

Use and interpretation of Schlichter's test on *Haemophilus influenzae*:

Relation of *in vitro* to *in vivo* results for cefamandole

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SUMMARY When *Haemophilus influenzae* infections are treated by an antibiotic acting on the bacterial wall, the adequacy of antimicrobial therapy can be assessed by Schlichter's test. This test may be carried out using Mueller Hinton broth (or Mueller Hinton broth with 50% pooled serum and a supplement of Ca⁺⁺ and Mg⁺⁺) supplemented with Fildes' enrichment and an inoculum adjusted to the 0.5 McFarland turbidity standard diluted 200×. However, correct reading of end points can be obtained only by phase contrast microscopic examination, which allows the establishment of good correlation between the *in vitro* and *in vivo* findings. In patients with lung infections successfully treated with cefamandole, the presence of spheroplasts in samples derived from Schlichter's tests correlates well with clinical improvement and eradication of the pathogenic organism checked by transtracheal aspiration.

The serum dilution test of Schlichter *et al.* (1949) is a method for the direct measurement of the antimicrobial activity of a patient's serum during antimicrobial therapy against the specific organism isolated from the infection. This method, which is being standardised according to Washington (1974), Washington and Barry (1974), Pien and Vosti (1974), Stratton and Reller (1977), and Reller and Stratton (1977), is related to the measurement of antibiotic sensitivity by the broth dilution method. The difficulties of this method in testing the genus *Haemophilus* are well known, as pointed out by Bottone *et al.* (1976), Gray *et al.* (1977), Marks and Weinmaster (1975), Roberts *et al.* (1974), Sykes *et al.* (1977), and Thornsberry and Kirven (1974):

- (1) The presence of slight turbidity, due to the formation of spheroplasts, makes reading difficult.
- (2) The presence of viable entities on replating after 18 hours frequently occurs when the inoculum reaches 10⁶ colony-forming units per ml.
- (3) The culture medium strongly influences the results.

In the present work Schlichter's tests were carried out on the sera of five patients treated with cefamandole for severe lung infection with *Haemophilus influenzae*. The object of the study was to choose the best criteria for reading the tests so as to give good correlation between *in vitro* and *in vivo* results.

Material and methods

CLINICAL STUDY

Five patients suffering from lung infection (severe exacerbation of chronic bronchitis) were included in a prospective study for the clinical and microbiological evaluation of cefamandole. Two grams of cefamandole nafate was diluted in 100 ml of 5% dextrose in water and perfused intravenously over 30 minutes six-hourly for five to seven days.

In addition to daily clinical examination, the progress of the temperature curve and chest radiographs were used to evaluate the efficiency of the treatment.

MICROBIOLOGICAL STUDY

Before antibiotic therapy, the pathogenic agent was isolated by transtracheal aspiration according to the technique of Kalinske *et al.* (1967), replacing physiological saline by Ringer's lactate. A second transtracheal aspiration was performed after seven days' treatment.

The determination of minimum inhibitory concentrations (MIC) was carried out by agar dilution on Mueller Hinton agar with the addition of 5% Fildes' enrichment (Difco) according to the method of Steers *et al.* (1959). Incubation was for 24 hours at 37°C in 10% CO₂. The beta-lactamase was detected

by using the chromogenic compound Glaxo 87/312, as described by O'Callaghan *et al.* (1972).

The concentration of cefamandole in blood was assayed at the peak and trough by the technique of Bennett *et al.* (1966) using cefamandole lithium as reference standard.

Determination of the inhibitory and bactericidal activities of the peak and trough serum samples (Schlichter's test) were first carried out in the routine laboratory. The patients' sera were diluted with Mueller Hinton broth (MHB) containing 5% Fildes' enrichment (Difco). Plastic plates (Microtiter, U-tubes, Cooke Engineering Co) were used with wells that contained 0.05 ml of a twofold dilution of the serum to be tested. The inoculum consisted of 0.05 ml of a 24-hour culture adjusted to a 0.5 McFarland turbidity standard and diluted 1:200. Incubation was for 18 hours at 37°C in 10% CO₂. The instructions used by the routine laboratory recommend as the limit of inhibitory activity the highest dilution with no visible growth at the bottom of the wells examined with a magnifying glass, and as the limit of bactericidal activity the absence of cultivable bacteria on spot subculture of 0.05 ml.

