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Influence of Clavulanic Acid on the Activity of Amoxicillin against an Experimental *Streptococcus pneumoniae-Staphylococcus aureus* Mixed Respiratory Infection

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An experimental respiratory infection caused by *Streptococcus pneumoniae* was established in weanling rats by intrabronchial instillation. Treatment of this infection with amoxicillin rapidly eliminated the pneumococci from the lung tissue. A β-lactamase-producing strain of *Staphylococcus aureus*, when inoculated in a similar manner, did not persist adequately in the lungs long enough to permit a reasonable assessment of the therapy, but staphylococcal survival was extended in the lungs of rats infected 24 h previously with *S. pneumoniae*. Amoxicillin therapy was relatively ineffective against the pneumococci in this polymicrobial infection and had no effect on the growth of *S. aureus*. In contrast, amoxicillin-clavulanic acid eliminated the pneumococci from the lung tissue and brought about a reduction in the numbers of staphylococci. The data illustrate the utility of this model for the study of polymicrobial lung infections and demonstrate the role of amoxicillin-clavulanic acid in the treatment of polymicrobial infections involving β-lactamase-producing bacteria.

Streptococcus pneumoniae remains the most common pathogen in community-acquired bacterial pneumonia. The antibiotics most frequently prescribed by general practitioners for this infection are ampicillin and amoxicillin (23). However, coinfection or superinfection of pneumococcal pneumonia has been recognized since the 1930s (10), and recent reports have described polymicrobial pyogenic pneumonia with *S. pneumoniae* and *Staphylococcus aureus* (8, 11, 20). In the majority of the cases reported, initial penicillin therapy directed against *S. pneumoniae* failed because of the presence of β-lactamase-producing staphylococci, which now account for more than 90% of community-acquired strains of *S. aureus* (9). The failure of penicillin therapy to eradicate penicillin-susceptible pathogens in the presence of β-lactamase-producing bacteria has been reported for a number of clinical situations (4, 14, 16).

The objective of this study was to develop an experimental model of mixed respiratory infection caused by a penicillin-susceptible strain of *S. pneumoniae* and a β-lactamase-producing strain of *S. aureus*, in order to study the role of the β-lactamase inhibitor clavulanic acid in such a situation. Clavulanic acid is a potent inhibitor of a wide range of bacterial β-lactamases, including those produced by *S. aureus* (18, 19), and has been reported to protect amoxicillin from inactivation by the β-lactamase activities of mixed bacterial cultures which are refractory to this penicillin in vitro and in vivo (2, 5, 22).

Experimental lung infections are not as easy to produce in laboratory rodents as are infections of other anatomical sites and frequently depend on the use of an infection-promoting agent (17). Bacterial inocula may be delivered by aerosol challenge or by the instillation of small volumes of suspension by the intranasal, intratracheal, or intrabronchial route. Of these techniques, intratracheal and intrabronchial inoculations permit the most accurate delivery of measured volumes into the lower respiratory tract but often require considerable skill and surgery under general anesthesia (7). The model reported here involves a novel, simple, nonsurgical technique for intrabronchial instillation in the weanling rat, which we have found to be more susceptible to experimental respiratory infection than adult animals.

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MATERIALS AND METHODS

Animals. Male or female Carlworth Farms Wyeth (CFY) Sprague-Dawley rats, 23 days old and weighing 60 to 80 g, were supplied by Interfauna U.K. Ltd., Huntingdon, England.

Organisms. *S. aureus* MB9, a β-lactamase-producing strain, and *S. pneumoniae* 1629, a penicillin-susceptible strain, were used. *S. aureus* was maintained on nutrient agar slants, and a broth culture was grown overnight for each experiment in double-strength veal infusion broth (Difco Laboratories, Detroit, Mich.). *S. pneumoniae* was grown overnight in Todd-Hewitt broth (Oxoid Ltd., London, England), and the culture was stored in aliquots at −70°C. One aliquot was used for each experiment. The antibiotic susceptibilities of these organisms are given in Table 1.

Compounds. Amoxicillin sodium (Amoxil; Bencard, Brentford, England) and potassium clavulanate (Beecham Pharmaceuticals, Worthing, England) were dissolved in sterile distilled water and 0.1 M citrate buffer (pH 6.5), respectively. The combination of amoxicillin and clavulanic acid consisted of 10 parts amoxicillin to 1 part potassium clavulanate and was prepared by mixing the solutions immediately prior to dosing.

Infective inocula. *S. pneumoniae* was diluted in Todd-Hewitt broth, and *S. aureus* was diluted in veal infusion broth. When given in 50-μl volumes to the rats, these suspensions resulted in inocula containing 3.0 to 5.0 $\log_{10}$ CFU of *S. pneumoniae* and 5.0 to 7.0 $\log_{10}$ CFU of *S. aureus* per animal. During the development of the model, the smaller inocula were used, but the larger inocula, which were found to result in a more reproducible infection, were used for all therapy studies.

