History and Development of Antimicrobial Susceptibility Testing Methodology

Philip F. Wheat

Mast Laboratories, Mast Group Limited, Mast House, Derby Road, Bootle, Merseyside, L20 1EA, UK

Tel: +44-151-933-7277; Fax: +44-151-933-2400;
Email: pfwheat@mastgrp.com.

Antimicrobial susceptibility testing (AST) is performed daily on bacterial isolates in clinical laboratories. The techniques employed are often taken for granted. This paper traces the history and development of some methods still in common use for routine AST, e.g. disc diffusion and agar dilution. It was quickly recognized by early investigators that there were many variables affecting the results of these tests. Consequently, there was recognition (as early as the late 1950's) that standardization of these techniques was required. This need has led to many organizations producing standardized AST methodologies. Although some disc diffusion techniques that generated results within 4-6 h were described, most relied on 18-24 h incubation before a result was available. The clinical and economic pressures for rapid methods with low labour input led to the development of semi-automated and automated AST methodologies in the 1970s. Until 10 years ago, AST techniques relied on phenotypically testing the bacteria isolated. However, to increase the speed and reliability of resistance testing, the use of a genotypic approach has been advocated. The limitations and benefits of this new approach are discussed.

The early pioneers of Microbiology, Pasteur, Koch and Ehrlich made many references to antibiosis.[1,2] Alongside these observations were descriptions of laboratory methodologies, which support the concept of antibiosis. These methodologies included the use of test tubes containing broth, loss of motility and animal protection studies. In the late nineteenth century, other workers also noted *in vitro* antibiosis well before the discovery of antibiotics. In 1874 William Roberts observed that liquid medium in which the mould *Penicillium glaucum* was growing, could not easily be contaminated with bacteria.[3] Two years later, John Tyndall made a similar observation that broth supported the growth of either bacteria or moulds, but rarely both.

It was Fleming who reported the inhibitory effect of what eventually became known as penicillin on solid media by observing an area of growth inhibition of staphylococcal colonies adjacent to a *Penicillium* contaminant on an agar plate. This, however, was not the first description of what was eventually termed agar diffusion as this was first utilized by Beijerinck in 1889 for studying the effect of different auxins on bacterial growth.[4] In addition to the discovery of penicillin, Fleming made two further significant contributions to the field of AST in the 1920s. In 1924 he introduced the use of the ditch plate technique for evaluating antimicrobial qualities of antiseptic solutions.[5] Reddish modified this technique when he cut wells into the agar and filled the wells with antiseptic solutions.[6] The principle of this technique was developed further by the cylinder plate synonymous with the 'Oxford cup', 'fish spine' or 'Heatley cup'.[7] Fleming's second contribution to modern AST was the development of a broth dilution technique using turbidity as an end point determination.[8] This has been described as a forerunner of contemporary minimum inhibitory concentration (MIC) methodology.[2] Fleming later developed the broth dilution method by using pH as an indicator instead of turbidity.[9]

Diffusion methods of AST were developed further in the 1940s. In 1940, Heatley introduced the use of absorbent paper for carrying antimicrobial solutions.[10] Filter paper discs incorporating penicillin were utilized by Vincent and Vincent[11] for assaying this newly discovered compound. Mohs introduced a "radial streak disc method " with 15 mm diameter discs.[12] This was the first description of the comparison of a test organism with a sensitive control on the same plate. This method has been described as the forerunner of the Stokes' technique, which until recently was still used in many laboratories in the UK.[2] More paper disc diffusion methods were described in the mid 1940s[13-16] and Hoyt and Levine[17] described a technique that utilized tablets incorporating penicillin instead of impregnated filter paper.[17] The one-quarter inch (6-6.5 mm) filter paper discs that are still commonly utilized today were first described by Bondi and co-workers in 1947.[18]

