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SCREENING APPROACHES FOR ANTIMICROBIAL AGENTS: AN OVERVIEW OF METHODS

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ABSTRACT: There are various screening methods which are used to evaluate antibacterial, antifungal, antiviral, and antiparasitic agents for treating various infections. These methods include both traditional observational methods and advanced techniques. For antibacterial and antifungal agents, the focus is on high-throughput methods that assess bacterial and fungal growth and viability. Antiviral screening methods involve testing compounds that inhibit viral replication and infection, employing cell culture systems and cytopathic effect assays. The review details susceptibility testing methods for parasitic infestations, tailored to specific parasites. For Plasmodium species, assays like radioactive hypoxanthine incorporation and SYBR Green staining are used to determine drug efficacy. *Leishmania* and *Trypanosoma* species are assessed through colorimetric assays and flow cytometry for intracellular amastigotes. *Giardia*, *Entamoeba histolytica*, and Cryptosporidium are evaluated using image-based high-throughput screens and ATP content-based bioluminescence assays. *Schistosoma* species are tested using automated methods like impedance-based mobility measurement and fluorescence assays. Filarial parasites are screened for drug efficacy using traditional motility observation and advanced software like WormAssay. These screening methods are critical for identifying effective treatments for infections, significantly contributing to ongoing efforts in managing and treating infectious diseases. The review highlights the diversity and specificity of screening methods required for different types of infections, underscoring their role in the discovery and evaluation of new antimicrobial agents. By providing a comprehensive overview of these techniques, the review aims to aid researchers and healthcare professionals in selecting appropriate methods for their specific needs, ultimately contributing to the advancement of infectious disease treatment and control.

INTRODUCTION: "Antimicrobial agents provide some of the most dramatic examples of the advances of modern medicine." Diseases once deemed incurable and life-threatening can now be effectively treated with antibiotics, demonstrating the transformative power of these medications.

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These drugs target essential components such as bacterial and fungal cell wall synthesis enzymes, bacterial ribosomes, enzymes involved in nucleotide synthesis and DNA replication, and viral replication machinery amongst others [1].

The emergence of drug-resistant pathogens and the persistent threat of infectious diseases highlight the critical need for effective antimicrobial agents [2, 3]. Hence, the development of robust drug screening methods tailored specifically for antimicrobial agents is paramount to identify novel compounds with potent antimicrobial activity, minimal toxicity, and broad-spectrum efficacy.

Through this review, we endeavor to encompass both *in-vitro* and *in-vivo* screening methods for bacteria, fungi, viruses, and parasites, providing an overview.

For Bacterial and Fungal Infections:

Agar Diffusion Tests: The agar disk diffusion test is a culture-based microbiology assay widely employed in diagnostic and drug discovery laboratories. It plays a crucial role in screening biological materials such as plant extracts and bacterial fermentation broths, along with drug candidates, for their antibacterial activity. This test is particularly significant in bioprospecting labs focused on identifying potential antimicrobial agents [4, 5].

The agar disk-diffusion method involves inoculating agar plates with a standardized amount of the test microorganism and placing filter paper discs containing the test compound on the agar surface. After incubation, the diameters of inhibition zones are measured to determine the antimicrobial activity qualitatively. However, this method cannot distinguish between bactericidal and bacteriostatic effects or determine the minimum inhibitory concentration (MIC) accurately due to difficulties in quantifying the diffused antimicrobial agent. Despite these limitations, the disk-diffusion assay is valued for its simplicity, low cost, scalability, and ease of result interpretation, making it a preferred choice for screening large numbers of microorganisms and antimicrobial agents [6].

In 1997, Magaldi introduced a modified version of the disc diffusion method known as the 'well diffusion' method. This technique involves placing drug-supplemented discs with various concentrations of the drug into wells cut in the agar. It allows for standardized drug concentrations across different fungal species. The well diffusion method is cost-effective, straightforward, and dependable for testing antifungal drug susceptibility in Candida spp., yielding results comparable to the disc diffusion test [7].

The agar plug diffusion method involves culturing the strain of interest on agar medium, allowing microbial cells to secrete diffusible molecules during growth. After incubation, agar plugs are cut and placed on plates inoculated with a test microorganism. The appearance of inhibition zones around the plugs indicates antimicrobial activity. This method is advantageous for testing various antibiotic production conditions. However, manual manipulation in agar-plug assays becomes cumbersome for screening large strain collections. A recent development includes a 96-well microplate-based system, facilitating efficient screening of actinomycete strains for bioactivity in agar-plug assays [8, 9].

The direct agar diffusion assay is primarily used to screen potential antibiotic producers like actinomycetes by exposing them to an indicator strain or a test strain on solid medium. Initially, the producer is incubated under optimal conditions. Subsequently, the agar plate is overlaid with a suspension of the indicator strain to assess antimicrobial activity [10].

In laboratory settings, the genetic and molecular mechanisms of pathogen resistance are often studied by exposing the pathogen to increasing antibiotic concentrations over time. Actinomycetes, known for their extensive secondary metabolism, can adaptively evolve in such conditions, potentially leading to the production of new antibacterial compounds not typically synthesized by the wild-type strain. Mutations activating antibiotic biosynthesis or enhancing production result in visible halos or larger zones of inhibition on agar plates.

