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REVIEW ARTICLE

Methods for *in vitro* evaluating antimicrobial activity

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ABSTRACT

The interest in researching and developing new antimicrobial agents from different sources to combat microbial resistance has been increasing in recent years. Therefore, greater attention has been paid to screening and testing methods of antimicrobial activity. Several bioassays such as disk-diffusion, well-diffusion and broth or agar dilution are well known and frequently used, but others such as cytofluorometric and bioluminescent methods are not widely used because they require specialized equipment and more reproducibility and standardization evaluation, even though they can provide rapid results of the effects of the antimicrobial agent and better evaluation for reproducibility and standardization. In this review article, an exhaustive list of *in vitro*-antimicrobial susceptibility testing methods and detailed details on their advantages and limitations are recorded.

INTRODUCTION

The science handling the study of the prevention and treatment of diseases caused by micro-organisms is understood as medical microbiology [1]. Its sub-disciplines are virology (study of viruses), bacteriology (study of bacteria), mycology (study of fungi), phycology (study of algae) and protozoology (study of protozoa). For the treatment of diseases inhibitory chemicals employed to kill micro-organisms or prevent their growth, are called antimicrobial agents. These are classified consistent with their application and spectrum of activity, as germicides that kill micro-organisms, whereas micro-biostatic agents inhibit the expansion of pathogens and enable the leucocytes and other defense reaction of the host to cope up with static invaders [2]. The germicides may exhibit selective toxicity counting on their spectrum of activity. They may act as viricides (killing viruses), bacteriocides (killing bacteria), algicides (killing algae) or fungicides (killing fungi). The beginning of modern chemotherapy has largely been due to the efforts of Dr. Paul Ehrlich (1910), who used salvarsan, as arsenic derivative effective against syphilis. Paul Ehrlich used the term chemotherapy for curing the communicable disease without injury to the host's tissue, referred to as chemotherapeutic agents like antibacterial, antiprotozoal, antiviral, antineoplastic, antitubercular and antifungal agents. Later on, Domagk (1953) prepared an important chemotherapeutic agent sulfanilamide. Bacteria are unicellular organisms present in entire biosphere. They have vital importance on earth and hence studied extensively in microbiology [3]. You can study about them in detail in microbiology courses in medicine, pharmacy and even basic biology. These bacteria are unicellular (single-celled) organisms and are of microscopic and invisible to the naked eye. The bacterial classification is one among the key factors to tackle them in disease. The classification is completed supported factors like their shape, nutrition requirement, cell membrane staining, the cell appendages, etc. of these bacteria; those harmful and useful to humans are widely studied in medicine and pharmacy while those pathogenic bacteria which cause the disease to plants and animals are extensively studied in

agriculture and farming sciences. Some of the aspects of bacterial classification also help within the identification of bacteria. A compound containing benzimidazole and benzene rings are used extensively for pharmaceutical purpose since 1960. 1-*H*-Benzimidazole rings, which exhibit remarkable basic characteristics thanks to their nitrogen content, comprise the active substances for several drugs. A number of biological activities have been attributed to these compounds. This ring system is present in numerous antiparasitic, anithelemintic and anti-inflammatory drug. An antimicrobial is an agent that kills microorganisms or stops their growth. Antimicrobial medicines are often grouped consistent with the microorganisms they act primarily against. For example, antibiotics are used against bacteria, and antifungals are used against fungi [4]. They can even be classified consistent with their function. Agents that kill microbes are microbicidal, while people who merely inhibit their growth are called biostatic. The use of antimicrobial medicines to treat infection is understood as antimicrobial chemotherapy, while the utilization of antimicrobial medicines to stop infection is understood as antimicrobial prophylaxis. The main classes of antimicrobial agents are disinfectants (non-selective agents, like bleach), which kill a good range of microbes on non-living surfaces to stop the spread of illness, antiseptics (which are applied to living tissue and help reduce infection during surgery), and antibiotics (which destroy microorganisms within the body).

