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Review article
Ανασκόπηση

Application of metagenomics in bacterial resistance in cattle production

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ABSTRACT: Microorganisms are widely found in nature, mostly in soil and water. For ruminant animals, microorganisms make up a high percentage of their rumen. In recent years, the inappropriate use of antibiotics has led to a gradual increase in antibiotic resistance among bacteria within the bovine digestive tract, which can spread to humans, animals, and the environment. With the rapid advancement of molecular biology techniques, metagenomics technology enables more efficient, fast, and accurate detection and identification of novel resistance genes within samples. This technology facilitates in-depth research into bacterial drug resistance in cattle. This paper primarily reviews the application of metagenomics technology in studying bacterial resistance in cattle, aiming to provide theoretical guidance for its clinical implementation in bovine production and for mitigating the emergence of bacterial resistance.

Keyword: Metagenomics; Cattle; Bacteria; Drug resistance; Application

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INTRODUCTION

Nowadays, classic antibacterial drugs - antibiotics, it can no longer meet the clinical requirements for combating bacterial infections[1]. Infections caused by monoresistance and multidrug resistance strains of bacteria have become an important challenge for the maintenance of public health, Widespread antimicrobial resistance genes have also emerged as one of the major threats to global health[2-3]. Since the discovery of penicillin in 1928, more than 100 antibiotics have been discovered and used, most of which were developed before 1970[4]. The misuse of antibiotics in animal husbandry has been cited by the World Health Organization (WHO) as a major threat to global public health security, seriously impeding the achievement of basic healthcare goals and exacerbating the global health crisis[5-6]. While China's cattle and sheep industry is developing rapidly, inappropriate use of antibiotics as growth promoters for livestock, etc., promotes the creation of bacterial drug resistance genes, this in turn leads to a gradual increase in the level of bacterial resistance in ruminants, disrupts the natural balance between bacteria and antibiotics[7-8]. In recent years, our research team has been working on studying bacterial drug resistance. In 2018, we isolated pathogenic *Bacillus cereus* from diseased and dead cattle from a large Simmental cattle farm in Tongliao City. The bacterium was tested to be resistant to nine drugs including penicillin, neomycin, and clindamycin[9]. In 2020, we also isolated Staphylococcus aureus from the organs of a sudden-death cow from an Angus cattle farm in Tongliao City, and drug sensitivity tests showed that the strain was resistant to penicillin[10]. Currently, nearly 100 strains of pathogenic bacteria of animal origin in our laboratory collection have severe bacterial resistance. Therefore, efficient and accurate detection techniques for the analysis of drug resistance genes have become a problem that needs to be solved urgently.

Metagenomics technology can detect pathogens that cannot be clarified by traditional testing methods such as culture and histopathology, to quickly identify bacterial drug-resistance genes and improve the accuracy of treatment[11]. The method shows strong advantages in finding new genes and obtaining species and abundance of a large number of microbial communities and related biological information. The aim of this paper is to provide a theoretical basis for the application of metagenomics technology in clinical cattle production. Through in-depth analysis of the potential of metagenomics technology in the study of animal microbiota drug resistance, disease mechanisms, and their interrelationships, it reveals its important role in cattle health management and drug resistance prevention and control.

OVERVIEW OF METAGENOMICS

Metagenomics, also known as environmental genomics or ecogenomics[12], involves the direct sequencing and analysis of microbial community DNA. It has rapidly become a standard method for characterizing the functional potential of microbial communities[13]. In 1998, handelsman of the University of Wisconsin first introduced the concept of Metagenomics[14]. When generating metagenomic datasets, sequencing read length requires careful consideration: longer reads facilitate the assembly of longer contigs, and sufficiently long contigs are essential for accurate gene prediction and functional annotation in subsequent steps[15]. Metagenomics can be used to construct a metagenomic library and perform deep sequencing on a selected functional gene (Fig.1). This technology enables deep sequencing of all DNA in an environment and analysis of the structure and function of microorganisms within that habitat. It can also be used to infer the biological functions of microbial communities. For instance, the association between the gut microbiome and host phenotypes and diseases, as revealed through metagenomic

