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Reviews

Natural release of virulence factors in membrane vesicles by *Pseudomonas aeruginosa* and the effect of aminoglycoside antibiotics on their release

J. L. Kadurugamuwa and T. J. Beveridge*

National Centre of Excellence–Canadian Bacterial Disease Network, Department of Microbiology, College of Biological Science, University of Guelph, Guelph, Ontario, Canada N1G 2W1

Pseudomonas aeruginosa **(and various other Gram-negative pathogens) liberate membrane vesicles during normal growth. These bilayered vesicles consist of endotoxin (lipopolysaccharide), outer membrane proteins and several potent hydrolytic enzymes including protease, alkaline phosphatase, phospolipase C and peptidoglycan hydrolase. The vesicles contain pro-elastase and alkaline phosphatase (which are periplasmic constituents) and so are important for packaging periplasmic components as they are liberated to the outside of the cell. Once liberated, the vesicles are capable of fusing with the membranes of epithelial cells and liberating their virulence factors into host cells where they degrade cellular components, thereby aiding infection by the pathogen. The aminoglycoside antibiotic, gentamicin, is thought to kill bacteria by inhibiting protein synthesis, yet this cationic antibiotic can also perturb the packing order of lipids, thereby destabilizing bilayered membranes. For pathogens with highly anionic lipopolysaccharide on their surface, such as *P. aeruginosa*, this membrane destabilization can be so serious that it can cause cell lysis; these cells are therefore killed by a combination of protein synthesis inhibition and surface perturbation. By destabilizing the membranes of *P. aeruginosa*, gentamicin increases the release of membrane vesicles three- to five-fold. This may help account for some of the bacterium-mediated toxicity encountered during patient treatment with aminoglycoside antibiotics.**

Introduction

Pseudomonas aeruginosa is an opportunistic, Gram-negative pathogen that can have serious consequences for immunocompromised and physically traumatized patients (e.g. those with burns or cancer[1,2]). It can also rapidly destroy the cornea[3] and, because it is commonly isolated from the lungs of cystic fibrosis patients, it has been implicated in contributing to the progression of this disease.[4] For many years, aminoglycoside antibiotics (e.g. tobramycin, gentamicin and amikacin), either alone or in combination, were the drugs of choice for these infections because of the pathogen's innate resistance to many other chemotherapeutic agents, yet aminoglycosides had to be used with care because of their well-recognized ototoxicity and nephrotoxicity.[5] Traditionally, these antibiotics have been considered to enter the pathogen, possibly by a self-promoted pathway,[6] and to interact with ribosomes so that protein synthesis is no longer possible.[7] Recent work suggests that this is only part of the story.

Surface interaction of gentamicin

Most Gram-negative bacteria have anionic (electronegative) surfaces because of the lipopolysaccharide (LPS) molecules that stud the outer face of the outer membrane.[8,9] Because of the abundant carboxyl groups of

*Tel: +1-519-824-4120 ext. 3366; Fax: +1-519-837-1802; E-mail: tjb@micro.uoguelph.ca

(some) O-side chains and the 'core region' keto-deoxy-octonates (KDOs), and of the phosphates of both the core and lipid A regions, LPS has a much higher charge per surface area than phospolipid (for details of an *Escherichia coli* K12 strain see references 10–12). These anionic sites on the LPS must interact with exogenous cations; indeed, Mg^{2+} and Ca^{2+} are strongly bound and form salt-bridges between adjacent LPS molecules, thereby stabilizing the bilayer and making it more rigid than the underlying plasma (cytoplasmic) membrane.[12] (Outer membrane lipoprotein interaction with the peptidoglycan layer also has a role in this.[8]) Because of the dependence of the outer membrane on these small metal cations, their displacement by other exogenous cations can seriously disturb normal bilayer structure.

Gentamicin is the generic name for a mixture of three almost identical compounds, gentamicins C_1, C_{1a} and C_2, which have a strong electropositive charge when in solution at neutral pH. Although gentamicin is much larger than either Mg^{2+} or Ca^{2+}, its strong electropositivity makes it highly competetive for the LPS sites occupied by these cations. In a general sense, this is similar to the action of polymyxin on the outer membrane. For gentamicin, the metal ions are displaced and the normal packing order of the outer membrane is perturbed so that the bilayer forms blebs which come off the cell.[13] Immediately beneath each outer membrane bleb, localized lesions can be seen in the peptidoglycan layer which help to cause cell lysis. The same cellular disruptions can be seen with other aminoglycosides such as amikacin.[14] These initial observations of the effect of aminoglycosides on the surface of *P. aeruginosa* suggested that ribosomes were not the only target of this group of antibiotics.

Kadurugamuwa *et al.*[15] have studied this surface phenomenon in more detail. *P. aeruginosa* PAO1 has two types of LPS on its surface, A- (or common antigen) and B- (or 05 serotype) band LPS[16]. The former has an O-side chain of α1–2-, α1–2- and α1–3-linked D-rhamnose[17, 18, 19] which is uncharged. B-band LPS contains a variable number of trimeric units comprised of two manno-uronic acids linked to *N*-acetylfucosamine.[20] This O-side chain can be up to 35 trimeric repeats in length[21] extending approximately 40 nm from the surface,[22] and is joined to a core polysaccharide and lipid A moiety which share 12 phosphates and three KDO (core) carboxylates. Accordingly, this B-band LPS is highly ionized at neutral pH and possesses a net electronegative charge.

When the gentamicin susceptiblity of the wild-type PAO1 strain was compared with that of isogenic LPS (A^+B^-, A^-B^+ and A^-B^-) mutants, those of the B^+ phenotype were more sensitive to the antibiotic (Figure 1).[15] This was thought to be the result of a strong interaction between gentamicin and B-band LPS which produced surface perturbation and lysis. To verify that this was the case and to distinguish between surface and ribosomal effects, an artificially large gentamicin, consisting of about 32 gentamicins plus a bovine serum albumin (BSA) molecule, was synthesized chemically (Figure 2a).[23] This macromolecule was too large to enter the bacterium and inhibit protein synthesis: it could only manifest the surface effect. Yet, it still lysed cells (Figure 2b) and reacted most strongly with those of the B^+ phenotype. This was unequivocal proof that aminoglycoside antibiotics not only affect protein synthesis but that they can also have a profound lytic effect on Gram-negative surfaces. Gentamicin is a highly charged cationic molecule that displaces Mg^{2+} and Ca^{2+} which salt-bridge anionic charges (carboxylates and phosphates, see above) between adjacent LPS molecules, thereby disrupting LPS packing order and perturbing the outer membrane.[13] In so doing, the stringent regulation of the cell's autolysins is disturbed, the peptidoglycan is hydrolysed and the bacterium lyses.[23,24] Since B-band LPS has more anionic sites than A-band, gentamicin has a greater effect on this LPS type.

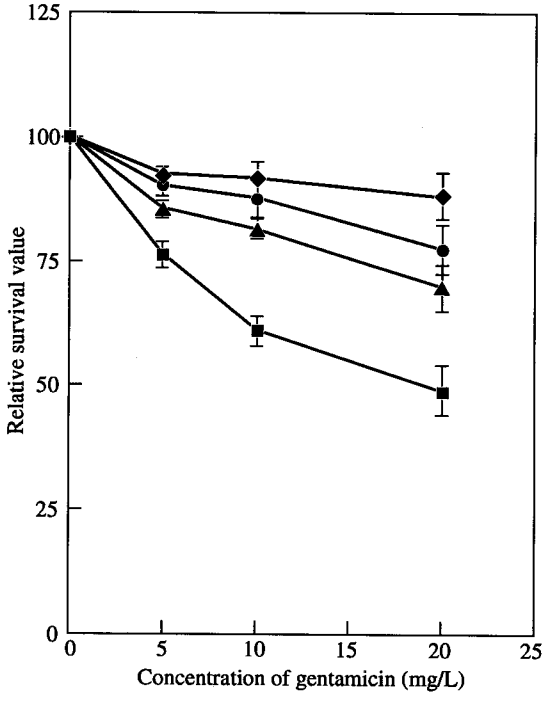

Figure 1. Antibacterial effect of gentamicin caused by ion-binding of *P. aeruginosa* PAO1 (A^+B^+; ■), AK 1401 (A^+B^-; ●), dps 89 (A^-B^+; ▲) and rd 7513 (A^-B^-; ♦). Means ± S.D. of three independent determinations are shown. Reproduced from reference 15.