The 1940s also saw the development of methods incorporating antimicrobial agents into agar; these subsequently became known as agar dilution techniques. As described earlier, Fleming had already diluted antimicrobial agents in liquid medium,[8] but Schmith and Reymann were the first to describe an agar dilution AST method.[19] They determined MICs of sulphapyridine for gonococci. Frisk then described incorporation of penicillin into agar when testing the susceptibility of *Streptococcus pneumoniae*.[20] Frank and co-workers utilized agar dilution for a study of numerous antibiotic-organism combinations.[21] It was also reported at that time that when agar diffusion and agar dilution techniques were performed correctly, similar results were obtained.[22] It was soon recognized, however, that performing agar dilution MIC estimations for routine bacterial isolates was too time consuming and cumbersome. The procedure was simplified by the introduction of multiple replication devices[23-24] and by replacing serial dilutions with one or more critical concentrations that separated organisms into resistant and susceptible categories.[25-26] This method is now commonly referred to as the "breakpoint" technique, a term first used by Ericsson and Sherris.[27] The widespread use of breakpoint techniques was also facilitated by the availability of convenient sources of antimicrobial agents in known quantities, firstly in filter paper strips,[28] then in filter paper pads[29] and finally presented as tablets or in glass vials where the agents have been freeze dried.[30]

At an early stage it became apparent to workers using diffusion or dilution techniques that there were many variables affecting AST methods. Early comprehensive studies assessing these variables were reported by many groups.[31-35] By the end of the 1950s it was apparent that there was a need to standardize AST. Several organizations and investigators began addressing this critical issue. The World Health Organization (WHO) published a report on AST methodology.[36] In 1966, significant progress in standardization of the disc method occurred when Bauer, Kirby and co-workers published their attempt to establish the disc diffusion technique as a practical method of testing with broad application to clinical laboratories.[37] In 1975 this method became the basis of the National Committee for Clinical Laboratory Standards (NCCLS) disc diffusion standards.[38] The results of a WHO sponsored, 10 year International Collaborative Study (ICS) of AST were reported in 1971.[27] The ICS

disc diffusion method was largely based on that originally described by Ericsson and colleagues.[39-40] Many others have followed these initial attempts at standardize AST methodology. Within Europe, at least six different systems for AST are used,[41] those of the Swedish Reference Group for Antibiotics,[42] the Deutsches Institut für Normung (in Germany),[43] the Werkgroep Richtlijnen Gevoeligheidsbepalingen (in the Netherlands),[44] the British Society for Antimicrobial Chemotherapy,[45] the Société Française de Microbiologie[46] and the NCCLS standards (in some countries).[38]

All the methods discussed so far have significant limitations. Although several authors have described methods that speed up the results obtained using disc diffusion techniques,[47-48] the methods currently used still rely mainly on 18-24 hours incubation. Also, most of the methods described do not lend themselves to automated integration into the electronic data processing systems now found in many modern clinical microbiology departments. Furthermore, the clinical and economic pressures for rapid methods with low labour input has resulted in the development of alternative AST methods.[49]

One of the first automated AST was the Autobac disc elution system, marketed by Pfizer Diagnostics and introduced in 1974.[50] This machine enabled some results to be reported within 4-6 h of inoculation. Another early automated system, introduced in 1977, was the Abbott MS-2 System,[51] which took 4 h and included organism identification. It was also a disc elution system and generated calculated MIC values. In the same year, the McDonnell Douglas Corporation introduced the AMS System.[52] This was the forerunner of what is known today as the Vitek System. This system utilized dehydrated reagents in sealed plastic cards and contained separate cards for AST and organism identification. It was also in 1977 that standardized microtitre trays containing antimicrobial agents were introduced. This innovation ultimately led to the development of such automated systems as Micro-Media Systems, Sensititre, BBL Sceptor and a whole line of products by Microscan, which started as a basic microtitre system then developed into more complex systems such as the TouchScan, the AutoScan and the MicroScan Walkaway System.[2]

All the susceptibility techniques described so far rely on phenotypically testing the bacteria isolated. Although these phenotypic susceptibility testing techniques are relatively simple, they require bacterial isolation, and hence the result is often not available until 2 days after treatment has started. Bergeron & Ouellette[53] highlighted other shortcomings of the phenotypic approach of susceptibility testing, such as the tests being highly dependent on experimental conditions and the fact that more than one method needs to be performed to obtain an accurate susceptibility profile. They also highlighted the fact that different bacterial species have different susceptibilities to the same antibiotic, and that there is no international agreement on breakpoints for interpretation of antimicrobial susceptibility tests.

