superfamily, MFS superfamily, MATE superfamily and SMR superfamily

ATP Binding Cassette (ABC) superfamily. The ATP binding box is a transport system involved in virulence and host-pathogen interactions, as well as nutrient absorption. During the primary active transport of antibacterial drugs, bacteria use bioenergy stored in the form of intact adenosine triphosphate (ATP) to export drugs through ATP hydrolysis using an antidrug concentration gradient. During the export of antibacterial agents from bacterial cells, ATP is hydrolyzed to activate the outward transmembrane transport of the drug through the transporter. The ABC transporter consists of four parts: two transmembrane domains (TMD1, TMD2) containing substrate binding pockets, and two nucleotide-binding domains (NBD1, NBD2) binding and hydrolyzing ATP to drive transport [25]. The cytoplasmic NBD domain is linked to TMD and is designed to keep proteins in bacterial membranes and acts as a motor domain. This domain is highly conserved in all ABC transporters, with at least seven ABC transporters identified, including the MacB transporter. Macb-tolc is an ABC transporter that acts as a tripartite efflux pump where MacB excrete various substrates by binding to MacA and TolC outer membrane proteins. MacAB-type transporters, originally described as macrolide exit efflux pumps, are one of the systems required for the exit of virulence factors in enterostreptococcus serotype typhimurium [26].

Major Facilitator Superfamily (MFS). MFS efflux pumps confer resistance to a variety of antimicrobial agents and are considered important molecular targets for resistance regulation. The MFS structure consists of 12 or 14 alpha-helices across the membrane segments, two seemingly symmetrical bundles, each belonging to the n-terminal or C-terminal, the so-called MFS fold consisting of adjacent triple alpha-helices, and a functionally highly conserved amino acid sequence motif. MFS pumps transport tiny molecules such as carbohydrates, amino acids, antimicrobials and nucleotides across cell membranes. To drive efflux function, they use their ion (H^+) gradient to cross the cell membrane. The efflux pumps of the MFS superfamily include the EmrA/B, MdfA, and SmvA proteins present in *Salmonella*, which are responsible for the transport of various substrates. The EmrA/B-TolC protein is a tripartite efflux pump, in which EmrB is an intima protein, EmrA is a membrane fusion protein found in the ectoplasm, and TolC is an outer membrane protein[27].

Multidrug and Toxic Compound Extrusion (MATE). The MATE efflux pump is a member of the multi-drug and oligosaccharide-lipid/polysaccharide transporter family first discovered in bacteria. The electrochemical gradient of hydrogen or sodium ions provides the energy needed for the exit. The MATE transporter is involved in homeostasis by removing metabolic waste and toxic compounds[28]. In *Salmonella*, the MdtK protein transports specific antibacterial molecules, such as norfloxacin, from the intima to the peripheral substance.

Small Multidrug Resistance (SMR) family. With the discovery of the SMR family and its subsequent inclusion in the larger DMT superfamily. It has been shown that this is an effective antibacterial transport model system. SMR-based efflux pumps differ from other efflux pumps in that the SMR family is involved in the transport of quaternary ammonium compounds, which are commonly found in preservatives, fungicides, and detergents. SMR efflux pump family has not been reported in the course of multidrug resistance in *Salmonella* [29].