Natural liberation of membrane vesicles

During normal growth, *P. aeruginosa* sloughs-off membrane vesicles (MVs) of approximately 50 nm in diameter (Figure 3).[25] ELISA, zymogram analysis and immunogold electron microscopy revealed that these MVs possessed a wide spectrum of virulence factors including protease,

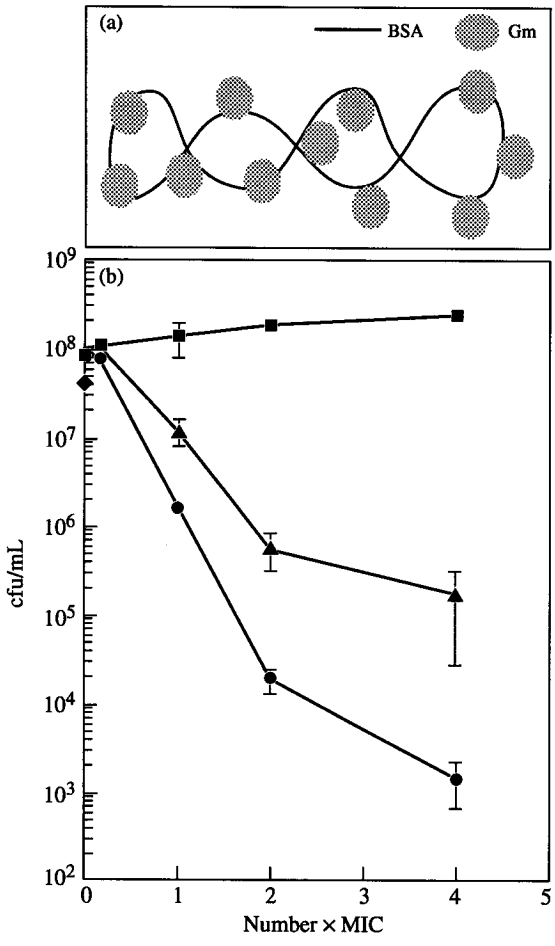

Figure 2. (a) A diagram representing a chemically synthesized macromolecule containing gentamicin (Gm) coupled to bovine serum albumin (BSA). (b) Viability of *P. aeruginosa* PAO1 incubated with different concentrations of gentamicin (●), gentamicin–BSA (▲) and a control without antibiotics (■). Panel b is reproduced from reference 23.

alkaline phosphatase, phospholipase C, peptidoglycan hydrolase and pro-elastase. Since many of these factors are associated with the periplasm[26] it is apparent that, as the outer membrane is shed from the surface, a discrete volume of periplasm is trapped within the lumen of the MV (this is the electron-dense material seen in Figure 3). The identification of pro-elastase within the lumen substantiates the fact that the lumen contains periplasmic components. This is because the enzyme is synthesized as pre-pro-elastase in the cytoplasm. Once it is translocated across the plasma membrane, the 'pre' signal peptide is cleaved off and pro-elastase remains within the periplasm until it is transferred across the outer membrane.[27] During this final transfer, the 'pro' peptide is removed and the enzyme is activated. It is probable that this liberation of MVs is a natural system developed by this opportunistic pathogen to package periplasmic components so that they can be conveyed in a concentrated form to susceptible tissue. Remarkably, natural MVs contain only serotype (B-band) LPS[25] which ensures a strong, specific, endotoxic response in the infected host. Preliminary experiments with epithelial tissue culture lines show that natural MVs fuse with host membranes, releasing virulence factors into epithelial cells and initiating cell degradation (J. L. Kadurugamuwa & T. J. Beveridge, unpublished data). It is apparent that natural MVs are a newly discovered, efficient way by which virulent strains of *P. aeruginosa* instigate infection. Although not as thoroughly studied as the *P. aeruginosa* system, MVs have also been seen by ourselves and other researchers in a wide-range of Gram-negative bacteria (Table). The wide number of genera producing natural MVs suggests that most Gram-negative bacteria release these membrane blebs. Not all do, though. Our preliminary experiments suggest that those bacteria whose outer membrane contains a high proportion of

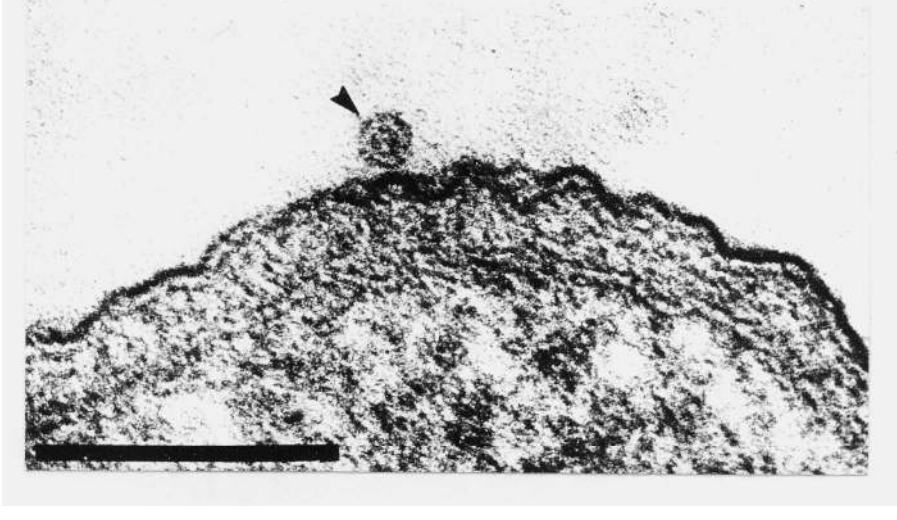

Figure 3. Thin section of the cell envelope of *P. aeruginosa* PAO1 showing a natural membrane vesicle (arrowed) above the cell surface. Bar represents 250 nm.

Table. Examples of other bacteria with membrane vesicles

Organism	Reference
Aeromonas sp.	W. Kay, T. Trust & T. J. Beveridge, unpublished
Aquaspirillum spp.	T. J. Beveridge, unpublished
Bacteroides spp.	32, 33
Borrelia sp.	37; J. Kreiling, N. Charon & T. J. Beveridge, unpublished
Campylobacter spp.	L. L. Graham & T. J. Beveridge, unpublished
Escherichia coli (enterotoxigenic)	36
Haemophilus spp.	30, 31
Magnetospirillum sp.	T. J. Beveridge, unpublished
Myxococcus sp.	T. J. Beveridge, unpublished
Neisseria spp.	28, 29; T. J. Beveridge, unpublished
Proteus sp.	A. J. Clarke & T. J. Beveridge, unpublished
Serratia sp.	J. L. Kadurugamuwa & T. J. Beveridge, unpublished
Treponema sp.	J. Ruby, N. Charon & T. J. Beveridge, unpublished
Vibrio sp.	34, 35

lipoproteins, covalently linked to the peptidoglycan layer, do not yield high concentrations of membrane blebs. The one exception to this are enterotoxigenic strains of *E. coli* (Table).[36] Therefore, pathogens, like *P. aeruginosa*, could also use their MVs as the first line of attack during infection.

Natural MVs from *P. aeruginosa* contain the major 26 kDa peptidoglycan hydrolase or autolysin of the bacterium[24] which can hydrolyse a variety of peptidoglycan chemotypes including Gram-positive bacteria.[38] Natural MVs can interact so strongly with other bacteria that they can lyse them, presumably allowing *P. aeruginosa* to feed on their remains. We have, therefore, coined the term 'predatory MVs' for MVs that contain the hydrolase. In a more chemotherapeutic sense, it is possible that these antibacterial properties of MVs could be used in a topical lotion to kill antibiotic-resistant pathogens. We recognize that MVs from pathogens contain virulence factors which may be injurious to the tissue, but our extended research on other non-pathogenic bacteria (e.g. *Myxococcus*, *Aquaspirillum* and *Magnetospirillum* spp.) also show that MVs are produced (some containing even more potent peptidoglycan hydrolases) and do not have potent virulence properties. We are currently assessing the necrotic potential of MVs from both pathogenic and non-pathogenic Gram-negative bacteria on several tissue lines.

Effect of gentamicin on the liberation of membrane vesicles

Because aminoglycoside antibiotics perturb the membranes of Gram-negative bacteria, they profoundly influence the liberation of MVs from the surface of these cells. For *P. aeruginosa*, gentamicin increases the production of MVs three- to five-fold (Figure 4).[25] It is possible that other Gram-negative pathogens could be encouraged to liberate more MVs by using sub-lethal aminoglycoside antibiotic concentrations. These gentamicin-induced MVs of *P. aeruginosa* contain all the usual constituents of natural MVs, but differ subtly in that they are larger (*c.*100–200 nm in diameter) and contain both A- and B-band LPS and (sometimes) cytoplasmic components such as DNA. Because aminoglycoside antibiotics substantially increase the production of MVs, it is possible that when these drugs are used to treat patients there is a similar high release of reactive material from the pathogen as its growth is diminished. Both A- and B-band LPS (common- and serotype-specific endotoxins) and other virulence factors would be excised from the bacteria in relatively large amounts and this could account for some of the pathogen-mediated toxicity commonly seen in patients treated with these antibiotics.[1,2,31]

Significantly, gentamicin-induced MVs also contain small quantities of gentamicin[25, 38] which gives them additional antimicrobial activity. Because MVs readily fuse with other biological membranes, gentamicin-induced MVs can be used to transport this (usually impenetrable) antibiotic into mammalian tissue to combat intracellular parasites such as *Shigella* and *Salmonella* spp. (J. L. Kadurugamuwa & T. J. Beveridge, unpublished). We are currently working on avirulent strains of *P. aeruginosa* in an effort to reduce both virulence-factor and endotoxic responses of gentamicin-induced MVs so that aminoglycoside antibiotics can be efficiently and safely incorporated into tissue by these vesicles.