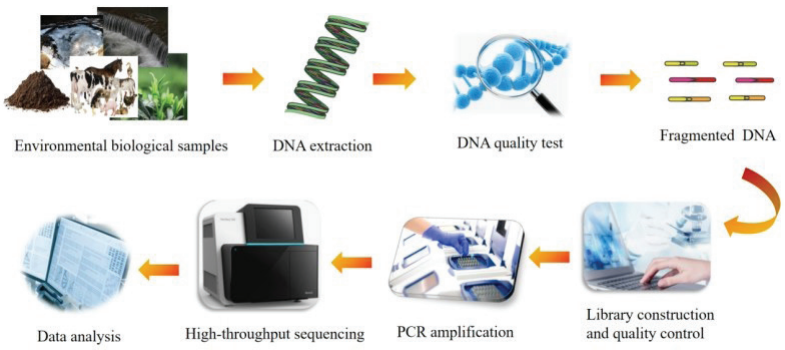

Figure 1. Metagenomics Sequencing Flowchart.

sequencing, is gradually being elucidated through metagenome-wide association studies[16].

With the continuous updating and iteration of sequencing methods, metagenomics has become one of the important areas of microbial ecology research[17]. From 16S rRNA marker gene analysis to characterize community composition to macro-genome sequencing and functional analysis, it can be applied to all genome types, and the method has been widely used for clinical testing in humans/animals[18] (Tab.1). This technology can deeply and systematically analyze and delineate the microbiome, reflecting the diversity, compositional and functional profiles of communities through homology and differences in nucleic acid sequences, and conduct large-scale comparative and correlation analyses of various microbiomes to further explore their significance to the entire ecosystem, thus assisting researchers to more comprehensively investigate the interaction mechanism between "microbiome this will help researchers to more comprehensively study the interaction mechanism between "microbiome and environment/host"[19]. However, due to the scarcity of gene annotation information and the incompleteness of reference genomes, the in-depth mining and effective utilization of metagenomic sequencing data are severely constrained, posing significant challenges to metagenomic analysis. In light of the above, researchers urgently need to establish comprehensive microbial gene catalogs and complete genome catalogs[20].

Application of metagenomics in the study of bacterial resistance in cattle

The mechanism of drug resistance changes in response to alterations in drug resistance genes, and the quality of livestock products is also impacted. Researchers are exploring antibiotic resistance from various angles, gaining deeper insights into resistance mechanisms at multiple levels[21-22] (Fig.2). Therefore, the discovery and characterization of microbial drug resistance genes and the analysis of the mechanisms of their transfer between species are urgent tasks to ensure human and animal health[23]. With the rapid development of molecular biology technology, metagenomics has become a hotspot in the study of bacterial drug resistance and other fields, and a powerful technique to identify new drug resistance genes from unknown microbial sequences[24].

Figure legend: The 5 key mechanisms of bacterial resistance: reduced membrane permeability, antibiotic inactivation, efflux pump activation, target modification, and metabolic pathway reshaping.

Application of Metagenomics in Drug Resistance of Cattle Fecal Samples

The presence of antibiotic-resistant genes in cattle is closely associated with microorganisms in the farming environment[25]. Macrogenomics technologies provide efficient methods in probing the structure and function of microbial communities.

Metagenomics techniques allow the exploration of rare or unknown microbial taxa in the bovine

Table 1. Application scope and advantages of high-throughput sequencing

Sequencing methods	Range of application	Advantages	Disadvantages
16S rRNA amplicon sequencing	It is used to study the diversity and distribution of microbial communities, reveal the species, relative abundance, and evolutionary relationship of microorganisms in environmental samples, and is widely used in the reconstruction of phylogenetic trees.	It has high accuracy, and high flux, can measure 1 - 2 hypervariable regions, is low cost, is a simple sample preparation method, and is a widely available bioinformatics tool. It is a generic-level study.	PCR amplification bias, 16s rRNA gene resolution is often too low to distinguish closely related species.
Metagenomics sequencing	It plays a key role in comprehensively revealing microbial species, genetic composition, microbial function, and maintaining cellular function.	It helps track bacterial populations in samples and can be used to correlate colonization trends of assembled genomes with the genomic environment for experiments (MAGs).	The cost is high and the gene expression information is not available.

