Mem. Inst. Oswaldo Cruz, Rio de Janeiro, Vol. *88* (3): 431-434, jul./set. 1993

THE EFFECT OF SUBMINIMAL INHIBITORY CONCENTRATIONS OF PENICILLIN ON GROWTH RATE AND HAEMOLYSIN ACTIVITY OF GROUP G *STREPTOCOCCUS*

VERÔNICA V. VIEIRA; LOUIS BARRUCAND & ANGELA C. D. CASTRO/+

Departamento de Microbiologia Médica, Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, Caixa Postal 68040, 21949-900 Rio de Janeiro, RJ, Brasil

The influence of the subminimal inhibitory concentrations (1/3 and 1/4 of the MIC) of penicillin on growth rate and on haemolysin production of a strain of group G Streptococcus *was studied. It was shown that 1/3 of the MIC almost completely inhibited the bacterial growth, but it was not able to inhibit haemolysin activity in the culture supernate. The generation time of bacteria grown in 1/4 of the MIC was approximately twice longer than that of the control culture. In all cultures, the haemolysin, after being produced (or liberated), reached a peak and decreased to low levels, which could suggest that group G* Streptococcus *produces some end products of metabolism that are able to inhibit haemolysin activity.*

Key words: group G *Streptococcus* – subminimal inhibitory concentrations of antibiotics – penicillin – haemolysin

It is known that antibiotic in subminimal inhibitory concentrations (sub-MICs) cause structural and metabolic alterations in microorganisms that can have some influence in virulence factors (Atkinson & Amaral, 1982). The haemolysins are among the virulence factors of streptococci. Extracellular enzyme (streptolysin 0) is well described for group A beta-haemolytic streptococci. This factor has not been well defined for group G streptococci, however, a toxin similar to streptolysin 0 of group A *Streptococcus* is present in culture supernates of serological group G (Alouf, 1980) and is considered a factor that probably contributes to pathogenic potential of the microorganism. Since the number of bacteria is extremelly important for the clinical outcome of certain infections, the growth rate of bacteria is an essential factor for virulence (Lorian, 1986). This paper describes the effect of penicillin in sub-MICs in growth rate and haemolysin production by a strain of group G streptococci because this microorganism can cause serious diseases in sites where the penicillin can not penetrate easily, which probably leads to the achievement of subminimal inhibitory concentrations.

Group G *Streptococcus*, an agent previously considered as causes of epizootics (Tillman et al., 1982) and an indigenous part of normal flora, has recently been recognized as an opportunistic and nosocomial pathogen (Vartian et al., 1985; Efstratiou, 1989). Several cases of serious infections caused by this microorganism have been described, including meningitis (Ashkenazi et al., 1988), septicaemia (Dickie et al., 1984; Finch & Aveline, 1984) and bacterial endocarditis (Barnham, 1980; Tuazon, 1980; Lam & Bayer, 1983; Rolston et al., 1985). Penicillin was chosen because it is the drug of choice for treatment of the majority of streptococcal diseases.

MATERIALS AND METHODS

Bacterial strain – Group G *Streptococcus* from oropharynx of a healthy carrier was isolated from a swab by culture on 5% sheep blood agar (Tryptose-blood-agar base, Difco) incubated at 37 °C. The serological group was determined by a precipitation test in capillary tubes (Lancefield, 1933) by use of antisera (produced in rabbits in accord with the techniques of Centers for Disease Control, USA) to Lancefield's groups A, B, C and G.

Determination of minimal inhibitory con-

This work was supported by grants from the Financiadora de Estudos e Projetos (FINEP) and the Conselho de Ensino para Graduados da UFRJ (CEPG-UFRJ).
+Research fellows.

Received
Accepted 26 April 1993.

centrations – MIC for penicillin was determined by a standard broth dilution technique according to Jones et al. (1985). Stock solutions of the antibiotics were prepared in Casoy broth (Merk). The inoculum was approximately 10^6 cfu/ml. A tube without antibiotic was inoculated in parallel as control.

Growth curves – An overnight culture, grown in Casoy broth, was diluted to contain approximately 10^6 cfu/ml. The diluted culture (100 µl) was transfered to a fresh medium (5 ml) to which penicillin at a final concentration of 1/3 or 1/4 of the MIC had been added. A control tube without penicillin was also inoculated. Growth at 35 °C was assessed by turbidity (optical density; Spectronic 20, Bausch & Lomb spectrophotometer) at 540 nm. Every hour a 100 µl sample was then removed for determination of viable cells.