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.

References

1. Lechevalier, H.A. & Solotorvsky, M. (1965). In *Three Centuries of Microbiology.*

2. McGraw-Hill Book Co., New York, NY.

3. Poupard, J.A., Rittenhouse, S.F. & Walsh, L.R. (1994). The evolution of antimicrobial susceptibility testing methods. In *Antimicrobial Susceptibility Testing*. (Poupard, J.A., Walsh, L.R. & Kleger, B., Eds), pp. 3-14. Plenum Press, New York, NY.

4. Roberts, W. (1874). Studies on biogenesis. *Philosophical Transactions of the Royal Society of London* **164**, 466.

5. Dufrenoy, J. (1947). Les methodes auxanographiques et leur application au dosage des antibiotiques. *Annales de Parasitologie Humaine et Comparee* **22,** 449-79.

6. Fleming, A. (1924). A comparison of the activities of antiseptics on bacteria and on leucocytes. *Proceedings of the Royal Society of London , Series B* **96,** 171-80.

7. Reddish, G.F. (1929). Methods of testing antiseptics. *Journal of Laboratory and Clinical Medicine* **14,** 649-58.

8. Abraham, E.P., Chain, E., Fletcher, C.M., Gardner, A.D., Heatley, N.G., Jennings, M.A. *et al*. (1941). Further observations on penicillin. *Lancet ii,* 177-88.

9. Fleming, A. (1929). On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of *B. influenzae. British Journal of Experimental Pathology* **10,** 226-36.

10. Fleming, A. (1942). In-vitro tests of penicillin potency. *Lancet i,* 732-3.

11. Heatley, N.G. (1944). Method for the assay of penicillin. *Biochemical Journal* **38,** 61-5.

12. Vincent, J.G. & Vincent, H.W. (1944). Filter paper disc modification of the Oxford cup penicillin determination. *Proceedings of the Society for Experimental Biology and Medicine* **55,** 162-4.

13. Mohs, F.E. (1945). A simple quantitative test for the penicillin sensitivity of bacteria: the 'radial streak ' method. *Journal of Laboratory and Clinical Medicine* **30,** 800-2.

14. Copeland, J.R. (1945). Method of testing sensitivity of micro-organisms to penicillin. *Bulletin of the United States Army Medical Department* **4,** 596-9.

15. Morley, D.C. (1945). A simple method of testing the sensitivity of wound bacteria to penicillin and sulphathiazole by use of impregnated blotting paper discs. *Journal of Pathology and Bacteriology* **57,** 379-82.

16. Kokko, U.P. (1947). Determination of bacterial sensitivity to sulphonamides by use of impregnated blotting paper disc method. *Annales Medicinae Experimentalis et Biologiae Fenniae* **25,** 79-93.

17. Kolmer, J.A. (1947). Laboratory controls in relation to penicillin and streptomycin therapy. *Southern Medical Journal* **40,** 72-6.

18. Hoyt, R.E. & Levine, M.G. (1947). Method for determining sensitivity to penicillin and streptomycin. *Science* **106,** 171.

19. Bondi, A., Spaulding, E.H., Smith, D.E. & Dietz, C.C. (1947). Routine method for rapid

determination of susceptibility to penicillin and other antibiotics. *American Journal of the Medical Sciences* **213,** 221-5.

20. Schmith, K. & Reymann, F.E. (1940). Expeimentelle og kliniske undersogelser over gonococcers folsomhed overfor sulfapyridin. *Nordisk Medicin* **8,** 2493-9.

21. Frisk, A.R. (1945). Bestamning av penicillin koncentration och penicillinkaslighet *Nordisk Medicin* **28,** 2249-52.

22. Frank, P.F., Wilcox, C. & Finland, M. (1950). *In vitro* sensitivity of coliform bacilli to 7 antibiotics (penicillin, streptomycin, bacitracin, polymycin, aerosporin, aureomycin, and chloromycetin). *Journal of Laboratory and Clinical Medicine* **35,** 188-204.