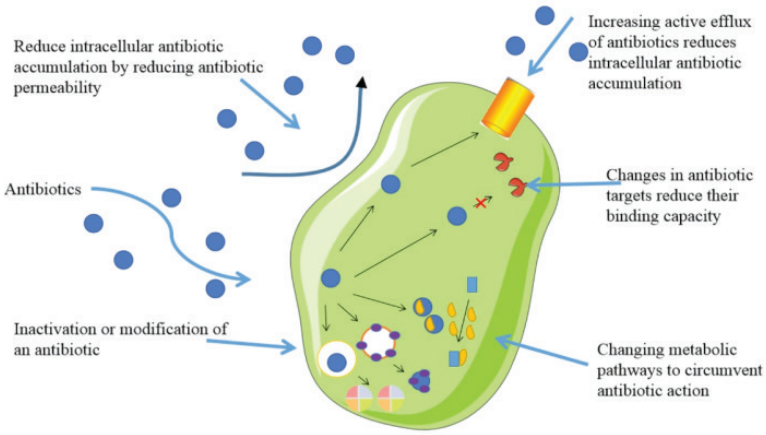

Figure 2. Mechanisms of antibiotic resistance in bacteria.

fecal microbiota[26]. Zhang Xu et al.[27] used macro-genome sequencing to obtain genome-wide information on three samples of piled manure and soil around the piled area in a dairy farm, and analyzed their pathogenic microbial enrichment by the NCBI-NR database. A comparison of the sequencing results revealed that a total of 78 dominant resistance genes belonging to 49 classes of antibiotics were annotated in the composted feces, which were higher than the 49 resistance genes in the soil. This may be due to the level of resistant genes in the piled-up feces spreading to the microbiota in the surrounding soil. Combined analysis of metagenomic sequencing technology and databases can reveal the distribution of drug-resistant genes within samples, underscoring the critical importance of refining these databases. To further understand the differences in resistance groups and microbial communities between the two, pre-weaned calves and lactating cows, Haley et al.[28] performed macro-genome sequencing on 34 fecal samples collected from 17 commercial dairy farms. Sequencing results showed significant differences in microbial community structure and antibiotic resistance between the two groups of calving and lactating dairy cows, indicating that age plays an important role in rumen microbiome dynamics. The abundance of resistance genes was significantly higher in calf feces than in lactating dairy cows, and trimethoprim, aminoglycoside, macrolide-lincosamide-streptomycin B, and β-lactam resistance genes were detected in calf samples and were significantly higher than resistance genes in dairy cow samples. Significant differences in microbial communities and drug-resistant bacteria between groups may be due to several factors, such as the gut microbiota of pre-weaned calves being influenced by the surrounding environment; Pre-weaning calves are still in the monogastric stage of digestion, and there are structural differences between them and the well-developed rumen of dairy cows; Differences in diets, with pre-weaned calves on colostrum and milk replacer, and dairy cows on forage. The study detected differences in the abundance of antibiotic resistance genes in the samples using metagenomic technology, sounding a necessary alarm for the cattle industry regarding the safety of feeding environments. Li et al. [29] tested the antibiotic resistance of 756 *E. coli* strains isolated from bovine feces. Followed by chloramphenicol (46.6%), chlortetracycline (45.2%), streptomycin (40.3%), gentamicin (12.8%), and narasin (12.8%). The dose and frequency of tetracycline use are the main reasons for its high rate of resistance. The assay detected multiple drug-resistant bacterial genes in the *E. coli* population and co-localized on the same mobile genetic element (MGE), corresponding to the drug-resistant phenotypes of the E. coli isolates. In contradiction to the above study, which reported that the addition of macrolide tylosin to feed reduced the incidence of bovine liver abscesses, the resistance profiles of bacteria in bovine feces were examined using macro-genomics techniques. It was found that the difference in bacterial resistance profiles in the feces of cattle consuming feed without added tylosin was not significant, which may be related to the geographical area in which they are kept, the feeding management practices, and the differences in the resistance groups of the feces themselves, and sometimes to the breed of cattle[30].