The term "antibiotic" originally described only those formulations derived from living microorganisms but is now also applied to synthetic agents, like sulfonamides or fluoroquinolones. The term also wont to be restricted to antibacterials (and is usually used as a synonym for them by medical professionals and in medical literature), but its context has broadened to include all antimicrobials. Antibacterial agents are often further subdivided into bactericidal agents, which kill bacteria, and bacteriostatic agents, which hamper or stall bacterial growth. In response, further advancements in antimicrobial technologies have resulted in solutions which will transcend simply inhibiting microbial growth. Instead, certain types of porous media have been developed to kill microbes on contact. The difference between gram-negative and gram-positive bacteria is due to cell wall peptidoglycan layer. Gram positive bacteria have a thicker peptidoglycan layer compared to gram negative bacteria. In this study, although gram positive bacteria were more resistant, the compound could still penetrate and resulting in inhibition zones. From the overall results, antibacterial activity of this compound show mild activity (8 to 10 mm) and indicate that this compound has antimicrobial properties and has a potential as an antibacterial agent [5].

- **Classification of Bacteria By Shape Or Cell Structure**

Cohn divided the bacterial into four types supported their shapes in 1872. They have a special cell structure, but most of them come under two basic shapes like bacillus or cocci. Check out the image below for an idea [6].

a) Bacillus:

These are rod-shaped or filament-shaped bacteria. They are of four types like;

i) *Monobacillus:* This is a single rod-shaped bacillus bacterium.

ii) *Diplobaciullus:* These are a pair of rod-shaped bacteria. Two bacteria cells stick together. They can even be present as four-celled as a tetrad.

iii) *Streptobacilli:* This is often a sequence of rod-shaped bacteria. Bacilli bacteria arranged like a long chain.

iv) *Palisade:* Here two cells of Bacillus are arranged side by side like stick.

Bacillus is rod-shaped, cocci are spherical, cholera bacteria is comma-shaped, cocci and bacilli can be in groups or chains and syphilis bacteria is spiral shaped.

b) Coccus:

These are spherical shaped bacteria or oval shaped. Based on the number and their arrangement they are divided

i) *Monococcus* which is a single-celled round-shaped bacteria.

ii) *Diplococci* are two spherical shaped bacteria existing as pairs.

iii) *Streptococci* may be a chain of the many round-shaped bacteria.

iv) *Staphylococci* are a group of spherical bacteria arranged like a bunch of grapes.

v) *Sarcina* may be a type where 8 round shaped bacteria are arranged in cubical shape.

c) Comma-shaped Bacteria:

The bacteria are slightly bent and appear sort of a comma.
Ex: Vibrio cholera bacteria causing cholera.

d) Spirillum Bacteria:

This is often an extended spiral-shaped bacterium. They are also called as spirochetes. These are spiral or hair like in shape.
Ex: syphilis-causing bacteria.

e) Pleomorphism:

Though most bacteria have a selected shape, some don't. They exist in multiple shapes. Examples include Acetobacteria [6].

➢ .**Classification of Antibacterial Agents**

The antibacterial agents are classified in three categories:

(I) Antibiotics and chemically synthesized chemotherapeutic agents.
(II) Non-antibiotic chemotherapeutic agents. (Disinfectants, antiseptics and preservatives)
(III) Immunological products.

(I) Antibiotics and Chemically Synthesized Chemotherapeutic Agents

They are produced by micro-organisms or they could be fully or partly prepared by chemical synthesis. They inhibit the growth of micro-organisms in minimal concentrations. Antibiotics could also be of microbial origin or purely synthetic or semisynthetic. They will be classified by manner of biosynthesis or chemical structure. Structurally, they are classified into different classes [7].

➢ **Classification of Antibiotics Consistent with their Chemical Structure**

1. **Carbohydrate-**containing antibiotics pure sugars
   - Aminoglycosides
   - Orthosymycins
   - N-Glycosides
   - C-Glycosides
   - Glycolipids
   - Nojirimycin
   - Streptomycin
   - Everninomicin
   - Streptothricin
   - Vancomycin
   - Moenomycin

2. **Macrocyclic-**
   - lactones Macrolide antibiotics
   - Polyene antibiotics
   - Erythromycin
   - Candicidin
   - Ausamycins
   - Macrotetrolides
   - Rifamycin
   - Tetranactin

3. **Quinones and Related Antibiotics-**
   - Tetracyclines
   - Anthracyclines
   - Naphthoquinones
   - Benzoquinones
   - Tetracycline
   - Adriamycin
   - Actinorhodin
   - Mitomycin

4. **Aminoalkanoic Acid and Peptide Antibiotics**
   Aminoalkanoic acid derivatives β-Lactum antibiotics, Peptide antibiotics. Chromopeptides Depsipeptides Chelate forming peptides.

