A Review on the Preparation of Antibiotics and Anti Microbial Sensitivity Test

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ABSTRACT:
Antibiotics have provided protection against life-threatening bacterial infections for more than a century. However, indiscriminate use of antibiotics and organism evolution have led to the emergence of multi-drug-resistant organisms (MDRO), and at times resistant to most or even all currently available antibiotic classes, extensively drug-resistant or pan-resistant organisms (XDRO, PDRO). Antimicrobials are probably one of the most successful forms of chemotherapy in the history of medicine. It is not necessary to reiterate here how many lives they have saved and how significantly they have contributed to the control of infectious diseases that were the leading causes of human morbidity and mortality for most of human existence. Contrary to the common belief that the exposure to antibiotics is confined to the modern "antibiotic era," research has revealed that this is not the case. Agar dilution involved an incorporation of different concentrations of the antimicrobial agent into a nutrient agar medium followed by swabbing of the standardized number of microbial cells with the sterile cotton swab on to the surface of the agar plate [13 - 16]. The plates were incubated for $18 - 24$ h at $35 - 37\ 0C$ and examined for the growth inhibited zones. PCR is one of the most efficient and rapid molecular tools for quantification and profiling of bacterially infectious genes. The first report on PCR diagnostic application was published by Saiki et al. The general methodology of PCR includes cycles of denaturation, annealing of the primers, and elongation of the primers by a thermostable DNA polymerase in a compatible buffer containing nucleotides, ions, and so on. Each cycle of amplification doubles the target DNA molecule
**Keywords:** Antibiotics, Antimicrobial, microbiological laboratories, clinical trials

I. INTRODUCTION :

Antibiotics have provided protection against life-threatening bacterial infections for more than a century. However, indiscriminate use of antibiotics and organism evolution have led to the emergence of multi-drug-resistant organisms (MDRO), and at times resistant to most or even all currently available antibiotic classes, extensively drug-resistant or pan-resistant organisms (XDRO, PDRO). Antibiotic resistance is a serious emerging global health threat and certain geographic areas might be affected more than others due to the pattern of antibiotic usage Thus, there is a great demand to search for novel antibiotics that are effective and safe. Antibiotic development has had several scientific and economic challenges over the years. A major hindrance for industrial support for new antimicrobial development is the low return of investment. That said, antibiotics are indispensable for global health. This paper reviews the anti-bacterial agents launched worldwide since 2017 and details their development status, mode of action, spectra of activity and the indications for which these antibiotics have been approved.

An ever-increasing demand for novel antimicrobials to treat life-threatening infections caused by the global spread of multidrug-resistant bacterial pathogens stands in stark contrast to the current level of investment in their development, particularly in the fields of natural-product-derived and synthetic small molecules. New agents displaying innovative chemistry and modes of action are desperately needed worldwide to tackle the public health menace posed by antimicrobial resistance. Here, our consortium presents a strategic blueprint to substantially improve our ability to discover and develop new antibiotics. We propose both short-term and long-term solutions to overcome the most urgent limitations in the various sectors of research and funding, aiming to bridge the gap between academic, industrial and political stakeholders, and to unite interdisciplinary expertise in order to efficiently fuel the translational pipeline for the benefit of future generations.
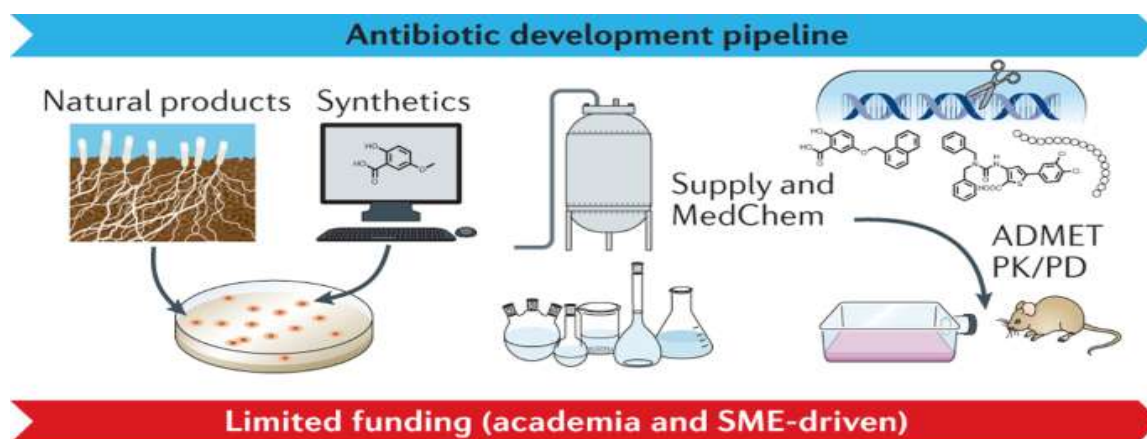
This article is conceived as a general roadmap with the central aim of promoting and accelerating translational science in the early stages of novel antibiotic discovery towards lead candidate development. The overuse and misuse of antibiotics in healthcare and agriculture, together with inappropriate waste management and environmental transmission, have led to substantially increased antimicrobial resistance (AMR) and associated bacterial persistence. This is of major public concern, since most areas of modern medicine are inconceivable without access to effective antimicrobial treatment. It is estimated that at least people worldwide die each year as a result of drug-resistant infections, and this could rise to as much as 10 million by 2050 if the problem of AMR is not addressed.

The anticipated death toll caused by drug-resistant infections over the next years and decades may be compared with the global fatality rate of the current SARS-CoV-2 (COVID-19) pandemic. which has already led to multibillion-dollar investments in vaccine development, repurposing existing drugs and antiviral discovery. A perhaps overlooked aspect of concern with the COVID-19 pandemic is the high numbers of secondary infections, often associated with multidrug-resistant bacteria, which are observed especially in hospitalized patients and those with already compromised immune systems. Associated with this problem is the massive use of antibiotics as a COVID-19 (co)treatment worldwide , which is predicted to add to the ongoing emergence of AMR. This multiplying effect of COVID-19 on the spread of bacterial resistance will most likely have further negative clinical, economic and societal consequences in the near future.