Metagenomics technology can efficiently and accurately detect drug resistance and the class of resistance genes belonging to cattle and their surroundings and can directly analyze the total DNA information in a sample, making it a highly efficient tool for analyzing microbial communities and functions. In summary, metagenomics analyses revealed

significant differences in the composition and abundance of resistance genes between ruminant species and populations (Tab.2). The application of this technology enables rapid and accurate analysis of drug resistance categories and abundance in large samples. Inspired by recent successes of artificial intelligence (AI) in computer vision, many pathologists anticipate applying AI to various tasks in digital pathology. Concurrently, the rapid advancement of deep learning further facilitates this synergy, making image-based diagnosis a reality within the digital pathology context[31]. This breakthrough also offers new insights into the application of metagenomics technology in detecting drug-resistant genes. Reports indicate that AI and machine learning have achieved certain research advances in predicting antimicrobial resistance in pathogenic microorganisms[32]. The integration of metagenomics technology with AI is expected to enable more timely and rapid detection of novel drug-resistant genes, predict their transmission pathways, and facilitate the implementation of intervention strategies to curb the spread of these genes. This approach can help the cattle industry avoid unnecessary economic losses.

Application of metagenomics in antibiotic resistance of bovine gut microbiota

The microorganisms present in the gut consist of a wide variety of eukaryotic and prokaryotic organisms, and these microbial communities work together with their hosts to form a mutually beneficial symbiotic whole, acting an important role in the protection of the health of the organism, the digestion of food and the absorption of nutrients[39].

Similar resistance genes are present in many animals within the same population. Zhu Zhen[40] systematically analyzed the differences in drug resistance genes in the intestinal flora of dairy cows, yaks, and beef cattle in different farming environments using metagenomics techniques. The results showed that the yak group had significantly lower levels than the dairy and beef groups in terms of diversity and abundance of drug resistance genes carried by the intestinal flora. Meanwhile, Screening of 51 strains of three-generation cephalosporin-resistant *Shigella spp*, 55 fluoroquinolone-resistant strains, and 96 resistance genes was conducted. Since yaks live in highland areas with little human influence[41], they are resistant to extreme living conditions[42]. Antibiotics are generally not used during feeding management, which is the main reason why they carry fewer resistance genes. Thus, yaks serve as a natural control group for the study of drug resistance in intestinal flora[43-44]. This study also underscores the critical importance of rearing environments and management practices, as improved husbandry conditions can reduce both

Table 2. Metagenomics analysis of drug resistance in bovine

Source	ARGs and associated antibiotics					Reference
	Macrolides	**Aminoglycosides**	***β*-lactam antibiotics**	**Tetracyclines**	**Others**	
bovine	erm35, ermC, mphE	Aph(3')-Ia aadA31 Aph(3'')-Ib Aph(6)-Id	blaBRO blaCARB blaROB	tet34, tetB, tetH, tetQ, tetW, tetX, tetY	lunC lsaB dfrA14	[33]
	emrB, emrC, emrF, emrQ, emrX		CTX-M	tet(40), tet(44), tet(W/N/W), tetA(P), tetB(P), tetO, tetQ, tetW		[34]
	erm(B)			tet(A), tet(B), tet(M)		[35]
			Cfx	tetQ, tetW, tetO, tet32, tet44		[36]
				tetM, tetA, tetD, tetC, tetB		[37]
				tetQ, tetW, tet40, tetO, tet32, tet44		[38]

the types and levels of bacterial resistance. Wang et al.[45] used the same methodology to study 40 fecal samples from yaks, beef cattle, and dairy cows to explore resistance due to antibiotic use in bacterial communities by analyzing the diversity and differences in antibiotic resistance genes within the gut microbiota of the three groups. The results showed that a total of 1,688 genes were annotated, including 734 subtypes of resistance genes. Part of the resistance genes are related to antibiotics widely used in humans or animals in clinical practice. They concluded that the emergence, prevalence, and differences in resistance genes in the intestines of yaks, beef cattle, and dairy cows may be due to selective pressure from different feeding practices. Additionally, although yak exhibits lower drug resistance gene abundance than beef cattle and dairy cattle, its integron abundance is higher than that of beef cattle and dairy cattle, which is consistent with the results of the Zhu Zhen experiment. Keijser et al.[46] showed that the addition of different doses of hygromycin to the feed caused significant changes in the intestinal microbiota of calves. After 21 days of medication, detection using macro-genomics revealed elevated abundance of the resistance genes tetM and mel in the medium-dose group (100-200μg of hygromycin orally every day for 7 weeks) and in the high-dose group (1g of hygromycin orally twice a day for 5 days), which was not observed in the low-dose group (no hygromycin taken). This suggests that duration of use can influence the microbiota and the abundance of antibiotic resistance genes in the gut. The findings underscore the complexity of antimicrobial resistance, which arises from interactions between the animal's environment, the dosage and duration of antimicrobial use, and the animal's own genetic resistance profile.