This methodology was applied to evolve a *Streptomyces clavuligerus* strain against methicillin-resistant *Staphylococcus aureus* (MRSA) N315, leading to the production of holomycin, which exhibited inhibitory effects against the pathogen. This approach highlights the potential of microbial evolution in the laboratory to discover novel antibacterial compounds from natural sources [11].

The poisoned food method is commonly used to assess antifungal activity against molds. In this method, the antifungal agent or extract is added to molten agar at a specific concentration and poured into Petri dishes. After pre-incubation, a mycelia disc is placed at the center of each plate, and fungal growth is monitored over time.

The antifungal effect is calculated using the formula:

$$\text{Antifungal activity (\%)} = (Dc - Ds) / Dc) \times 100$$

Where, Dc is the diameter of growth in the control plate and Ds is the diameter of growth in the plate with the antifungal agent. Positive and negative controls with known antimicrobial agents are essential for result comparison [12]. In the Oxford cup method, small cylinders containing penicillin are placed onto a layer of bacteria spread across agar plates, and the plates are then incubated. The sizes of inhibition zones, delineating bacterial growth suppression, are measured from the edge of the disk to the boundary of the clear zone formed.

The improved Filter Paper Disc Modification of the Oxford Cup Penicillin Determination Method: Utilizes thick filter paper discs saturated with penicillin samples instead of small cylinders. This modification allows for quicker setup (about 6 discs per minute) on seeded plates, offering enhanced flexibility during plate manipulation for reading. Comparatively, the zones of inhibition observed with these discs demonstrate greater consistency and sensitivity to penicillin content variations than those obtained using the cup method. This improvement is attributed to improved contact of the penicillin solution with agar and more uniform diffusion from the disc. *Staphylococcus aureus* H (Oxford strain) grows granularly in nutrient broth but diffusely in peptone broth, ensuring even seeding on test plates [13].

The Epsilometer test (E-test) is a method for determining antimicrobial resistance using an exponential gradient. It quantifies microbial susceptibility directly by combining antibiotic dilution and diffusion. On Mueller Hinton Agar (MHA), a bacterial suspension is cultured using a sterile cotton swab. An E-test strip, carrying antibiotics, is then placed on the agar. Antibiotics diffuse into the agar immediately upon contact. After incubation, bacterial growth is visible, forming symmetrical inhibition ellipses along the strip. The Minimum Inhibitory Concentration (MIC) is determined by reading where the ellipse intersects the strip's scale, measured in µg/ml [14].

Thin Layer Chromatography-Bioautography: Assessing antimicrobial compounds in plants involves segregating plant extract components, exposing them to a test microorganism, and ascertaining if any compound inhibits microbial growth. Thin-layer chromatography (TLC) separations are advantageous as they allow numerous compounds to be separated on a flat surface. Separation hinges on polarity, where certain compounds bind strongly to the adsorbent and migrate less compared to others [15].

Contact bioautography involves the diffusion of antimicrobial agents from a developed TLC plate or paper to an inoculated agar plate. After placing the chromatogram face down on the agar layer for diffusion and subsequent removal, the agar is incubated. Zones of inhibition on the agar, corresponding to spots on the chromatographic plates, indicate antimicrobial substances. Challenges of contact bioautography include achieving complete agar-plate contact and preventing adsorbent adherence [16]. Improved bioautography aided in isolating an antimicrobial bromoditerpene from a marine alga [17]. Antifungal activity-guided fractionation of *Bridelia retusa* stem bark extracts yielded active compounds on TLC bioautography, demonstrating the method's sensitivity [18]. High-performance thin-layer chromatography (HPTLC) offers enhanced resolution and sensitivity compared to TLC [19].

In direct TLC bioautography, the developed TLC plate is treated with a fungal or bacterial suspension. The suspension is sprayed or dipped onto the plate, typically using specific absorbance or CFU/mL measures for different microbes. After incubation, tetrazolium salts are applied to visualize microbial growth, indicating antimicrobial activity by clear white zones against a purple background on the TLC plate [16]. Bioautography revealed that the CH_2Cl_2 extract showed higher antifungal activity against *Cladosporium cladosporioides* and *C. sphaerospermum* compared to the isolated chromenes [20].

Agar overlay combines contact and direct bioautography. The chromatogram is covered with molten agar medium containing microorganisms, followed by incubation and staining. Visualization of inhibition or growth bands occurs after solidification [16].

Annatto, derived from the seeds of the achiote tree, contains carotenoid-type pigments and exhibits antimicrobial activity against *Staphylococcus aureus*, attributed to its major components 9'-cis-norbixin and all-trans-norbixin as revealed by bioautography [21].

Dilution Methods: Dilution testing quantitatively determines the minimal concentration of an antimicrobial agent required to inhibit or kill bacteria. Dilution methods are preferred for determining MIC values as they allow estimation of antimicrobial agent concentration in agar (agar dilution) or broth medium (macrodilution or microdilution). Broth micro- or macro-dilution involves creating two-fold dilutions of the antimicrobial agent in liquid medium in tubes or 96-well microtitration plates. These are then inoculated with standardized microbial suspensions and incubated under suitable conditions. Time–kill methodology, using different concentrations of the agent, is another approach for broth macrodilution, assessing viability over time by colony counting.