History:

Antimicrobials are probably one of the most successful forms of chemotherapy in the history of medicine. It is not necessary to reiterate here how many lives they have saved and how significantly they have contributed to the control of infectious diseases that were the leading causes of human morbidity and mortality for most of human existence. Contrary to the common belief that the exposure to antibiotics is confined to the modern "antibiotic era," research has revealed that this is not the case. The traces of tetracycline, for example, have been found in human skeletal remains from ancient Sudanese Nubia dating back to 350–550 CE (Bassett et al., 1980; Nelson et al., 2010). The distribution of tetracycline in bones is only explicable after exposure to tetracycline-containing materials in the diet of these ancient people. Another example of ancient antibiotic exposure is from a histological study of samples taken from the femoral midshafts of the late Roman period skeletons from the Dakhleh Oasis, Egypt. These samples showed discrete fluorophore labeling consistent with the presence of tetracycline in the diet at that time . The postulated intake of tetracycline in these populations possibly had a protective effect because the rate of infectious diseases documented in the Sudanese Nubian population was low, and no traces of bone infection were detected in the samples from the Dakhleh Cook et al.

The first antibiotic, Silverman, was deployed in 1910. In just over 100 years antibiotics have drastically changed modern medicine and extended the average human lifespan by 23 years. The discovery of Penicillin in 1928 started the golden age of natural product antibiotic discovery that peaked in the mid-1950s. Since then, a gradual decline in antibiotic discovery and development

and the evolution of drug resistance in many human pathogens has led to the current antimicrobial resistance crisis. Here we give an overview of the history of antibiotic discovery, the major classes of antibiotics and where they come from. We argue that Of future antibiotic discovery looks bright as new technologies such as genome mining and editing are deployed to discover new natural products with diverse bioactivities. We also report on the current state of antibiotic development, with 45 drug currently going through the clinical trials pipeline, including several new classes with novel modes of action that are in phase 3 clinical trials. Overall, there are promising signs for antibiotic discovery, but changes in financial models are required to translate scientific advances into clinically approved antibiotics.

METHOD:

Antimicrobial peptides are endogenous peptide antibiotics forming important components of the innate immune system [1]. They usually contain less than 50 amino acids, approximately 50% of which are hydrophobic, and have a net positive charge due to an excess of basic residues. Antimicrobial peptides have two attractive properties. First, they demonstrate a broad range of antimicrobial activities. Secondly, they mainly target microbial membranes, impeding the ability of microbes to develop resistance against them. As a result, these peptides are thought to be promising candidates for new antibiotics [2]. However, a number of fundamental issues such as mechanisms, efficacy and safety must be addressed before the peptides can be brought to clinical trials. Answering these questions requires extensive functional and structural studies, the progress of which partially relies on the availability of pure peptides. Moreover, peptide availability is one of the major factors that determine the feasibility of their widespread usage as antibiotics.

Preparation of CAM:

For the preparation of CAM, the aforementioned commercially available antibiotic formulations were procured and their working stocks were prepared in water. From the working stock, the calculated amount volume of antibiotics were mixed. The details for the preparation of working stock and the final volume used for the preparation of 5 l Middlebrook 7H11 agar medium, sufficient for around 200 culture plates, are given in .

Table 1.
Antibiotics and their concentration used for the preparation of CAM.

Serial number	Antibiotics/Name of commercial formulation	Concentration of antibiotics per pack	Concentration of working stock solution	Required amount antibiotic for 5 l agar media	Cost (INR) per pack * (for 5 l agar media)	Volume of working stock used for 5 l media
1.	Polymyxin B/POLY-B™	~500,000 IU per 580 mg	3.5 mg/1000 µl	30,000 IU	1600/pack (10)	1000 µl
2.	Amphotericin B/AMPHOTRET™	50 mg per pack	10 mg/1000 µl	3,000 µg	300/pack (60)	300 µl
3.	Nalidixic acid/GramoNeg®	500 mg per tab	1 tab/10 ml	12,000 µg	25/10Tab (2.5)	240 µl
4.	Trimethoprim/Bactrim®	160 mg per tab	1 tab/10 ml	3,000 µg	20/10 Tab (2)	187.5 µl
5.	Azlocilin/Azenam	1 g per pack	10 mg/1000 µl	3,000 µg	650/pack (6.5)	300 µl

Open in a separate window
*65 INR ≈ 1 USD.

Typically, one tablet each of nalidixic acid (GramoNeg®; Best laboratories Pvt. Ltd.) and Trimethoprim (Bactrim®; Piramal Enterprises Limited) tablets were dispersed in 10 ml water and kept on rocker shaker for 15–20 mins, the mixture was then centrifuged at 600 g for 10 min. Supernatant was aspirated and used for the preparation of the CAM.

Required amounts shown in of Polymyxin B (POLY-B [TM]; Samarth Life Sciences Pvt. Ltd.), Amphotericin B (AMPHOTRET [TM]; Bharat Serum and Vaccine Limited) and Azlocilin (Azenam; ARISTO Pharmaceuticals Pvt. Ltd.) were weighed and dissolved in water. The CAM was prepared by mixing appropriate volumes of working stock solutions and was filtered through a 0.2-µm syringe filter.

Preparation of sustained-release tablets

Formulations I and II were prepared by wet granulation technique . All the powders were passed through 100 mesh. Required quantities of drug and excipients were mixed thoroughly, and a sufficient volume of wetting agent (80% or 90% Ethanol) was added slowly. After enough cohesiveness was obtained, the mass was sieved through 20 mesh. The granules were dried at 60 °C for 2 h. Then, the granules were retained on 20 mesh. Magnesium stearate was finally added as gliding and Lubricant. Finally, the tablets were compressed. Tablet hardness was controlled between 8.0 and 10.0 N, and the tablet weight was 1.0 g. Each tablet contained 500 mg of AZI and other pharmaceutical ingredients as listed in . Compositions of 500 mg AZI sustained-release tablets.

Ingredients (per tablet)	F-I	F-II
AZI (mg)	500	500
HPMC K100LV (mg)	80	90
Xanthan gum (mg)	–	20
Latin (200 screen) (mg)	260	220
MCC (mg)	150	160
Magnesium stearate (mg)	10	10
Ethanol (80%)	qs	–
Ethanol (90%)	–	qs

*sq. indicates quantity sufficient

most modern antibacterials are semisynthetic modifications ofvarious natural compounds.These include, for example, the beta-lactam antibiotics, which include thepenicillins (produced by fungi in the genus Pointillism), the cephalosporin, and the carbapenems.Compounds that are still isolated from living organisms are the aminoglycosides, whereas otherantibacterials—for example, the sulfonamides, the quinolones, and the oxazolidinones—are producedsolely by chemical synthesis. Many antibacterial compounds are relatively small molecules with a molecular weight of less than 1000 Daltons. All formulations had passed assay test successfully. Content uniformity was in an acceptable range, which shows powders and granules were uniformly mixed. The OVIVA (oral versus intravenous antibiotics for bone and joint infections) study is currently investigating the possibility of oral administration in bone infections, including spondylodiscitis; the results of this study are still pending. In the case of culture-negative spondylodiscitis, antibiotics that cover the most common pathogens (especially S. aureus, streptococci, and E. coli) should be administered . When deciding on antibiotics and oral administration, one also needs to take bioavailability and bone penetration into consideration in the course of treatment.. Main constraints are related to energy cost, catalyst management and potential residual toxicity in treated effluents. The main advantages are related to the full mineralization of target compounds or the ability to increase their relative biodegradability. Future challenges include Nano-based green synthetized catalysts maximizing the use of solar radiation for energy saving. Generally,