2.3. Modification of Antibiotic Targets

Antibacterial targets play a crucial role in the growth or survival of microorganisms and can therefore serve as potentially useful targets for reducing infection. Bacterial species have evolved mechanisms to alter or modify antibiotic targets to become resistant to antibiotics[30]. Antibiotics interfere with specific target sites and impede their function, thereby stopping cellular processes. Bacterial cell walls are one of the important lines of defense against antimicrobial agents, a structure that was originally thought to have evolved to protect against inflationary pressures inside cells and also acts as a physical barrier that isolates the cytoplasm and cell membranes from the outside world. Prokaryotic cell walls consist of linear glycan chains crosslinked with small peptides that help limit which substances can continue their journey to the membrane and eventually into the cytoplasm. Peptidoglycan also plays a crucial role in bacterial growth and proliferation, and while the cell wall helps protect cytoplasmic antibacterial targets, it also eventually became a target for penicillin, the first natural antibiotic, which prevents the complete formation of this barrier by inhibiting the occurrence of peptide crosslinking [31]. This protective mechanism is compromised by the introduction of beta-lactam antibiotics. Beta-lactamases help protect peptidoglycan cell walls from beta-lactam antibiotics. Specifically, beta-lactamases help endow resistant bacteria with phenotypes because they work by hydrolyzing the beta-lactam ring of such antibiotics. The resulting chemical structure no longer obstructs the synthesis of bacterial cell walls. Modifications to drug targets may interfere with drug target interactions, examples include: DNA gyrase, targets of quinolones; Targets such as tetracycline are successfully modified by bacteria to prevent antibiotics from entering to do their job[32]. Membrane proteins and porins are important targets for drugs that need to be transported across cell membranes in order to function. Bacteria can manipulate and alter the cell membrane to protect themselves. These

modifications also allow targets to perform their functions and thus develop antibiotic resistance. Antibiotic resistance is usually acquired by manipulating a target site through point mutations that cause a single residue change at the target site.

DNA gyrase and topoisomerase IV. Fluoroquinolones (FQS) are the most common antibiotics used to treat salmonellosis, but because of their frequent use, strains of Salmonella have now developed resistance to fluoroquinolones [33]. Two fluoroquinolone antibiotic targeting enzymes: DNA gyrase (gyrA and gyrB) and DNA topoisomerase IV (parC and parE). DNA gyrase (GyrA/B) plays an important role in DNA replication and transcription and is the primary target of fluoroquinolones. DNA topoisomerase IV mediates DNA strand replication and untangles superhelix strands. Quinolones target DNA gyrase and topoisomerase IV to block the pathway of DNA strands during replication, thereby generating double-strand breaks in DNA and thus bactericidal activity[34]. Quinolone resistance is caused by chromosomal mutations in QRDRs (quinolone resistance determinant regions) of DNA gyrase and topoisomerase IV. The mutation sites of gyrA gene QRDR were located at amino acid sites 83 and 87, and the main mutation mode was Ser83→Phe12 and Asp87→Asn, followed by Asp87→Tyr and Ser83→Gly. The single mutation of QRDR could reduce the susceptibility of Salmonella to fluoroquinolones[35]. Double mutations of these two residues were also observed.

Alteration in Penicillin-Binding Proteins (PBPs). PBPs are transpeptidases or carboxypeptidases that catalyze the cross-linking of sugar chains, thereby mediating their polymerization. PBPs are responsible for later maturation and are used to engineer bacterial peptidoglycans and ultimately confer resistance to beta-lactam antibiotics. These antibiotics use PBPs as their target[36]. The specific experimental mutagenesis of PBP changes the target and reduces the affinity of antibiotics to the active site of PBP. Bacterial species have also evolved beta-lactamase activity to inactivate antibiotics.

2.4. The Permeability of the Cell Membrane Changes

The important mechanism of bacterial resistance to antibacterial drugs is to prevent the drug from penetrating and entering the internal cell environment. Gram-negative bacteria are less intrinsically permeable to antibiotics than Gram-positive bacteria, so they are more resistant to antibiotics[37]. The outer membrane and inner membrane selectively permeate substrates such as antibiotics, bile salts and nutrients. Phospholipids and endometrial proteins make up the majority of the inner membrane, and the components of the outer membrane, namely phospholipids and lipopolysaccharides, are produced either on the inner membrane or on bacterial cytosol. They are further transported to the outer membrane by the MsbA protein, an ABC transporter[38]. On the other hand, the outer membrane composed of lipopolysaccharides and OMPs, consisting of lipid A, oligosaccharides and antigenic determinants, plays a crucial role in reducing the entry of antibiotics. They are mainly involved in protecting bacterial cells from the environment.

