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Chapter 4

Emergence of Antimicrobial Resistance, Causes, Molecular Mechanisms, and Prevention Strategies: A Bovine Perspective

Muhammad Ashraf, Behar-E -Mustafa, Shahid-Ur -Rehman, Muhammad Khalid Bashir and Muhammad Adnan Ashraf

Additional information is available at the end of the chapter

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Abstract

Emergence of the resistance in microbial population is a major threat to both animal and human health. In bovine, the development of microbial resistance is a persistent threat for health especially in the form of zoonotic pandemics due to viral and multidrug bacterial resistance. Mechanisms of antimicrobial resistance in microbes are of natural as well as acquired origin. There are half dozen molecular mechanisms identified that possibly cause the emergence and transfer of antimicrobial resistance within and between different bacterial genera. These mechanisms include degradation of the antibacterial drug by the bacterial enzymes, reduced permeability of the drug by bacteria, increased efflux of the drug, modification of drug target and use of alternative pathways by bacterial cells. Various assays viz. disk diffusion test and E-test, focusing on minimum inhibitory concentration of antimicrobials, have been employed to detect the antimicrobial resistance in microbes. The most important factor responsible for the development of multidrug resistance in bovine pathogenic microbes is irrational use of the antibiotics. Antibiotics are necessary evil, so judicious use of antibiotics, early detection of infections, vaccination, use of immune-modulators and medicinal plants or their derivatives are some of the strategies to reduce the possible emergence of antimicrobial resistance.

Keywords: microbes, resistance, pandemic, zoonotic, antibiotic

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1. Introduction

Antimicrobial compounds include antibiotics as well as many other substances which are used to kill or inhibit the growth (multiplication) of bacteria. But nowadays, many bacteria causing diseases of pandemic (e.g., tuberculosis) importance are increasingly developing the resistance against a myriad of the antimicrobial compounds which, in terms, is leading to the ineffective treatment of many fatal human and animal disease outbreaks.

1.1. Antimicrobial resistance in microbes

The idea of using antimicrobial compounds was originally proposed by Paul Ehrlich in 1908 who proposed to use some chemicals which he originally thought as "magic bullet" to kill the bacteria specifically with a minimum harm to the host (animals and humans). He used SilverSan to treat an infectious disease in humans transmitted through sexual contact known as syphilis. This was the first time that a chemical was used to treat the microbial infections. Since the discovery of the first antibiotic, penicillin (1920s), researcher worked diligently to find new antibiotics, and this leads to the discovery of many new antibiotics, e.g., tetracycline, gentamicin, and chloramphenicol. Antibiotics are compounds which are produced by one microbe, and they are used to kill the other microbial spp. So, till 1950 a majority of infectious diseases in humans were treatable by using these antibiotics [1].

However, unfortunately, soon after the clinical use of antibiotics, a phenomenon was found in *Staphylococcus aureus* by means of which it was no longer susceptible to penicillin. It started producing an enzyme named as penicillinase, which can easily break down the beta lactam ring of penicillin. This ring is necessary to bind bacteria (penicillin binding proteins; PBPs) and therefore its bacterial killing ability. This effect was named as antimicrobial/antibiotic resistance in bacteria. Since then, many different types of bacteria are becoming increasingly resistant to many new antibiotics. The phenomenon of antibiotic resistance in bacteria is a persistently ongoing process and is on rise with every new day. This process can further be increased by humans, e.g., the inappropriate use of antibiotics, following reduced doses of antibiotics than required/standards, using antibiotics as a precautionary measures in viral infections, using antibiotics as growth promoters, prescribing broad-spectrum antibiotics, using antibiotics without using the antimicrobial sensitivity testing, and finally by noncompliance of the animal owner. There has been a surge in the use of antibiotics for treatment of a variety of infections. In fact, nowadays, the use of a variety of anti-infection therapy has become an invaluable tool for the treatment of a variety of bovine infections. Using anti-infective agents has greatly reduced the mortality as well as the morbidity against a variety of microbial infections in animals and humans. However, their frequent use has also led to a major problem in human and animal health, the development of antimicrobial resistance in microbes (ability of a microorganism to tolerate and even grow in the presence of the normal inhibitory concentration), and its transmission to a variety of other microbes (within same or different genera) against these anti-infective agents. The development of the resistance in microbes against anti-infective agents was predictable as the discoverer of the first antibiotic, Dr. Alexander Fleming, discussed this issue in his Nobel prize winning lecture (1945) [2, 3].

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The emergence of the antibiotic-resistant strains has been associated with an increased occurrence of the morbidity and mortality by the antibiotic-resistant isolates than caused by the nonresistant isolates in both humans and animals. In the last 6 decades, there has been a tremendous increase in the number of the multidrug-resistant (MDR) isolates in bacterial community, which have been associated with an increase in hospital stay in humans. Nowadays, with an advent of a variety of molecular biology techniques, there have been several mechanisms reported for the development of the resistance in the bacterial populations infecting humans and animals, and this process is ever increasing. Therefore, it is extremely important to know the factors that cause antibiotic resistance in humans and animals, molecular mechanisms of the antimicrobial resistance in different microbes, different methods of the genetic transfer of the resistance among different microbes, and the development of a variety of strategies for the control of bacterial resistance against antimicrobials, so that a better control of the infections in humans as well as in animals can be made.