The agar dilution method incorporates varying concentrations of the antimicrobial agent into molten agar medium using serial two-fold dilutions. A defined microbial inoculum is then spread on the agar plate surface, and the MIC endpoint is the lowest concentration inhibiting growth under suitable incubation conditions. This method is effective for both antibacterial and antifungal susceptibility testing [12].

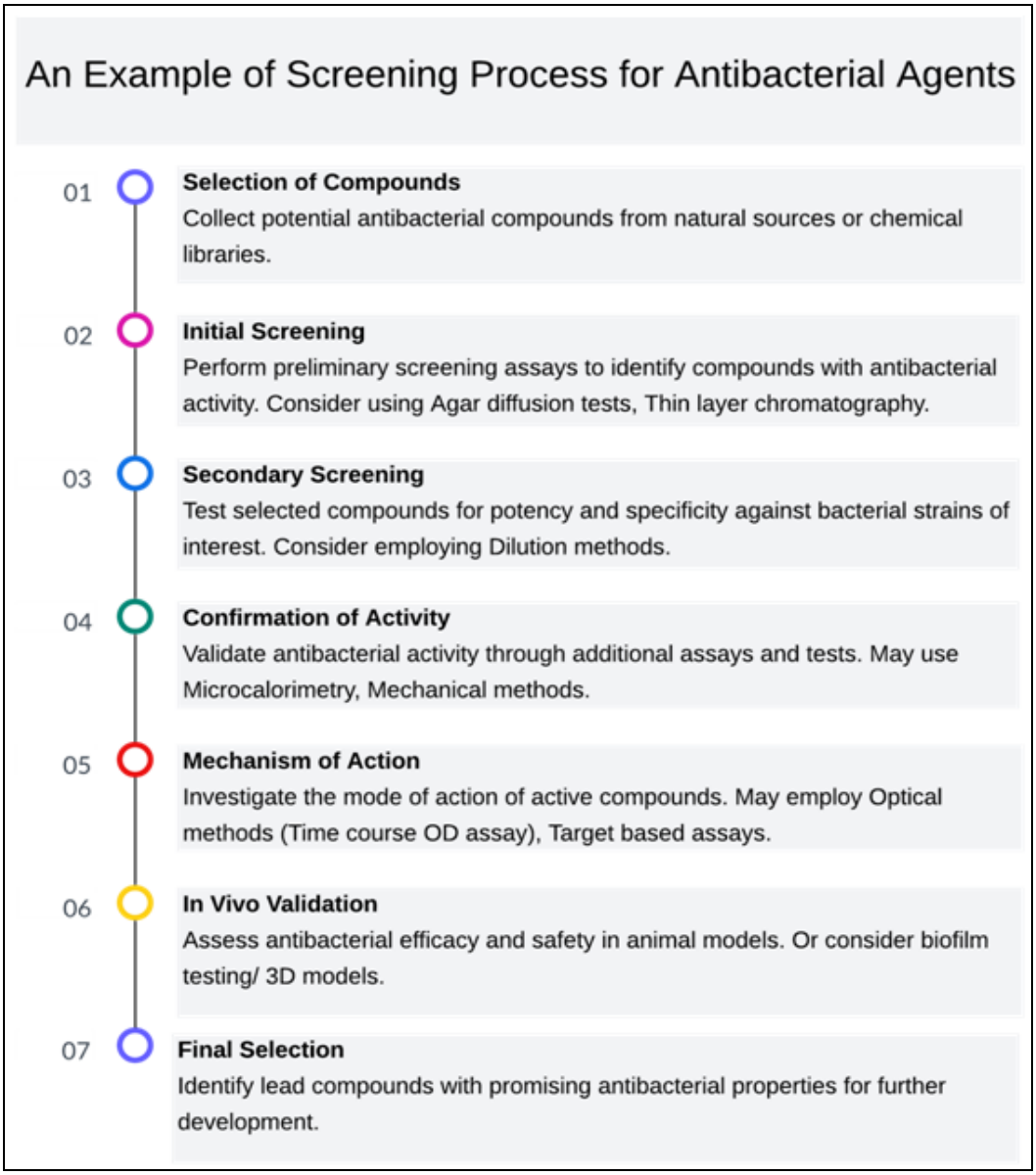

FIG. 1: AN EXAMPLE OF SCREENING PROCESS FOR ANTIBACTERIAL AGENTS

TABLE 1: SCREENING METHODS FOR ANTIBACTERIAL AND ANTIFUNGAL DRUGS

Method	Description
Agar diffusion tests	Widely used in diagnostic and drug discovery labs for screening antibacterial activity. Methods include: Agar disk diffusion. Well diffusion. Agar plug diffusion. Direct agar diffusion assay
Thin layer chromatography-bioautography	Separates plant extract components. Bioautography methods: - Contact bioautography. Direct TLC bioautography. Agar overlay
Dilution methods	Determines minimal concentration of antimicrobial agent required to inhibit or kill bacteria. Methods: - Broth micro/macro-dilution. Agar dilution
Target based assays	Screens compounds targeting cell wall, DNA synthesis, and protein synthesis. Methods:- Whole-cell assays. High-throughput bioluminescence screens - Universal model mRNA translation assay
Microcalorimetry	Detects bacterial susceptibility based on heat generated during growth. Used to determine MICs and identify MRSA
Mechanical methods	Measures changes in bacterial mass and density. Methods: - Asynchronous Magnetic Bead Rotation (AMBR) sensor. Vibrating cantilevers
Optical methods	Measures turbidity of bacterial suspension to assess antimicrobial activity - Methods: - Optical density (OD). Time course OD assay
Microfluidics method	Utilizes microfluidic systems for rapid antimicrobial susceptibility testing. Allows for point-of-care diagnostics
In-vivo model	Assesses antibacterial activities using mouse lung infection model. Examines effects of antimicrobial agents on infection outcomes
Biofilm model	Studies drug efficacy against biofilms using dynamic biofilm systems. Mimics natural conditions for better assessment of drug efficacy
3D Model	Utilizes organoids and organ-on-a-chip technology for studying antimicrobial effects and interactions with pathogens. Offers alternatives to traditional models.

Target Based Assays: Antibiotic discovery often focuses on targeting the cell wall due to its importance in bacterial growth, division, and recycling. Whole-cell assays, such as the 14C-labeled UDP-N-acetyl glucosamine assay, are used to screen compounds inhibiting peptidoglycan (PG) biosynthesis. Antibiotics like fosfomycin, bacitracin, and flavomycin demonstrated efficacy in inhibiting PG biosynthesis, while compounds not targeting PG enzymes showed no effect in the assay [22]. Inhibition of DNA synthesis is critical for preventing cell propagation in bacteria, with DNA gyrases being prominent targets due to their roles in DNA replication, recombination, and transcription. Quinolone antibiotics like nalidixic acid act as potent DNA gyrase inhibitors. Screening assays for DNA synthesis inhibitors include cell-based high-throughput bioluminescence screens, such as one involving a luciferase operon fused to a promoter that responds to reduced gyrase levels in Pseudomonas aeruginosa. This reporter assay, sensitive to low levels of ciprofloxacin, enables high-throughput screening for gyrase inhibitors and other DNA-damaging agents, identifying potential new antibacterials with diverse mechanisms of action