The role of outer membrane proteins (OMPs) in antibiotic resistance. Another important molecular mechanism for resistance through reduced permeability relates to pore-proteins, which are intact outer membrane proteins with water-filled porous channels that are used for passive transport of hydrophilic compounds, allowing passage of molecules with a defined size and charge. The outer membrane porpoproteins are divided into specific and non-specific porpoproteins based on their activity, (1) specific porpoproteins, which allow transport of specific substrates such as the sugar-specific channel LamB, and (2) non-specific porpoproteins, which allow passive diffusion of low molecular weight hydrophilic molecules. OMPs have a variety of functions, such as adhesion, nutrient uptake, membrane integrity, selective penetration of hydrophilic compounds, protein secretion, and excretion of toxic compounds. The most common members of OMPs include OmpF, OmpC, OmpA, OmpD, PhoE, LamB, etc. The OmpF structure of E. coli is one of the earliest and best understood porins. Figure 3 depicts the structure of the outer membrane protein OmpF of *Salmonella*.

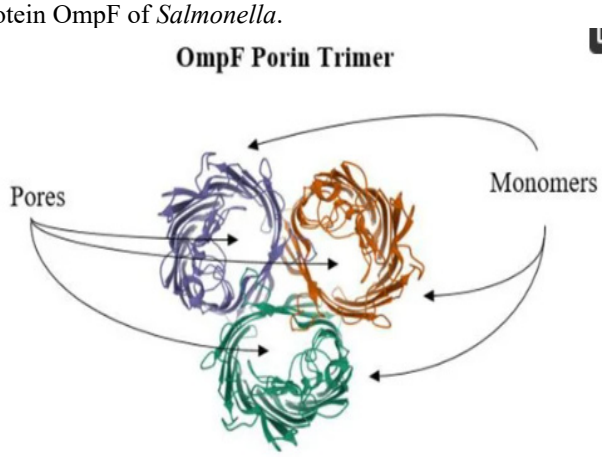

Figure 3. The outer membrane protein OmpF of *Salmonella*

2.5. Antibiotic Resistance is Acquired through Horizontal Gene Transfer

Resistance genes are carried and spread by plasmids, integron transposons, and prophages, and bacteria develop resistance to antibiotics by acquiring exogenous genetic material through horizontal gene transfer. The mechanism of antibiotic resistance acquired through horizontal gene transfer is shown in Figure 4. The Plasmid is extrachromogenetic DNA that can confer corresponding characteristics on the host bacterium, with a variety of determinant clusters, which make it easier for the host to survive in an unfavorable environment[39]. The plasmid encoding antibiotic resistance (R plasmid) is the most common, and the genes encoding resistance exist in clusters on the R1 plasmid. Plasmid mediated qnr genes involved in quinolone and fluoroquinolone resistance in Gram-negative pathogens such as Enterobacteriaceae have been observed in *Salmonella* typhus. PMQR (plasmid mediated quinolone resistance) is mediated by: (1) QepA as a quinolone efflux pump, (2) Qnr protein, and (3) AAC (6 ')-Ib. Efflux pumps QepAB and OqxAB are combined with quinolones for the purpose of exporting antibiotics outside bacterial cells. Qnr protein disrupts DNA cyclotron quinolone interaction, increases the expulsion of quinolones from bacterial cells, protects DNA cyclotron enzyme and topoisomerase IV from quinolones, and is encoded by plasmid genes qnrA, qnrB, qnrC, qnrD, qnrS and qnrVC. Among them, qnrA, qnrB and qnrS genes are the most common. *aac(6')-Ib-cr* is a typical aminoglycoside acetyltransferase variant responsible for quinolone acetylation[40]. Many plasmid-mediated antibiotic

resistance genes of other kinds are also associated with *Salmonella*, such as trimethoprim-sulfamethoxazole resistant *dfrA7*, *dfrA15*, *sul1*, *sul2* genes; *catA1* gene is associated with chloramphenicol resistance, and $bla_{\text{TEM-1}}$ gene is associated with amoxicillin resistance.