2. Common bovine infections and their treatment

Currently, in Indo-Pak numerous infectious diseases are prevalent in bovines. Because these are the major source of income for the poor farmers (who merely raise about 4–5 animals), there is massive irregular use of a variety of anti-infectious agents to save their life and thus family earnings. Whereas, in **Table 1**, a list of diseases and their possible treatments in bovines is given.

Infection/disease	Etiology	Treatment options
Hemorrhagic septicemia	*Pasteurella multocida*	Oxytetracycline
Anthrax	*Bacillus anthracis*	Penicillin
Black leg/quarter	*Clostridium chauvoei*	Penicillin
Contagious bovine Pleuropneumonia	*Mycoplasma mycoides*	Tylosin

Table 1. Common bovine infections and their treatment.

3. Factors associated with antimicrobial resistance in animals

Since the discovery of the first antibiotic, penicillin, by Alexander Fleming, there has been a tremendous use of antibiotics in farm animals. In animals, antibiotics have been used as growth promoters as prophylaxis as well as metaphylaxis. Both approaches involve the administration of antibiotics in animals either by injection or through feed/water.

The primary goal of antibiotics was to treat the infectious diseases in animals and, thereby, improve the overall health of animals and humans. However, an unexpected thing observed in chickens during the 1940s was that antibiotic use may also cause an increase in the growth rate

in animals. This finding has led to their use as growth promoters. Antibiotics have been used as growth promoters for decades. This aspect is of tremendous controversy since the beginning. The practice employs the use of antibiotics at subtherapeutic levels, with the main purpose to control the enteric and respiratory diseases in farm animals associated with poor management. The exact mechanism by which antibiotic may enhance the growth rate in animals is yet unknown precisely; however, it is postulated that antibiotics may increase the animal growth rate by either one of the following mechanisms:

a. Killing the pathogenic bacterial species in the intestine and thereby reducing the inflammatory conditions

b. Reduced inflammation in the intestinal mucosa leading to the increased absorption of the variety of nutrients from the intestinal tract

c. Increasing the amount of beneficial microbes in the intestinal tract leading to an optimal intestinal environment

d. Synthesis of a variety of bacteriocins (bacterial products made by beneficial bacteria or probiotics which are used to inhibit other pathogenic microbes) and vitamins by the probiotics further increasing animal health

Currently, China, the USA, and Brazil are the leading countries of antibiotic utilization in animals. Approximately, 40 antibiotics have been approved for animals in the USA, out of which more than 30 are currently being used in humans. Majority of antibiotic use in animals falls in either one of the three uses:

3.1. For the treatment of infectious diseases in animals (therapeutic use)

Therapeutic treatment usually involves the treatment of either single or whole herd/flock with specific doses of specific antimicrobial drugs. One typical care should be kept in mind in this aspect is that each animal should receive the complete dose of antibiotics and the antibiotic treatment should be continued for at least 72–120 h. But there may be problems in this aspect of antibiotic use as the diseased animal has greatly reduced appetite, and this thing may lead to the reduced intake of the specific antibacterial drug. This may lead to the potential problem of developing the resistance in microbial communities against these antibiotics [4].

3.2. For the prevention of a variety of infections in animals (prophylactic use)

Traditionally many antibiotics were being used in the feed lot and dairy cattle to prevent against a variety of diseases. However, this practice is discouraged.

3.3. The use of antibiotics as growth promoters

Although the use of antibiotics in animals has greatly contributed in increasing the production in farm animals, however, their use as growth promoter was an issue of great controversy as this practice has greatly enhanced the development of resistance in a variety of microbial species not only in animals but also in humans. Therefore, nowadays, several

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countries have banned the use of the latest antibiotics as growth promoters in animals (to be intended for human use only in order to minimize the chances for development of antimicrobial resistance) [5, 6].

There are some exceptions in this aspect of development of the resistance in microbial communities against antimicrobial use as AGPs. The typical example is the use of ionophores which are mainly used to reduce the incidence of coccidiosis as well as improve the microbial communities in rumen (favor the microbial communities toward Gram-positive bacteria). Furthermore, latest studies have also shown that the use of the AGPs may contribute toward the disturbance of normal microflora in animals, thereby greatly affecting the immune system of the animals [7–10].

4. Molecular mechanisms of the resistance against major antimicrobial drugs

Although, till now, different types of the resistance mechanisms have been reported in bacteria against different antibiotics, they can be broadly classified as follows.