Metagenomics technology has been widely used in the study of antibiotic resistance in the intestinal tract of cattle. It can reliably describe the antibiotic resistance genes present in the intestinal bacterial community and dynamically monitor antibiotic resistance through metagenomics. Timely measures can be taken to improve livestock hygiene and adjust feeding management methods according to the current situation, which can effectively reduce the increase in the abundance of antibiotic resistance genes. It can also carry out research on genetic diversity of pathogens, cross-species transmission mechanisms, potential pathogenicity and epidemiological analyses, as well as establish a rich database of drug resistance genes. Building upon this foundation, integrating metagenomics with other technologies can significantly enhance both testing speed and accuracy. For instance, Oxford Nanopore technology reduces the time from sample collection to diagnosis. When combined, these approaches improve target sequencing coverage, strengthen the reliability of the technology, enable more precise monitoring of pathogens and their resistance genes, and provide robust data support for clinical diagnosis[47].

Metagenomics in bovine rumen flora drug resistance

The rumen is a complex ecosystem composed of microorganisms such as phages, fungi, and various bacteria[48]. These microorganisms work closely together to provide the energy needed for host metabolism. The application of metagenomics technology provides researchers with advanced tools for conducting more in-depth and comprehensive studies on antimicrobial resistance within the rumen microbiome[49].

The rumen, as an important digestive organ, can effectively digest roughage with a high content of fibrous material and plays a vital role in ruminant activity. Therefore, the study of rumen resistance is indispensable. Jing et al.[50] analyzed antimicrobial resistance genes and their co-occurrence patterns in bovine rumen microorganisms using 4941 rumen microbial genomes and 20 macrogenomic samples by birdshot metagenomics and network analysis. The results showed that 999 genomes (20.22%) were identified as drug-resistant genes, which were mainly concentrated in the phylum *Anabaena*, *Actinobacteria*, and *Thick-walled bacteria*. Among them, the *Actinobacteria phylum* (especially *Bifidobacteriaceae*) showed significant resistance gene carriage characteristics, and the detection rate of resistance genes in strains of this family reached 100%, the genomes of the Corynebacterium genus contain 50.86% of antibiotic resistance genes. In Aspergillus phylum and Anaplasma phylum, resistance genes were concentrated in Vibrio *Succinivibrionaceae* (100%) and *Prevonellae* (77.54%), respectively. This study employed a dual-technology detection and analysis approach to conduct precise analysis of drug-resistant genes within a large sample, revealing co-occurrence patterns both within and between types of antibiotic resistance genes. Xue et al.[51] conducted a trial with a group of mid-lactation Holstein cows in order to investigate the effect of feed intake on rumen antagonist resistance genes. Research has found

that the rumen-resistant genome encompasses resistance potential against 26 classes of antimicrobial drugs, with tetracycline resistance genes exhibiting the highest abundance. Additionally, the multidrug resistance genes carried by this microbiome can confer host tolerance to multiple classes of antimicrobial agents. These genes may be excreted via saliva and enter the environment through oral-nasal pathways, or they may migrate with intestinal contents into feces, constituting potential routes for the dissemination of resistance genes into the external environment. This study revealed the distribution of ARGs in the rumen of dairy cows, elucidated the mechanism of interaction between host and rumen resistance groups, and provided basic theoretical and empirical evidence for the reduction of antimicrobial resistance (AMR) in ruminant livestock as well as interventions to regulate ARGs. Sun et al.[52] found significant differences in bacterial species in the rumen of buffaloes and dairy cows using a macrogenomic approach and different resistance profiles between the two. Of the 505 ARGs identified in buffalo and dairy cattle together, 18 AMGs were detected only in buffalo. Among them, tcmA not only showed higher prevalence in buffaloes, but also was highly positively correlated with 93 co-expressed ARGs in the rumen. This study identified unique ARGs with their associated bacteria in buffalo, providing new insights into further exploration of the microbiome and resistance groups of buffalo and laying the foundation for preventing the spread of ARGs.

