COMBINED EFFECTS OF AMPICILLIN AND METHICILLIN ON CELL WALL MORPHOLOGY OF A METHICILLIN-RESISTANT STAPHYLOCOCCUS

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Some semi-synthetic penicillins and cephalosporins are resistant to hydrolysis by beta-latamases. Hence, penicillin-resistant staphylococci exhibiting beta-lactamase activity become susceptible to these beta-lactam antibiotics. Methicillin (2,6-dimethoxyphenyl penicillin) in combination with ampicillin (D(−)-alpha-aminobenzyl penicillin) showed either a synergistic (14 strains) or an additive (3 strains) effect (never antagonistic), against 17 methicillin-resistant staphylococcal strains. One of these strains did not produce beta-lactamase. This strain No. 667 grew well in broth containing either 50 mcg/ml of methicillin or half the minimum inhibitory concentration of ampicillin which indicated that inhibition of cell wall synthesis was not apparent. However, ampicillin in combination with methicillin, using half of the above concentrations, exhibited synergism* against the strain 667. In the presence of sub-lethal doses of either ampicillin or methicillin, *Escherichia coli* became thread-like, while spheroplasts of *E. coli* were formed under similar conditions when these 2 beta-lactam antibiotics were combined. Since methicillin has no inhibitory action on strain 667, it can be concluded that the inhibition of growth by the combined penicillin might be due to a change in mechanism or site of action (or change of pathways). This is more suggestive as (a) the staphylococcal strain 667 did not produce any beta-lactamase and (b) spheroplasts of *E. coli* were formed only when the 2 penicillins were combined.

Methicillin is much less susceptible to the hydrolytic activity of beta-lactamase because of its low affinity for the enzyme and its bulky groups attached to the carbon in the side chain[6,7,9,16,20]. The high degree of resistance of methicillin to hydrolysis is also due to a very weak binding of methicillin to the beta-lactamase and as a result, the enzyme beta-lactamase is gradually inactivated[6]. However, methicillin-resistant staphylococci are also encountered[12]. There are several reports in the literature[1,3,5,8,19] indicating that semi-synthetic penicillins resistant to beta-lactamase are capable of inhibiting the enzyme. Methicillin and isoxazolyl compounds of penicillin, on the contrary, do not inhibit beta-lactamase produced by staphylococci but do inhibit beta-lactamase produced by gram-negative bacilli[11,18,21]. The activity of beta-lactamase produced by a staphylococcal strain is not destroyed by methicillin, hence, if methicillin is combined with a beta-lactamase sensitive penicillin, it is obvious that the enzyme will hydrolyse the penicillin though methicillin may not be affected. In this case, synergism may not be noticed.

* Synergism is defined as the condition in which no visible growth is observed when 50 mcg/ml concentration of methicillin is combined with one-half minimum inhibitory concentration of ampicillin (the test organism in this case being methicillin-resistant staphylococci).

A bacterial cell wall is composed of a complex structure consisting of a number of highly polymerized compounds such as mucopeptide, teichoic acid, lipoprotein, lipopolysaccharide and protein[15,17,22]. The mucopeptide component confers rigidity to the cell. As penicillin bears a structural analogy to N-acetyl muramic acid[4], interference in mucopeptide synthesis and thereby interference in the bacterial cell wall structure by penicillin is envisaged.

In our laboratories, on an average one strain out of every 1,500 strains of staphylococci isolated from human lesions was found to be resistant to methicillin. Since the morphology of these strains was not changed in the presence of 10 mcg/ml of methicillin, the following work was undertaken to determine the effect of methicillin on cell wall synthesis in high concentrations.

Materials and Methods

Cultures: Seventeen methicillin-resistant staphylococci isolated from patients were maintained in semi-solid nutrient agar. These cultures were plated on agar plates and a loopful of culture obtained from 5 colonies from each of the agar plates was inoculated into 10 ml of nutrient broth. These broth cultures were used as inocula after incubation at 37°C for 18 hours. *Escherichia coli* (local isolate No. 754) was maintained in a similar manner.

***In Vitro* Tests:** One millilitre of varying concentrations of antibiotics (methicillin* or ampicillin*) was added to tubes containing 9.0 ml of nutrient broth to give final concentrations ranging from either 10, 20, 40, 60, 80 and 100 mcg/ml or ranging from 1, 2, 4, 6, 8 and 10 mcg/ml depending upon the activity and the cultures used. The test tubes were inoculated with one drop of 18-hour broth culture adjusted by photometer readings to contain 2×10^6 cells/ml and were incubated at 37°C for 24 hours. The end point was determined by visual examination. To study the effect of combining methicillin and ampicillin, tubes containing varying amounts of each antibiotic, alone and in combination, were prepared and inoculated by the procedures as described previously. When *E. coli* No. 754 was used as the inoculum, cell morphology was examined microscopically using GRAM's stain.