4.1. Degradation of the antibacterial drug by the bacterial enzymes

Several bacterial beta lactamases can degrade the beta lactam antibiotics. Beta lactamases do it so by degrading the beta lactam ring of the antibiotics. Also, some antibiotics can be degraded by the addition of the specific group, e.g., chloramphenicol molecule possesses hydroxyl group that can be easily acetylated by the incorporation of acetyl-CoA, a reaction that is catalyzed by acetyl transferases. Moreover, aminoglycosides can also be easily degraded by the addition of phosphate, acetyl-CoA, and adenylyl group, and these additions are carried out by the phosphotransferases, acetyl transferases and the adenyl transferases [11, 12].

4.2. Reduced permeability to a drug by bacteria

For example, most of the Gram negatives contain extremely small-sized porin in their outer membrane, and this imparts a major permeability barrier to a large number of antibiotics to cross that barrier. In similar manner, *Mycobacterium* also offers a major permeability barrier to most of the antibiotics as they contain a thick layer of the mycolic acid, and this waxy material hinders the entry of the majority of drugs [13].

4.3. Increased efflux of the drug

Several bacterial species possess the efflux pumps that actively pump out the drug by bacterial cells. Such pumps effectively reduce the bactericidal concentration of a particular antibiotic drug, and they have been actively implicated in the emergence of resistance against a myriad of the antibiotics particularly against tetracyclines, aminoglycosides, and sulfonamides. The

examples of bacteria containing these types of pumps include *Pseudomonas*, *Staphylococcus aureus*, and *E. coli* [14].

4.4. Modification of the drug target

Several drugs act by their initial binding to a particular site within bacterial cells in order to initiate their bactericidal/bacteriostatic activity. So when a drug binding target is changed, it may lead to the development of the resistance in bacteria against that drug. This phenomenon has been incriminated in the emergence of the resistance against antibiotics such as macrolides and phenolics (Chloramphenicol) in which alternations in the binding sites at ribosome drastically reduce the antibacterial activity of these drugs [15].

4.5. Use of an alternative pathway by bacterial cells

Some of the microbes use alternate pathways, and they change the previous pathway which is inhibited by a particular antibiotic. Typical example is that some of the bacteria use the preformed folic acid rather than synthesizing it, which is inhibited by sulfonamides by a phenomenon known as competitive antagonism.

4.6. Specific analysis of antibiotic resistance at molecular level

With an advent of the molecular biology techniques, many mechanisms for the development of antibiotic resistance have been observed in the last half century [2]. One of the major threats nowadays is by the extended spectrum beta lactamases (ESBLs) producing Gram-negative bacteria [11]. Till now, approximately 13,000 different types of the ESBLs have been reported, and they are mainly classified into three different functional groups. Most of the ESBLs are encoded by a gene known as bla, and the most important ones are bla CTX, bla TEM, bla SHV, bla KPC, and bla OXA. One of the major issues is the origin of the carbapenemase enzymes which are mainly associated with the development of the resistance in the carbapenems (e.g., IMP, KPC, VIM beta lactamases, and OXA), major drugs which are used for the treatment of multidrug-resistant (MDR) Gram-negative infections. These enzymes also act on the third- and fourth-generation cephalosporins, e.g., cefquinome and ceftiofur, thus referred as the extended spectrum cephalosporinases (ESCs). One of the major features associated with many ESBLs/ESCs is that they are located in the moveable genetic elements, e.g., plasmids [12]. In similar way, resistance to macrolides is also associated with the development of the resistance in a variety of closely related drugs, i.e., lincosamides, pleuromutilins, and streptogramins, and thus they are referred as PLSA phenotype [16, 17] and MLSB phenotype [18].

Another main point associated with the aminoglycoside resistance is that the same gene in bacteria is responsible for resistance against the gentamicin and apramycin. Moreover it has also been observed that the gene causing the resistance against the aminoglycoside is also responsible for the resistance against the fluoroquinolones [19].

The table below illustrates the different mechanisms of the development of the resistance against a variety of anti-infection drugs used in veterinary medicine. This table clearly depicts

that each bacterium possesses a unique set of the resistance mechanisms, and virtually resistance against antibiotics is present in nearly all species. One such example is that the resistance against the macrolide is different in *Staphylococcus* than observed in the *Campylobacter*.

One of the major emerging issues nowadays is the resistance against multiple drugs, i.e., fluoroquinolones and cephalosporins; another such issue is the emerging MDR *E.coli* ST 131 [20, 21]. Some of the old antibiotics, e.g., colistin, which was previously widely used for the control of *E. coli* [21], have been withdrawn so that it may be used to treat a variety of infections caused by the MDR Gram-negative bacterial spp. [22]. However, recently it has been observed in China that bacteria possess the mcr-1 gene which plays an important role in conferring resistance against colistin, and this observation has severely raised questions on the use of colistin for the treatment of a variety of bacterial infections [23–25]. Just immediately afterward, in Belgium, mcr-2 gene was also observed in a bacterium that did not contain the mcr-1 gene and that gene was also responsible for conferring the resistance against colistin in those bacteria [26, 27].