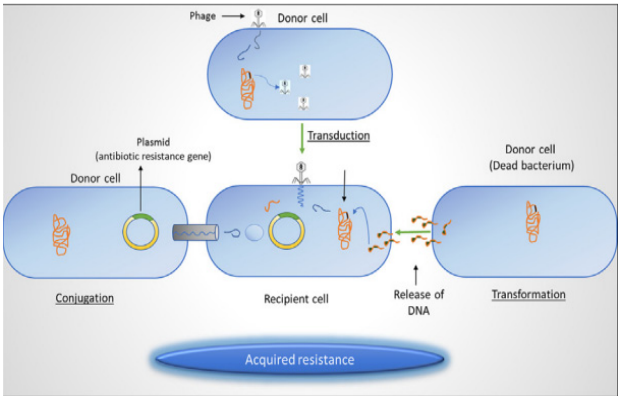

Figure 4. Schematic diagram of horizontal gene transfer (HGT) mechanism in *Salmonella*

2.6. Adaptive Antibiotic Resistance: Biofilms

Biofilms are self-produced networks of extracellular polysaccharides, extracellular DNA, proteins, and lipids that form a surfacing attached population. The presence of biofilms prevents drugs from entering the interior of bacteria. *Salmonella* can produce biofilms that can survive harsh conditions such as toxic compounds, high salt concentrations, high temperatures and antibiotics. Biofilm formation has been shown to increase resistance to antibiotic therapy. There are several factors in the formation of biofilms: (1) Physiological changes of bacterial cells due to environmental stress and nutrient deprivation; (2) Phenotypic changes; (3) quorum sensing; (4) extracellular stress such as antibiotics; (5) Expression of efflux pump [41].

Quorum sensing is a form of communication within a bacterial population that coordinates gene expression in response to the environment and population. Once a bacterium receives a signal when it detects a toxic extracellular compound, it can initiate cell-to-cell communication through adhesion, and finally reduce the influx of antibiotics by producing biofilms and fighting them through other factors. Autosensing is the signal generated during quorum sensing. Biofilm formation by synthetic AI-2 of *Salmonella*. The LuxS enzyme is encoded by the *luxS* gene and is involved in the production of AI-2 signaling. Inactivation of the *luxS* gene has been shown to increase biofilm formation and thus antibiotic resistance to *Salmonella*.

3. Traditional Methods for Detecting Drug Resistance in Salmonella

Traditional AMR assay methods focus on the identification and quantification of bacteria, and they are considered standard techniques for identifying specific microorganisms, mainly due to their high sensitivity. The commonly used AMR detection methods are minimum inhibitory concentration (MIC), disk diffusion, and E-test[42].

3.1. Minimum Inhibitory Concentration (MIC)

The most common technique used to determine antimicrobial resistance is to measure MIC. The aim of the method is to quantify the minimum concentration of antibacterial agents that inhibit the apparent growth of bacteria when cultured in agar or broth. A certain concentration of bacteria (the standard is usually 0.5 McGrath turbidimetric tube) was inoculated on agar or broth containing different gradient antibiotic concentrations. After culture, it was observed whether there was microbial growth in agar or broth containing different gradient antibiotics, so as to determine the MIC value of antibiotics and adjust the therapeutic concentration of antimicrobials for effective treatment[43]. In general, this is a semi-quantitative technique, the advantages are simple operation, low cost, no need for specialized equipment, the disadvantage is that it may not be possible to determine the exact MIC value.

3.2. Kirby-Bauer

Disk diffusion method is composed of antibacterial agent impregnated on a paper disc diffusing on agar. Bacteria with a fixed concentration are uniformly coated on agar medium. The paper carrying a specific concentration of antibiotics is stuck on the surface of agar and cultured. This creates a growth inhibition zone around the paper. Inhibitory zone size was negatively correlated with antibiotic concentration. This is a qualitative method that classifies samples as resistant, intermediate, or sensitive. This method is suitable for fast growing bacteria, but has some limitations, such as antibiotics not diffusing well in agar, and difficulty in accounting for finicky and anaerobic microorganisms[44].

3.3. E-test

E-test is a combination of the above two methods, which not only uses disk-like diffusion, but also determines MIC values. A rectangular device is placed on an AGAR plate with an antibacterial concentration gradient on one side of the gadget and an interpretive standard on the other. Although E-test has the limitations of the two previously mentioned tests, it shows a fixed antibacterial gradient on the scale and guarantees a simpler way to directly quantify the sensitivity of microorganisms, especially those that are difficult to culture, even anaerobic bacteria[45].