5. **Heterocyclic Antibiotics-** containing oxygen Polyether antibiotics Monensin.

6. **Heterocyclic Antibiotics-** containing nitrogen Nucleoside antibiotics Polyoxin.

7. **Aromatic Antibiotics-** Cycloalkane derivatives Steriod antibiotics Cycloheximide Fusidic acid.

8. **Aromatic Antibiotics-** Benzene derivatives Condensed aromatic antibiotics Aromatic ether Chloramphenicol Griseofulvin Novobiocin.

9. **Aliphatic Antibiotics-** Compounds containing phosphorous Fosfomycins Synthetic antimicrobial agents include sulfonamides, diamino pyrimidine derivatives, antitubercular compounds, nitrofuran compounds, 4-quinoline antibacterials, imidazole derivatives, flucytosine etc [8].

(II)Non-antibiotics

The second category of antibacterial agents includes non-antibiotic chemotherapeutic agents which are as follows:

1) **Acids and their Derivatives**
   Some organic acids like sorbic, benzoic, lactic and propionic acids are used for preserving food and pharmaceuticals. Salicyclic acid has strong antiseptic and germicidal properties because it may be a carboxylated phenol. The presence of –COOH group appears to reinforce the antiseptic property and to decrease the destructive effect. Benzoic acid is used externally as an antiseptic and is employed in lotion and ointment. Benzoic acid and salicylic acid are used to control fungi that cause disease such as athlete's foot. Benzoic acid and sodium benzoate are used as antifungal preservatives. Mandolic acid possesses good bacteriostatic and bactericidal properties [9].

2) **Alcohols and Related Compounds**
   They are bactericidal and fungicidal, but aren't effective against endospores and a few viruses. Various alcohols and their derivatives are used as antiseptics e.g. ethanol and propanol. The antibacterial value of open chain alcohols increases with a rise within the relative molecular mass and beyond C8- the activity begins to fall off. The isomeric alcohol shows a drop by activity from primary, secondary to tertiary. Ethanol has extremely numerous uses in pharmacy.

3) **Chlorination and Compound Containing Chlorine**
   Chlorination is extensively used to disinfect drinking water, swimming pools and for the treatment of effluent from industries. Robert Koch in 1981 first mentioned the bactericidal properties of hypochlorites. N-chloro compounds are represented by amides, imides and amidines wherein one or more hydrogen atoms are replaced by chlorine [10].

4) **Iodine Containing Compounds**
   Iodine containing compounds are widely used as antiseptic, fungicide and amoebicide. Iodophores are used as disinfectants and antiseptics. The soaps used for surgical scrubs often contain iodophores [11].

5) **Heavy Metals**
   Heavy metals like silver, copper, mercury and zinc have antimicrobial properties and are utilized in disinfectant and antiseptic formulations. Mercurochrome and merthiolate are applied to skin after minor wounds. Zinc is used in antifungal antiseptics. Copper sulfate is used as algicides.

6) **Oxidising Agents**
   Their value as antiseptics depends on the liberation of oxygen and every one are organic compounds.

7) **Dyes**
   Organic dyes are extensively used as antibacterial agents. Their medical significance was first recognized by Churchman in 1912. He reported inhibitory effect of gentian violet on Gram-positive organism. The acridines exert bactericidal and bacteriostatic action against both Gram-positive and Gram negative organisms [12-16].

8) **8-Hydroxyquinolines**
   8-Hydroxyquinoline or oxine is exclusive among the isomeric hydroxylquinolines, for it alone exhibits antimicrobial activity. This attributes to its ability to chelate metals, which the other isomers do not exhibit.

9) **Surface Active Agents**
   Soaps and detergents are used to remove microbes mechanically from the skin surface. Anionic detergents remove microbes mechanically; cationic detergents have antimicrobial activities and may be used as disinfectants and antiseptics.

(III) Immunological Products

Certain immunological products such as vaccines and monoclonal antibodies are used to control the diseases as a prophylactic measure [17-20].