Another important fact is that the emergence of the resistance against one member of a class of antibiotic may result in the development of the resistance against other members of that class. The other mechanism is known as co-selection. This is a phenomenon in which the genes for resistance against a myriad of antibiotics are located in the single plasmid or the mobile genetic element. In this case this mobile genomic element or the plasmid confers the resistance against all the antibiotics. In contrary to this, the other mechanism is that when one bacterium becomes resistant against one microbe, then it exhibits an increased susceptibility against the other antibiotic [28].

4.6.1. Mechanisms of the antibacterial drug resistance

Table 2 illustrates the mechanisms of the bacterial resistance against different drugs. It also depicts various genes involved in the emergence of resistance against that drug.

4.7. Mechanisms of antibacterial resistance transfer in bacteria

One of the major problems is that bacteria not only become resistant to many antibacterial drugs by a variety of phenomena, but they are also capable of transferring this resistance to other bacteria of same as well as with other genera. The main reason for transfer of genetic resistance is because the genes for antibacterial resistance are mainly present on the moveable genomic elements (MGE), e.g., integrons, plasmids, and transposons. But the changes at gene level may also occur in chromosomes although they are extremely rare (except in *Mycobacterium*). This often occurs because there is a change in the drug target and, thus, the antibacterial drug cannot bind to the appropriate binding site, leading to the loss of efficacy of that particular antibiotic. If the use of antibiotics is inappropriate, e.g., not following the well-established dosage regimen for a particular drug against a particular disease, it can dramatically increase the chances of the development of the resistance against that drug because of the selective advantage of changing their genomic elements.

One of the main important locations of antibacterial resistance is the bacterial plasmids. Plasmids are single covalently closed circular pieces of the DNA which are not important in the survival of the bacteria, but they are extremely important in offering an added advantage

Antibiotic class	Members	Target	Resistance mechanism	Genes involved (examples)
Polypeptide	Bacitracin	Cell wall (inhibits peptidoglycan synthesis)	Efflux	*Bcr*
Tetracycline	Tetracycline Oxytetracycline Chlortetracycline Doxycycline Minocycline	Ribosome (30 S)	Drug efflux Change in binding site Drug modification by bacterial enzymes	*Otr*, *tet*
Streptogramins	Virginiamycin	Ribosome	Efflux Change in drug target Drug inactivation by bacterial enzymes	Erm, Isa, Vgb, Vga, cfr, vat
Macrolides	Tylosin Tilmicosin Tiamulin Erythromycin Azithromycin Clarithromycin Spiramycin Tulathromycin	Ribosome (50 S; inhibit the translocation of the aminoacyl tRNA from P site to A site)	Glycosylation Hydrolysis Phosphorylation Efflux Altered drug target	Ere, erm, msr, mef, mph, cfr
Pleuromutilins	Valnemulin	Ribosomes	Altered drug target Efflux of the drug	Sal, cfr, vga, isa
Lincosamides	Lincomycin Clindamycin	Ribosome (50 S; Inhibit the translocation of the aminoacyl tRNA from P site to A site)	Drug efflux Inactivation of the drug Altered drug target	Erm, inu, vga, inu, cfr, msr,
Beta lactams	Penicillin Amoxicillin Ampicillin Cloxacillin Cefuroxime Cefalexin Ceftiofur Cefquinome	Cell wall (inhibit the synthesis of peptidoglycan)	Efflux, Hydrolysis Altered drug target	Ade, mec, bla, mex, cme
Aminoglycosides	Gentamicin Kanamycin Neomycin Apramycin Spiramycin Framycetin	Ribosomes	Inactivation by bacterial enzymes Efflux Altered drug target	Aph, ant, acc, rmt, ade, arm, spd, apw
Fluoroquinolones	Enrofloxacin Marbofloxacin Orbifloxacin Ibafloxacin Pradofloxacin	DNA replication (inhibit the DNA gyrase)	Altered drug target Drug efflux Inactivation of the drug by the bacterial enzymes	Aac (6′), oqx, qnr, gyr A, parC
Phenicols	Florfenicol Chloramphenicol	Ribosomes (50 S; inhibit the peptidyltransferase)	Drug efflux Inactivation of the drug by enzymes Altered drug target	Cat, cfr, cml, flo, optr

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Antibiotic class	Members	Target	Resistance mechanism	Genes involved (examples)
Sulfonamides	Sulfamethoxazole	Folic acid synthesis	Altered drug target Increase efflux of the drug	acr, sul
Nitrofurans	Nitrofurazone Nitrofurantoin	DNA	Modification of the target in bacteria Changes in porins	Omp, nfs
Pyrimidines	Trimethoprim	Folic acid synthesis	Modification of the target in bacteria Enhance drug efflux	Dhfr, bpr-opc
Cationic polypeptides	Colistin Polymyxin B	Cell membrane	Modification of the drug target Increased efflux of the drug	pmr AB, mgr

Table 2. Mechanisms of the antibacterial drug resistance.

to the microbes when present. They do it by providing the genes for resistance against many antibacterial drugs. Moreover, they can also make the bacteria resist the toxins and heavy metals. Because of the fact that they are important in providing the bacteria resistance against many factors, that is why they are also called as R factor. Plasmids are extremely important in the development of the resistance against the drugs as they can easily be transferred from one to the other bacteria by means of the pilus. This process is known as conjugation. This process can occur in many species including *Clostridium*, *E. coli*, *Bordetella*, *Salmonella*, *Proteus*, *Streptococcus*, and *Shigella*. One important factor should be kept in mind that plasmids may not only be transmitted within a genus but also between genera. This fact is of extreme danger to the bovine as well as public health as resistance in bovine microbial spp. can transfer the resistance in many microbes of public health importance, sometimes even leading to the pandemic spread of the zoonotic and public health pathogens.

