MODIFIED ANTIMICROBIAL DISC SUSCEPTIBILITY TESTING FOR NUTRITIONALLY-VARIANT STREPTOCOCCI

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Abstract. Streptococci that were dependent for their growth upon staphylococci were isolated from a patient with sub-acute bacterial endocarditis and subsequently identified as nutritionally-variant streptococci (NVS). Failure of the isolate to grow on agar media supplemented with pyridoxal hydrochloride or L-cysteine, the known supporting growth factors for NVS, made conventional antimicrobial disc diffusion assay impossible. We modified the assay by co-inoculating *Staphylococcus aureus* resistant to the drugs being tested as a helper to support the growth of the NVS. Streaking *S. aureus* closely to the antibiotic discs that were placed above NVS resulted in the growth of satellite colonies of NVS that orbited the *S. aureus* and that produced a pattern of interrupted zones of growth inhibition. Using an alternative method – adding staphylococcal secreting factor(s) to a 10% staphylococcal cell-free culture supernatant and adding this to an antibiotic susceptibility testing medium, – we found that the NVS formed colonies that formed clear zones of growth inhibition around the disc. When the sizes of the growth inhibition zones produced by both these methods were compared with those recommened by the NCCLS, the NVS were found to be susceptible to penicillin, vancomycin, erythromycin, chloramphenicol, cefoperazone, cefamandole and ofloxacin and resistant to co-trimoxazole, gentamicin and tetracycline. Based on these findings, vancomycin was selected for treatment and the patient was cured of endocarditis. The correlation between the *in vitro* drug susceptibility testing and the *in vivo* clinical response indicated that the modified antibiotic susceptibility test is an appropriate method for establishing antibiotic regimens.

INTRODUCTION

Nutritionally-variant streptococci (NVS), also known as *Abiotrophic* spp (Kawamura *et al*, 1995), were first isolated from streptococci which formed satellite colonies around other microorganisms (Frenkel and Hirsch, 1961) and have since been characterized as fastidious streptococci which require thiol groups or pyridoxal hydrochloride for growth (Carey *et al*, 1975; Cayeux *et al*, 1971; Schiller and Roberts, 1982). NVS are not uncommon pathogens found in blood culture (Frenkel and Hirsch, 1961; Stein and Nelson, 1987) and account for 10% of all streptococcal endocarditis (Wilson and Geraci, 1985). Penicillin, either alone or in combination with an aminoglycoside, is generally accepted as the antibiotic of choice (Bisno *et al*, 1989; Carey *et al*, 1977; Cooksey and Swensen, 1979; Henry *et al*, 1986; Saleh-Mghir *et al*, 1992).

In this study, we isolated NVS from a penicillin-allergic patient with sub-acute bacterial endocarditis; the patient could not be treated with penicillin for fear of an anaphylactic reaction. In order to provide appropriate antibiotic treatment, we determined the susceptibility of the NVS to various antibiotics. However, the nutritional requirements of isolated NVS differed markedly from those of previously described NVS: the growth of our NVS could be supported only by helping bacteria (*Staphylococcus aureus*) and not by the known supporting growth factors for NVS (*ie* L-cys-

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teine or pyridoxal hydrochloride) (Bouvet, 1995; Carey *et al*, 1975; Cayeux *et al*, 1971; Holloway and Denkert, 1982; Schiller and Roberts, 1982). Neither thioglycollate broth nor Todd Hewitt broth supported growth of our isolate in spite of prior success with these media (Cooksey and Swenson, 1979). The antimicrobial disc diffusion method, described by Bauer *et al* (1966) and Thornsbery *et al* (1988), could not be used for our strain of NVS.

We went on to develop a modified antibiotic disc diffusion assay for the staphylococcal-dependent NVS using either live staphylococci or their secreting factors to support the growth of the NVS on antibiotic testing media.

MATERIALS AND METHODS

Bacterial strains

The NVS used in this study were isolated from the hemoculture of a penicillin-allergic patient with sub-acute bacterial endocarditis; they were identified as *viridans*-NVS because of their growth pattern as α-hemolytic satellites around colonies of *S. aureus* and because of their tendency to form catalase-negative chains of Gram-positive cocci that showed no growth in response to factors V and X. What was more, the NVS were not attributed to a Lancefield group.

Antibiotic disc diffusion assay

The antibiotic disc diffusion assay was performed as described by Bauer *et al* (1966) and Thornsberry *et al* (1988) with some modification. A NVS inoculum was prepared from the cells grown in the blood culture bottle by collecting the red-cell-free upper portion; the inoculum was spread onto a brain heart infusion (BHI)-blood agar plate and two antibiotic discs per plate were added. A strain of *S. aureus* with intrinsic resistance to the drugs being tested was streaked closely to the discs in a radial (4-6 radial streaks) formation (Fig 1) to support the growth of the NVS. Alternatively, a 10% staphylococcal cell-free culture supernatant (0.45μm filtrate) containing secreting factor(s) was added directly to the BHI-blood agar. All plates were incubated overnight at 35ºC and measured for growth inhibition zones. Sizes of the inhibition zones were used to determine the sensitivity or resistance of the NVS to antibiotics according to the zone sizes recommended by the NCCLS (1995).