Another intriguing possibility is that gentamicin-induced MVs could be used against Gram-negative

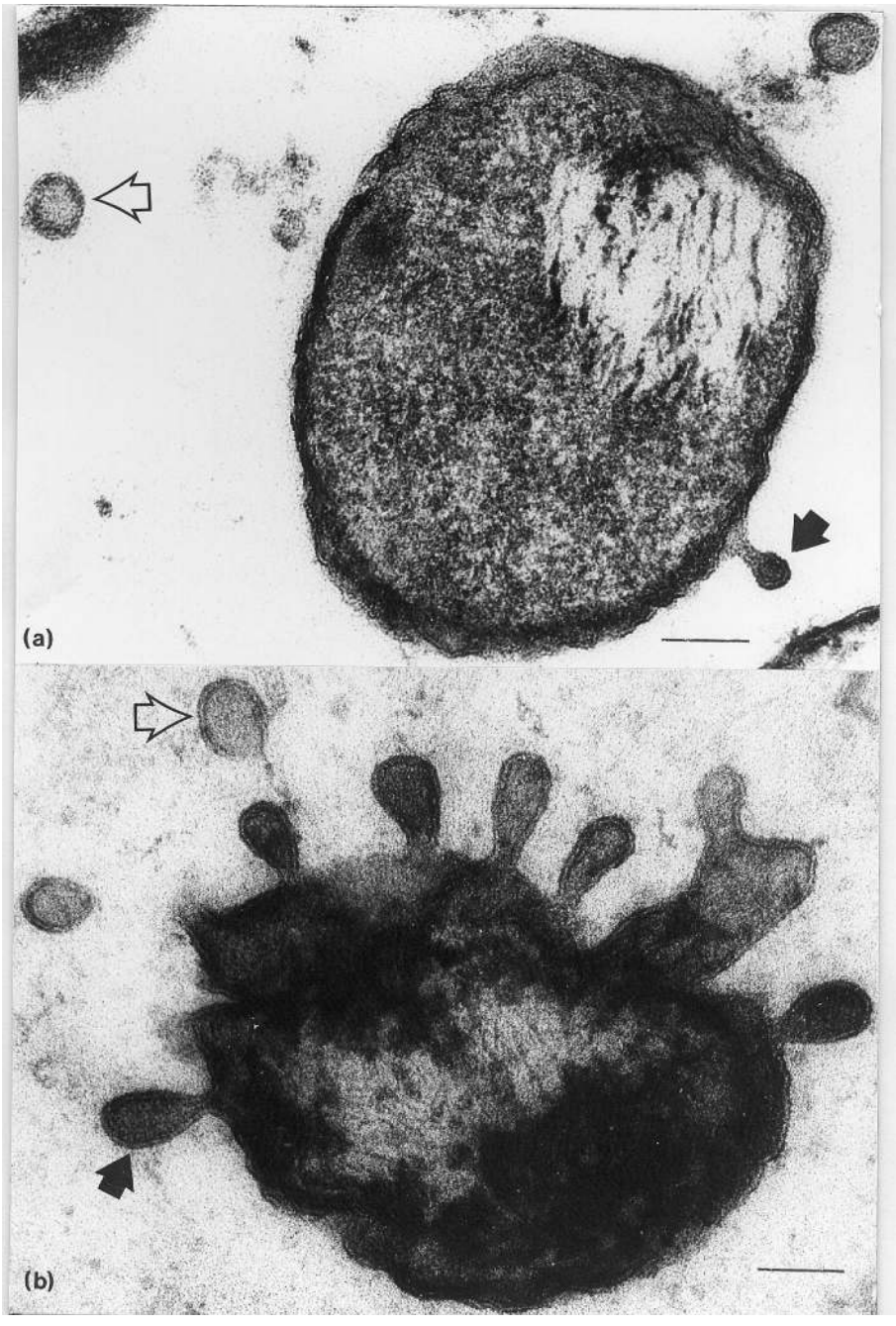

Figure 4. Thin sections of *P. aeruginosa* PAO1 showing the formation of MVs (solid arrowheads) and free MVs (open arrows) in growth medium. (a) Control cell forming natural MVs and (b) a cell exposed to 4 × MIC of gentamicin. Note that more MVs are formed by cells exposed to gentamicin than by untreated cells. Also note that electron-dense material has been trapped in the developing and free vesicles. Bars represent 100 nm. Reproduced from reference 25.

pathogens with 'permeability-type' antibiotic resistance. For example, *P. aeruginosa* strain 8803 is not sensitive to gentamicin because the drug cannot pass through the outer membrane,[39] yet when this strain is treated with gentamicin-induced MVs, it is readily killed (Figure 5).[38] Gentamicin-induced MVs fuse with the outer membrane and deliver their luminal contents (including gentamicin) into the periplasm of the 8803 strain where the antibiotic can inhibit protein synthesis. Since this strain is not closely related to the PAO1 strain from which the gentamicin-induced MVs were derived, the peptidoglycan hydrolase contained in the vesicles is unregulated and can digest the bacterium's peptidoglycan sacculus, thereby increasing the killing power of the gentamicin-induced MVs.[38]

Over the last decade there has been much research on the use of artificial liposomes as delivery devices for thera-

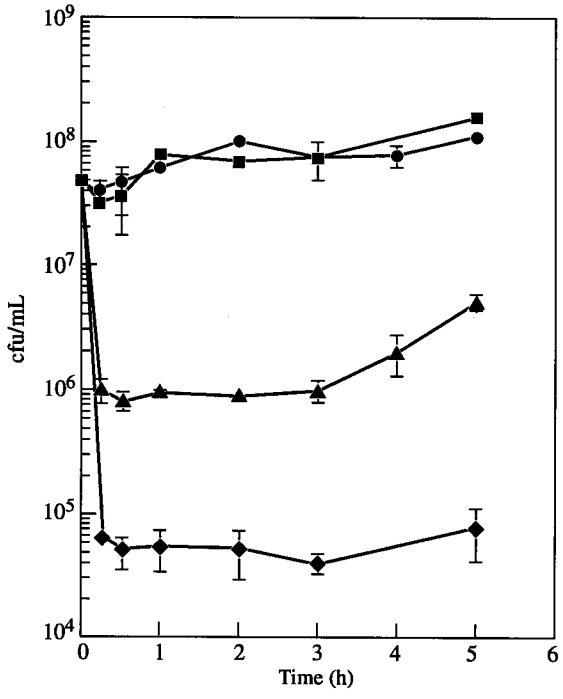

Figure 5. Bactericidal effect of natural MVs (▲), gentamicin-induced MVs (♦) and gentamicin (●) on *P. aeruginosa* 8803. Natural MVs, gentamicin-induced MVs or gentamicin were added to viable cells and viability was monitored over 5 h. The control (■) contained cells with no MVs or gentamicin. The soluble gentamicin concentration for the gentamicin experiment was 128 mg/L. The natural MVs and the gentamicin-induced MVs were added at a protein concentration of 100 mg/L. Strain 8803 is 'permeability-resistant' to soluble gentamicin. Reproduced from reference 38.

peutic agents. These require special equipment and 'blends' of amphipathic lipids for their production. This can be expensive, the uniformity of size and content is poor, and liposomes have only a short lifetime in body fluids, even if exotic lipid mixtures are used.[40] The size and composition of natural MVs are constant, they can easily be fused with live, attenuated oral vaccine strains of bacteria to make an antigenic mosaic (liposomes are not compatible with Gram-negative outer membranes), and they are more resilient in body fluids than liposomes. Clearly, the disadvantage of MVs is their potential toxicity but we believe this can be overcome. Because MVs are natural products, they can be generated easily and efficiently during the normal growth of Gram-negative bacteria and certainly warrant increased study.

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References

1. Hurley, J. C. (1992). Antibiotic-induced release of endotoxin: a reappraisal. *Clinical Infectious Diseases* **15**, 840–54.

2. Prins, J. M., van Deventer, S. J. H., Kuijper, E. L. & Speelman, P. (1994). Clinical relevance of antibiotic-induced endotoxin release. *Antimicrobial Agents and Chemotherapy* **38**, 1211–8.

3. Howe, T. R. & Iglewski, B. H. (1984). Isolation and characterization of alkaline protease-deficient mutants of *Pseudomonas aeruginosa in vitro* and in a mouse eye model. *Infection and Immunity* **43**, 1058–63.

4. Govan, J. R. W. & Harris, G. S. (1986). *Pseudomonas aerugi - nosa* and cystic fibrosis: unusual bacterial adaptation and pathogenesis. *Microbiological Sciences* **3**, 302–8.

5. Ngeleka, M., Beauchamp, D., Tardif, D., Auclair, P., Gourde, P. & Bergeron, M. G. (1990). Endotoxin increases the nephrotoxic potential of gentamicin and vancomycin plus gentamicin. *Journal of Infectious Diseases* **161**, 721–7.

6. Hancock, R. E. W. (1981) Aminoglycoside uptake and mode of action—with special reference to streptomycin and gentimicin. I. Antagonists and mutants. *Journal of Antimicrobial Chemotherapy* **8**, 249–76.

7. Reden, J. & Durckheimer, W. (1979). Aminoglycoside antibiotics. Chemistry, biochemistry, structure–activity relationships. *Topics in Current Chemistry* **83**, 105–70.

8. Beveridge, T. J. (1981). Ultrastructure, chemistry, and function of the bacterial wall. *International Review of Cytology* 7**2**, 229–317.

9. Beveridge, T. J. & Graham, L. L. (1991). Surfaces of bacteria. *Microbiological Reviews* **55**, 684–705.

10. Ferris, F. G. & Beveridge, T. J. (1984). Binding of a paramagnetic metal to *Escherichia coli* K12 lipopolysaccharide. *FEMS Letters* **24**, 43–6.

11. Ferris, F. G. & Beveridge, T. J. (1986). Site specificity of metallic ion binding in *Escherichia coli* K-12. *Canadian Journal of Microbiology* **32**, 52–5.

12. Ferris, F. G. & Beveridge, T. J. (1986). Physicochemical roles of soluble metal cations in the outer membrane of *Escherichia coli* K-12. *Canadian Journal of Microbiology* **32**, 594–601.