AOPs application is part of a more structured wastewater treatment process including operating units at various technological contents. It is seen from the available literature that β-lactams, Quinolones, Sulphonamides, Tetracycline and Ionophores are generally not easily degraded and could be easily adsorbed (Li and Zhang, 2010). Only 48–77% antibiotics are treated by conventional treatment plants (Oberoi et al., 2019). The prevalence of antibiotics in consumable water leads to expansion of antibiotic resistant bacterial community.

This paper, we summarized the use of antibiotics in the poultry sector, persistence of antibiotics in animal body, and their release into environment. Transfer mechanism of antibiotics and their metabolites to the human body and their fatal effects have been investigated. Developments of ARB and ARGs in the soil due to excessive use of veterinary antibiotics . The risk of antibiotics to human health generally manifests itself in two ways – adverse drug reaction ADR and potential prevalence of antibiotic resistance via exerting selective pressure on bacteria of clinical importance. Allergic reaction is one type of ADR and the common symptoms are urticarial, antinarcotic edema, gastrointestinal reactions, aplastic anemia, as well as shock and death if serious (Bousquet, 2009, Solensky and Smolensk, 2012). A large proportion of antibiotics In Europe, several surveys have studied the pattern of antibiotic prescribing in the treatment of endodontic diseases. Amoxicillin was the first-choice antibiotic prescribed in endodontic infection in most of the surveys (Palmer et al. 2000, Dailey & Martin 2001, Tulip & Palmer 2008, Mainjot et al. 2009, Rodriguez-Núñez et al. 2009, Segura-Elea et al. 2010, Skučait≐ et al. 2010, Kaptan et al. 2013, Pyric et al. 2015). Only in Turkey was it reported that ampicillin was the first-choice antibiotic for endodontic infections (Kandemir & Ergo 2000). In allergic patients, clindamycin (Rodriguez-Núñez et al. 2009, Segura-Elea et al. 2010, Kaptan et al. 2013, Pyric et al. 2015) and erythromycin (Mainjot et al. 2009, Dailey & Martin 2001) were the preferred antibiotics. Doctors' socio-demographic and personal factors did not appear to exert much influence. Complacency (fulfilling what professionals perceived as being patients'/parents' expectations) and, to a lesser extent, fear (fear of possible complications in the patient) were the attitudes associated with misprescription of antibiotics. The viscosity-lowering excipients are sodium chloride and the amino acids arginine, glycine, proline, and lysine. Arginine may also function to adjust ionic strength and minimize aggregation. Human serum albumin is used in 2 products for intravenous infusion. Other excipients include methionine as an anti-oxidant, and EDTA or DTPA as chelating agents. The maximum volume of subcutaneous injection is 15 mL administered over 3-5 minutes, but the typically volume is 0.5-2 Mr. Five fixed-dose combinations have recently been approved and four contain harmonicas to assist the large volume subcutaneous injection of up to 15 mL, while one is a fixed-dose combination for intravenous with three antibodies. Prefilled autoinjectors and syringes are becoming more common and many come affixed with a needle of 27-gauge or 29-gauge, while a few have a 26-gauge or a 30-gauge needle. Recent advancements include harmonicas to assist the large subcutaneous injection volume of 5-15 mL, fixed-dose combinations, buffer-free formulation, and smaller subcutaneous injection volume (0.1 mL). In 2019 three antibody drug conjugates were approved Emhart®, Policy™, and Padcev™ and all contain a peptide linker and a common spacer attachment group.[9] The spacer attachment group consists of caprice acid attached to the drug and maleimide attached to the antibody. In 2020 Trodelvy™ was approved and has a similar drug as Enhertu®. Trodelvy™ contains sacituzumab govitecan-hziy a recombinant, humanized anti-Trop-2 (trophoblast cell-surface antigen-2) monoclonal antibody in which the topoisomerase inhibitor SN-38, the active metabolite of irinotecan, is attached at the C20 position via a pH-sensitive hydrolysable benzyl carbonate linker (called CL2A). Attachment at the 20th position of SN-38 in the lactone ring stabilizes the ring from opening to the less active carboxylate form. "Unlike most ADCs that use ultratoxic drugs and stable linkers, sacituzumab govitecan-hziy uses a moderately toxic drug with a moderately stable carbonate bond between SN-38 and the linker.".[10] "The CL2A linker also has a short PEG (polyethylene glycol) residue to aid in solubility since SN-38 is hydrophobic.".[6] Mild reduction of the antibody reveals eight thiol sites and all are conjugated with the maleimide moiety of the CL2 linker thus the DAR is 7-8. This audit recommends commonplace immune response definitions and these can be utilized as a decent beginning stage in plan improvement of another immunizer. The regular immune response detailing contains an immunizer, an excipient to change constitution or osmolality, a lyoprotectant (lyophilized powders), a

buffer (s), and a surfactant. Immune response details can be isolated into intravenous or subcutaneous, and each course of organization can be additionally separated into an answer or a lyophilized powder definition. A regular intravenous arrangement detailing has 10 mg/mL immune response, is isotonic by 9 mg/mL sodium chloride, cradled at pH 6 by 0.01 M histidine, has the surfactant polysorbate 80 at 0.01 mg/mL, and is bundled a solitary portion vial. A commonplace intravenous lyophilized plan has after reconstitution 10 mg/mL immunizer, the lyoprotectant sucrose at 50 mg/ mL, cradled at pH 6 by 0.01 M histidine, the surfactant polysorbate 80 at 0.01 mg/mL, and is bundled a solitary portion vial. A commonplace subcutaneous arrangement plan has 100 mg/mL immunizer, sucrose at 50 mg/mL, cradled at pH 6 by 0.01 M histidine, has the surfactant polysorbate 80 at 0.01 mg/mL, the thickness brought down with 10 mg/mL arginine, and is bundled a prefilled needle. A commonplace subcutaneous lyophilized plan has after reconstitution 100 mg/mL immunizer, the lyoprotectant sucrose at 50 mg/mL, cradled at pH 6 by 0.01 M histidine, the surfactant polysorbate 80 at 0.01 mg/mL, the thickness brought down with 10 mg/mL arginine, and is bundled a solitary portion vial.