23. Jackson, G.G. & Finland, M. (1951). Comparison of methods for determining sensitivity of bacteria to antibiotics in vitro. *Archives of Internal Medicine* **88,** 446-60.

24. Garrett, S.D. (1946). A multiple - point inoculating needle for agar plates. *Transactions of the British Mycological Society* **29,** 171-2.

25. Steers, E., Foltz, E.L. & Graves, B.S. (1959). An inocula replicating apparatus for routine testing of bacterial susceptibility to antibiotics. *Antibiotics and Chemotherapy* **9,** 307-11.

26. Hutchison, J.G.P. (1954). Antibiotic sensitivity tests : a rapid method suitable for multiple cultures. *Journal of Clinical Pathology* **7,** 350-1.

27. Tolhurst, J.C., Buckle, G. & Williams, S.W. (1963). Agar dilution tests. In *Chemotherapy with Antibiotics and Allied Drugs.* 2nd edn., pp. 153-4. National Health and Medical Research Council of Australia, Canberra.

28. Ericsson, H., & Sherris, J.C. (1971). Antibiotic sensitivity testing . Report of an international collaborative study. *Acta Pathologica et Microbiologica Scandinavica Section B, Suppl. 217,* 1-90.

29. Rolinson, G.N. & Russell, E.J. (1972). New method for antibiotic susceptibility testing. *Antimicrobial Agents and Chemotherapy* **2,** 51-6.

30. Shafi, M.S. (1975). Determination of antimicrobial MIC by paper diffusion method. *Journal of Clinical Pathology.* **28,** 989-92.

31. Snell, J.J.S., Danvers, M.V.S. & Gardner, P.S. (1984). Comparison of antibiotic susceptibility results obtained with Adatab and disc methods. *Journal of Clinical Pathology* **37,** 1059-65.

32. Heatley, N.G. (1949). Methods for measuring the sensitivity of micro-organisms to antibiotics. In *Antibiotics*, Volume 1. (Florey, H.W., Chain, E., Heatley, N.G., Jennings, M.A., Sanders, A.G., Abraham, E.P. *et al* ., Eds), pp. 200-14. Oxford University Press, London.

33. Erlanson, P. (1951). Determination of the sensitivity in vitro of bacteria to chemotherapeutic agents with special reference to routine tests. *Acta Pathologica et Microbiologica Scandinavica Suppl. 85,* 1-162.

34. Waterworth, P.M. (1951). A comparative study of methods of testing sensitivity to antibiotics and of the factors influencing the results. *Journal of Medical Laboratory Technology* **9,** 65-85.

35. Gould, J.C. & Bowie, J.H. (1952). The determination of bacterial sensitivity to antibiotics. *Edinburgh Medical Journal* **59,** 178-99.

36. Anderson, T.G. & Troyanosky, A. (1960). Antibiotic susceptibility testing by the disc method. In *Antibiotics Annual 1959-1960.* (Marti-Ibanez., F., Ed), pp. 587-95. Antibiotica Inc., New York, NY.

37. World Health Organisation. (1961). *Standardization of Methods for Conducting Microbic Sensitivity Tests. Second report of the Expert Committee on Antibiotics. WHO Technical Report Series, No. 210.* WHO, Geneva.

38. Bauer, A.W., Kirby, W.M.M., Sherris, J.C. & Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology* **45,** 493-6.

39. National Committee for Clinical Laboratory Standards. (1975). *Performance Standards for Antimicrobial Disk Susceptibility Tests*; Approved Standard *M2-A7 ASM-2*. NCCLS, Villanova , PA.

40. Ericsson, H., Hogman, C. & Wickman, K. (1954). A paper disk method for determination of bacterial sensitivity to chemotherapeutic and antibiotic agents. *Scandinavian Journal of Clinical Laboratory Investigation* **6,** *Suppl. 11*, 21-6.

41. Ericsson, H. & Svartz-Malmberg, G. (1959). Determination of bacterial sensitivity *in vitro* and its clinical evaluation. *Antibiotica et Chemotherapia* **6,** 41-77.