Metagenomics technology breaks the barriers of conventional detection methods and promotes the research progress in rumen resistance detection, which can rapidly and comprehensively understand the diversity and abundance of ARGs in the rumen, and then discover the transmission pattern of ARGs among different strains. This technology is crucial for veterinary clinical diagnosis and pathogen detection, and will also be a powerful reference for identifying animal infectious diseases and pathogens[53-54]. In addition, the full application of macro-genomics technology in the field of animal pathogen detection and research is more helpful for the prevention and control of human and animal infectious diseases and the prevention of epidemic outbreaks. Additionally, researchers have employed a combined approach of genome-wide association studies (GWAS), transcriptome-wide association studies (TWAS), and metagenomic data to analyze interactions between host genes and rumen microbiota. If this multi-omics integrated analysis method is fully applied in animal pathogen detection research, it will provide a powerful diagnostic tool for preventing and controlling zoonotic diseases and averting epidemic outbreaks[55].

CONCLUSIONS AND PERSPECTIVES

With the rapid development of animal husbandry, the inappropriate use of antibiotics has led to the widespread emergence of bacterial multidrug resistance. The rational use of antibiotics and reduction of bacterial resistance are crucial for enhancing the quality and efficiency of livestock farming and promoting the healthy, high-quality development of the cattle industry. The application of metagenomics technology holds immense potential for investigating antimicrobial resistance in animal microbiomes, as well as the mechanisms and interrelationships of diseases. This technology can identify microbial diversity, novel functional genes, microbial pathways, and antibiotic resistance genes. This paper systematically reviews the application of metagenomics technology in studying antimicrobial resistance in bovine fecal samples, intestinal, and rumen microbiota. It thoroughly explores the advantages of this technology in elucidating the mechanisms of antimicrobial resistance within animal microbiomes and their association with disease. Although metagenomics is accelerating the elucidation of microbial ecology and drug resistance mechanisms, it remains in its "tool development phase." Leveraging multi-omics (transcriptome-proteome-metabolome) synergy enables high-throughput identification of novel drug resistance profiles at the "microbe-drug-host" level, while simultaneously uncovering functional microbes and probiotics. This approach delivers precision microbiological solutions for healthy cattle farming. Combining metagenomics technology with AI-driven gene prediction enables efficient forecasting of bacterial drug resistance, achieving high-precision prospective determination of resistant phenotypes and tracing their evolutionary trajectories. When combined with nanopore real-time sequencing, it enables the immediate capture of dynamic expression and structural variations of drug-resistant genes in clinical samples. This hybrid sequencing strategy can further enhance assembly quality, analyze mobile genetic elements such as antibiotic resistance genes (e.g., β-lactamases and tetracycline resistance genes), and identify novel plasmid-borne resistance genes, significantly improving the efficiency of resistance gene mining and pathogen detection. Strategies combining metagenomics technology with other

tools provide a new technical paradigm for the early diagnosis and treatment of antimicrobial-resistant bacteria in the cattle industry, enabling precise interventions.

LIST OF ABBREVIATIONS

Abbreviations

ARGs	Antimicrobial resistance genes
MGE	Mobile genetic element
AMR	Antimicrobial resistance
HGT	Horizontal gene transfer
MLS	Macrolides-lincosamidsstreptogramins
MIC	Minimum inhibitory concentration
AI	Artificial intelligence
ML	Machine learning

DECLARATIONS

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Availability of data and materials
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Data availability
Not applicable.

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Authors' contributions
X.Z., J.L. and X.W. conceived the idea and laid out the outline of this review. Y.C., W.J., and Q.M. generated the figures. Z.L., Z.J., and J.C. generated the tables. All authors participated in the interpretation of initial ideas and writing the manuscript. All authors read and approved the final manuscript.

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