- **Mode of Action**

Antimicrobial medications meddle synthetically with the combination of capacity of essential parts of small scale living beings, the cell structure and elements of eukaryotic cells of the

human body. These distinctions give us specific harmfulness of chemotherapeutic operators against microscopic organisms. Antimicrobial medications may either murder microorganisms by and large or essentially forestall their development. There are different manners by which these specialists display their antimicrobial action. They may repress:

(1) Cell-divider amalgamation
(2) Protein blend
(3) Nucleic corrosive combination
(4) Enzymatic action
(5) Folate digestion or
(6) Damage cytoplasmic film [21]

➢ **Bacteriostatic Colours**

Stearn and Stearn credited the bacteriostatic movement to triphenylmethane colors. Fischer and Munzo7 have discovered the connection between their structure and adequacy of such colors. Various medications are metal-restricting operators. The chelates are the dynamic type of medications. The site of activity inside the cell or on the cell surface has not been built up. The site of activity of oxine and its analogs has been recommended inside the bacterial cell8 or on cell surface [22].

➢ **Detoxification of Antibacterials**

P-Aminobenzoic corrosive is a development factor for certain smaller scale living beings and seriously restrains the bacteriostatic activity of sulfonamides. The metabolites recognized in man are p-amino-benzoylglucoronide; p-aminohippuric corrosive, p-acetylaminobenzoic corrosive. 8-Hydroxyquinoline (oxine) and 4-hydroxyquinoline are excerted as sulfate esters or glucorinides [23].

➢ **Staining**

Cell membrane of bacteria differs supported the layers in it. Gram stain is employed to classify these bacteria supported the variation within the layers. This bacterial cell membrane is formed of three materials generally viz. carbohydrates, proteins (peptidoglycan) and lipids (lipopolysaccharide). But there is variation in the quantity of peptidoglycan and lipopolysaccharides ratio among them. The grams staining helps in the classification of bacteria as gram positive & gram negative bacteria. In gram +ve bacteria there is a thicker peptidoglycan layer while gram -ve has less peptidoglycan and more of glycolipid membrane. So when stained with grams stain, crystal violet, peptidoglycan retains it giving violet color to gram +ve bacteria. Gram-ve bacteria cannot retain this gentian violet and instead retains saffron colour. The Gram's Method consists of coloring dyes like gentian violet and saffron. When a bacteria culture is added with Gram's stain, gram-positive bacteria show violet color, while gram-negative bacteria show saffron color. So, the bacterial species which take up the blue color on grams stain is termed gram +ve bacteria. The one which takes up an orange color is named gram -ve bacteria [24].

➢ **Evalution Techniques**

The accompanying conditions must be met for the screening of antimicrobial action:

- ✓ There ought to be private contact between the test living beings and substance to be assessed.
- ✓ Required conditions ought to be accommodated the development of microorganisms.
- ✓ Conditions ought to be same through the examination.
- ✓ Aseptic/sterile condition ought to be kept up.

A few laborers have used various techniques every once in a while to test the antimicrobial movement. The evaluation should be possible through the accompanying techniques [25]:

- ✓ Turbidometric strategy.
- ✓ Agar streak weakening strategy.
- ✓ Serial weakening strategy.

Following techniques are utilized as agar dispersion strategy:

- ✓ Agar Cup strategy.
- ✓ Agar Ditch technique.
- ✓ Paper Disk technique.

In order to assess the antibacterial activity, we have used the Agar cup process. It is one of the studies for *in vitro* bacterial helplessness that is not mechanized. For the measurement of antimicrobial specialists, this great technique yields a zone of restriction in mm result that is supposed to impede the production of explicit microorganisms. It is done with petri dishes. The bacteriostatic property of the mixes was attempted by the technique of plate dispersion as portrayed by the strategy of Bauer Kirby [26].

[A] Preparation of Mueller-Hinton Agar

(1) Beef imbuement: 300 g

(2) Acid hydrolysate of casein: 17.5 g

(3) Starch: 1.5 g

(4) Agar: 17 g

(5) Distilled water: 1 Lit.

The above constituents were gauged and disintegrated in water. The blend was warmed on water shower till agar broke up. This was then cleaned in an autoclave at 15 lbs pressure and 121^{o}C for fifteen minutes. The sanitized medium (20 ml) was poured in cleaned Petri dishes under aseptic condition, permitting them to set on a plane table [27].