Detection of Beta-Lactamase Production: Staphylococcal strain No. 667 was inoculated into a 250 ml conical flask containing 50 ml of peptone water (10 g of peptone and 5 g of sodium chloride were dissolved in one litre of 0.2 M phosphate buffer at pH 7.4 by gentle warming. The medium was autoclaved at 15 lbs. pressure for 15 minutes and the final pH was adjusted to 7.4 if required). The flask was incubated at 37°C on a rotary shaker and the culture was filtered through a 0.45 mμ millipore filter using a pre-filter after 24 hours of incubation. The filtrate (A) was tested for penicillinase activity.

In order to ascertain that staphylococcal strain No. 667 did not produce beta-lactamase as an adaptive enzyme, 50 ml of peptone water was inoculated with the strain No. 667 and after 12 hours of incubation on the shaker, 100, 200, 400, 600, 800, 1,600 and 2,400 units of penicillin G were added aseptically at regular intervals of 6 hours. After 54 hours of total incubation, the culture was filtered as stated earlier and the filtrate (B) was collected and tested for penicillinase (beta-lactamase) activity.

Beta-lactamase activity was tested by the microbiological assay method[10,13] using *Staphylococcus aureus* 209 P as the test organism. Fifteen millilitres of the melted agar was maintained at 44°C. To this agar, 0.1 ml of an 18-hour broth culture of the test organism was added. Thirty units of penicillin G was incorporated into this agar and

* Ampicillin (Ampicin) and Methicillin (Staphcillin) were kindly supplied by Bristol Laboratories of Canada Limited.

immediately poured in a petri dish after mixing. Six stainless cups were placed on the agar surface. To the first 2 cups, filtrate A and B were added. To cup No. 3, a known penicillinase solution (2 units/ml) was added to serve as a positive control. To cup No. 4, the above penicillinase solution was added after boiling for 2 hours. To cup No. 5, peptone water and to cup No. 6, an equal-volume mixture of 2 units/ml of penicillin G and penicillinase was added. Cups 4, 5, and 6 were negative controls. Six agar plates were used in each experiment and the above experiment was repeated using one unit of penicillin G per ml of the agar. The results were read after overnight incubation at 37°C.

Results

All the 17 strains of staphylococci produced a heavy turbid growth in 100 mcg/ml of methicillin. The minimum inhibitory concentrations (M.I.C.) of ampicillin against these 17 strains ranged between 2 to 20 mcg/ml. No antagonism between methicillin and ampicillin was observed. With all these 17 methicillin-resistant strains synergism was observed with 82 % of the strains while 18 % of the strains showed an additive effect. Out of 17 strains of staphylococci, only one (strain No. 667) did not produce detectable beta-lactamase. The M. I. C. of ampicillin against this staphylococcal strain No. 667 was 10 mcg/ml. Methicillin and ampicillin in combination gave synergism against this staphylococcal strain No. 667. The results are recorded in Table 1.

Instead of strain No. 667, *E. coli* strain No. 754 was used and the morphology of *E. coli* was studied after growing them in different concentrations of ampicillin and methicillin alone and in combination. The results of this experiment are recorded in Table 2. Photomicrographs of the culture subjected to these antibiotics are shown in Plates 1, 2 and 3.

A zone of growth exhibition was seen around the cup No. 3 as the penicillinase neutralized the effect of penicillin on *Staphylococcus aureus* 209 P. All the other cups did not show any zone of growth indicating that strain No. 667 did not produce any detectable beta-lactamase.

Table 1. Combined action of methicillin and ampicillin.

Antibiotics (concentrations mcg/ml)		Results
Methicillin	Ampicillin	
100	0	Growth
0	10	No growth
0	5	Growth
25	2.5	No growth

Culture used : Staphylococcal strain No. 667, resistant to methicillin. Incubated at 37°C for 22 hours.

Plate 1. Morphology of *E. coli* strain No. 754 without the influence of any antibiotics.