The growth rate (µ) was given by the formula:

$$\log N - \log N_0 = (\mu/2.303)\ (T - T_0)$$

were N and N_0 represented the number of cfu/ml at the end and at the beginning of the exponential growth phase, respectively. T and T_0 corresponded to the time at the end of the growth and to the time at the beginning of the growth, respectively. Doubling time (G) was calculated by the formula: $G = 1n2/\mu$

Haemolysin determination – Oxygen sensitive streptococcal haemolysin was determined in cultures supernates during bacterial growth by a method previously described (Castro et al., 1990).

Briefly, to 3 ml of culture supernate were added 0.14 ml of 0.03M 2-mercaptoethanol and 10mM sodium phosphate buffer pH 6.8 to make a final volume of 7 ml. This mixture was applied on 1% sheep red blood cells button, homogenized and incubated for 45 min at 35 °C. After the incubation period, the red cells which were not lysed were harvested by centrifugation at 500 g for 20 min and the optical density of the supernates was measured in a spectrophotometer at 540 nm. Haemolytic activity was considered as lysis percentage in relation to the water lysis (100%). Specific haemolytic activity was the ratio between haemolytic activity and the bacterial growth assessed by turbidity.

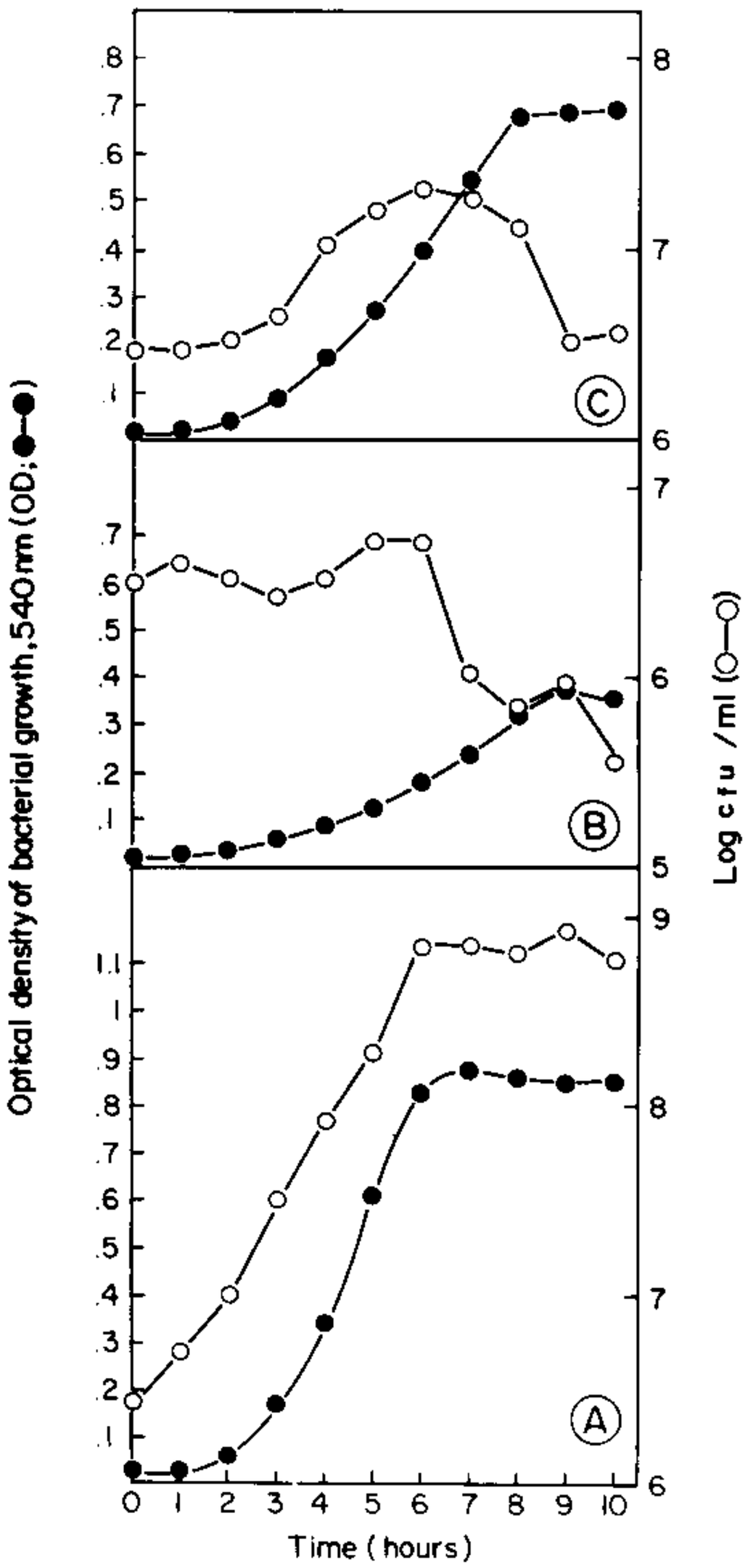

Fig. 1: growth curves of a group G *Sreptococcus* strain grown in the presence of subminimal inhibitory concentrations of penicillin. A: control curve; B: one third of the MIC; C: one quarter of the MIC.