[B] Preparation of Antibacterial Solution

All the mixes were disintegrated in dimethyl formamide (DMF). Legitimate medication controls were utilized. Compound was taken at grouping of 100µg/mL for testing antibacterial movement. The compound diffused into the medium delivered a fixation inclination. After the hatching time frame, the zones of restraint were estimated in mm. The arranged outcomes speak to the genuine readings control [28].

[C] Test Societies

Adhering to normal standard strains was utilized for screening of antibacterial and antifungal.

- Escherichia coli [Gram negative] MTCC– 443
- Pseudomonas aeruginosa [Gram negative] MTCC– 424
- Staphylococcus aureus [Gram positive] MTCC– 96
- Streptococcus Pyogenes [Gram positive] MTCC– 442
- Candida albicans [Fungus] MTCC– 227
- Aspergillus Niger [Fungus] MTCC– 282

[D] Inoculum's Arrangement

The inoculum was normalized at 1* 106 CFU/mL contrasting and turbidity standard (0.5 MacFarland tube)

[E] Swabs Planning

A flexibly of cotton fleece swabs on wooden tool sticks was readied. They were sanitized in tins, culture tubes, or on paper, either in the autoclave or by dry warmth [29].

[F] Experimental Strategy

✓ **Agar Disk-diffusion Method**

The official method used in many clinical microbiology laboratories for rou-tine antimicrobial susceptibility testing is Agar disk-diffusion testing, developed in 1940. The Clinical and Laboratory Standards Institute (CLSI) for bacteria and yeast research is currently publishing many agreed and approved standards. While this method does not reliably detect all fastidious bacteria, standardization has been made to test such fastidious bacterial pathogens, such as streptococci, Haemophilus influenzae, Haemophilus parainfluenzae, Neisseria gonorrhoeae and Neisseria meningitidis, using clear culture media, different conditions of incubation and interpretive requirements for inhibition zones. Agar plates are inoculated with a standardized inoculum of the test microorganism in this well-known method. Then filter paper discs (about 6 mm in diameter) are mounted on the agar surface containing the test com-pound at the desired concentration. In suitable conditions, the Petri dishes are incubated. The antimicrobial agent typically diffuses into the agar and inhibits the germination and development of the test microorganism and then tests the diameters of the growth zones of the inhibition. However, since bacterial growth inhibition does not imply bacterial death, bactericidal and bacteriostatic effects cannot be separated by this process. Moreover, the method of agar disk diffusion is not sufficient to establish the minimum inhibitory concentration (MIC) since the volume of the antimicrobial agent diffused into the agar medium cannot be quantified. Nevertheless, by comparing the inhibition zones with the stored algorithms, an estimated MIC can be determined for certain microorganisms and antibiotics. Nonetheless, disk-diffusion research provides several benefits over other techniques: flexibility, low cost, the ability to evaluate massive quantities of microorganisms and antimicrobial agents, and the ability to interpret outcomes provided. Moreover, several studies have shown the great interest in patients who suffer from bacterial infection of an antibiotherapy based on the antibiogram of the causative agent. The good correlation between *in vitro* data and *in vivo* evolution is the reason for this fact. The disk diffusion method was already used to test posaconazole against filamentous fungi, micafungin against Aspergillus, and caspofungin against Aspergillus and Fusarium before its

standardization. Actually, to test non-dermatophytefila-mentous fungi, a standardized antifungal disk-diffusion technique is used. The above-mentioned advantages of this method have led to its popular use for the antimicrobial screening of plant extracts, essential oils and other drugs, primarily in terms of simplicity and low cost [30].