13. Martin, N. L. & Beveridge, T. J. (1986). Gentamicin interaction with *Pseudomonas aeruginosa* cell envelope. *Antimicrobial Agents and Chemotherapy* **29**, 1079–87.

14. Walker, S. G. & Beveridge, T. J. (1988). Amikacin disrupts the cell envelope of *Pseudomonas aeruginosa* ATCC 9027. *Canadian Journal of Microbiology* **34**, 12–8.

15. Kadurugamuwa, J. L., Lam, J. S. & Beveridge, T. J. (1993). Interaction of gentamicin with the A-band and B-band lipopolysaccharides of *Pseudomonas aeruginosa* and its possible lethal effect. *Antimicrobial Agents and Chemotherapy* **37**, 715–21.

16. Wilkinson, S. G. (1983). Composition and structure of

lipopolysaccharide from *Pseudomonas aeruginosa*. *Review of Infectious Diseases* **5**, *Suppl. 5*, S941–9.

17. Arsenault, T. L., Hughes, D. W., Maclean, D. B., Szarek, W. A., Kropinski, A. M. B. & Lam, J. S. (1991). Structure studies on the polysaccharide portion of 'A-band' lipopolysaccharide from a mutant (AK1401) of *Pseudomonas aeruginosa* strain PAO1. *Canadian Journal of Chemistry* **69**, 1273–80.

18. Lightfoot, J. & Lam, J. S. (1991). Molecular cloning of genes involved with expression of A-band lipopolysaccharide, an antigenetically conserved form, in *Pseudomonas aeruginosa*. *Journal of Bacteriology* **173**, 5624–30.

19. Rivera, M. & McGroarty, M. E. (1989). Analysis of a common-antigen lipopolysaccharide from *Pseudomonas aeruginosa*. *Journal of Bacteriology* **171**, 2244–8.

20. Knirel, Yu. A., Vinogradov, E. V., Kocharova, N. A., Paramonov, N. A., Kochetkov, N. K., Dmitriev, B. A. *et al.* (1988). The structure of O-specific polysaccharides and serological classification of *Pseudomonas aeruginosa*. *Acta Microbiologica Hungarica* **35**, 3–24.

21. Rivera, M., Bryan, L. E., Hancock, R. E. W. & McGroarty, J. E. (1988). Heterogeneity of lipopolysaccharides from *Pseudomonas aeruginosa*: analysis of lipopolysaccharide chain length. *Journal of Bacteriology* **170**, 512–21.

22. Lam, J. S., Graham, L. L., Lightfoot, J., Dasgupta, T. & Beveridge, T. J. (1992). Ultrastructural examination of the lipopolysaccharides of *Pseudomonas aeruginosa* strains and their isogenic rough mutants by freeze-substitution. *Journal of Bacteriology* **174**, 7159–67.

23. Kadurugamuwa, J. L., Clarke, A. J. & Beveridge, T. J. (1993). Surface action of gentamicin on *Pseudomonas aeruginosa*. *Journal of Bacteriology* **175**, 5798–805.

24. Li, Z., Clarke, A. J. & Beveridge, T. J. (1996). A major autolysin of *Pseudomonas aeruginosa*: subcellular distribution, potential role in cell growth and division, and secretion in surface membrane vesicles. *Journal of Bacteriology* **178**, 2479–88.

25. Kadurugamuwa, J. L. & Beveridge, T. J. (1995). Virulence factors are released from *Pseudomonas aeruginosa* in association with membrane vesicles during normal growth and exposure to gentamicin: a novel mechanism of enzyme excretion. *Journal of Bacteriology* **177**, 3998–4008.

26. Beveridge, T. J. (1995). The periplasmic space and the periplasm in Gram-positive and Gram-negative bacteria. *ASM News* **61**, 125–30.

27. Kessler, E. & Safrin, M. (1988). Synthesis, processing, and transport of *Pseudomonas aeruginosa* elastase. *Journal of Bacteriology* **170**, 5241–7.

28. Devoe, I. W. & Gilchrist, J. E. (1973). Release of endotoxin in the form of cell wall blebs during *in vitro* growth of *Neisseria meningitidis*. *Journal of Experimental Medicine* **138**, 1156–67.

29. Dorwood, D. W., Garon, C. F. & Judd, R. C. (1989). Export and intercellular transfer of DNA via membrane blebs of *Neisseria gonorrhoeae*. *Journal of Bacteriology* **171**, 2499–505.

30. Kahn, M. E., Barany, F. & Smith, H. O. (1983). Transformasomes: specialized membranous structures that protect DNA during *Haemophilus* transformation. *Proceedings of the National Academy of Sciences of the USA* **80**, 6927–31.

31. Wispelwey, B., Hansen, E. J. & Scheld, W. M. (1989). *Haemophilus influenzae* outer membrane vesicle-induced blood–brain barrier permeability during experimental meningitis. *Infection and Immunity* **57**, 2559–62.

32. Forsberg, C.W., Beveridge, T. J. & Hellstrum, A. (1981). Cellulase and xylanase release from *Bacteroides succinogenes* and its importance in the rumen environment. *Applied Environmental Microbiology* **42**, 886–96.

33. Mayrand, D. & Holt, S. C. (1988). Biology of asaccharolytic black-pigmented *Bacteroides* species. *Microbiological Reviews* **52**, 134–52.

34. Kondo, K. A., Takade, A. & Amako, K. (1993). Release of the outer membrane vesicles from *Vibrio cholera* and *Vibrio parahaemolyticus*. *Microbiology and Immunology* **37**, 149–52.

35. Chatterjee, S. N. & Das, J. (1967). Electron microscopic observations on the excretion of cell-wall material by *Vibrio cholerae*. *Journal of General Microbiology* **59**, 1–11.

36. Wai, S. N., Takade, A. & Amako, K. (1995). The release of outer membrane vesicles from strains of enterotoxigenic *Escherichia coli*. *Microbiology and Immunology* **39**, 451–6.

37. Whitmire, W. M. & Garon, C. F. (1993). Specific and non-specific responses of murine B cells to membrane blebs of *Borrelia burgdorferi*. *Infection and Immunity* **61**, 1460–7.

38. Kadurugamuwa, J. L. & Beveridge, T. J. (1996). Bacteriolytic effect of membrane vesicles from *Pseudomonas aeruginosa* on other bacteria including pathogens: conceptually new antibiotics. *Journal of Bacteriology* **178**, 2767–74.

39. Bryan, L. E., O'Hara, K. & Wong, S. (1984). Lipopolysaccharide changes in impermeablity-type aminoglycoside resistance in *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* **26**, 250–5.

40. Choquet, C. B., Patel, G. B., Beveridge, T. J. & Sprott, G. D. (1994). Stability of pressure-extruded liposomes made from archaeobacterial ether lipids. *Applied Microbiology and Biotechnology* **42**, 375–84.

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Antibiotic susceptibilities of mycoplasmas and treatment of mycoplasmal infections

David Taylor-Robinson[a] and Christiane Bébéar[b]

[a]MRC Sexually Transmitted Diseases Research Group, Department of Genitourinary Medicine, the Jefferiss Wing, Imperial College School of Medicine at St Mary's, Paddington, London W2 1NY, UK; [b] Centre Hospitalier Régional de Bordeaux, Place Amélie Raba Léon, 33076 Bordeaux, France

Mycoplasmas are the smallest free-living microorganisms, being about 300 nm in diameter. They are bounded by a triple-layered membrane and, unlike conventional bacteria, do not have a rigid cell wall. Hence, they are not susceptible to penicillins and other antibiotics that act on this structure. They are, however, susceptible to a variety of other broad-spectrum antibiotics, most of which only inhibit their multiplication and do not kill them. The tetracyclines have always been in the forefront of antibiotic usage, particularly for genital tract infections, but macrolides are also widely used for respiratory tract infections. Indeed, in comparison with the tetracyclines, erythromycin, the newer macrolides, the ketolides and the newer quinolones have equal or sometimes greater activity. The two latter antibiotic groups also have some cidal activity. The antibiotic susceptibility profiles of several mycoplasmas of human origin are presented, those of *Mycoplasma pneumoniae* and *Mycoplasma genitalium* being similar. Apart from the penicillins, mycoplasmas are innately resistant to some other antibiotics, for example the rifampicins. In addition, some may develop resistance, either by gene mutation or by acquisition of a resistance gene, to antibiotics to which they are usually sensitive. Resistance of mycoplasmas to tetracyclines is common and due to acquisition of the *tetM* gene. The antibiotic susceptibility pattern may be influenced greatly by the source of the mycoplasma; for example, one recovered from a contaminated eukaryotic cell culture that has been subjected to extensive antibiotic treatment may have an antibiotic profile quite different from the same mycoplasmal species that has been recovered directly from a human or animal source. Mycoplasmas may be difficult to eradicate from human or animal hosts or from cell cultures by antibiotic treatment because of resistance to the antibiotic, or because it lacks cidal activity, or because there is invasion of eukaryotic cells by some mycoplasmas. Eradication may be particularly difficult in immunosuppressed or immunodeficient individuals, particularly those who are hypogammaglobulinaemic. The regimes that are most likely to be effective in the treatment of respiratory or genitourinary mycoplasmal infections are presented.