The tests of bactericidal activity were repeated (a) using the same medium as before (MHB + Fildes), (b) using Mueller Hinton broth enriched with Ca⁺⁺ (50 mg/l) and Mg⁺⁺ (20 mg/l) combined with pooled human serum (which had been inactivated by heating at 50°C for 30 minutes) in a 1:1 ratio, as recommended by Stratton and Reller (1977). This medium (MHB-S/HS) was also supplemented to a concentration of 5% Fildes' enrichment (Difco) to ensure good growth of *haemophilus*.

Incubation was for 18 hours in 10% CO₂. The subcultures for measurement of bactericidal activity were made by subculturing samples of 0.05 ml onto the surface of a 9 cm plate containing Mueller Hinton agar with the addition of 5% Fildes' enrichment (Difco). The colony counts were made after 48 hours' incubation at 37°C in 10% CO₂. Samples of each well were also examined after 18 hours' incubation by phase contrast microscopy to obtain evidence of

morphological change, particularly the transformation of bacillary forms to spheroplasts.

Results

As a result of this cefamandole therapy, all the patients improved clinically, as indicated by a fall in temperature, reduction of sputum volume, and improvement of the radiological picture.

In all patients, direct examination of the initial aspirates showed numerous Gram-negative coccobacilli surrounded by numerous polymorphonuclear neutrophils. Dense cultures of *H. influenzae* were obtained. The MICs of these strains were all at or within one doubling dilution of 0.2 µg/ml. No strain produced beta-lactamase. After seven days' treatment with cefamandole, transtracheal aspiration no longer showed Gram-negative coccobacilli by direct examination and on culture.

The concentrations of cefamandole in the serum samples submitted to Schlichter's test were > 100 µg/ml at the peak (mean 161 µg/ml) and > 10 µg/ml at the trough (mean 16 µg/ml). However, the Schlichter's tests carried out and interpreted by the routine laboratory were initially recorded as 'absence of inhibitory activity' because of a haziness observed at the bottom of the wells. The persistence of cultivable bacteria in the spot subculture onto solid medium justified the comment 'absence of bactericidal activity'.

Results of the Schlichter's tests carried out on two different sera containing, respectively, 100 µg/ml (sample A) and 10 µg/ml (sample B) of cefamandole, when adopting the criteria of evaluation recommended by Bottone *et al.* (1976) for the determination of MIC end points, are presented in Tables 1 and 2 (which follow the data presentation of these authors). The visual observation of microbial growth in all the wells, including those containing the highest concentrations of antibiotic, corresponded to microbial forms of great variety. Spheroplasts were present in the sample containing 100 µg/ml (sample A) in a dilution ranging from 1:1

Table 1 Results obtained by macroscopic and microscopic observations of Schlichter's test carried out on two sera diluted with Mueller Hinton broth supplemented with 5% Fildes' enrichment (MHB)

Determination	Sample	Tube dilution (MHB)										Control
		1	2	4	8	16	32	64	128	256	512	
Status of visual clarity	A	'Growth'—										
	B	'Growth'—										
Microscopic morphology	A	Spherical bodies—										Coccobacillus
	B	Spherical bodies—										Coccobacillus—
No. of colonies after subcultures to agar medium	A	3	2	4	12	9	10	50	50	50	50	Confluent—
	B	4	8	10	50	200	Confluent					

Samples A and B, respectively, contain 100 µg/ml and 10 µg/ml of cefamandole.