targeting DNA synthesis [23]. Inhibition of protein synthesis is crucial for stopping bacterial growth, as proteins are essential for various cellular functions. A screening assay targets the initial steps of protein synthesis in bacteria, such as mRNA binding to ribosomes, which is necessary for translating genetic instructions into proteins. Compounds that interfere with this process can be identified using this approach. Additionally, by specifically focusing on inhibitors of the S1-dependent pathway of translation initiation, the screening assay helps pinpoint compounds that disrupt this critical step, potentially leading to the development of new antibiotics with targeted mechanisms against bacterial protein synthesis.

The S1-dependent pathway of translation initiation refers to a specific mechanism in bacteria where the ribosomal protein S1 plays a crucial role in promoting the initial interaction between mRNA and the ribosome during translation. This pathway is important for the efficient translation of most mRNAs in bacteria. In contrast, leaderless mRNAs, which lack a 5′-UTR (untranslated region) and directly start with the AUG start codon, follow a different pathway for translation initiation, bypassing the need for S1. By targeting the S1-dependent pathway, researchers can focus on compounds that specifically interfere with bacterial protein synthesis, making them potential candidates for new antibacterial drugs [24].

The assays for identification of inhibitors targeting specific translational steps were developed to pinpoint inhibitors that selectively target bacterial or yeast translational processes, focusing on early protein synthesis stages. Using a universal model mRNA and cell-free extracts from *E. coli*, *S. cerevisiae*, and HeLa cells, researchers translated an immunogenic peptide, quantifying it to identify selective bacterial or fungal inhibitors while sparing human systems. High-throughput screening of 25,000 natural products via this assay revealed two specific bacterial translation inhibitors, showcasing its effectiveness in discovering novel antibacterial agents [25].

Microcalorimetry: Microcalorimetry is a method that utilizes the heat generated during bacterial growth to detect bacterial susceptibility. Recent studies have employed microcalorimetric assays to determine the minimum inhibitory concentrations (MICs) of various strains of *S. aureus* and Escherichia coli, as well as to identify methicillin-resistant *S. aureus* (MRSA). As bacteria enter the exponential growth phase, their metabolic activity increases, leading to a corresponding rise in heat production. Calorimeters can measure this heat flow, and plotting the heat pattern over time allows researchers to generate bacterial growth curves [26].

Mechanical Methods: The Asynchronous Magnetic Bead Rotation (AMBR) sensor utilizes self-assembled magnetic particles to which bacteria adhere. When a magnetic field is applied, these magnetic beads start to rotate. As bacteria grow, the viscosity changes, affecting the rotational behavior of the beads, which can be measured as changes in rotational periods. This sensor is effective for monitoring bacterial growth dynamics in real-time [27]. Vibrating cantilevers use buoyant mass to detect bacterial mass and density changes, offering single-cell analysis for growth rates and adhesion studies. They show promise for rapid antimicrobial susceptibility testing without pre-culture and potential high-throughput screening, but further validation is needed for various bacterial strains and co-culture scenarios [28, 29].