Introduction

All organisms in the class Mollicutes ('soft skin') are here referred to trivially as mycoplasmas. Their characteristics and a molecular explanation for their pathogenicity have been reviewed quite recently.[1] In brief, they possess a triple-layered limiting membrane but no rigid bacterial cell wall and, therefore, tend to be pleomorphic, although some have a well-defined appearance with a terminal structure by which they attach to eukaryotic cells. The smallest viable forms are about 300 nm in diameter and, although they do not possess flagella or pili, many are motile. Growth occurs in nutrient media in the absence of living tissue cells. Organisms of the genera *Mycoplasma*, *Ureaplasma*, *Entomoplasma*, *Anaeroplasma* and most *Spiroplasma* spp. require sterol for growth, whereas species in the genera *Acholeplasma*, *Asteroleplasma*, *Mesoplasma* and a few *Spiroplasma* spp. do not. Apart from the strictly anaerobic mycoplasmas (anaeroplasmas and asteroleplasmas), most other mycoplasmas are facultatively aerobic, growth often being optimal anaerobically or in an atmosphere containing added CO_2. Multiplication of most species on solid media results in the formation of small colonies that have a characteristic 'fried egg' appearance,

Table I. Primary sites of colonization, metabolism and pathogenicity of mycoplasmas isolated from humans

Species	Primary site of colonization		Metabolism of		Pathogenicity
	oropharynx	genitourinary tract	glucose	arginine	
Mycoplasma buccale	+	−	−	+	non-pathogenic[a]
Mycoplasma faucium	+	−	−	+	non-pathogenic
Mycoplasma fermentans	+	±[b]	+	+	detected in joints in inflammatory arthritides and in lungs in HIV infection
Mycoplasma genitalium	±	+	+	−	a cause of acute and chronic non-gonococcal urethritis (NGU)
Mycoplasma hominis	±	+	−	+	a possible cause of pelvic inflammatory disease; causes infections in immunodeficiencies
Mycoplasma lipophilum	+	−	−	+	non-pathogenic
Mycoplasma orale	+	−	−	+	non-pathogenic
Mycoplasma penetrans	−	+	+	+	associated serologically with HIV infection
Mycoplasma pirum	?	?	+	+	non-pathogenic
Mycoplasma pneumoniae	+	±	+	−	a cause of atypical pneumonia and sequelae
Mycoplasma primatum	−	+	−	+	non-pathogenic
Mycoplasma salivarium	+	−	−	+	non-pathogenic, but has caused arthritis in hypogammaglobulinaemia
Mycoplasma spermatophilum	−	+	−	+	non-pathogenic
Ureaplasma urealyticum[c]	+	+	−	−	a probable cause of acute NGU; causes chronic NGU, and arthritis in hypogammaglobulinaemia; detected in joints in inflammatory arthritides
Acholeplasma *laidlawii*	+	−	+	−	non-pathogenic
Acholeplasma *oculi*	?	−	+	−	non-pathogenic

[a] 'Non-pathogenic' means that no evidence for pathogenicity is available.
[b] ± = primary site occasionally.
[c] Metabolizes urea.

the smallest colonies being produced by organisms of the genus *Ureaplasma*. The latter are unique in hydrolysing urea, other species fermenting carbohydrates and/or hydrolysing arginine. Some species are pathogenic, causing diseases mainly in the respiratory tracts and genital tracts of vertebrates, or diseases in plants and insects. A large cluster of the plant pathogenic mycoplasmas (now termed phytoplasmas), which are transmitted by insect vectors or grafting, have not been successfully cultured on artificial medium. Many species of mycoplasma occur as part of the normal vertebrate or plant/insect flora. The mycoplasmas of human origin, their characteristics and pathogenicity have been reviewed recently[2,3] and some of the important features are shown in Table I. Growth of all mycoplasmas is inhibited by broad-spectrum antibiotics, and the effect that antibiotics have on those of human origin, together with a review of current approaches to treatment is the focus of this article.

Antibiotic susceptibility tests

The antibiotic susceptibilities of mycoplasmas may be determined *in vitro* by two basic methods: the agar dilution method[4] and the broth dilution method, usually in the form of the metabolism inhibition test.[4–6]

Agar dilution method

If a standard agar dilution method is used to determine the antibiotic susceptibility of mycoplasmas, then the lowest concentration of antibiotic completely preventing colony development after incubation at 37°C is usually regarded as the MIC. Investigators often disregard a single colony or a few colonies within the inhibition zone, but it may be unwise to do so since these may represent an antibiotic-resistant strain in a mixture of sensitive and resistant ones. Indeed, an advantage of the agar dilution method over the broth dilution method is that, in using an uncloned inoculum, resistance can be detected in this way. Nevertheless, the agar dilution method is time-consuming and labour-intensive. Two modifications are rapid and easy to undertake. The first of these involves the use of filter paper discs. Organism suspensions are spread on agar medium, allowed to dry, and filter paper discs containing serial two-fold decreasing concentrations of antibiotic are added. After incubation, discs are sought around which there are zones of colony inhibition and the lowest concentration of antibiotic causing a zone is the MIC. The second method is the Etest.[7] As before, organism suspensions on agar medium are allowed to dry and then strips containing antibiotics in a concentration gradient ranging from, for example, 0.016 mg/L to 256 mg/L are applied. After incubation, MICs are defined as the antibiotic concentration on the strip at the point of intersection with the zone of colony inhibition.

Broth dilution method

Incubation of decreasing concentrations of an antibiotic with a suspension of organisms in broth medium, followed by application of aliquots of the mixtures to agar medium and further incubation to determine whether there is inhibition of colony development, is a feasible approach to antibiotic susceptibility testing. However, for mycoplasmas a modification of this broth dilution method in the form of the metabolism inhibition test is usually used. This is much simpler and is, in fact, a simple modification of the metabolism inhibition method used for measuring antibody,[8] with the antibody replaced by antibiotic. Decreasing concentrations of antibiotics are mixed with a standard concentration of organisms (usually 10^4/mL) in broth medium and the mixtures incubated. Multiplication of the organisms results in metabolism of glucose, arginine or urea with the consequent change in pH of the medium made visible as a colour change by incorporation of a pH indicator (usually phenol red); the antibiotic (or antibody) inhibits the colour change.[8] Several commercially available kits are based on this principle. The MIC is the highest dilution of antibiotic that inhibits the colour change at the time when the change in the control without antibiotic has just developed;[4–6] some investigators regard the end-point as the dilution at which there is $\geqslant$50% reduction (not absence) of the colour seen in the control. Continued incubation results in an increasing MIC value so that, in effect, it is possible to record a final inhibitory concentration some time after the initial reading.[6] It is clear that results and reproducibility are strongly influenced by the time of reading and by the number of organisms in the inoculum and that some effort to standardize is desirable, otherwise varying results in a laboratory and, particularly, differences in results from one laboratory to another will continue. Nevertheless, even if attention is not paid to these aspects it is usually possible within a laboratory to distinguish a strain that is susceptible to an antibiotic from one that is not. However, resistant organisms in a mixture of resistant and sensitive ones will multiply and may obscure those that are sensitive. The penalty of not having an inoculum of cloned organisms is obvious, although cloning is not always practised. Despite the difficulties mentioned in using the metabolism inhibition method, it is preferred by many investigators, particularly when ureaplasmas are being tested, since colour changes caused by these organisms are easier to demonstrate than colony development. Nevertheless, a particular problem may be experienced in testing the susceptibility of ureaplasmas to erythromycin,[9] since the MIC value is affected greatly by pH, the antibiotic being much more active at pH 7 than at pH 6–6.5 (the pH of the medium used in the test). A corollary of this is the failure of erythromycin to eradicate ureaplasmas from the vagina[10] as a result of the vaginal secretions being so acidic (pH $<$ 4.5). It is unproven but interesting to speculate that eradication of vaginal ureaplasmas with erythromycin might be

achieved in women who have bacterial vaginosis, when the vaginal pH can rise to ⩾7.0.

Tests of mycoplasmacidal activity

Apart, perhaps, from the quinolones, antibiotics active against mycoplasmas tend not to be cidal, at least in concentrations that can be achieved *in vivo*. Lack of cidal activity is seen, as mentioned above, by a 'creeping' increase in the MIC value on continued incubation of the metabolism inhibition test. However, more detailed information may be gained by removing the mixture of organisms and antibiotic, at whatever concentration of the latter is considered to be inhibitory, and determining whether the organisms are still capable of multiplication once the antibiotic has been diluted in growth medium beyond its inhibitory concentration.[6] Alternatively, the mixture may be passed through a 0.2 μm pore-size filter to trap the organisms, the filter washed by passing clean medium through it, and then placed in growth medium to culture viable organisms.[6]

In summary, there is no agreed usage of a single test and expediency often dictates which method is used. The agar dilution method has some advantages, as outlined, and has its proponents,[4,11,12] but the broth dilution method in the form of the metabolism inhibition test is probably used more often. Furthermore, it is invaluable in assessing mycoplasmacidal activity (see below), since such activity can not be determined adequately by methods that do not allow the antibiotic to be separated from the organisms at some stage of the test.