RESULTS

Effect of penicillin on bacterial growth – The MIC of penicillin for the strain studied was 0.02 mg/l. Growth curves of group G *Streptococcus* subjected to two different antibiotic concentrations are shown in Fig. 1. These results are the mean of at least four and six experiments to the growth curves obtained with the optical density and cfu/ml measurements, respectively. Growth curves obtained with the viable counting method and with the optical density method led to comparable profiles when the culture was not exposed to antibiotic (Fig.

1A). However, when penicillin in subMICs was added to the growth medium the situation was quite different. In the culture grown in the presence of 1/3 of the MIC (Fig. 1B), only a very slight increase in the growth rate could be observed. After the 6th hr of incubation, the number of viable cells reduced until the end of incubation period. The growth assessed by turbidity presented a slight, but continuous enhacement. When the microorganism grew in the medium containing 1/4 of the MIC for penicillin (Fig. 1C) it was observed a lag phase during the first 2 hr of incubation followed by an increase of the growth rate until the 6th hr. After this period of time, a decrease of the number of cfu/ml was perceived. The optical density of the culture increased until the 8th hr. The growth rate and generation time for control culture and for culture grown at 1/4 of the MIC are shown in Table.

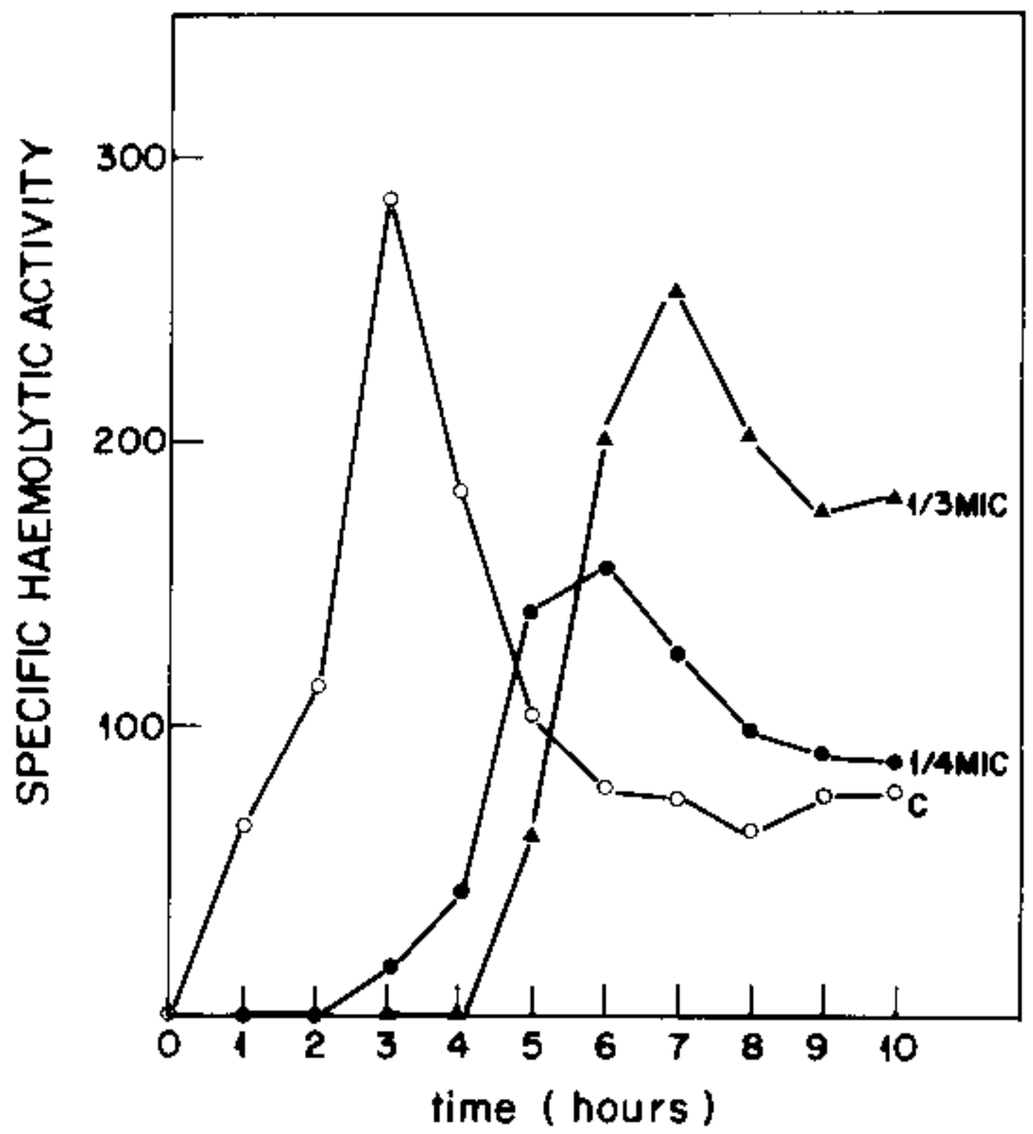

Fig. 2: haemolysin production by a group G *Streptococcus* strain grown in the presence of subminimal inhibitory concentrations of penicillin. Specific haemolytic activity is the ratio between haemolytic activity (lysis percentage in relation to the water lysis, 100%) and the bacterial growth assessed by turbidity.

TABLE

Growth rate and generation time of a group G *Streptococcus* strain grown in the presence of 1/4 of the MIC of penicillin

Antibiotic level	Growth rate[a] (μ)	Generation time[b] (min)
0	0,90	45
1/4 MIC	0,40	103

a: calculated by the formula log N-log N_0 = (μ/2.303) ($T-T_0$) as described in methods.
b: calculated by the formula G = 1n2/μ.