Table 2 Results obtained by macroscopic and microscopic observations of Schlichter's test carried out on two sera diluted with Mueller Hinton broth supplemented with Ca⁺⁺, Mg⁺⁺ Fildes' enrichment, and pooled human serum (MHB-S/HS)

Determination	Sample	Tube dilution (MHB-S/HSF)											Control
		1	2	4	8	16	32	64	128	256	512	1024	
Status of visual clarity	A	'Growth'											
	B	'Growth'											
Microscopic morphology	A	Spherical bodies							Pleomorphic forms			Coccobacillus	
	B	Spherical bodies			Pleomorphic forms		Coccobacillus						
No. of colonies after subcultures to agar medium	A	3	3	5	5	1	60	60	60	Confluent			
	B	3	4	2	100	200	Confluent						

Samples A and B, respectively, contain 100 µg/ml and 10 µg/ml of cefamandole.

to 1:512 when using MHB, and from 1:1 to 1:64 when using MHB-S/HS. For sample B, spheroplasts were noted, respectively, in samples diluted 1:32 and lower and 1:4 and lower. Paralleling the presence of spheroplasts, subcultures onto agar resulted in the development of a small number of colonies even for samples containing the highest concentration of cefamandole. In contrast, subcultures of 0.05 ml of the broths containing pleomorphic or typical coccobacillary forms gave rise to confluent growth.

Discussion

Schlichter's test has been recommended for monitoring severe infections usually caused by staphylococci, streptococci, and various Gram-negative bacteria belonging to the Enterobacteriaceae or non-fermentative groups (Klastersky *et al.*, 1974; Bryan *et al.*, 1975). However, no one has reported the usefulness of this test for assessing the adequacy of antimicrobial therapy of severe *H. influenzae* infections (pneumonia, meningitis, arthritis, etc). Indeed, many investigators have drawn attention to the marked effects of media and inocula on the activities of beta-lactam antibiotics against this bacterial species (Roberts *et al.*, 1974; Washington, 1974; Washington and Barry, 1974; Sykes *et al.*, 1977). In fact, when testing cell wall-acting antibiotics on *H. influenzae* by a broth dilution method, MIC end points are difficult to determine by macroscopic examination of the growth milieu because of the frequent observation of a haziness in tubes containing these agents. MBCs are not easier to determine because subculturing the hazy broths often results in the growth of colonies which, at an inoculum of 10⁶ organisms/ml, are more numerous than for most other bacterial species (Washington, 1974).

For the patients treated with cefamandole for severe haemophilus pulmonary infection, Schlichter's tests (the principle of which is closely related to MIC and MBC determinations), when carried out and

interpreted by conventional methods, were initially recorded as demonstrating a lack of antibacterial activity.

The discrepancy between, on the one hand, the development of a favourable clinical response, the eradication of the causative organism confirmed by transtracheal aspiration, the MIC of 0.2 µg/ml, the peak blood level of > 100 µg/ml of cefamandole, and, on the other hand, Schlichter's test, asserting no activity, justified the re-evaluation of this test. Only microscopic examination, carried out on the various dilutions of serum, allowed the definition of a level of activity compatible with the observed clinical results. This criterion is applicable both to the MHB medium and to the MHB-S/HS medium recommended by Stratton and Reller (1977) and Reller and Stratton (1977), the loss of activity of cefamandole in this latter medium being four doubling dilutions.

The role of spheroplasts of *H. influenzae* in pathology is ill defined. Although, for certain authors, this bacterial form can be the origin of relapses (Lapinski and Delle Flakas, 1967), the present study shows that antibiotic concentrations capable of generating them *in vitro* (Klein and Luginbuhl, 1977) do not prevent the development of a favourable clinical response and the eradication of the pathogenic organisms *in vivo*.

In conclusion, the criteria of Bottone *et al.* (1976) (microscopic examination and determination of the level of activity on the basis of the presence of spheroplasts), recommended for the determination of MIC in broth, must be applied to Schlichter's test as the only criteria allowing good correlation between *in vitro* and *in vivo* results. This finding could probably be applied to all antibiotics that are active on the cell wall (Thornsberry and Kirven, 1974).

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