Susceptibility profiles

It has long been recognized that mycoplasmas are normally susceptible to antibiotics that inhibit protein synthesis and are resistant to those that act on bacterial cell wall components (because of the absence of the latter). The susceptibility of *Mycoplasma pneumoniae*, *Mycoplasma genitalium*, *Mycoplasma hominis*, *Mycoplasma fermentans* and *Ureaplasma urealyticum* to a range of antibiotics is shown in Table II. The concise representation hides the fact that the susceptibilities shown are drawn from numerous studies in which there is a wide range of MICs of any particular antibiotic.[4] As a consequence, some investigators may find that, when they test a particular antibiotic, its MIC does not fall precisely within the category presented in Table II. However, overall, the representation of the antibiotic susceptibility profiles is likely to be correct, as is the order in which the antibiotics have been placed. It is noteworthy that antibiotics other than tetracyclines and erythromycin, particularly the streptogramins, such as pristinamycin[13] and RP59500,[14] some of the newer macrolides, such as clarithromycin and azithromycin, and the newer quinolones,

Table II. Susceptibilities of *M. pneumoniae*, *M. genitalium*, *M. hominis*, *M. fermentans* and *U. urealyticum* to various antibiotics[a]

Antibiotic(s)	*M. pneumoniae*	*M. genitalium*	*M. hominis*	*M. fermentans*	*U. urealyticum*
Tetracyclines	++	++	++[b]	++	++[b]
Erythromycin	++	++	–	+	++
Clarithromycin	++	++	–	–	++
Azithromycin	++	++	–	++	+
Pristinamycin	++	++	++	?	++
Streptomycin	++	++	–	–	+
Spectinomycin	++	?	+	+	+
Gentamicin	+	?	+	+	+
Chloramphenicol	+	+	+	+	+
Clindamycin	+	+	++	++	–
Lincomycin	+	+	++	++	–
Sparfloxacin	++	++	++	++	++
Ciprofloxacin	+	+	++	++	+
Difloxacin	+	?	++	?	+
Nalidixic acid	–	–	–	–	–
Cephalosporins	–	–	–	–	–
Penicillins	–	–	–	–	–
Rifampicin	–	–	–	–	–

[a] ++, susceptible (MIC < 1 mg/L); +, partially susceptible (MIC = 1–10 mg/L); –, resistant (MIC > 10 mg/L). Results are presented mostly in order of diminishing activity for *M. pneumoniae*.

[b] Organisms within this species that carry the Tet M determinant are not susceptible to tetracyclines.

such as sparfloxacin,[15] are active gainst *M. pneumoniae*.[16] In addition, the ketolides, which constitute a new and distinct class of macrolide derivatives, are highly active against *M. pneumoniae* and some of the other mycoplasmas[17] (see below); compound RU 004 seems to be the most active. The results for the small number of strains of *M. genitalium* that are currently available indicate that this mycoplasma has an antibiotic susceptibility profile similar to that of *M. pneumoniae*, being susceptible to the tetracyclines and highly susceptible to a range of macrolides and streptogramins.[17,18]

In contrast to *M. pneumoniae* and *M. genitalium*, *M. hominis*, although partially susceptible to the ketolides,[17] is not susceptible to erythromycin or some of the other macrolides, but is susceptible to clindamycin and lincomycin, whereas the reverse is true for *U. urealyticum*. Indeed, lincomycin has been incorporated in medium to inhibit the growth of bacteria and select out ureaplasmas from animal sources.[19] *M. fermentans* shows some resistance to erythromycin,[20,21] but not the complete resistance exhibited by *M. hominis*, and is at least partially sensitive to the ketolides.[17] Otherwise, *M. hominis* and *M. fermentans* have similar susceptibility patterns. The antibiotic susceptibility profiles of other mycoplasmas of human origin are not available in such detail.

Mycoplasmastatic and mycoplasmacidal effects and eradication

It is important to emphasize that most antibiotics that are used successfully in treating mycoplasmal infections (see below) have a static effect on the organisms. The greatest cidal activity is exhibited perhaps by the newer quinolones, for example sparfloxacin,[15] which inhibit the replication of DNA, and by the ketolides.[17] However, the general inability of antibiotics to kill mycoplasmas, despite the fact that they may suppress their growth, is one of the reasons why eradication from the host tissues is often slow. The intracellular location of some mycoplasmas, by affording protection against an antibiotic, may also be a reason for slow eradication. Less than 10 years ago, it was dogma that mycoplasmas did not gain entrance to cells other than phagocytes. In the intervening period, however, it has been demonstrated that *M. fermentans*,[22] *M. hominis*,[22] *M. genitalium*,[23,24] *M. pneumoniae* and *Mycoplasma penetrans*[25] do enter eukaryotic cells and, in the case of the two latter species, there has been evidence for intracellular multiplication. The same may be true for *U. urealyticum*.[26] A delay in eradication from the host ensues, even though the results of in-vivo testing indicate that an active antibiotic has been given in sufficient dosage. Another problem is that the diagnosis of a mycoplasmal infection, in particular infection by *M. pneumoniae*, is often delayed so that infection is well-established by the time antibiotic therapy is initiated, further compromising eradication of the organisms and accounting for the occurrence of relapse. This, in turn, is a plausible reason for starting antibiotic therapy for respiratory mycoplasmal disease on the basis of clinical suspicion and for recommending extended treatment rather than a short course. Of course, as discussed below, if there is innate resistance to an antibiotic or resistance develops, eradication and clinical improvement are not expected.

Antibiotic resistance

Mycoplasmas as a whole are innately resistant to certain antibiotics, such as the penicillins, cephalosporins and the rifamycins, in whatever dosage. In the case of the rifamycins, insusceptibility seems to be related to the presence of a single amino acid, at position 526, in the β subunit of RNA polymerase, as determined from the sequences of the *rpoB* gene of *Spiroplasma citri*[27] and from those of different other mycoplasmal species including *M. genitalium*.[28] It is such insusceptibility that argues against the claim for the existence of mycoplasma-like organisms in various human diseases that are reported to be responsive to rifampicin.[29–31] Some mycoplasmal species are selectively innately resistant to an antibiotic to which other species are sensitive. An example of this is *M. hominis*, all strains of which are resistant to erythromycin. Mycoplasmas also develop resistance to antibiotics to which they are usually considered sensitive. Such resistance to streptomycin is common and may develop as a one-step process.[32] Complete resistance to this and other aminoglycosides has been seen in strains of *M. fermentans* isolated from cell cultures in which such antibiotics have been used,[21] although resistance of this kind is not seen with *M. fermentans* strains that have been isolated directly from human sources. In this regard, it is interesting to note that the aminoglycoside resistance of the first strain of *M. fermentans* (strain '*incognitus*'), recovered from patients with the acquired immunodeficiency syndrome via the use of eukaryotic cell cultures,[33] was used as an argument to suggest that it was derived from the cells and not from the patients.[21] That the source of a mycoplasma isolate is a factor that may influence the results of antibiotic susceptibility tests means that results may be obtained that are not always in keeping with the data shown in Table II.

Resistance of *M. hominis* to fluoroquinolones, as for other bacterial species, is associated with a *gyrA* mutation at Ser83.[34] Resistance of *M. hominis* to tetracyclines[35,36] probably assumes more importance because of the widespread use of these drugs for genital tract infections, and in some areas the frequency of resistant strains has increased to 30% or more.[37] The reason for this, apparently, is the acquisition of a streptococcal *tetM* gene.[38] *U. urealyticum* strains may also become resistant to tetracyclines[39] for the same reason.[40] The *tetM* gene encodes a protein which binds to ribosomes and in the case of *U. urealyticum* it has been demonstrated to be associated, on the

chromosome, with *Tn*916, a conjugative transposon.[4] In London, the proportion of tetracycline-resistant ureaplasmal strains isolated from patients attending sexually transmitted disease (STD) clinics during the decade 1973–83 remained at about 10%;[39] whether the proportion has altered subsequently has not been assessed. Erythromycin-resistant ureaplasmal strains in the same area also comprised about 10%[39] but strains resistant to both antibiotics were very infrequent. It is noteworthy that strains of *M. hominis* known to be resistant to various tetracyclines because of the TetM determinant have been shown to be as susceptible to the glycylcyclines (new tetracycline derivatives) as the tetracycline-susceptible strains; tetracycline-resistant strains of *U. urealyticum* have shown variable susceptibility to the glycylcyclines,[41] but seem to be universally susceptible to the ketolides.[17]

Erythromycin-resistant strains of *M. pneumoniae* have been isolated from treated patients. In erythromycin-resistant mutants selected *in vitro*, the resistance affected several macrolide–lincosamide–streptogramin B (MLS) antibiotics, and was demonstrated to occur as the result of point mutations in the 23S rRNA gene.[42] The elimination of such resistant strains by erythromycin therapy is, of course, not expected. However, the difficulty of eradicating even erythromycin-sensitive *M. pneumoniae* strains from the respiratory tract[43] indicates that the promise of in-vitro tests does not always correlate with clinical outcome.

Role of the immune system

As for other infections, there are unquestionable difficulties in controlling mycoplasmal infections in patients with immune deficiencies[44] and of eradicating such infections from nude mice as opposed to their immunocompetent counterparts (D. Taylor-Robinson & P. M. Furr, unpublished data). In the case of the former, although clinicians treating mycoplasma-infected immunodeficient patients may not always experience a problem, failure to respond microbiologically and clinically has at times created serious problems. The persistence for years of *M. pneumoniae* in the respiratory tract[45] and of ureaplasmas in the urethra,[46] joints and other sites[47,48] of hypogammaglobulinaemic patients has occurred despite multiple courses of antibiotics, sometimes given intravenously. In some patients, the administration of high titre anti-ureaplasmal antibody prepared in goats, together with antibiotic, seems to have been responsible for clinical recovery.[44] The ability to detect *M. fermentans* by a polymerase chain reaction (PCR) assay in the blood of HIV-positive patients over many months, despite courses of various antibiotics for other intercurrent infections, is also noteworthy (J. Ainsworth & D. Taylor-Robinson, unpublished data). This, by inference, means that successful chemotherapeutic intervention in a mycoplasmal infection depends to a large extent on the ability of the host to mount an adequate immune response.[49] Support for this concept also comes from the difficulties experienced in controlling mycoplasmal infection in plants[50] and of eradicating contaminating mycoplasmas from cell cultures, both situations where a functioning immune system does not exist.