However, it should be kept in mind that although the plasmids are often exchanged between bacteria by the process of conjugation, however, they may also be transmitted from one to the other bacteria by means of other methods of genetic transfer, and they may include the transduction and transformation. In transformation, the genetic components of one bacterium are transmitted to the other bacterium when placed in a medium. However, it should be kept in mind that this process is poorly understood, yet at molecular level and moreover, it occurs in limited microbes under specific conditions (the presence of divalent ions or electrical pulses).

Transduction is defined as the transfer of genetic components from one to another bacterium through a bacteriophage (viruses infecting bacteria). When a virus infects the bacterial cells, there is possibility that during integration with the host cell genome, it may take away some components of those bacterial cells in the process when it leaves the cells. Two types of transduction are possible. One is generalized transduction in which any component of the

bacterial cell may be transmitted. On the other hand, the specialized transduction is one in which the virus carries only the specific component of the bacterial cells (mostly surrounding portion of the integration site). When the same bacteriophage may infect the other cell, it can transfer the genes of previous bacteria into the new bacteria. The process of transduction occurs with the greatest frequency in gram-positive bacteria.

A single plasmid may carry the genes for antibacterial resistance against many drugs (3–9); thus, the whole population of the bacteria may become multidrug resistant by the process of plasmid transfer although the animals may be treated with only one drug. A single plasmid has been found to carry the genes of antibacterial drug resistance at against least nine different drugs.

Other MGEs may also play an important role in the emergence of antibacterial resistance in microbial populations. Many transposons may also carry the genes for antibacterial drug resistance, and they are rapidly moving within the bacteria leading to the emergence of antibacterial drugs resistance in microbes. Some examples of the transposons include the Tn05 (bleomycin, kanamycin, and streptomycin), Tn21 (spectinomycin, streptomycin, and sulfonamides), and Tn4001 (tobramycin, gentamicin, and kanamycin).

A genetic component that carries many genes against many antibiotics together in the form of gene cassettes is also known as integrons. An integrin is normally a nonreplicating piece of the DNA that is often associated with a plasmid, chromosomes, or integrons. The integrons also play an important role in the spread of antibiotic resistance against many microbes as they are also easily transmitted from one to the other bacterial spp.

4.8. Determination of antibiotic susceptibility

Although antibiotics have been made many years ago, however, still little information is available on how to use antibiotics in which way. Although there are many guidelines about the treatment of specific infections with specific antimicrobial agents, however, still it is often extremely difficult to select an appropriate antibiotic to treat a particular disease. This happens primarily as many factors contribute to the selection of an appropriate antibiotic, and they include animal species affected (some antibiotics are contraindicated in bovine because of public health issues, or they are too much toxic for animals),the infection (aerobic or anaerobic, soft tissue or hard tissue infection), microorganism susceptibility to a particular agent, drug behavior within the body (pharmacodynamics, e.g., where the drug distributes more effectively and toxicity profile of the drug within the body), dosage regimen of particular antibiotic, route of administration, and various drug residues. Majority of the mentioned parameters are studied in depth while designing and final approval of the drug, but still pharmacodynamics of a particular drug are often unpredictable in some animals and the site of infection also matters in the selection of antibiotics.

So, testing antimicrobial compounds to determine the antimicrobial susceptibility in various bacteria becomes an extremely important parameter to be determined, before making a final decision about the antibiotic use in animals. On the basis of the results of the antibiotic sensitivity testing, the organisms may be classified as the susceptible, intermediate, or resistant microorganisms. However, an important aspect should be kept in mind that antibiotic is not

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equally effective against all bacteria even if they belong to the same spp. A typical example is *Pasteurella multocida*, which causes the respiratory infection in bovines. When its (isolated from many animals from different countries) antibiotic susceptibility was done against ampicillin, then different isolates demonstrated different levels of the susceptibility and resistance against the same drug (Penicillins). In addition, it is possible that an antibiotic is initially highly effective in the treatment of a particular infection; however, with the passage of time, its effectiveness may become lower. This problem may arise if the animal is treated many times with the same antibiotic or is exposed to the suboptimal concentration of a particular antibiotic.