❖ **Antimicrobial Sensitivity Test** :
Biofilm micro-organisms in the form of solid cultures have traditionally been used by microbiologists as a means of recognising contaminant organisms and maintaining pure stock cultures. For the majority of regulatory tests the inocula are passaged several times on solid media to ensure purity and subsequently harvested by washing off the plates with a suitable medium. Superficially, this procedure appears admirable.

However, different workers will inoculate the plates to different colony densities, either by using different streak patterns or by being heavy or light-handed during the primary inoculation. All microbiologists will have observed that in areas of plates where colony density is high then colony size is small. This relates to nutrient-availability and/or production of inhibitory substances by individual colonies. An additional consequence of this will be that large colonies might well be oxygen-limited at the core yet depleted by some other nutrient at their periphery. Growth-rate will also differ at different locations within the colony, a process contributing towards colony morphology. Thus, different densities of colony on a plate will produce individual cells of different nutritional status and large colonies will be more heterogenous in this respect than small ones. Al-Haiti & Gilbert, (1983) evaluated such effects with respect to P. aeruginosa and Escherichia coli and their sensitivity to chlorhexidine diacetate. They observed that sensitivity decreased markedly as the colony density of the inoculum was increased towards confluence (Figure 1), and that reproducibility determined from replicate experiments was greatest for liquid cultures and also increased with increasing colony density. Besides high costs, major drawbacks of molecular methods are detection of the resistance genes targeted only by the known probes and overestimating resistance because the resistance gene is not necessarily associated with the expression of a resistance phenotype. Because of a significant rise in multi- and pan-drug-resistant infections, there is an urgent need for a more rapid and reliable test to improve infection diagnosis and support evidence-based antimicrobial prescribing . The currently used methods for AST are summarised in.

Methods of antimicrobial susceptibility testing
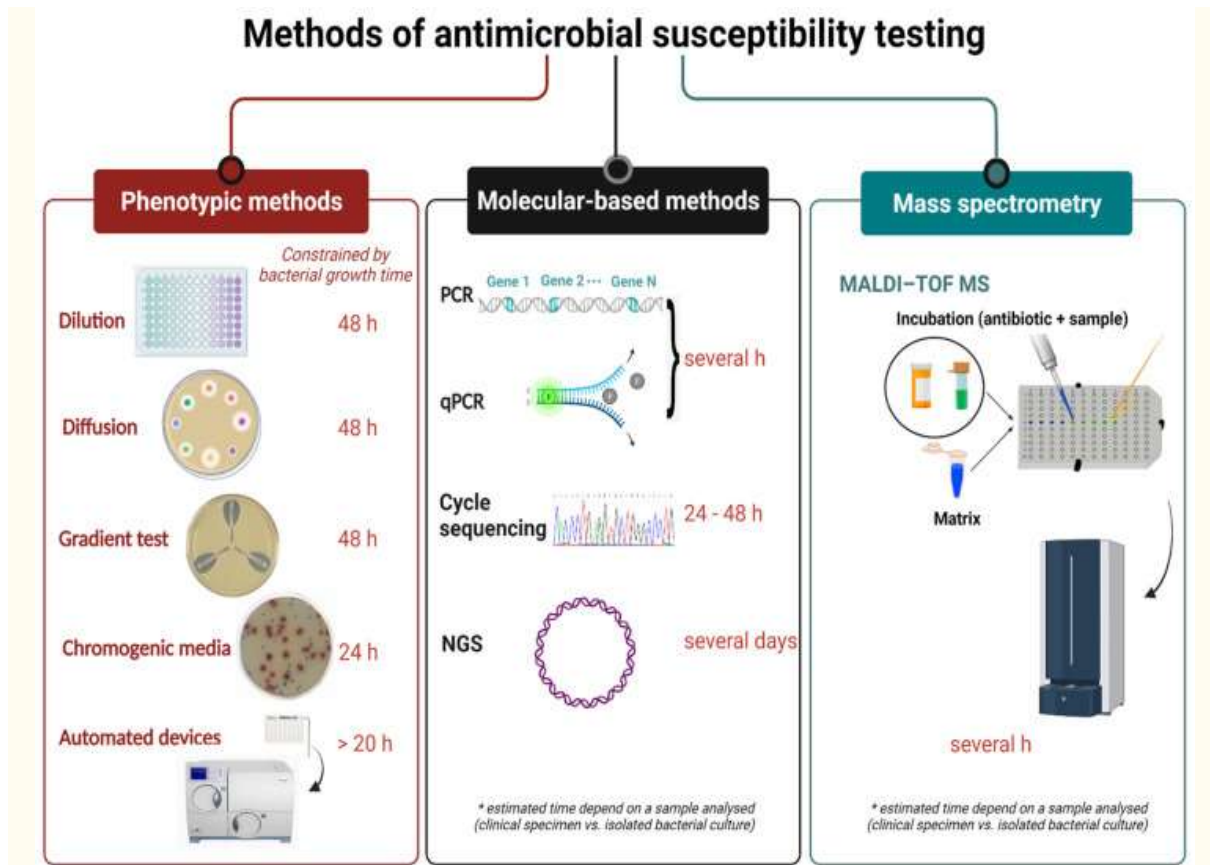
Broth dilution tests. One of the earliest antimicrobial susceptibility testing methods was the macrobroth or tube-dilution method . This procedure involved preparing two-fold dilutions of antibiotics (eg, 1, 2, 4, 8, and 16 μg/mL) in a liquid growth medium dispensed in test tubes . The antibiotic-containing tubes were inoculated with a standardized bacterial suspension of $1-5 \times 10^5$ CFU/mL. Following overnight incubation at 35°C, the tubes were examined for visible bacterial growth as evidenced by turbidity. The lowest concentration of antibiotic that prevented growth represented the minimal inhibitory concentration (MIC). The precision of this method was considered to be plus or minus 1 two-fold concentration, due in large part to the practice of manually preparing serial dilutions of

Disk Diffusion Test

Since its development in 1940, the disk diffusion (DD) test has remained the most widely the anttibiotics. Used routine AST in clinical microbiological laboratories . It has been standardised to test the susceptibility of the most common and clinically relevant bacteria that cause

human diseases . The standardisation is a continuous process, and DD for many microorganisms/antimicrobials is an ongoing diffuses radially outward through the agar medium, producing an antibiotic concentration gradient. After the inhibition zones are established process . The method is based on placing different antibiotic-impregnated disks on previously inoculated agar with bacterial suspension. The antibiotic within 24 h of incubation at 35 ± 1 °C, the zone diameters of each tested antibiotic are measured by the naked eye or using an automated system . Obtained results should be interpreted and categorised according to the recommended clinical breakpoint of the standard in use Disk diffusion is the most widely used AST method in microbiology laboratories because of its low cost and ease of performance and applicability of numerous bacterial species and antibiotics The choice of antibiotic disks is flexible and enables the clinical laboratory to make different combinations according to the bacterial species and the type of sample the isolate was obtained from. Simple interpretation allows the detection of atypical phenotypes and visibility of contamination.