Optical Methods: The optical density (OD) method involves measuring the turbidity of a bacterial suspension at regular time intervals using a spectrophotometer. Turbidity, indicated by the OD value, correlates with the bacterial concentration in the sample. The method utilizes an inoculated bacterial sample, typically containing gram-positive (e.g., *Streptococcus, Staphylococcus*, *Enterobacter, Bacillus*) or gram-negative organisms (e.g., *Escherichia, Enterobacter*, *Hemophilus, Klebsiellae, Moraxella, Pasteuella*, *Pseudomonas, Legionella*). The OD600 measurement, taken at a wavelength of 600 nanometers, provides a baseline bacteria level.

To assess antimicrobial activity, a candidate compound is added to the bacterial sample, and turbidity levels are measured at multiple time intervals. This measurement can indicate bacterial growth, death, or stagnancy, allowing for testing of both bacteriostatic and bactericidal compounds.

The intervals for turbidity measurements may vary, typically spaced at intervals such as four or eight hours apart, commencing four to eight hours after the assay components are combined. A preferred time course includes measurements at 8, 12, 16, 20, and 24 hours. The time course approach enhances sensitivity, allowing for the detection of antimicrobial compounds that may not be readily detected using conventional single-time-point assays. By evaluating changes in percentage inhibition of bacteria at different time points for different compounds, the method identifies preferred time points for effective drug screening.

Furthermore, the time course assay can be integrated into high-throughput screening assays, facilitating rapid screening of numerous potential compounds. In such assays, bacteria are inoculated in growth media, and cells are diluted to the appropriate concentration for turbidity measurements. The cells are then added to plates containing the compounds to be tested, and turbidity is measured at the specified time intervals.

The time course measurement scheme enables the detection of inhibitory or uninhibitory compounds at each time interval. This enhanced sensitivity is valuable for identifying compounds that exhibit antimicrobial activity only at specific time points during the assay. Overall, the time course OD assay provides a comprehensive understanding of antimicrobial activity over time, offering insights into the dynamics of bacterial growth and response

to antimicrobial compounds [30]. High-throughput screens that compare growth rates of arrays of distinct micro-organism cultures on solid agar provide a rapid, effective method for quantifying genetic interactions. This involves inoculating cultures onto agar and measuring cell densities at various times after inoculation, typically using plate-scanning or photography during the exponential growth phase. To parametrize a dynamic growth model such as the logistic growth model, a robust image analysis tool capable of capturing a wide range of cell densities from plate photographs is necessary. Colonyzer is a set of image analysis algorithms designed for automatic quantification of size, granularity, color, and location of micro-organism cultures grown on solid agar. It is highly sensitive to low cell densities after dilute liquid culture inoculation, using a mixed Gaussian model for plate-wide thresholding based on pixel intensity. The tool is robust against experimental variations and adjusts for lighting gradients that could introduce spatial bias in cell density estimates. It is flexible enough to quantify cultures in various formats, such as pinning with dense inoculum or spotting with irregular morphology.

Colonyzer is developed with open-source packages such as Python, RPy, and the Python Imaging Library. The source code and documentation are available under the GNU General Public License. Colonyzer can adapt to specific needs, including automatic detection of cultures at irregular locations on streaked plates for robotic picking, or reducing analysis time by disabling certain features [31].

An optical tweezers technique is used to evaluate the impact of chemical agents on single bacterial cells, specifically monitoring the viability of trapped Escherichia coli by observing its flagellar motility in the presence of different concentrations of ethyl alcohol. The "killing time" of the bacterium is determined through the correlation statistics of the positional time series recorded from the trap. This method minimizes bacterial photodamage and aligns with previous ethanol toxicity findings from conventional culture-based methods. This approach can be extended to assess other pairwise combinations of drugs and motile bacteria, offering precise measurement of single-cell response times [32].

Microfluidics Method: Use of microfluidics for rapid antimicrobial susceptibility testing at the point of care has been reported. The large surface-to-volume ratio in microfluidic systems accelerates the growth of uropathogenic *E. coli* in gas-permeable polymeric microchannels. For microchannels with a depth of 250 micrometers or less, effective oxygenation allows *E. coli* to grow to over 10^9 cfu/ml without additional agitation or oxygenation. This method eliminates the need for bulky equipment and speeds up bacterial growth for antimicrobial susceptibility testing at the point of care. The testing is applicable in culture media and urine with clinical bacterial isolates showing different antimicrobial resistance profiles.

The rapid testing allows antimicrobial resistance patterns to be determined in as little as 2 hours, a significant improvement over the days required by standard clinical procedures. This advancement facilitates quicker diagnostics at the point of care [33].

***In-vivo* Model:** The *in-vivo* approach involves assessing the antibacterial activities of various antimicrobial agents against multi-drug resistant *Acinetobacter baumannii* using a mouse lung infection model. This model is established through ultrasonic atomization, which allows for a precise and controlled introduction of the infection.

The *in-vivo* assessment examines the effects of different antimicrobial agents, either alone or in combination, on infection outcomes. Measurements include changes in white blood cell counts, lung tissue inflammation, and mortality rates. The data reveal that certain drug combinations, such as minocycline with either rifampicin or amikacin, yield higher efficacy compared to single-agent treatments. Throughout the course of the in vivo study, observations include lung tissue inflammation, vasodilation, and congestion with hemorrhage during the initial infection phase. However, after 3 days of anti-infective therapy, the lung tissue shows signs of recovery and structural clarity. This in vivo technique serves as a valuable method for screening the effectiveness of various

antimicrobial treatments and understanding the infection mechanism [34].