RESULTS

Disc diffusion test

Co-cultivation of *S. aureus* with intrinsic resistance to the antibiotics being tested allowed the NVS to grow and form satellite colonies in radial fashion around the antibiotic discs, resulting in easily visible clear zones of growth inhibition (Fig 1). The sizes of the inhibition zones were used to determine the sensitivity or resistance of the organisms to the antibiotics and followed the cut-off sizes recomended for *Streptococcus* by the NCCLS (1995). According to these criteria, the NVS were sensitive to penicillin, erythromycin, vancomycin, chloramphenicol, cefoperazone, cefamandole, and ofloxacin. The NVS showed resistance to co-trimoxazole, gentamicin and tetracycline. This same pattern of sensitivity was found when *S. aureus* cell-free culture filtrate, instead of staphylococcal radial streaks, was used to support growth of the NVS (Fig 2).

DISCUSSION

This study describes a modified method for antimicrobial disc susceptibility testing for the NVS by allowing for the provision of supporting growth factors in two different assays. Whereas both modified methods gave the same drug susceptibility pattern, their advantageous and applications are different. Using staphylococcal secreting factor(s) as a growth supporting factor has the advantage in that any *Staphylococcus* strain can be used. On the other hand, the use of live *S. aureus* must be

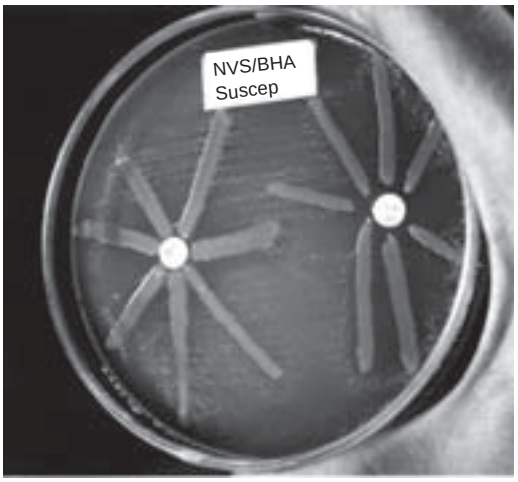

Fig 1–A modified disc diffusion test of the NVS. Multidrug-resistant *S. aureus* was streaked radially around the discs over the previously spread NVS.

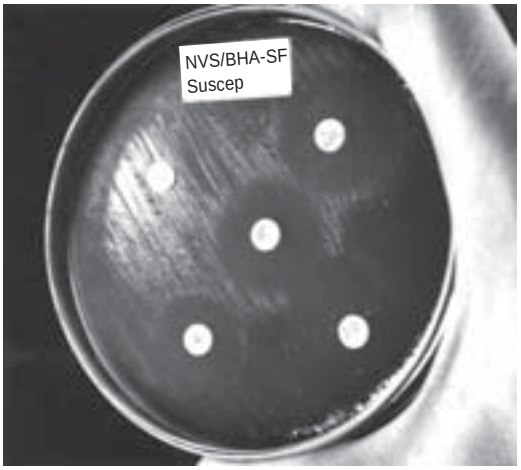

Fig 2–NVS spread onto a BHI plate supplemented with 10% staphylococcal cell-free culture filtrate; antibiotic discs added subsequently. Growth inhibition of the NVS by antibiotics is shown as clear zones around the discs.

based upon the resistance of these live organisms to the antibiotics being tested. The latter method has advantages, however: it is convenient, quick (the assay and the detection of NVS in hemoculture can be done in 24 hours -a whole day sooner than expected for the former method). Vancomycin was used successfully to treat the patient in this study, an outcome which indicates a correlation between *in vitro* susceptibility testing and *in vivo* clinical response.

The NVS used in this study were later revert to normal streptococci that did not require *Staphylococcus* as a helper: this reversion was seen after several subcultures. When a standard disc diffusion method was tested, the reverted NVS gave the same drug susceptibility pattern as that given by the original NVS (data not shown). We concluded that the modified disc diffusion technique described here could be used as a reliable method for drug susceptibility testing. Furthermore, we successfully used staphylococcal cross-streaking to determine the viability of the NVS in broth containing various concentrations of antibiotics: we discovered that minimal bactericidal concentration (MBC) of penicillin was 0.125 μg/ml. The MBC is needed for the effective treatment of patients suffering from serious infections, such as endocarditis.

To our knowledge, the modified disc diffusion test using staphylococcal radial streaking has never been described before. The method serves as a convenient and rapid means for the determination of the antimicrobial susceptibility of NVS.

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