So because of this intense variation in antibiotic response against different infections, it becomes imperative to accurately test the antibiotic susceptibility routinely prior to make any decision. The results provided by some of the routinely used antibiotic susceptibility tests can provide some guidance about the susceptibility profile of bacteria against different antibiotics. However, it should also be kept in mind that although these in vitro antibiotic profile may provide us with an idea of bacterial antibiotic susceptibility testing, however, still they do not provide any guarantee to be effective in vivo. A simpler scenario may be that when animals is suffering from an anaerobic infection in which *E. coli* is primarily the causative organisms, in vitro test may show that aminoglycosides possess good antibacterial activity against the *E. coli*; however, if only the interpretation of that result is kept in mind and aminoglycosides are used to threat that infection, there will be treatment failure. This is because of the fact that aminoglycosides need oxygen-based uptake within the bacteria, and in anaerobic environment, there is no uptake of the aminoglycosides leading to the failure of treatment. Similarly, different drugs have different affinities for different tissues, and thus if some drug is unable to reach to a particular tissue, it is unable to demonstrate its microbial killing or inhibition activity. Moreover, nowadays a very hot research issue is how to control bacterial population in biofilms. Different bacteria make biofilms which are collections of millions of the microbes. In biofilms, microbial population becomes extremely resistant to a particular drug, as it does not allow the drug to penetrate the microbial populations. In short various factors are responsible for an antimicrobial drug to demonstrate different antibacterial effects in vitro and in vivo.

Although the assays for the determination of the antimicrobial sensitivity were developed just after the discovery of the first antibiotic, penicillin, however, they were poorly designed assays, and they exhibited a greater variability not only among different communities but within the same hospital. So there was a pressing issue for the development of the test which exhibits greater reproducibility and may be applied universally across the globe. So after a lot of hit and trial method, researcher became successful in devising an assay which is used till now as one of the most preferred methods for the determination of the antibiotic susceptibility testing in microbes. This test was developed by Bauer in 1966 and is known as disk diffusion assay of antimicrobial sensitivity in microbes.

4.8.1. Disk diffusion test for antimicrobial sensitivity

This is one of the most widely assays currently being used in veterinary medicine because this assay can be used to test the antimicrobial activity of many drugs against any bacterial spp., and it is also economical.

The test is based on the use of disk impregnated with antibacterial compound and placing it on the agar plate (Muller Hinton agar) which is cultured with 1×10^8 microorganisms/ml of the suspension. When the antibiotic disk is placed on the agar surface, it allows the antibiotic to move in a lateral position due to diffusion. This results in the generation of the antibiotic gradient along the agar surface. Near the antibiotic disk, the concentration of the antibiotic is higher, but as the distance increases from the disk, it causes a reduced concentration of the antibiotic. This results in the production of the zone of inhibition of the bacterial growth. This zone gives an indirect idea for the estimation of the minimum inhibitory concentration of the drug that is required against particular bacterium.

The disk diffusion method can also be affected by many different types of factors. The first one is the thickness of the agar in the petri plate. The recommended thickness of agar medium is 4 mm. If the thickness of the agar medium is more than the recommended thickness, it will result in the reduced diffusion of the drug in lateral direction, resulting in false interpretation of the antimicrobial sensitivity test. If the thickness of the plate is less than the recommended thickness, it will result in increased drug diffusion in the lateral direction. The second factor is the bacterial concentration. If the bacterial concentration in the media is less than the recommended, it will result in an increased zone of inhibition, which leads to the false interpretation of the test. Similarly, if the concentration of the bacterial suspension is more than the standard one, it will also mislead the final interpretation. If the generation time of the microorganism is too long, e.g., *Arcanobacterium*, it will result in an increased zone of inhibition leading to a false interpretation. Another factor to be kept in mind while conducting antibiotic sensitivity testing is the placement of the disks on agar. The standard distance is 24 mm. If the distance is too small between two antibiotic disks, it may lead to overlap among various zones of inhibition making the interpretation of the assay difficult.

One major disadvantage of this test is that this assay is mainly qualitative. Although it gives us an idea about the minimum inhibitory concentration of a particular drug against a particular microbe, however, still it is one of the most commonly used particularly in developing countries, where infrastructure is poor at local veterinary hospitals. The other advantage of this test is that it is an extremely simpler test to carry out with a minimum cost. The results of this assay are available within 24–48 h for most of the bacterial pathogens of bovine importance [29].

4.8.2. E-test

The other method of determination of antibiotic sensitivity is the E-test. The disadvantage of the disk diffusion assay can be mitigated by using another similar test which almost involves the same technique; however, it gives a nearly realistic value of the MIC of particular antibiotics against a particular bacterium. The test is named as E-test.

The principle of the E-test is based on the lateral diffusion of the drug from a plastic strip which is impregnated with the different conc. of the antibiotics usually starting from 0. Along the length of the plastic strip, the concentration of the antibiotic increases gradually, thus creating a continuous gradient of antibiotic concentration along the plastic strip.

So at the beginning where the concentration of antibiotic is zero, there is usually no zone of the inhibition. As the concentration of the antibiotic increases, there is a proportional increase in the zone of inhibition around the plastic strip.

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Although E-test gives us quantity data regarding the MIC against a particular bacterium, however, it is expensive to carry out, which makes it difficult to be carried out under field conditions; however, it may be used for research purpose against a particular bacterium [30].