However, the main disadvantages are the inability to determine the MIC and delays in getting the results. Reduction in turnaround time and timely treatment are of great importance for critically ill patients. In addition, the biological properties of lag and log phase of bacterial growth and their expression on antibiotic influence should be considered . Nevertheless, methods to reduce incubation time for DD were suggested decades ago . A revival of that idea led to the development of automated systems (WASPLab, Copan, Murrieta, CA, USA and BD Kiestra, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for an acceleration of AST by DD The automatisation of the AST by DD leads to a shortening of the required time to obtain results and produce the final report EUCAST has defined a methodology of disk diffusion rapid AST (RAST), which is performed directly from positive blood culture bottles, with breakpoints for short incubations of 4, 6, and 8 h . RAST can be implemented in routine laboratories without major investments. The method has been validated for a limited number of bacterial species and antibiotics so far. Furthermore, the combined use of a MALDI-TOF MS for the identification of bacteria and RAST directly from positive blood bottles enables reporting AST results within less than 24 h, which significantly reduced the turnaround time compared with the 24–48 h needed for culturing and classical AST methods, such as DD. The predictive value of direct DD testing from positive blood cultures has been reported to have an important influence on AMS .

Disk diffusion test. The disk diffusion susceptibility method [2, 11, 12] is simple and practical and has been well-standardized. The test is performed by applying a bacterial inoculum of approximately $1–2\times10^8 CFU/mL$ to the surface of a large (150 mm diameter) Mueller-Hinton agar plate. Up to 12 commercially-prepared, fixed concentration, paper antibiotic disks are placed on the inoculated agar surface (Figure 3). Plates are incubated for 16–24 h at 35°C prior to determination of results. The zones of growth inhibition around each of the antibiotic disks are measured to the nearest millimeter. The diameter of the zone is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium. The zone diameters of each drug are interpreted using the criteria published by the Clinical and Laboratory Standards Institute (CLSI, formerly the National Committee for Clinical Laboratory Standards or NCCLS) [13] or those included in the US Food and Drug Administration (FDA)-approved product inserts for the disks. The results of the disk diffusion test are "qualitative," in that a category of susceptibility (ie, susceptible, intermediate, or resistant) is derived from the test rather than an MIC. However, some commercially-available zone reader systems claim to calculate an approximate MIC with some organisms and antibiotics by comparing zone sizes with standard curves of that species and drug stored in an algorithm .

A disk diffusion test with an isolate of Escherichia coli from a urine culture. The diameters of all zones of inhibition are measured and those values translated to categories of susceptible, intermediate, or resistant using the latest tables published by the CLSI. Well diffusion method: In this agar well diffusion method, a suitable agar medium was prepared, once the agar is solidified the medium was inoculated and swabbed with bacterial suspension of approximately $1\text{-}2 \times 10^8$ CFU/mL using cotton swab. The wells were prepared by punching with a six millimeters diameter standard sterile cork borer made up of solution/s. to be tested. Well diffusion test has been used for susceptibility testing of antifungals like fluconazole, itraconazole [9, 10].

Agar dilution method: Agar dilution involved an incorporation of different concentrations of the antimicrobial agent into a nutrient agar medium followed by swabbing of the standardized number of microbial cells with the sterile cotton swab on to the surface of the agar plate [13 - 16]. The plates were incubated for 18 – 24 h at 35 – 37 0C and examined for the growth inhibited zones. The MIC is expressed as the highest dilution which inhibited growth by measuring the zone of inhibition. Agar dilutions are most often prepared in petri dishes and have advantage that it is possible to test several organisms on each plate. The dilutions are made in a small volume of water and added to agar which has been melted and cooled to not more than 60oC. Blood may be added and if 'chocolate agar' is required, the medium must be heated before the antibiotic is added. The pH of the agar must be between 7.2 and 7.4 at room temperature. Supplemental cations must not be added to the agar. It may be supplemented with 5% defibrinated sheep blood or lysed horse blood. The reproducibility of the results and satisfactory growth of most nonfastidious organisms can be expected advantages from agar dilution method. However, its disadvantages include the labor

required to prepare the agar dilution plates and their relatively short shelf life.

E Test (Diffusion and dilution): The principle of E test (also known as Epsilmeter test) method is based on antimicrobial concentration gradient in an agar plate. An 'E' in E test refers to the Greek symbol epsilon (ε).The E test (bioMerieux AB Biodisk) is a quantitative method for antimicrobial susceptibility testing applies both the dilution and diffusion of antibiotic into the medium. A predefined stable antimicrobial gradient is present on a thin plastic inert carrier strip. These strips are impregnated on the underside with a dried antibiotic concentration gradient and are labeled on upper surface with a concentration scale "Figure 6". When this E test strip was placed onto an inoculated agar plate, there was an immediate release of the drug. Following overnight incubation, a symmetrical inhibition ellipse was produced. The MIC value over a wide concentration range (> 10 dilutions) is determined by intersection of the lower part of the ellipse shaped growth inhibition area with the test strip. Some investigators have reported an excellent correlation between E-test results and broth dilution or agar dilution methods

Broth macrodilution

The broth macrodilution (or tube-dilution) method was one of the earliest AST methods, but should be superseded by the broth microdilution technique (Moreno et al. . Its principal disadvantages include the relatively large amount of space and reagents required, its labour-intensive nature, and the possibility of errors in preparation of the antibiotic solutions for each test. However,

the advantage of this method is in providing quantitative results—the MIC (Jorgensen and Ferraro .

The procedure is performed by preparing twofold dilutions of the antimicrobial agent (expressed in $\mu g \ ml^{-1}$) in a liquid growth medium dispensed in test tubes containing a minimum volume (2 ml) of the standardized microbial suspensions adjusted to 0·5 McFarland turbidity scale (Balouiri et al. . Following overnight incubation at 37°C for 24 h (bacterial strains) or at 25°C for 4–10 days (fungal strains), the tubes are examined for the presence of visible microbial growth by turbidity. The lowest concentration of the antimicrobial agent where the growth was completely inhibited (no turbidity) represents the MIC (Salem and Ali. The precision of broth macrodilution was considered to be plus/minus one twofold dilution concentration, due to the manual preparation of the dilutions.