Effect of penicillin on haemolysin production – Haemolysin production occurred during the bacterial growth is shown in Fig. 2. It was observed that in control culture the haemolysin production did not parallel the bacterial growth. The maximal of expression was observed at the 3rd hr of growth, and when the growth was maximal the haemolysin activity in supernate of culture had reached its minimal measured value. In the culture grown in medium to which 1/4 of the MIC was added, haemolysin expression nearly paralleled the bacterial growth. At 1/3 of the MIC of penicillin a great expression of haemolysin activity was verified although an enhacement of bacterial growth rate was not observed.

DISCUSSION

The inhibition of bacterial growth by betalactam agents in subminimal inhibitory concentrations does not seem to be due bacterial death. It seems to be a result of a multiplication without a division as demonstrated by the formation of filaments in *Escherichia coli* (Opferkuch et al., 1985) and by the giant cells that appear in *S. aureus* cultures (Lorian, 1986). The present work shows that no decrease in bacterial growth was observed in all of the three cultures until the 6th hr of incubation. However, by this time the cultures grown in the presence of antimicrobial drug reached the death phase whereas the control culture started a stationary phase of growth. Despite of the decrease in the number of colony forming units, no decrease in optical density measurements was observed. This can suggest that a nonlytic mechanism of death is present in streptococcus studied. Absence of autolytic activity in penicillin-induced nonlytic death in a group A strain was described by McDowell & Lemanski (1988).

Influence of some products on the haemolysin of group A *Streptococcus* is well defined. Streptolysin 0 is only activated in the presence of a reduced atmosphere. It is reversibly inhibited by oxygen and irreversibly inhibited by cholesterol. Another haemolysin (streptolysin S) is stimulated by serum or by the presence of a variety of other substances such as serum albumin, α-lipoprotein, ribo-

nucleic acid or detergents such as Tween (Alouf, 1980). Subminimal inhibitory concentrations of antibiotics produce significant alterations in haemolysins of microorganisms. All pneumococci have the potential to produce beta-haemolysin when exposed to sub-MICs of oxacillin, methicillin and vancomycin. A ring of beta-haemolysis appeared around the zone of inhibition surrounding most beta-lactam antibiotics when *S. aureus* was tested on blood agar plates (Lorian, 1986). Lincomycin and clindamycin at sub-MICs where shown to inhibit the synthesis of streptolysin S in *Streptococcus pyogenes* (Shibl & Al-Sowaygh, 1979).