4. Molecular Methods for Detection of Drug Resistance in Salmonella

4.1. Polymerase Chain Reaction (PCR)

PCR is a method of amplifying specific sequences of DNA and RNA in vitro and is widely used due to its high specificity. PCR is a rapid assay to identify bacteria, virulence genes, and drug resistance genes from a variety of environments. PCR has been shown to be suitable for detecting the presence of point mutations associated with broad-spectrum AMR genes. PCR optimization methods include multiple reactions, improved methods of traditional PCR, and qPCR[46]. In this method, several primers are used in the solution mixture, so it is possible to analyze up to nine different DNA targets in a single run. In this study, PCR was used to detect multidrug-resistant strains of Staphylococcus aureus isolated from milk, meat and other animal products in supermarkets. *blaZ*, *msrA*, *ermB*, *ermC* and *tetK* genes were identified in 125 strains of Staphylococcus aureus. The results showed that 19 strains were multidrug-resistant to penicillin, enteromycin, kanamycin and tetracycline [47]. The significant advantage over traditional culture is the possibility of amplifying genes from unculturable and/or dead microorganisms that cannot be identified by traditional methods. The biggest advantage is that it reduces cost and time by amplifying different genes

simultaneously. However, the key to PCR technology still depends on internal and external factors such as: circulation conditions, temperature, primer concentration and even DNA extraction solution.

4.2. DNA Microarray Technology

Microarray technology is another important technique for studying resistance to drug resistance in bacteria. This technique allows the study of gene expression through hybridization of oligonucleotide sequences, purification and amplification of specific RNA molecules in samples, which makes it versatile, mainly for identifying the function of certain genes. The application of microarrays in AMR gene detection can be optimized through multiple hybridization processes and performed simultaneously on the same substrate with multiple probes (such as: glass, film, or gel pad). Another advantage is that no prior bacterial culture is required, as DNA samples can be isolated directly for microarray detection, but one limitation of microarray technology is the need for prior knowledge of the genomic region to be studied[48].

Baumgartner et al. used microarray technology to identify resistance genes in ready-to-eat foods and found that the resistance genes were methicillin (*mecA*), Vancomycin (*vanB*), macrolide (*msr*), tobramycin (*aadD*), tetracycline (*tet*), and chloramphenicol (cat). Sadouki et al. used the technique to detect genes resistant to chloramphenicol (*cmlA1-like*), sulfonamides (*sul*), tetracycline (*tet*), and trimethoprim (*dfrA*) [49].

4.3. Whole Genome Sequencing (WGS) Technology

WGS technology is the assembly of DNA extracted from test samples based on De Bruijn programs, such as: SPAdes, Velvet, ABySS and SOAPdenovo, which are assembled from small sequence reads into contigs, which can be annotated for searching resistance genes. The search for resistance genes was mainly accomplished by methods such as BLAST and USEARCH, which took into account the similarity between contigs and the genes contained in the reference database, such as Resfinder, ARG-ANNOT, RGI and ARGs-OAP. The choice of databases depends both on the purpose of each study (i.e., resistance genes, virulence genes, and proteins) and on the level of confidence of the sequences held in each database[50]. Due to recent improvements in the cost-benefit ratio of sequencing techniques, whole genome sequencing has become readily available and an effective tool for predicting antibiotic resistance, and there have been several studies based on the consistency of AMR predictions for detecting genetic markers and phenotypic resistance. Hong et al., by using a custom antibiotic resistance gene (ARG) database, WGs-based antibiotic resistance prediction has been shown to be highly accurate for *Salmonella* and other microorganisms [51]. Tyson et al. used WGS to identify drug-resistant genotypes of multidrug-resistant Escherichia coli, and more than 30 drug-resistant genes and multiple drug-resistant mutations were identified in the study to observe whether genotypes were related to phenotypes. The specificity and sensitivity of the resistance genotypes to the phenotype were 97.8% and 99.6%, and WGS also revealed rare information about the mechanism of resistance, such as structural mutation of the ampC chromosome leading to resistance to the third generation of cephalosporins[50]. Zhao et al. used in vitro antibiotic susceptibility test and whole genome sequencing technology to evaluate the correlation between drug resistance phenotype and genotype. The results showed that phenotypic resistance to specific drugs was highly correlated with the existence of one or more corresponding drug resistance genes. The correlation between phenotype and genotype of tetracycline, ciprofloxacin and erythromycin was 100%. The correlation between azithromycin, clindamycin and tilrimycin was 95.4% to 98.7%.