Agar dilution

The agar dilution method is a manual method not frequently used in the researched literature. This technique is standardized by the National Committee for Clinical Laboratory Standards . The agar dilution method is performed by incorporation of different concentrations of the antimicrobial agent into a molten agar medium, usually using serial twofold dilutions, followed by the inoculation of a standardized microbial inoculum to the surface of the agar plate The agar plates are evaluated by visually comparing varying strains in the series, and thereafter, the MIC endpoint is determined as the lowest antibiotic concentration that inhibits bacterial growth.
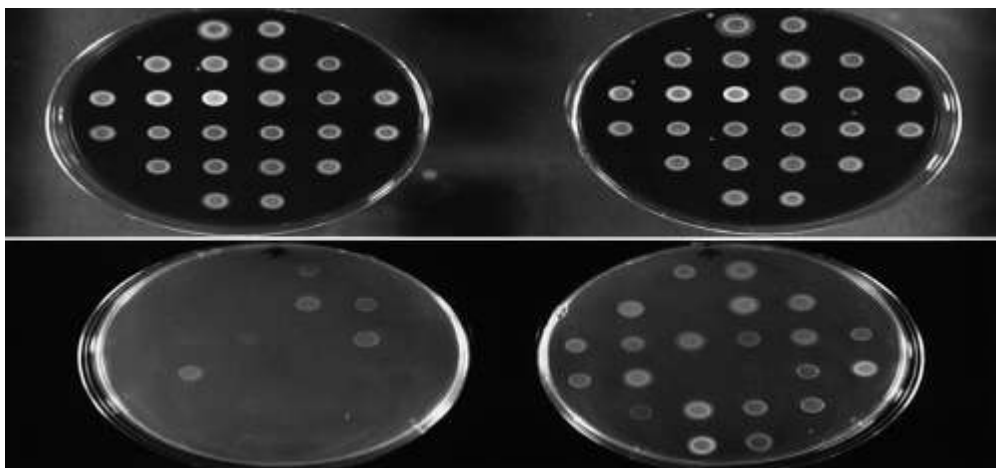

Figure 3

Open in figure viewer Power Point

Series of agar dilution plates. Twenty-four spots per plate represent a different Legionella sp. on buffered charcoal yeast extract (BCYE; on top) and buffered starch yeast extract (BSYE; below) agar plate with 10^4 CFU per spot of inocula (left) and 10^6 CFU per spot of inocula (right). Incubation was performed at 35°C/48 h. Spots where there is no visible growth are considered as the MIC. Reprinted with permission from Pendland et al. Copyright (2018) American Society for Microbiology.

The method is suitable for antibacterial as well as antifungal susceptibility testing and is recommended as a standardized AST method for fastidious organisms, such as anaerobes and Helicobacter sp. It has been also used for AST against Candida sp., Aspergillus sp., Fusarium sp. and dermatophytes Mock et al. The agar dilution technique preferred to broth dilution if a single antimicrobial agent is tested against an array of isolates or if the compound may influence the detection of microbial growth in a broth medium because of All antimicrobial agent-reference organism combinations using NA were unacceptable compared to MHA. All antibiotic-organism combination showed more than three misreading over 30 successive days; hence, none of the results would be accepted according to CLSI guidelines. its colouring.

Here, we present the identification and antimicrobial susceptibility testing (AST) results from isolates from clinical specimens from 13 microbiology laboratories participating in VINARES between June 2016 and May 2017. These results provide an insight in the dynamics of AMR and an update on the earlier results published based on data from the VINARES for the 2012–2013 period

Antibiotic susceptibility testing results of Gram-positive bacteria
Antimicrobial susceptibility testing results of bacteria from all specimens and from invasive infections or stool are shown in Tables , respectively. Additional file Table a and 2b shows AST results from ICUs.

Since not all isolates were tested for all listed antibiotics, the denominator of each susceptible proportion test was different and smaller than the total number of isolates collected. There were 4,833 S. aureus isolates, including 715 (15%) from blood and CSF. 690 isolates (14%) were from ICU. 73% (3,302/4,515 isolates) of S. aureus were MRSA, 71% of S. aureus (476/674) from blood and CSF were MRSA. Among the

isolates from ICU, the proportion of MRSA was 75% (478/640). The proportion reported as non-susceptible to vancomycin was low (2% (45/2,680) in all specimens and 1% (7/565) in blood and CSF). No confirmatory testing for vancomycin resistance was reported. The proportion resistant to macrolides was 83% (38,61/4,661) in all specimens.

E. faecium was isolated from 296 specimens; among which 51 (17%) were blood and CSF and 65 (22%) were from ICU. 34/46 tested isolates (74%) were high level aminoglycoside-resistant, 7/9 isolated from blood and CSF. 99/290 isolates (34%) of E. faecium were resistant to vancomycin (VRE) (19% of VRE tests were done by MIC method). 22 of 64 isolates (36%) from ICU were reported as vancomycin-resistant.

1,367 S. pneumoniae were isolated among which 160 (12%) were from blood and CSF and 184 isolates (13%) were from ICU. The penicillin-resistant S. pneumoniae proportion was 58% (663/1,136) in all specimens, and lower in blood and CSF (37%, 42/114 isolates) and among isolates from specimens collected in ICU (29%, 42/146 isolates). 691/794 (87%) of penicillin susceptibility tests were done by MIC method. 58/356 (16%) S. pneumoniae isolates were cephalosporin-resistant; this proportion was lower among ICU isolates (11%, 10/94). Two isolates (0.2%) were recorded as resistant to vancomycin, none of them were from blood/CSF or ICU.

. Genotypic AST Methods
Molecular or genotypic AST are the effective direct methods that eliminate tedious bacterial cultures, long incubation, chances of contamination, and the spreading of deadly infections . PCR, DNA microarray and DNA chips, and loop-mediated isothermal amplification (LAMP) are some of the genotypic techniques for the detection of antibiotic resistance. Mutational assessment of methicillin resistance in Staphylococcus spp., vancomycin resistance in Enterococcus spp., and multi-antibiotic (isoniazid, rifampin, streptomycin, pyrazinamide, and the fluoroquinolones) resistance in Mycobacterium spp. have been successfully estimated through various genotypic techniques.