Treatment of infection

Mycoplasma pneumoniae *infection*

The value of antibiotic therapy in *M. pneumoniae*-induced disease was shown first in a controlled trial of dimethylchlortetracycline undertaken in marine recruits in the USA, the duration of fever, pulmonary infiltration, and other signs and symptoms being reduced significantly.[51] Subsequently, other trials provided evidence for the effectiveness of various tetracyclines, as well as erythromycin and other macrolides.[52] It should be noted, however, that antibiotics tend to be more effective in planned trials than they are in routine clinical practice, probably because disease has become more established in routine practice before treatment is instituted. This should not be construed as meaning that antibiotic therapy is not worthwhile, although clinical improvement is not always accompanied by early eradication of the organisms from the respiratory tract.[43] The likely reason for this, as mentioned previously, is that almost all antibiotics have only static activity against mycoplasmas. The quinolones are an exception, having cidal qualities, although the earlier ones have only moderate activity against *M. pneumoniae*.[16] Failure to kill is also an explanation for clinical relapse in some patients and a plausible reason for recommending a 2–3 week course of antibiotic treatment rather than a shorter course. It is a moot point whether early treatment might prevent some of the complications but, nevertheless, it should commence as soon as possible. If facilities for rapid laboratory diagnosis, namely a PCR assay, are not available, confirmation of a *M. pneumoniae* infection will inevitably be slow. A raised cold haemagglutinin and/or single serum antibody titre ($1:\geqslant 64$) that can be obtained quickly might provide some diagnostic assurance but, nevertheless, it would seem wise to start suitable antibiotic treatment on the basis of the clinical evidence alone. The antibiotics used most widely are the macrolides (erythromycin, roxithromycin) and the tetracylines, doxycycline in particular. Erythromycin is more active against *M. pneumoniae* than against some of the other mycoplasmas of human origin (see Table II). Fortunately, it is also active against some of the other bacteria, for example *Legionella* spp., that cause atypical pneumonia. In the case of pregnant women and children, it is certainly advisable to use a macrolide rather than a tetracycline, roxithromycin being tolerated better than erythromycin, and for the reasons given macrolides have the edge over tetracyclines in adults. Overall, there should be no difficulty with therapeutic options because *M. pneumoniae* is also inhibited by the newer macrolides, such as clarithro-

mycin and azithromycin, and to some extent by the quinolones, such as ciprofloxacin.[16]

Genitourinary infection

Some disease syndromes are caused not only by mycoplasmas but also by various other microorganisms. Since it is usually impossible to define rapidly which one is responsible, the antibiotic sensitivity of all of them must be taken into account when empirical therapy is prescribed. Thus, for example, in the case of non-gonococcal urethritis, patients should receive a tetracycline that inhibits *Chlamydia trachomatis*, *M. genitalium* and *U. urealyticum*. Doxycycline is often used, given in a dose of 100 mg twice daily for 7 days. However, as mentioned before, at least 10% of ureaplasmal strains isolated from patients attending STD clinics in London are resistant to tetracyclines[39] and patients who fail to respond should be treated with erythromycin (0.5 g daily for 7 days), to which most tetracycline-resistant ureaplasmas are sensitive. A tetracycline should also be included in the antibiotic regimen for pelvic inflammatory disease, so that *C. trachomatis* and *M. hominis* strains are covered. However, since the proportion of *M. hominis* strains that are resistant to tetracyclines has been increasing ($\geqslant$20%),[37] other antibiotics such as lincomycin, clindamycin or fluoroquinolones (often ofloxacin) may sometimes need to be used. Azithromycin, which is being used increasingly to treat non-gonococcal urethritis and other infections in which *C. trachomatis* might be involved, is also active against a wide range of mycoplasmas. If mycoplasma-induced maternal fever occurs after abortion or after vaginal delivery of a live baby and does not subside rapidly, tetracycline treatment should be started, but keeping tetracycline resistance in mind. Erythromycin would be the first choice in neonatal infection.

Immunocompromised patients

Treatment of *M. pneumoniae* and other mycoplasmal and ureaplasmal infections in patients who are immunodeficient may prove particularly challenging (see above). As a consequence of the difficulties sometimes experienced in treating hypogammaglobulinaemic patients, particularly those with arthritis, the following recommendations have been proposed:[44] (i) the likelihood of mycoplasmal involvement should always be considered when arthritis occurs in such a patient; (ii) a synovial mycoplasmal isolate should be tested immediately against a wide range of antibiotics *in vitro*; (iii) the most inhibitory antibiotic should be given as soon as possible by the most appropriate route (intravenously, if possible); (iv) such therapy should be prolonged and terminated only if there is no reasonably rapid clinical and/or microbiological response, and (v) administration of specific antiserum should be considered, perhaps together with another antibiotic, in those cases that do not respond.

References

1. Maniloff, J., McElhaney, R. N., Finch, L. R. & Baseman, J. B. (Eds) (1992). *Mycoplasmas: Molecular Biology and Pathogenesis*. American Society for Microbiology, Washington, DC.

2. Krause, D. C. & Taylor-Robinson, D. (1992). Mycoplasmas which infect humans. In *Mycoplasmas: Molecular Biology and Pathogenesis* (Maniloff, J., McElhaney, R. N., Finch, L. R. & Baseman, J. B., Eds), pp. 417–44. American Society for Microbiology, Washington, DC.

3. Taylor-Robinson, D. (1995). Mycoplasma and Ureaplasma. In *Manual of Clinical Microbiology* (Murray, P. R., Barron, E. J., Pfaller, M. A., Tenover, F. C. & Yolken, R. H., Eds), pp. 652–62. American Society for Microbiology, Washington, DC.

4. Roberts, M. C. (1992). Antibiotic resistance. In *Mycoplasmas: Molecular Biology and Pathogenesis* (Maniloff, J., McElhaney, R. N., Finch, L. R. & Baseman, J. B., Eds), pp. 513–23. American Society for Microbiology, Washington, DC.

5. Taylor-Robinson, D. (1967). Mycoplasmas of various hosts and their antibiotic sensitivities. *Postgraduate Medical Journal* **43**, *Suppl.*, 100–4.

6. Taylor-Robinson, D. & Furr, P. M. (1982). The static effect of rosaramicin on *Ureaplasma urealyticum* and the development of antibiotic resistance. *Journal of Antimicrobial Chemotherapy* **10**, 185–91.

7. Waites, K. B., Crabb, D. M., Duffy, L. B. & Cassell, G. H. (1996). Etest: a novel method for screening *Mycoplasma hominis* for tetracycline resistance. IOM Letters. Vol. 4. In *Program and Abstracts of the 11th International Congress of the International Organization for Mycoplasmology, Orlando, 1996*. pp. 408–9.

8. Taylor-Robinson, D. (1983). Metabolism inhibition tests. In *Methods in Mycoplasmology*, Vol. I (Razin, S. & Tully, J. G., Eds), pp. 411–7. Academic Press, London.

9. Kenny, G. E. & Cartwright, F. D. (1993). Effect of pH, inoculum size, and incubation time on the susceptibility of *Ureaplasma urealyticum* to erythromycin *in vitro*. *Clinical Infectious Diseases* **17**, *Suppl. 1*, S215–8.

10. Eschenbach, D. A., Nugent, R. P., Rao, A. V., Cotch, M. F., Gibbs, R. S., Lipscomb, K. A. *et al*. (1991). A randomized placebo-controlled trial of erythromycin for the treatment of *Ureaplasma urealyticum* to prevent premature delivery. *American Journal of Obstetrics and Gynecology* **164**, 734–42.

11. Kenny, G. E., Cartwright, F. D. & Roberts, M. C. (1986). Agar dilution method for determination of antibiotic susceptibility of *Ureaplasma urealyticum*. *Pediatric Infectious Disease* **5** (6), *Suppl.* S332–4.

12. Kenny, G. E., Hooton, T. M., Roberts, M. C., Cartwright, F. D. & Hoyt, J. (1989). Susceptibilities of genital mycoplasmas to the newer quinolones as determined by the agar dilution method. *Antimicrobial Agents and Chemotherapy* **33**, 103–7.

13. Bebear, C., Renaudin, H., Maugein, J., de Barbeyrac, B. & Clerc, M.-T. (1990). Pristinamycin and human mycoplasmas: *in vitro* activity compared with macrolides and lincosamides, *in vivo* efficacy in *Mycoplasma pneumoniae* experimental infection. In *Recent Advances in Mycoplasmology* (Stanek, G., Cassell, G. H., Tully, J. G. & Whitcomb, R. F., Eds) *Zentralblatt für Bakteriologie*, *Suppl. 20*, pp. 77–82. Gustav Fischer Verlag, Stuttgart.

14. Renaudin, H., Boussens, B. & Bebear, C. (1991). *In vitro* activity of RP59500 against mycoplasma. In *Program and*

Abstracts of the Thirty-First Interscience Conference on Antimicrobial Agents and Chemotherapy, Venice, 1991. Abstract 897, p. 248. American Society for Microbiology, Washington, DC.