Biofilm Model: Biofilms are associations of microorganisms that tightly adhere to surfaces, encased in an extracellular matrix of polysaccharides, DNA, and other molecules. These biofilms pose challenges to *in-vivo* drug susceptibility testing (DST), as their ECM hinders antibiotic penetration and fosters antibiotic resistance. Dynamic biofilm systems, such as flow cell models and CDC biofilm reactors, mimic natural conditions more closely, allowing for better modulation of parameters like nutrients, gas, and extracellular matrix components. These systems enable researchers to assess drug efficacy against biofilms in a more realistic in vivo-like environment [35].

3D Model: Organoids offer a promising alternative to traditional *in-vivo* and *in-vitro* models for studying the effects of antimicrobial agents and interactions with pathogens. These three-dimensional structures mimic the architecture, cellular diversity, and function of human organs, providing a more accurate representation of the *in-vivo* environment [36].

Recent studies have used lung organoids to investigate infections with various pathogens, such as *E. coli* and SARS-CoV-2, allowing for the screening of antiviral compounds and exploration of host-pathogen interactions. Though limited, research involving Mycobacterium tuberculosis in airway organoids has demonstrated the potential for modeling infection and host responses. However, challenges such as the integration of immune cells and vascularization remain. Organ-on-a-chip technology complements organoids for high-throughput screening and real-time monitoring, offering a comprehensive approach for studying antimicrobials and pathogens across multiple organ systems. *In-vivo* mycobacterial infections involve immune aggregates called granulomas, which form a protective barrier against antibiotics. In vitro 3D granuloma models replicate this environment by culturing peripheral blood mononuclear cells (PBMCs) in controlled conditions. These models enable drug testing against active and dormant mycobacteria, offering a cost-effective and scalable alternative to *in-vivo* studies [37].

For Viral Infections:

Cell Culture: Vero cells are a widely used mammalian continuous cell line derived from the kidney of an African green monkey in the 1960s. These cells are anchorage-dependent and have been extensively utilized in virology studies, as well as in the propagation and study of intracellular bacteria, parasites, and for assessing the effects of chemicals, toxins, and other substances on mammalian cells.

Vero cells have been licensed for the production of live and inactivated vaccines and have been used worldwide for the production of several viruses, including rabies, reovirus, and Japanese encephalitis virus. The cells require careful handling to maintain sterility and avoid contamination, especially with mycoplasma. They also need to be maintained in specific conditions, including regular subculturing and monitoring their growth [38].

In studying Hepatitis B virus (HBV) infection and its life cycle, researchers use various cell culture systems, each with its own advantages and challenges. Hepatoma cell lines, such as HepG2 and Huh7, are derived from liver cancer cells and are useful for studying HBV replication. However, they lack some key functions of normal liver cells. Primary human hepatocytes (PHH) are actual liver cells that can be infected by HBV, but their limited availability and short lifespan in culture pose challenges.

Differentiated hepatoma cell lines, like HepaRG, can support HBV infection and produce viral particles, though they require complex preparation. NTCP-expressing cell lines, by adding NTCP to HepG2 and Huh7 cells, allow researchers to enable them to be infected by HBV. NTCP (sodium-taurocholate cotransporting polypeptide) is a liver cell protein that serves as a receptor for HBV, making these cell lines a promising platform for studying HBV infection. However, this system still needs improvement for full HBV life cycle studies. Inducible pluripotent stem cells (iPSCs) can be reprogrammed to become liver-like cells and support long-term HBV infection studies. Micropatterned co-cultured cells (MPCCs) support HBV infection and virus-host interactions, but may not yield highly infectious viral particles.

Liver organoids mimic the liver's structure and function, providing a more natural setting for HBV infection studies [39].

Cell Viability Tests: Cell-based assays are used to screen compounds for effects on cell proliferation or direct cytotoxicity. They can measure receptor binding and signaling events, such as the expression of genetic reporters and cellular component trafficking. Determining viable cell count at the end of an experiment is important and can be estimated using methods like tetrazolium reduction, resazurin reduction, protease markers, and ATP detection.

Tetrazolium reduction, resazurin reduction, and protease activity assays measure general metabolism or enzymatic activity as markers of viable cells. These methods involve incubating cells with a reagent that converts a substrate into a colored or fluorescent product detectable by a plate reader. Dead cells lose the ability to convert the substrate, providing the basis for cell viability assays.

The ATP assay immediately ruptures cells upon reagent addition, eliminating the need for an incubation period. Tetrazolium reduction assays, such as MTT, MTS, XTT, and WST, measure viable cells by producing a colored formazan product, detected by a spectrophotometer. Longer incubation times increase sensitivity but may be limited by cytotoxicity [40].