PCR is one of the most efficient and rapid molecular tools for quantification and profiling of bacterially infectious genes. The first report on PCR diagnostic application was published by Saiki et al. The general methodology of PCR includes cycles of denaturation, annealing of the primers,

and elongation of the primers by a thermostable DNA polymerase in a compatible buffer containing nucleotides, ions, and so on. Each cycle of amplification doubles the target DNA molecule. The amplified target can be confirmed for the presence of resistance genes through electrophoresis, southern blotting, restriction fragment-length polymorphism, single-strand conformation polymorphism (SSCP), DNA

fingerprinting, molecular beacons, and other DNA sequencing analysis methods Another tool developed on the basis of PCR is LAMP, which has also been used for the evaluation of AST. In LAMP, the gene of interest is amplified at a constant temperature of 60–65 °C using a DNA polymerase instead of Taq polymerase because of strong strand displacement activity (required in isothermal techniques) .
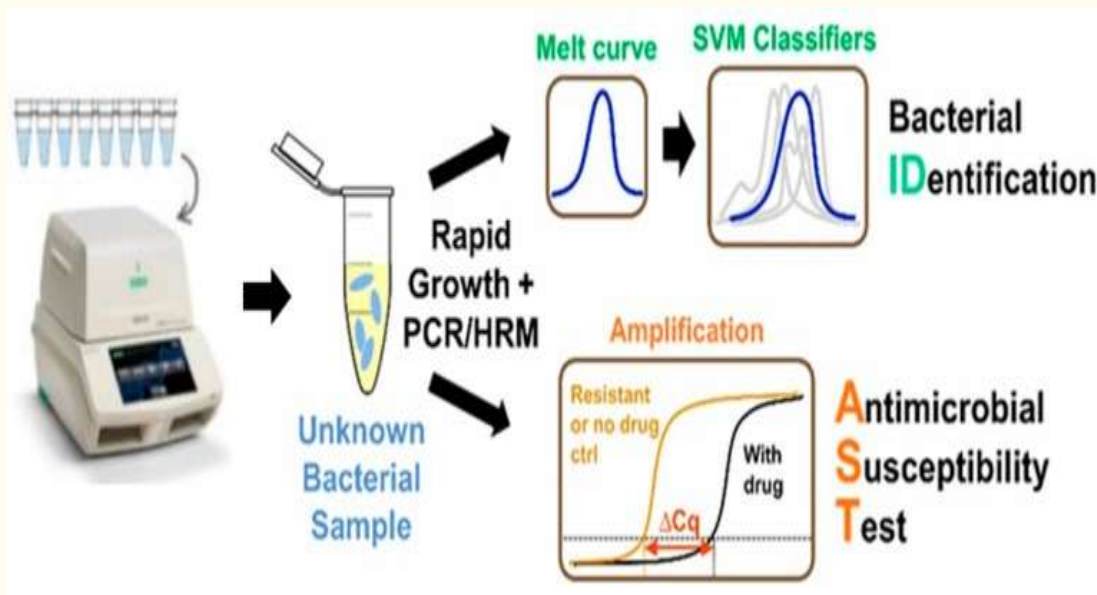
Digital PCR-High Resolution Melt analysis (HRM)-based bacterial identification from mixed bacterial samples, reproduced with permission from , published by American Chemical Society, 2017. (SVM: Support-vector machine)

DNA microarrays and DNA chips are the other promising technologies utilized for screening susceptibility .DNA arrays employ cDNA fragment probes on nylon membrane, where each DNA chip has a glass or silicon platform for probe binding. The specific hybridization of the labeled probe with the target and its recognition help to determine the resistance. Determination of isoniazid resistance in M. tuberculosis has been carried out successfully through DNA microarrays and chips . Colorimetric detection and multiplexing are the attractive features of these techniques.

MIC Determination

The lowest concentration of an antibiotic that completely inhibited visible growth as detected by the unaided eye was recorded as the MIC. MICs were determined using the broth microdilution reference method as described by the Clinical and Laboratory Standards Institute (CLSI). MICs were determined by examining the growth of each strain for at least three replicates. If two of the three results were the same, that MIC was used as the composite ("voted") result. If the three results differed, the middle result was used. CLSI breakpoints were applied to determine category interpretations, i.e., susceptible or resistant

Growth Conditions

The media utilized were lysogeny broth (LB) broth and LB agar (Difco, Lawrence, KS, USA). Bacteria from a frozen glycerol stock were inoculated in 10 ml of LB medium in 50 ml centrifuge polypropylene tubes (Sardtedt, Numbrecht, Germany). Bacteria were grown in an agitated, liquid LB medium at 37°C overnight. This culture was diluted 10^4-fold in LB medium and grown again overnight to obtain a starter culture for the antibiotic treatments.

Antibiotic Treatment Procedure

Fresh LB medium was inoculated with bacteria from the starter culture to give an initial

inoculum of 0.01 OD_{600}, which was allowed to continue to grow for 1 h, and the culture was then divided into equal volumes and placed into tubes in the presence or absence of an antibiotic. The following antibiotics were used: ampicillin (Totapem) (Unipex, Paris, France), cefoxitin (Mefoxin) (Sigma-Aldrich, Saint-Louis, MO, USA), imipenem (Tienam) (bioMérieux Inc Saint-Louis, MO, USA), gentamicin (Gentalline) (Unipex, Paris, France), and ciprofloxacin hydrochloride (Ciflox) (MP Biomedical, Santa Ana, CA, USA). Each sample was incubated for at least 4 h at 37°C. Each hour, aliquots of cells were taken, stained with the dyes described below, and analyzed via FC. The number of colony forming units (CFUs) were also counted.

CFU Analysis
To establish the number of CFUs per ml of liquid culture, aliquots of cells, or of the diluted cells, were spread on LB agar plates (agar 15 g/liter) and incubated overnight at 37°C, and the number of CFUs was then counted. Minimum inhibitory concentration (MIC): carbapenem

The electrical MIC obtained using iFAST was compared with the MIC determined using standard BMD for three different strains of K. pneumoniae [susceptible (strain 18397); susceptible, increased exposure (strain KS11); or resistant (strain K14)] exposed to six different Meropenem concentrations, measured according to protocol 1 . Figure shows a set of scatter plots of electrical opacity vs. electrical diameter for these isolates. For the susceptible strain (18397) changes in electrical properties are observed even at the lowest concentration of antibiotic, whilst K14 (resistant) shows no changes at up to 8 mg/L. The MIC of the strains determined by BMD (in triplicate) was K14 = 128 mg/L, KS11 = 8 mg/L, and 18397 < 0.25 mg/L. Figure shows the electrical MIC for ten different strains of K. pneumonia that have a range of different MICs (see Supplementary Table for details of strains). The data is plotted as the % cells within a contour (or gate) defined by the unexposed population vs. antibiotic concentration (for three biological replicates). Qualitatively the data shows that there are three different "classes" of response. The three resistant strains (red) all demonstrate no change in the exposed vs. unexposed gate. The five susceptible strains (blue) all demonstrate a large change in the scatter plot and absolute cell count for the lowest concentration of antibiotic

(0.25 mg/L).
The susceptible, increased exposure strains (orange) fall to >50% cell count within the gate at an antibiotic concentration >2 mg/L. The accepted definition for a BMD MIC is inhibition of growth visible by eye, but there is no equivalent EUCAST standard for fast MIC tests. Assuming a doubling time of 30 min, a bacteriostatic agent with no biophysical changes would approximately halve the number of cells compared with a control gate. If the MIC is calculated at an assumed threshold of 50%, all strains have a MIC within a single twofold dilution of the BMD results.