15. Renaudin, H. & Bébéar, C. (1995). *In vitro* susceptibility of mycoplasmas to a new quinolone, BAY Y 3118. *Drugs* **49**, *Suppl. 2*, 243–5.

16. Bébéar, C., Dupon, M., Renaudin, H. & de Barbeyrac, B. (1993). Potential improvements in therapeutic options for mycoplasmal respiratory infections. *Clinical Infectious Diseases* **17**, *Suppl. 1*, 202–7.

17. Renaudin, H., Aydin, M. D. & Bébéar, C. (1995). Ketolides and mycoplasmas: *in vitro* evaluation of RU 004. In *Program and Abstracts of the Thirty-Fifth Interscience Conference on Antimicrobial Agents and Chemotherapy*, *San Francisco CA*, 1995. Abstract F 168, p. 142. American Society for Microbiology, Washington, DC.

18. Renaudin, H., Tully, J. G. & Bebear, C. (1992). In vitro susceptibilities of *Mycoplasma genitalium* to antibiotics. *Antimicrobial Agents and Chemotherapy* **36**, 870–2.

19. Koshimizu, K., Ito, M., Magaribuchi, T. & Kotani, H. (1983). Selective medium for isolation of ureaplasmas from animals. *Japanese Journal of Veterinary Science* **45**, 263–8.

20. Hayes, M. M., Wear, D. J. & Lo, S.-C. (1991). In vitro antimicrobial susceptibility testing for the newly identified AIDS-associated *Mycoplasma: Mycoplasma fermentans* (incognitus strain). *Archives of Pathology and Laboratory Medicine* **115**, 464–6.

21. Hannan, P. C. T. (1995). Antibiotic susceptibility of *Mycoplasma fermentans* strains from various sources and the development of resistance to aminoglycosides *in vitro. Journal of Medical Microbiology* **42**, 421–8.

22. Taylor-Robinson, D., Davies, H. A., Sarathchandra, P. & Furr, P. M. (1991). Intracellular location of mycoplasmas in cultured cells demonstrated by immunocytochemistry and electron microscopy. *International Journal of Experimental Pathology* **72**, 705–714.

23. Mernaugh, C. R., Dallo, S. F., Holt, S. C. & Baseman, J. B. (1993). Properties of adhering and nonadhering populations of *Mycoplasma genitalium. Journal of Clinical Infectious Diseases* **17**, *Suppl. 1*, S69–78.

24. Jensen, J. S., Blom, J. & Lind, K. (1994). Intracellular location of *Mycoplasma genitalium* in cultured Vero cells as demonstrated by electron microscopy. *International Journal of Experimental Pathology* **75**, 91–8.

25. Baseman, J. B., Lange, M., Criscimagna, N. L., Giron, J. A. & Thomas, C. A. (1995). Interplay between mycoplasmas and host target cells. *Microbial Pathogenesis* **19**, 105–16.

26. Mazzali, R. & Taylor-Robinson, D. (1971). The behaviour of T-mycoplasmas in tissue culture. *Journal of Medical Microbiology* **4**, 125–38.

27. Gaurivaud, P., Laigret, F. & Bové, J. M. (1996). Insusceptibility of members of the class *Mollicutes* to rifampin: studies of the *Spiroplasma citri* RNA polymerase β-subunit gene. *Antimicrobial Agents and Chemotherapy* **40**, 858–62.

28. Fraser, C. M., Gocayne, J. D., White, O., Adams, M. D., Clayton, R. A., Fleischmann, R. D. *et al.* (1995). The minimal gene complement of *Mycoplasma genitalium. Science* **270**, 397–403.

29. Editorial. (1990). Do human MLO exist? *Lancet* **335**, 1068–9.

30. Johnson, L., Wirostko, E., Wirostko, W. & Wirostko, B. (1996). Mycoplasma-like organisms in Hodgkin's disease. *Lancet* **347**, 901–2.

31. Taylor-Robinson, D. (1996). Mycoplasmas and oncogenesis. *Lancet* **347**, 1555.

32. Blyth, W. A. (1958). An investigation into the aetiology of non-gonococcal urethritis with special reference to the role of pleuropneumonia-like organisms. PhD Thesis, University of London.

33. Lo, S.-C., Shih, J. W.-K., Newton, P. B., Wong, D. M., Hayes, M. M., Benish, J. R. *et al.* (1989). Virus-like infectious agent (VLIA) is a novel pathogenic mycoplasma: *Mycoplasma incognitus. American Journal of Tropical Medicine and Hygiene* **41**, 586–600.

34. Bébéar, C. M., Bové, J. M., Bébéar, C. & Renaudin, J. (1997). Characterization of *Mycoplasma hominis* mutations involved in resistance to fluoroquinolones. *Antimicrobial Agents and Chemotherapy* **41**, 269–73.

35. Taylor-Robinson, D., Thomas, B. J., Furr, P. M. & Keat, A. C. (1983). The association of *Mycoplasma hominis* with arthritis. *Sexually Transmitted Diseases* **10**, *Suppl. 4*, 341–4.

36. Bygdeman, S. M. & Mårdh, P.-A. (1983). Antimicrobial susceptibility and susceptibility testing of *Mycoplasma hominis*: a review. *Sexually Transmitted Diseases* **10**, *Suppl. 4*, 366–70.

37. Koutsky, L. A., Stamm, W. E., Brunham, R. C., Stevens, C. E., Cole, B., Hale, J. *et al.* (1983). Persistence of *Mycoplasma hominis* after therapy: importance of tetracycline resistance and of coexisting vaginal flora. *Sexually Transmitted Diseases* **10**, *Suppl. 4*, 374–81.

38. Roberts, M. C., Koutsky, L. A., Holmes, K. K., LeBlanc, D. L. & Kenny, G. E. (1985). Tetracycline-resistant *Mycoplasma hominis* strains contain streptococcal *tetM* sequences. *Antimicrobial Agents and Chemotherapy* **28**, 141–3.

39. Taylor-Robinson, D. & Furr, P. M. (1986). Clinical antibiotic resistance of *Ureaplasma urealyticum*. *Pediatric Infectious Diseases* **5** (6), *Suppl.* S335–7.

40. Roberts, M. C. & Kenny, G. E. (1986). Dissemination of the *tetM* tetracycline-resistance determinant to *Ureaplasma urealyticum. Antimicrobial Agents and Chemotherapy* **29**, 350–2.

41. Kenny, G. E. & Cartwright, F. D. (1994). Susceptibilities of *Mycoplasma hominis*, *Mycoplasma pneumoniae*, and *Ureaplasma urealyticum* to new glycylcyclines in comparison with those to older tetracyclines. *Antimicrobial Agents and Chemotherapy* **38**, 2628–32.

42. Lucier, T. S., Heitzman, K., Liu, S. K. & Hu, P. C. (1995). Transition mutations in the 23S rRNA of erythromycin-resistant isolates of *Mycoplasma pneumoniae. Antimicrobial Agents and Chemotherapy* **39**, 2770–3.

43. Smith, C. B., Friedewald, W. T. & Chanock, R. M. (1967). Shedding of *Mycoplasma pneumoniae* after tetracycline and erythromycin therapy. *New England Journal of Medicine* **276**, 1172–5.

44. Furr, P. M., Taylor-Robinson, D. & Webster, A. D. B. (1994). Mycoplasmas and ureaplasmas in patients with hypogammaglobulinaemia and their role in arthritis: microbiological observations over twenty years. *Annals of the Rheumatic Diseases* **53**, 183–7.

45. Taylor-Robinson, D., Webster, A. D. B., Furr, P. M. & Asherson, G. L. (1980). Prolonged persistence of *Mycoplasma pneumoniae* in a patient with hypogammaglobulinaemia. *Journal of Infection* **2**, 171–5.

46. Taylor-Robinson, D., Furr, P. M. & Webster, A. D. B. (1985).

Ureaplasma urealyticum causing persistent urethritis in a patient with hypogammaglobulinaemia. *Genitourinary Medicine* **61**, 404–8.

47. Webster, A. D. B., Taylor-Robinson, D., Furr, P. M. & Asherson, G. L. (1978). Mycoplasmal (ureaplasma) septic arthritis in hypogammaglobulinaemia. *British Medical Journal i*, 478–9.

48. Taylor-Robinson, D., Furr, P. M. & Webster, A. D. B. (1986). *Ureaplasma urealyticum* in the immunocompromised host. *Pedi - atric Infectious Disease* **5** (6), *Suppl.* S236–8.

49. Gelfand, E. W. (1993). Unique susceptibility of patients with antibody deficiency to mycoplasma infection. *Clinical Infectious Diseases* **17**, *Suppl. 1*, S250–3.

50. McCoy, R. E., Caudwell, A., Chang, C. J., Chen, T. A., Chiykowski, L. N., Cousin, M. T. *et al*. (1989). Plant diseases associated with mycoplasma-like organisms. In *The Mycoplasmas*, Vol. 5 (Whitcomb, R. F. & Tully, J. G., Eds), pp. 545–640. Academic Press, New York.

51. Kingston, J. R., Chanock, R. M., Mufson, M. A., Hellman, L. P., James, W. D., Fox, H. H. *et al*. (1961). Eaton agent pneumonia. *Journal of the American Medical Association* **176**, 118–23.

52. Shames, J. M., George, R. B., Holliday, W. B., Rasch, J. R. & Mogabgab, W. J. (1970) Comparison of antibiotics in the treatment of mycoplasmal pneumonia. *Archives of Internal Medicine* **125**, 680–4.

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