42. Baquero, F. (1990). European standards for antibiotic susceptibility testing: towards a theoretical consensus. *European Journal of Clinical Microbiology and Infectious Disease* **9,** 492-5.

43. The Swedish Reference Group for Antibiotics. (1977). A revised system for antibiotic sensitivity testing. *Scandinavian Journal of Infectious Diseases, Supp.* 105.

44. Deutsches Institut fur Normung. (2000). Methoden zur Empfindlichkeitspufung von bakteriellenKrankheitserregern (auber Mycobacterien) gegen Chemotherapeutika. DIN 58940.

45. Werkgroep Richthjnen Govoligheidsbepaligen Report. (1990). Standaardisatie van Gevoeligheidsbepalingen. WRG, Bilthoven.

46. Andrews. J.M. for the BSAC Working Party on Susceptibility Testing. (2000). BSAC standardized disc susceptibility testing method. *Journal of Antimicrobial Chemotherapy* **48,** *Suppl. 1,* 43-57.

47. Comité de l'Antibiogramme de la Société Française de Microbiologie. *Clinical Microbiology and Infection* **2,** *Suppl.* S1–49.

48. Jackson, J.L.W., Dye, W.E. & Mitchell, R.B. (1954). Use of haemoglobin indicator for rapid method of determining antibiotic sensitivity of microorganisms. *Texas Reports on Biology and Medicine* **12,** 171-2.

49. Bartlett, R.C. & Mazens, M.F. (1979). Rapid antimicrobial susceptibility test using tetrazolium reduction. *Antimicrobial Agents and Chemotherapy* **15,** 769-74.

50. Spencer, R.C. & Wheat, P.F. (1986). Novel mechanisms for determining antibiotic susceptibilities. *Journal of Antimicrobial Chemotherapy* **17,** 404-7.

51. Mckie, J., Borovoy, R., Dooley, J., Enavega, G., Mendoza, G., Meyer, F. *et al.* (1974). Autobac I – A three hour automated antimicrobial susceptibility system. In *Automation in Microbiology and Immunology,* (Heden, C. & Illeni, T., Eds). John Wiley, New York, NY.

52. Spencer, H.J., Stockert, J., Welaj, P., Wilborn, R., & Price, B. (1977). Automated antimicrobial susceptibility testing with the MS-2 system. In *Rapid Methods and Automation in Microbiology.* (Johnston, H.H. & Newsom, S.W.B.), pp 262-3. John Wiley, New York, NY.

53. Aldridge, C., Jones, P.W., Gibbson, S., Lanham, J., Myer, M., Vannest, R. & Charles, R. (1977). Automated microbiological detection/identification system. *Journal of Clinical Microbiology* **6,** 406-13.

54. Bergeron, M.G. & Ouellette, M. (1998). Preventing antibiotic resistance through rapid genotypic identification of bacteria and of their antibiotic resistance genes in the clinical microbiology laboratory. *Journal of Clinical Microbiology* **36,** 2169-72.

55. Courvalin, P. (1991). Genotypic approach to the study of bacterial resistance to antibiotics. *Antimicrobial Agents and Chemotherapy* **35,** 1019-23.

56. Tenover, F.C. (1992). Bauer and Kirby meet Watson and Crick: antimicrobial susceptibility testing in the molecular era . *ASM News* **58,** 669-72.

57. Arlet, G. & Philippon, A. (1994). PCR-based approaches for the detection of bacterial resistance. In *PCR-based Diagnostics in Infectious Diseases,* (Ehrlich, G.D. & Greenberg, S.J. Eds), pp. 665-87. Blackwell Scientific Publications, Oxford.

58. Persing, D.H., Relman, D.A. & Tenover, F.C. (1996). Genotypic detection of antimicrobial resistance. In *PCR protocols for Emerging Infectious Diseases,* (Persing D.H. Ed), pp. 33-57. ASM Press, Washington DC.

59. Carroll, K.C., Leonard, R.B., Newcomb Gayman, P.L., & Hillyard, D.R. (1996). Rapid detection of the staphylococcal mecA gene from BACTEC blood culture bottles by the polymerase chain reaction. *American Journal Clinical Pathology* **106,** 600-5.