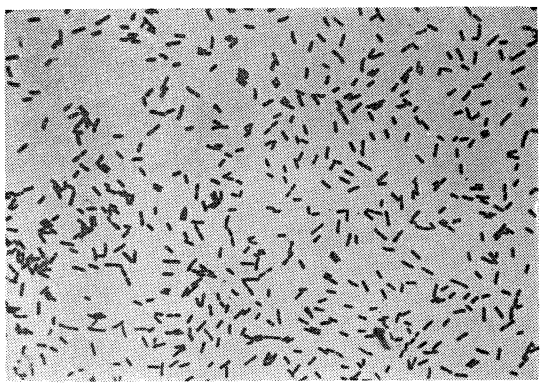


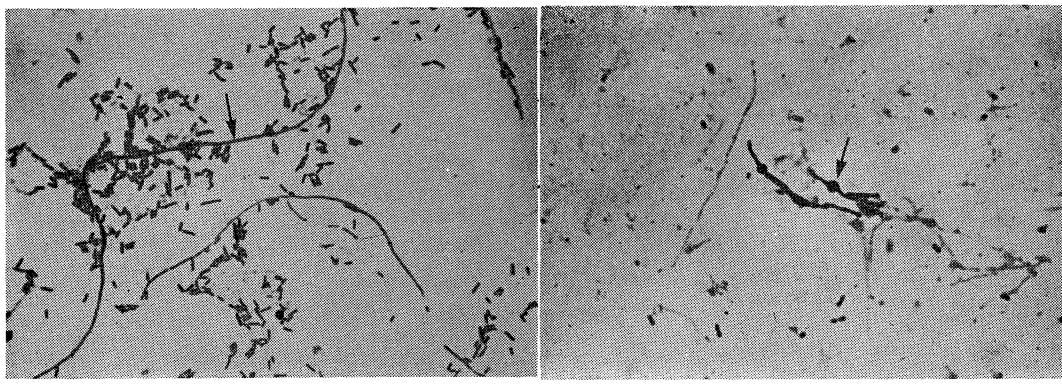
Table 2. Morphology of *E. coli* No. 754 in ampicillin and methicillin.

Methicillin (mcg/ml)	Ampicillin (mcg/ml)	Results
25	0	Growth-no elongated forms
50	0	Growth-few elongated forms
100	0	Growth-many elongated forms
0	100	Growth-elongated forms
0	200	Growth-many elongated forms
0	300	Growth-many elongated forms
25	100	Growth-many elongated forms and spheroplasts
0	0	Growth-no elongated forms

Smears from all the tubes were made after 20 hours incubation at 37°C.

Plate 2. Morphology of *E. coli* strain No. 754 grown in 100 mcg/ml of ampicillin. Similar morphology was revealed with 50 mcg/ml of methicillin.

Plale 3. Morphology of *E. coli* strain No. 754 grown in combination of ampicillin and methicillin in sub-lethal doses.



Discussion

It is not very common to isolate methicillin-resistant staphylococci in large numbers from patients. One out of every 1,000 or 2,000 staphylococci isolated in this laboratory was found to be methicillin-resistant. Out of the 17 strains isolated, only one strain No. 667, did not produce any detectable beta-lactamase. All the 17 strains were coagulase negative. It is interesting to note that methicillin and ampicillin in combination gave synergism (82 %) or an additive effect (18 %) and showed no antagonism against the 17 methicillin-resistant strains. These 17 staphylococci, though resistant to methicillin, were comparatively sensitive to ampicillin.

Staphylococcal strain No. 667 grew luxuriantly in the presence of 5 mcg/ml (1/2 M.I.C.) of ampicillin. The cell synthesis of this strain was apparently not affected. Since the strain is resistant to methicillin, 25 mcg/ml concentration of methicillin also did not affect the cell wall. When methicillin was added to ampicillin, it was in effect, adding an inactive substance to 1/4 M.I.C. of ampicillin (concentrations of either of them were far too low to interfere with the cell wall synthesis) and these two penicillins in combination did interfere with the cell wall synthesis.

To substantiate the above results, *E. coli* was chosen as a model to demonstrate interference of cell wall synthesis for one of the following reasons: (a) The cell wall of gram negative bacilli consists of three layers, *e. g.* (i) protein, (ii) lipopolysaccharide and (iii) lipoprotein above the mucopeptide layer in cell wall anatomy[14]. These three layers protect gram-negative bacilli from the action of penicillins on the mucopeptide layer hence spheroplast formation rather than protoplasts is normally expected. (b) It is difficult to demonstrate spheroplast formation alone in gram positive cocci as (i) often protoplasts predominate in the culture and (ii) protoplasts and spheroplasts are sensitive to osmotic shock. (c) Spheroplasts of *E. coli* No. 754 were found to be comparatively stable in the experimental conditions. The results on *E. coli* spheroplast formation is a comparative study under similar conditions.

E. coli became elongated in the presence of either methicillin or ampicillin. BURDASH *et al.*[2] reported that *Proteus vulgaris* exposed to cephalothin showed long filamentous (elongated) forms. Electron microscopy revealed no difference except in length between these forms and normal cells. They reported no evidence of a cell wall membrane partitioning the elongated forms. In the case of *E. coli* No. 754, the elongated forms were transformed into spheroplasts under similar conditions except the cells were exposed to the 2 antibiotics in combination.

As the staphylococcal strain No. 667 did not produce beta-lactamase, it appears that

synergism between methicillin and ampicillin did not occur by inactivation of beta-lactamase. This is further supported by the fact that staphylococcal bata-lactamase is not inactivated by methicillin[11,18,21].

The above findings suggest that mechanism of action might have been changed or altered in such a way that the cell wall formation was affected. It might be due to a change in permeability factor or in case of *E. coli*, one or more of the upper three layers above the mucopeptide layer might have been affected.

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