Antiviral Activity: Determining the antiviral activity of a drug involves evaluating its efficacy against viruses through a variety of assays and methods. Cell-based and pseudovirion assays use effector and target cells, chimeric viruses, and fluorescence-based techniques to monitor viral fusion and screen inhibitors. Techniques such as ultracentrifugation and assays involving cellular restriction factors help investigate the early stages of the viral life cycle. Polymerase inhibitors target DNA and RNA polymerases and can be monitored using real-time PCR (qPCR) and quantitative PCR (qRT-PCR) to measure viral RNA or DNA levels, assessing the impact of the drug on viral replication. Assays such as radioactivity-based and fluorescence-based methods study enzymes that modify viral RNA, providing insights into the drug's ability to hinder viral replication. Monitoring helicase activity with fluorescence and colorimetric methods helps assess how effectively the drug disrupts the unwinding of nucleic acid strands during replication. Inhibitors targeting viral and cellular kinases can block essential pathways for viral replication and are evaluated using fluorescence and mass spectrometry for precise measurement of kinase activity and drug efficacy. Similarly, drugs that target lipid kinases, such as sphingosine kinase 1, provide insights into their potential to affect viruses like dengue virus [41]. These methods are critical for drug discovery and understanding viral infection mechanisms, ultimately leading to potential antiviral therapies.

***In-vivo* Model:** *In-vivo* antiviral screening involves assessing the efficacy and safety of potential antiviral compounds in animal models that mimic human diseases caused by various viruses. Superior compounds showing antiviral activity in vitro can be tested in female mice by administering different doses in deionized distilled water with a solvent. Observations may include monitoring body weight loss and mortality over seven days, and collecting sera to assess liver and kidney functions. The screening process may involve administering the compounds via inhalation once daily for five days, starting four hours after infection with different influenza viruses. Mice are often intranasally inoculated with the influenza virus in PBS (phosphate-buffered saline) and monitored for body weight loss and mortality over 10 days post-infection. Viral titers can be determined from lung tissue homogenates, and nasal washes can be collected to study virus shedding. Standard antiviral drugs serve as drug controls [42].

Different animal models, such as mice, hamsters, and guinea pigs, are used depending on the virus being studied. For example, mice are valuable for investigating coronaviruses and influenza strains, while hamsters and guinea pigs are useful for studying specific diseases like arenaviral hemorrhagic fever. Non-human primates, particularly African green monkeys, provide crucial insights into infectious diseases due to their close genetic similarity to humans, aiding in the evaluation of vaccines and antiviral drugs [43, 44]. Transgenic (Tg) and knock-out (KO) mice models are employed to overcome species barriers that

arise due to differences in viral entry receptors and innate immune responses. Tg mice express human genes coding for viral entry receptors, while KO mice lack certain innate immune response components. Humanized mouse models are another approach, involving the engraftment of functional human cells and tissues into immunodeficient mice. These models closely replicate human diseases, making them essential for studying human viral infections and evaluating the effectiveness of vaccines and antiviral agents [45].

Other animal models such as Syrian hamsters, ferrets, cats, dogs, pigs, bats, rabbits, and guinea pigs offer various benefits depending on the virus or treatment being studied. For instance, ferrets are excellent models for respiratory viruses like influenza, while pigs share physiological similarities with humans, making them suitable for studying certain viruses [46]. Collectively, these animal models provide critical data for the development of effective antiviral therapies and vaccines.

TABLE 2: SCREENING METHODS FOR ANTIVIRAL DRUGS

Method	Description
Cell Culture	Utilizes various cell lines to propagate viruses and study their replication
Cell Viability Tests	Assesses compound effects on cell proliferation and cytotoxicity using methods like tetrazolium reduction and ATP detection
Antiviral Activity	Evaluates drug efficacy against viruses through various assays targeting viral fusion, polymerase inhibition, helicase activity, and kinase activity
In-vivo Model	Assesses drug efficacy and safety in animal models, including mice, hamsters, and non-human primates

For Parasitic Infestation: Susceptibility Testing

***Plasmodium* sp.:** Anti-parasitic screening methods for malaria focus on various stages of the disease to identify effective treatments and prevent transmission. Susceptibility testing for *Plasmodium falciparum* includes a range of assays such as radioactive hypoxanthine incorporation, ethanolamine incorporation, enzyme-linked immunosorbent assays (ELISAs), and nucleic acid stain-based methods like SYBR (Synchronized in vitro Binding Reaction) Green. These methods help determine the IC_{50} and IC_{90} levels, which are drug concentrations that kill 50% and 90% of the parasites, respectively [47].

The Ring-Stage Survival Assay (RSA) targets the early ring stage of *P. falciparum* and is used to determine resistance to artemisinin by exposing parasites to the drug at specific life cycle stages. This assay is essential for understanding how certain drugs affect the parasite's survival and resistance patterns [48].

Transmission-blocking drug tests aim to block the transmission of malaria by targeting the gametocyte stages of the parasite. These tests involve gametocyte culture and assays measuring exflagellation or using metabolic readouts to assess the impact of drugs on the transmission potential of malaria. The ultimate goal is to reduce the spread of malaria at the population level [49].

Antihypnozoite drug tests focus on the liver stage of malaria, specifically targeting hypnozoites in *P. vivax* and *P. ovale* infections. Current drugs like primaquine and tafenoquine have associated risks, such as glucose-6-phosphate dehydrogenase (G6PD) deficiency, necessitating careful use and accompanying diagnostic tests. Recent progress in developing *in-vitro* human hepatocyte assays and murine models offers potential for testing new drugs against liver-stage parasites, providing opportunities for improved treatments [50].