5. Discussion

Most antibiotics on the market today are from the 1980s, the golden age of antibiotic treatment. We are currently experiencing a huge imbalance between the demand for new drugs and the supply. At the same time, according to the World Health Organization, the post-antibiotic era has begun, with irregular and frequent use of broad-spectrum antibiotics without detailed knowledge of the biology of the pathogen leading to increased resistance to the drugs used[52]. The resistance of pathogens to antibacterial compounds is based on several major strategies, such as inactivation of the compound, the mechanism by which it is removed from the cell, modification of the site of action, and changes in the permeability of the cell sheath. Molecular mechanisms such as gene expression, post-transcriptional modification and protein translation are important pathways of multidrug resistance in pathogenic bacteria. In *Salmonella*, multiple signaling pathways actively regulate the expression of resistance genes in response to specific environmental stimuli, such as the presence of certain antibiotics and metal ions[53]. Therefore, it makes sense to conduct a more comprehensive study on the molecular mechanism by which the pathogen strains acquire resistance at the molecular level. Perhaps, over time, the right medical strategy will return not to pharmacology but to biological approaches to fighting pathogenic bacteria.

Many resistance genes have been found in various bacteria, which correspond to different resistance mechanisms. The rate at which resistance develops involves the selective pressure exerted by the antimicrobial agent and the resistant genes available to the bacteria. These resistance mechanisms apply to bacteria from humans as well as those from animals. Loss of acquired resistance is usually a cumbersome process, mainly affected by selection pressure, but also by the coordination of resistance genes on plasmid or chromosome DNA and the resistance genes in the multi-resistance gene cluster or integron structure. When resistance gene clusters or integrons bind together, the loss of resistance genes may be unpredictable even in the absence of direct selection pressure[54]. Because we know that bacterial resistance can arise from the use of every antimicrobial substance, careful use of antimicrobial agents in both human and veterinary medicines, especially in food animal production, is strongly recommended to maintain the efficacy of antimicrobial agents in treating bacterial infections in animals.

In this paper, the main characteristics, advantages and limitations of traditional and genetic AMR detection methods are reviewed, and their applications in basic research are discussed. Table 1 summarizes the advantages and disadvantages of several methods commonly used in bacterial AMR detection. This suggests that a combination approach is needed to obtain the most accurate results when identifying resistance genes. There is a need to use multiple genetic analysis methods to identify the determinants of bacterial resistance and to provide a sound scientific basis for the

molecular monitoring of AMR bacteria and the determinants of resistance on a global scale [59]. In order to provide the necessary confidence and accuracy in the genetic AMR determinants, metagenomics is considered a more complete approach than other molecular approaches because it can obtain all the information about the microbiome in the study sample. However, there is no distinction between good or bad molecular assay methods in AMR determination, and the optimal combination of assay methods used depends on the purpose of the study [60]. In general, the regulated and rational use of antimicrobial drugs is an effective means of controlling the increase and spread of antibiotic resistance in bacteria, especially with regard to drugs used to treat animals and humans. With greater awareness of the risks of antimicrobial abuse, future problems in treating pathogen infections can be alleviated.

Table 1. Summarizes the advantages and disadvantages of the most commonly used conventional and molecular methods for microbial and drug-resistant gene identification

Method	Advantages	Disadvantages	References
Traditional	Low cost; high sensitivity; gold standard for microorganism identification	It takes time; Fastidiosa is difficult; Low specificity	[55]
PCR	Amplification of a living caustic bacterium gene	Does not detect cell viability	[56]
Microarray	Several resistance genes were detected simultaneously	Need previous knowledge about genomic regions to be studied	[57]
WGS	Detection of whole microbiome; no previous culture required, discovery of non-cultivable microorganisms; New genes and microorganisms	Need prior knowledge in bioinformatics; high cost; challenge of achieving deep sequencing of more complex microbiomes	[58]

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