✓ **Antimicrobial Gradient Method (Etest)**

To assess the MIC value, the antimicrobial gradient approach blends the concept of dilution methods with that of diffusion methods. It is based on the probability of the antimicrobial agent tested in the agar medium generating an a concentration gradient. A commercial variant of this technique is the Etests (BioMérieux). In the process, a strip impregnated from one end to the other with an increasing concentration gradient of the antimicrobial agent is deposited on the surface of the agar, previously inoculated with the tested microorganism. For the MIC determination of antibiotics, antifungals and antimycobacterials, this approach is used. At the junction of the strip and the growth inhibition ellipse, the MIC value is determined. It is easy to implement; it is also routinely used to fulfill the requirements of clinicians. Strips from Etests, however, cost around $2-3 each. M. About Balouiri et al. This strategy, therefore, becomes expensive if multiple drugs are tested. There has been a strong association between the MIC values calculated by Etest and those obtained by the process of broth dilution or agar dilution in several previous studies. To investigate the antimicrobial activity between two drugs, this technique can also be carried out. An Etest strip, impregnated with an initial antibiotic, is put on a pre-inoculated agar plate surface to research the combined effect of two antibiotics. The strip is removed after one hour and replaced with another one that is impregnated with a second antibiotic. The synergy is observed by a reduction of at least two dilutions of the combination's MIC relative to that of the most active antibiotic measured alone. The Etest strips can also be deposited on the agar medium throughout formation with a 90° angle at the intersection between the scales at the respective MICs for the microorganism tested for the same reason. The FICI was interpreted as an addition between 0.5 and 1 and as a difference between 1 and 4 [31].

➢ **Other Diffusion Methods**

In microbiology re-search laboratories, more diffusion methods are used to screen extracts, fractions or pure substances for their antimicrobial potency or to investigate antagonism between microorganisms. The most popular among these techniques are described below.

✓ **Agar Well Diffusion Method**

The method of Agar well diffusion is commonly used to test the antimicrobial activity of microbial extracts or plants. The agar plate surface is inoculated by spreading a volume of the microbial inoculum over the whole agar surface, similarly to the technique used in the disk-diffusion process. A hole with a diameter of 6 to 8 mm is then aseptically punched with a sterile cork borer or a tip, and a volume (20-100 mL) is injected into the well at the desired concentration of the antimicrobial agent or extracts solution. Then, depending on the examined microorganism, agar plates are incubated under acceptable conditions. The agar medium diffuses the antimicrobial agent and prevents the growth of the tested microbial strain.

✓ **Agar Plug Diffusion Method**

To illustrate the antagonism between microorganisms, the method of Agar plug diffusion is often used and the technique is close to that used in the method of disk diffusion. It involves developing an agar culture through tight streaks on the plate surface of the strain of interest on its acceptable culture medium. Microbial cells secrete molecules which diffuse into the agar medium during their development. An agar-plot or cylinder is aseptically cut with a sterile cork borer after incubation and deposited on the agar surface of another plate previously inoculated by the microorganism studied. From the plug, the substances diffuse to the agar medium. Then, the appearance of the inhibition zone around the agar plug detects the antimicrobial activity of the microbial secreted molecules.

✓ **Cross Streak Method**

To quickly test microorganisms for antagonism, the cross-streak technique is used. A single strip in the center of the agar plate seeds the microbial strain of interest. After an incubation time depending upon the microbial strain, the plate is seeded with the microorganisms examined by

single streak perpendicular to the central streak. After more incubation, by observing the inhibition zone scale, the antimicrobial interactions are analyzed.

- ✓ **Poisoned Food Method**

To test the antifungal activity against molds, the poisoned food approach is mostly used. The antifungal agent or the extract is incorporated into the molten agar at a desired final concentration and combined well. Then, into Petri dishes, the medium is poured. After pre-incubation overnight, an amycelia disc ranging from 2 to 5 mm, which is deposited in the center of the plate, may be inoculated. After further incubation under acceptable conditions for the fungal strain examined, the fungal diameters are thinly monitored and the sample plates are measured [32-33].

CONCLUSION

At present, microbial infections, with substantial associated morbidity and mortality, have become an important clinical danger, primarily due to the emergence of microbial resistance to existing antimicrobial agents. Methods for testing anti-microbial susceptibility and the discovery of novel anti-microbial agents have therefore been used extensively and continue to be created. The CLSI and EUCAST were subjected to standardization by some techniques, marking the main remarkable steps in this area. However, certain changes to standardized protocols are also required when evaluating natural products. Therefore, by diluting the cultural media and using a highly concentrated inoculum, it is important to be careful not to alter the fundamentals of microbiology. In addition, if we consider the use of solvents that can influence the growth of the tested microorganism, we may suggest that making minor methodological adaptations to standardized protocols may be a solution to ensure a precise experimental approach and enable other researchers to compare outcomes.

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