Breakpoint analysis
Clinical breakpoints are based on the epidemiological cut-off values taken from bacterial culture collections, and define antibiotic concentrations that enable interpretation of the results of MIC tests to classify bacterial isolates as susceptible (S); susceptible, increased exposure (I); or resistant (R) to that antibiotic when used therapeutically. Breakpoints reflect drug potency against a population of potential pathogens, the pharmacokinetics/pharmacodynamics of the antibiotic and the dosing regimens that may be achievable in the clinic. For example, isolates of Enterobacteriaceae with an MIC of 2 mg/L Meropenem or lower are defined by EUCAST as susceptible, and an MIC greater than 8 mg/L is defined as resistant. A MIC > 2 mg/L, but no more than 8 mg/L is in an intermediate category that may require an increased Meropenem dose for some infections caused by this bacterial isolate. We tested the utility of the iFAST technology to rapidly measure the breakpoint for different antibiotics and priority pathogens (see Supplementary Table 1) to see if it could correctly classify strains as susceptible or resistant, using protocol 2 (Supplementary. In these experiments we measured growth, cell volume and membrane biophysical changes after incubation with Meropenem at the clinical breakpoints (2 mg/L; susceptible and 16 mg/L; resistant). The data was quantified by measuring the number of cells in the unexposed contour after 30 min incubation with Meropenem . Figure summarises the results for strains measured at the S/I boundary (inhibition of growth at 2 mg/L indicates a susceptible strain), whilst Fig. summarises measurements where strains were exposed to a higher concentration (16 mg/L). This concentration was selected because growth at >8 mg/L indicates resistance according to EUCAST guidelines. The bars are coloured

according to standard BMD data with red indicating resistant strains and blue for susceptible strains.

We used several data collection strategies. Through our large collaborator networks, we obtained datasets not previously available for AMR research, including hospital and laboratory data, as well as datasets published previously and those outlined in research articles.

Each component of the estimation process had different data requirements and, as such, the input data used for each modelling component differed. The diverse data sought included the following sources: pharmaceutical companies that run surveillance networks, diagnostic laboratories, and clinical trial data; high-quality data from researchers including large multisite research collaborations, smaller studies, clinical trials, and well established research institutes based in low-income and middle-income countries (LMICs); data from public and private hospitals and public health institutes providing diagnostic testing; global surveillance networks; enhanced surveillance systems; national surveillance systems; and surveillance systems for specific organisms such as Mycobacterium tuberculosis and Neisseria gonorrhoeae (all sources are listed by data type in the .

Total sample size and fraction of countries covered for each modelling component by GBD region. The units for sample size are deaths for sepsis and infectious syndrome models; cases for case-fatality ratios; cases, deaths, or isolates for pathogen distribution; pathogen–drug tests for fraction of resistance; and pathogen–drug tests for relative risk. Sample sizes reflect model-specific selection criteria, resulting in lower totals for the sepsis, infectious syndrome, case-fatality ratio, and pathogen distribution models in this table than those in Totals for fraction of resistance and relative risk are higher in this table than in because of the difference in units for certain source types, such as microbial data (isolates in pathogen–drug tests here). Several data sources inform multiple components; therefore, data points should not be summed across a row as that will lead to duplication. More information on the data types used and the components that they inform is presented in the . GBD=Global Burden of Diseases, Injuries, and Risk Factors Study.

To increase the speed and reliability of resistance testing, the use of a genotypic approach has been advocated recently,53 and numerous DNA – based assays have been developed for detection of bacterial resistance genes.54-56 Although this approach has been described as a true revolution, it does require a good understanding of resistance mechanisms and the genes involved.53 Bergeron & Ouellette also highlight other limitations to this approach, as clinical studies will be required to validate the genotypic approach to testing for resistance, the presence of a resistance gene may not always be indicative of resistant bacteria, and conversely, if a gene coding for resistance to an antibiotic is not detected, it may not mean that the bacteria are susceptible to that particular agent. One approach comparing the results of genotypic testing with the results obtained using conventional phenotypic testing of >500 strains of antibiotic-resistant staphylococci, showed that there was an excellent correlation between the resistance genotype and phenotype for methicillin and erythromycin. As we move into a new millennium, most AST methodologies in operation within routine diagnostic clinical microbiology laboratories will be based on techniques first described at the beginning of the twentieth century. If the limitations of these methods are understood and the procedures standardized, the results generated should offer a reliable guide to susceptibility in a cost effective manner. The newer, genotypic resistance testing methods need to be practical for routine use and competitively priced before they can be considered for routine testing. They also need to identify the organism carrying the resistance gene to avoid reporting on commensals or insignificant organisms in a mixed population. However, they may reduce the risk of increasing bacterial resistance by permitting the withholding of antibiotics from patients who will not benefit from them. It is argued that this will be achieved due to the more rapid identification of bacteria and consequent use of targeted antibiotics, and the fact that broad-spectrum antibiotics will be needed only when dealing with resistant organisms.53 Obviously, for this to become a reality the new methods will have to yield very rapid results for treatment to be delayed in the case of serious infection.

II. CONCLUSION

Biodiversity, the variety of life on Earth, is a fundamental pillar of our planet's health and resilience. It encompasses not only the multitude of species, but also the genetic diversity within those species and the complex ecosystems they form. Biodiversity ensures the provision of essential

ecosystem services, such as clean air and water, pollination of crops, and disease control. Moreover, it plays a crucial role in supporting human livelihoods, as many communities rely on biodiversity for food, medicine, and cultural traditions. However, biodiversity faces unprecedented threats from habitat destruction, climate change, pollution, and overexploitation. As we witness the ongoing loss of species and habitats, we must recognize the urgency of preserving biodiversity. Our future well-being, both ecological and economic, is intricately linked to the protection and restoration of the intricate web of life on our planet.

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