4.9. Strategies to reduce the antimicrobial resistance

As development of antibiotic resistance in major microbes is posing a major threat to animal and human populations, it requires efforts at gross root level to reduce this problem. Several different strategies may be adopted which are extremely important for the reduction in the emergence of antibacterial resistance. This may involve the early detection and diagnosis of the infectious diseases, the use of alternatives to antibiotics, estimation of the cost and relative benefit analysis, and using immunomodulation.

4.9.1. Early detection and diagnosis of diseases

This strategy may also aid a lot in the reduction of antimicrobial use and thus develop resistance as discussed; see [29]. Several diseases have different parameters, which may give an important indicator about the disease occurrence at an earlier stage. For example, several liver enzymes and kidney enzymes start to alter their serum conc. And this may provide an initial indicator for a variety of infectious diseases at earlier stages. Several infectious diseases produce systemic indicators, such as increase in the serum proteins, and this effect is collectively known as acute phase response to an infection. In acute phase response, several acute phase proteins are greatly increased in their serum concentration. Similarly at initial stages of viral and bacterial infection, there are changes in the conc. of the specific cytokines in the serum of an animal. All of these acute phase proteins and changes in the levels of the different cytokines can easily be detected by a variety of laboratory tests (e.g., ELISA). Although this approach requires a state-of-the-art diagnostic laboratory facility, using this approach can help in the reduction of the emergence of the antibiotic residues in bovines. All of these changes in earlier stages of infection are the consequences of systemic inflammatory response which can easily be controlled by the use of a variety of anti-inflammatory drugs, i.e., nonsteroidal anti-inflammatory drugs. This treatment can be augmented by using the strategy of nutritional modulation in dairy animals by supplementing feed with omega-3 fatty acid, phytochemicals (e.g., *Aloe arborescence*, *Echinacea purpurea*), and antioxidants. It has been observed that using this strategy can lead to the reduction in the incidence of many reproductive diseases, ketosis, and somatic cell count in milk.

The above strategy may be adopted with a greatest success in terms of the reduction of the antibiotics use in animals, but this strategy also necessitates the routinely strict monitoring and inspection of the dairy herd. A variety of animal welfare and health parameters should be regularly monitored, and they include recording the dry matter intake of animals, milk somatic cell count, rectal temperature, the presence of blood or fibrin clots in milk, and noting any teat lesions. It has been observed that routine monitoring of these parameters can result in earlier detection of the disease and therefore earlier and simpler treatments, often not requiring antibiotics (e.g., by improving the ruminal activity with the use of yeast or glucogenic supplementation).

4.9.2. Using antibiotic alternatives

Recently, it has been proposed in the international OIE conference that an emphasis should be placed on the use of antibacterial drug alternatives such as bacteriophages, biological response modifiers (BMRs), antibacterial peptides of natural origin, prebiotics, probiotics, and the use of proper vaccination schedule in animals (given in the table below); probiotics, prebiotics, and BRMs are responsible for the development of the optimal intestinal microbial flora which can overall improve the animal health along with reducing the incidence of the several infectious diseases in dairy animals; see [31–33]. Bacteriophages are extremely specific in infecting particular bacteria, and that is why they pose a minimum toxicity danger in humans and animals, yet this approach is mainly on theoretical grounds and needs many successful clinical trials and intensive knowledge before they may be adapted in animals for treating infectious diseases. Similarly following an appropriate vaccination program in animals (keeping in view of the serotype/strain of the agent to be used in a particular area) can dramatically reduce the occurrence of many primary as well as secondary infections in bovines.

Table 3 illustrates a variety of diseases and their treatment.

Vaccinating the bovine against many infectious diseases can definitely reduce the incidence of majority of bacterial and viral infections, thereby further reducing the usage of the antimicrobials in bovine.

Similarly, using a variety of phytochemicals may also be useful in the treatment of variety of infections. These chemicals derived from different plants act systemically for the treatment of diseases.

For treating the infections of gastrointestinal tract, *Allium sativum* stem is crushed, is boiled in the water, and then is provided to the animals. Its effect is mainly due to the compound present in it also known as allicin which is well known for its antibacterial activity [34, 35]. Also *Cassia fistula* is also being used in treating a variety of GIT maladies of bovines; particularly, it is effective in treating *E. coli* infections effectively. This activity may be due to the presence of several compounds of pharmacological importance including terpenoids, saponins, steroids, anthraquinone, phenolic compounds, and steroids present in them [36]. Similarly seeds of the *Foeniculum vulgare* are mixed with the root of *Glycyrrhiza glabra*, and they are also used to treat the GIT infections [37, 38]. *Morus alba* leaves are given to the animals for

Brucellosis (breeding animals)	4–12 months of age
Vibriosis (breeding animals)	Before breeding
Leptospirosis (breeding animals)	Before breeding
Black leg	March
Anthrax	February–August
Hemorrhagic septicemia	June–December
Foot-and-mouth disease	March–September

Table 3. Vaccination schedule of bovine.