The enhacement of generation time observed in culture exposed to 1/4 of the MIC could lead one to think that there is a probable decrease of bacterial virulence *in vitro*. However, it is important to emphasize that when the production of a virulence factor of streptococci was studied it could be noted that even in the abscence of a true growth this factor (haemolysin) was produced in great amounts. It is interesting to observe that at the end of incubation at 1/3 the MIC the amount of haemolysin liberated in relation to the bacterial growth was greater than the haemolysin liberated by control culture. This result is in accordance with a previous work (Castro et al., 1990). This work presents an interesting observation in respect to the haemolysin production by group G *Streptococcus* strain. Haemolysin specific activity paralelled the bacterial growth in cultures exposed to the antibiotic, but in control culture the result was quite different. The haemolysin specific activity decreased after the middle of exponential phase. This result can suggest that Group G *Streptococcus* must form some end products of metabolism that are capable of inhibiting the haemolysin activity.

REFERENCES

ALOUF, J. E., 1980. Streptococcal toxins (streptolysin O, streptolysin S, erythrogenic toxin). *Pharmacol. Ther., 11:* 661-717.

ASHKENAZI, S.; FRENUK, R.; WANGER, A.; MURRAY, B. E. & KOHL, S., 1988. Group G streptococcal meningitis in childhood. *Pediatr. Infect. Dis. J., 7:* 522-523.

ATKINSON, B. A. & AMARAL, L., 1982. Sublethal concentrations of antibiotics, effects on bacteria and the immune system. *Crit. Rev. Microbiol., 9:* 101-138.

BARNHAM, M., 1980. Rapidly fatal group B and G streptococcal infections in adults. *J. Infection, 2:* 279-281.

CASTRO, A. C. D.; FERREIRA, B. T. & BENCHETRIT, L. C., 1990. Correlation between growth in antibiotic-medium and hemolytic activity of group C and G streptococci. *Zbl. Bakt-Int. J. Med. Microbiol., 272:* 283-289.

DICKIE, A. S.; BREMMER, D. A. & SAY, P. J., 1984. Group G streptococcal septicaemia: report of six cases. *J. Infection, 8:* 173-176.

EFSTRATIOU, A., 1989. Outbreaks of human infections caused by pyogenic streptococci of Lancefield groups C and G. *J. Med. Microbiol., 29:* 207-219.

FINCH, R. G. & AVELINE, A., 1984. Group G streptococcal septicaemia: clinical observations and laboratory studies. *J. Infection, 9:* 126-133.

JONES, R. N.; BARRY, A. L.; GAVAN, T. L. & WASHINGTON II, J. A., 1985. Susceptibility tests: microdilution and macrodilution broth procedures, p. 972-977. In E. H. Lennette; A. Ballows; W. J. Hausler Jr. & H. J. Shadomy, Manual of clinical microbiology. American Society for Microbiology, Washington, DC.

LAM, K. & BAYER, A. S., 1983. Serious infections due to group G streptococci. Report of 15 cases with *in vitro - in vivo* correlation. *Amer. J. Med., 75:* 561-570.

LANCEFIELD, R., 1933. A serological differentiation of human and other groups of hemolytic streptococci. *J. Exp. Med., 57:* 571-595.

LORIAN, V., 1986. Effect of low antibiotic concentrations on bacteria: Effects on ultrastructure, their virulence, and susceptibility to immunodefenses, p. 596-661. In V. Lorian, *Antibiotics in Laboratory Medicine,* Williams & Wilkins, Baltimore.

McDOWELL, T. D. & LEMANSKY, C. L., 1988. Absence of autolytic activity (peptidoglycan nicking) in penicillin-induced nonlytic death in a group A streptococcus. *J. Bacteriol., 170:* 1783-1788.

OPFERKUCH, W.; BUSCHER, K. H.; KARCH, H.; LEYING, H.; PAWELZIK, H.; SCHUMANN, U. & WIEMER, C., 1985. The effect of sublethal concentrations of antibiotics on the host-parasite relationship. *Zbl. Bakt. Suppl., 13:* 165-177.

ROLSTON, K. V. I.; CHANDRASEKAR, P. H. & LE FROCK, J. L., 1985. Clinical features and antimicrobial therapy of infection caused by group G streptococci. *Infection, 13:* 203-206.

SHIBL, A. M. & AL-SOWAYGH, I. A., 1979. Differential inhibition of bacterial growth and hemolysin production by lincosamide antibiotics. *J. Bacteriol., 137:* 1022-1023.

TILLMAN, P. C.; DODSON, N. D. & IDIVERY, M., 1982. Group G streptococcal epizootic in a closed cat colony. *J. Clin. Microbiol., 16:* 1057-1060.

TUAZON, C. U., 1980. Group G streptococcus. *Amer. J. Med. Sci., 279:* 121-124.

VARTIAN, C.; LERNER, P. L.; SHLAES, D. M. & GOPALAKRISHNA, K. V., 1985. Infections due to Lancefield group G streptococci. *Medicine, 64:* 75-88.