These anti-parasitic screening methods play a critical role in combating malaria, advancing our understanding of resistance, and ultimately improving patient outcomes by identifying effective drugs and preventing the spread of the disease.

***Leishmania* & *Trypanosoma*:** *Leishmania*, *Trypanosoma cruzi*, *and Trypanosoma brucei* are parasites causing severe global health issues like leishmaniasis, Chagas disease, and human African trypanosomiasis (HAT). Identifying compounds to effectively target parasites at different life cycle stages is crucial. For *Leishmania*, screening drugs against intracellular amastigotes is a primary focus, using colorimetric assays and flow cytometry. For *Trypanosoma cruzi*, assessing drug susceptibility in Chagas disease is challenging, requiring advanced imaging techniques such as bioluminescence

mouse models and real-time PCR. In the case of HAT, caused by *Trypanosoma brucei*, testing drugs for different disease stages is vital. Researchers explore new drugs like fexinidazole and the role of aquaglyceroporins in drug resistance.

Refining screening methods can enhance throughput and accuracy, leading to safer and more effective treatments for parasitic infections.[51]

Giardia, *Entamoeba histolytica* & Cryptosporidium: Susceptibility testing for Giardia traditionally involved closed tubes and manual counting, which was labor-intensive. Modern methods use image-based high-throughput screens with DAPI (4',6-diamidino-2-phenylindole) stain and bioluminescence assays based on ATP content. These assays require follow-up microscopic studies. For *Entamoeba histolytica*, testing was previously tedious due to microscopy and radioisotopes. A new microtiter plate-based assay under anaerobic conditions uses ATP bioluminescence, providing fast and sensitive testing. In Cryptosporidium testing, methods simulate the small intestine environment and include quantifying parasite mRNA, high-content imaging assays, and exploring CRISPR-Cas9 technology and luciferase-tagged parasites for screening [47].

Schistosoma & Filariae: Schistosomiasis, caused by Schistosoma species, is a neglected tropical disease with praziquantel as the primary treatment. Concerns include limited protection from reinfection and potential praziquantel resistance. Automated methods for drug testing such as impedance-based mobility measurement and fluorescence assays have been developed [54].

Filariae cause diseases like lymphatic filariasis and river blindness. Traditional *in-vitro* drug testing involves observing worm motility and other methods like MTT-formazan colorimetry. Advanced software like WormAssay and *in-vitro* systems that model worm environments are being researched to improve drug screening [47, 52].

TABLE 3: SCREENING METHODS FOR ANTIPARASITIC DRUGS

Method	Description
Plasmodium sp.	Screens drugs for malaria susceptibility using assays like radioactive hypoxanthine incorporation and Ring-Stage Survival Assay (RSA)
Leishmania & Trypanosoma	Tests compounds against intracellular amastigotes and different disease stages using colorimetric assays, bioluminescence, and advanced imaging techniques
Giardia, Entamoeba histolytica & Cryptosporidium	Utilizes high-throughput screens with stains and ATP assays for Giardia and *Entamoeba histolytica*. Explores mRNA quantification and CRISPR-Cas9 technology for Cryptosporidium
Schistosoma & Filariae	Tests drugs using impedance-based mobility measurement and fluorescence assays for Schistosoma. Utilizes in vitro systems and advanced imaging for Filariae

CONCLUSION: In conclusion, the diverse array of screening methods discussed in this review underscores the multifaceted approach required for evaluating antibacterial, antifungal, antiviral, and antiparasitic drugs effectively. These methods, ranging from traditional techniques to cutting-edge technologies, provide a comprehensive toolkit for researchers and clinicians alike in the battle against infectious diseases. In the realm of antibacterial and antifungal screening, techniques such as agar diffusion tests, thin layer chromatography-bioautography, dilution methods, target-based assays, microcalorimetry, mechanical and optical methods, microfluidics, and in-vivo models offer insights into the efficacy of antimicrobial agents. These methods not only assess the growth inhibition or killing of bacteria and fungi but also delve into the mechanisms underlying their action, facilitating the discovery of novel drug targets. Similarly, antiviral screening encompasses a wide range of approaches, including cell culture systems, cell viability tests, and assays targeting viral fusion, polymerase inhibition, helicase activity, and kinase activity. These methods enable researchers to evaluate the ability of compounds to inhibit viral replication and infection, shedding light on potential therapeutic interventions against viral diseases. In the context of antiparasitic drug screening, diverse methodologies are employed to address the complex life cycles and drug susceptibilities of parasites. Assays such as radioactive hypoxanthine incorporation and the RSA provide insights into the susceptibility of Plasmodium species to antimalarial drugs, while

colorimetric assays, bioluminescence, advanced imaging techniques, high-throughput screens, mRNA quantification, CRISPR-Cas9 technology, impedance-based mobility measurement, and fluorescence assays offer avenues for assessing drug efficacy against *Leishmania, Trypanosoma, Giardia, Entamoeba histolytica, Cryptosporidium, Schistosoma,* and *Filariae*.

To summarize, these screening methods serve as invaluable tools in the ongoing fight against infectious diseases, offering detailed insights into the mechanisms of action of antimicrobial agents and paving the way for the development of novel drug therapy. Continued advancements in technology hold the promise of further enhancing the accuracy and efficiency of these screenings, ultimately leading to improved management and treatment options for a wide range of infectious diseases.

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