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5 days in order to treat constipation. This anticonstipation activity is mainly reported because of the presence of the quercetin, rutin, and apigenin [39, 40]. *Punica granatum* peel has also demonstrated its activity against majority of the GIT pathogens which may be because of the presence of the flavonoids and phenolic compounds in it [41].

One of the most economically devastating diseases of bovine, mastitis, can also be treated by using a variety of phytochemicals. Here again, *Allium sativum* is also routinely being used in the treatment of mastitis. It has shown promising results even against those pathogens which are resistant to many antibiotics [42]. *Zingiber officinale* is also being used to control mastitis in dairy animals [43]. Similarly, *Allium cepa* and *Trachyspermum ammi* mixture was also being routinely used to control mastitis successfully in dairy herds [44].

Bovine often do suffer from a myriad of respiratory infections particularly when kept in a closed humid confinement in winter season and with poor ventilation. Again some compounds are extremely useful in treating respiratory diseases of animals, and they include *Glycyrrhiza glabra* (stem) and *Hordeum vulgare* (seeds). Both of these plants possess a variety of compounds such as saponins, volatile compounds, tannins, and alkaloids which are beneficial in treating a variety of mild to moderate respiratory infections (when treated for 5–7 days) [45, 46].

Similarly for the treatment of a variety of bovine skin infections, many compounds are routinely used, and they include *Aloe barbadensis* leaves (given orally alone or mixing with salts), *Tamarix aphylla* leaves (applied topically), *Citrullus colocynthis* (extracts from fruit and seed), and *Azadirachta indica* (leaves). All of these plants are extremely useful in treating a variety of inflammatory/infectious diseases of bovine skin [47–49].

4.9.3. Estimation of the cost and relative benefit analysis

Although currently there is a great emphasis on the reduction of antibiotic use in animals, however, its use is still needed in some cases of infectious diseases. But in such cases, it is better to prioritize the basis of cost of antibiotics in animals and the benefits achieved. For example, in the case of mastitis, treatment efficacy with the help of gram-negative bacteria depends on several factors including the bacterial spp. involved and the acuteness or chronicity of the disease. Some cases have good outcomes following treatment, while the treatment of the chronically affected animals is often futile. Similarly, some bacterial spp., e.g., *Streptococcus agalactiae* and *Staphylococcus aureus*, along with coagulase-negative *Staphylococcus aureus*, are extremely difficult to treat. This also implies that culturing of the mastitis-infected milk along with antibiotic susceptibility testing should be a routine practice while treating the animals; see [50].

Similarly if the animal is repeatedly suffering from clinical mastitis, particularly after the third lactation, then mostly cost of the treatment becomes extremely higher than the benefits.

4.9.4. Immunomodulator use

Animals often suffer from chronic stress when their welfare is compromised. Stress may be in the form of overcrowding, nutritional deficiency, poor ventilation and temperature control, transportation, and high production. Under stress body physiology is altered, and it results in

secretion of cortisol from the adrenal cortex. Under the influence of the adrenal cortisol, the body immune system is greatly compromised. This predisposes the animals to a variety of infectious diseases. So it is better to use immunomodulators in animals under the conditions of stress. This strategy has been discussed in detail; see [51, 52]. These immunomodulators may include the use of vitamins A, C, and E along with minerals such as selenium, copper, and many other minerals. This can dramatically reduce the incidence of many infectious diseases in dairy animals leading toward a reduced use of antibiotics in animals.

5. Conclusion

There are numerous aspects of antibiotic resistance in animals, which are important public health threats for humans, and therefore it is imperative to make as many possible efforts to reduce the emergence of antibiotic resistance in farm animals. It may involve maintaining the animal welfare and good nutritional and immunological status, routine examination of the herd, early detection and appropriate diagnosis of infectious diseases in animals, appropriate therapy at time and the use of antibiotic alternatives such as bacteriophages and vaccination, the use of phenomena of competitive exclusion (by using pre-, pro-, and synbiotics), biological response modifiers, and appropriate immunomodulation in animals. Implementing such programs at national level requires huge financial and political support for ongoing efforts to effectively control the emergence of antibacterial resistance in major bacterial spp. Although using these approaches may not completely assure the avoidance of antibiotics and, therefore, the possibility of emergence of antibiotics, yet, these approaches may aid in slowing down the antibiotic resistance to a greater extent in animals leading toward a great step in improving the public health.

Conflict of interest

Authors have no conflict of interest.

Author details

Muhammad Ashraf[1], Behar-E-Mustafa[2], Shahid-Ur-Rehman[3], Muhammad Khalid Bashir[1]* and Muhammad Adnan Ashraf[4]

*Address all correspondence to: mkhalidbashir@uaf.edu.pk

1 Animal Breeding and Genetics, University of Agriculture, Faisalabad, Pakistan

2 Microbiology, University of Agriculture, Faisalabad, Pakistan

3 Molecular Biology, University of Agriculture, Faisalabad, Pakistan

4 Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan

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