Route of infection. Rats were anesthetized with fentanyl fluanisone (0.06 mg/kg of body weight) (Hypnorm; Janssen Pharmaceutica, Crown Chemical Co., Lamberhurst, England) and diazepam (0.03 mg/kg) (Valium; Roche Products

TABLE 1. Susceptibility of *S. pneumoniae* 1629 and *S. aureus* MB9 to amoxicillin, amoxicillin-clavulanic acid, and clavulanic acid

Organism	MIC (μg/ml)[a]		
	Amoxicillin	Amoxicillin-clavulanic acid	Clavulanic acid
S. pneumoniae 1629	0.01	0.01/0.005	16
S. aureus MB9	256	1.0/0.5	16

[a] Serial dilution in 5% blood agar; inoculum, 5×10^5 CFU per spot. MICs were determined after 18 h at 37°C.

Ltd., Welwyn Garden City, England). The drugs were prepared in sterile distilled water, and 0.05 ml of each was injected intramuscularly into separate hind legs. Each anesthetized rat was placed on its back, and a curved metal cannula 12 cm long with a bore of 1 mm was introduced into the larynx. An intravenous 19-gauge plastic cannula (Portex Ltd., Hythe, England) attached to a 1 ml syringe containing the inoculum was passed through the metal cannula 2 cm past the tip. Then, 0.05 ml of the *S. pneumoniae* suspension was instilled into the lower left lung. Both cannulae were removed together immediately after instillation, and the rat was placed on its front to aid respiration while still unconscious. Consciousness was regained within half an hour. The procedure described above was repeated 24 h later for the inoculation of *S. aureus*.

Therapy. To permit the establishment of the staphylococcal component of the mixed infection, therapy by the subcutaneous route commenced 6 h after infection with *S. aureus* (i.e. 30 h after inoculation of *S. pneumoniae*). Groups of 30 rats were treated with 200 mg of amoxicillin or 200/20 mg of amoxicillin-clavulanic acid per kg. A second dose was given at 36 h, and further doses were given at 48, 54, 72, and 78 h. A total of 35 control rats received saline subcutaneously.

Assessment. A group of five untreated animals and groups of five treated animals were sacrificed at 24, 30, 36, 48, 58, 72, and 96 h (i.e., at least 4 h after the previous dose). Blood was taken from the posterior vena cava. The lungs were removed aseptically and homogenized in Todd-Hewitt broth in a Colworth stomacher (A. J. Seward & Co. Ltd., London, England). Serial dilutions of the blood and homogenates were plated in triplicate onto cysteine lactose electrolyte-deficient agar (Oxoid) to determine the numbers of viable staphylococci. The pneumococci were counted on blood agar containing 1 μg of neomycin per ml to prevent the growth of *S. aureus*. Animals which died during any 24-h period prior to sampling were considered to have an estimated bacterial lung count of 8.7 $\log_{10}$ CFU per rat (based on data obtained from moribund animals). A statistical analysis of the data with the Mann-Whitney test was carried out, and lung sections were taken for histological analysis with hematoxylin and eosin staining.

Distribution. In separate experiments, rats were infected with *S. pneumoniae* 1629 followed by *S. aureus* MB9 24 h later. After another 24 h, 35 rats were dosed subcutaneously with amoxicillin (200 mg/kg) and 35 were dosed with amoxicillin-clavulanic acid (200/20 mg/kg). At intervals up to 3 h after dosing, samples of plasma, pleural fluid, and lung tissue were taken for microbiological assay. At each time point, five rats per treatment were sacrificed with an intraperitoneal injection of pentobarbital. Blood was taken from the posterior vena cava, heparinized, and centrifuged for 1 min at 15,000 × *g* to obtain plasma. Sterile 6-mm blank filter paper

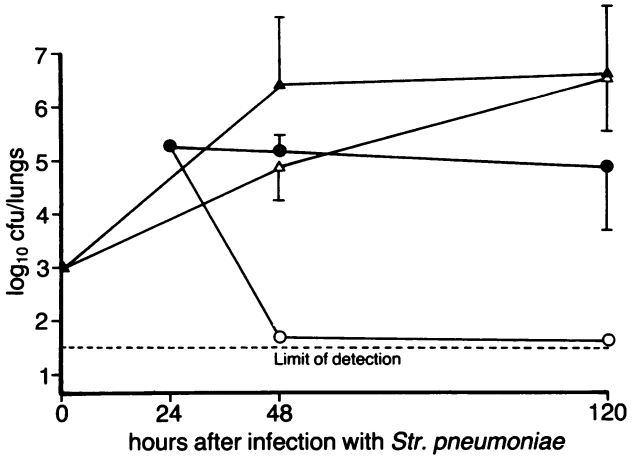

FIG. 1. Growth of *S. pneumoniae* 1629 (△, ▲) and *S. aureus* MB9 (○, ●) in the lungs of rats following intrabronchial instillation. Inocula were either mono-infections (△, ○) or mixed infections (▲, ●).

disks (Difco) were inserted into the lower thorax to absorb pleural fluid. Finally, the lungs were removed, washed in sterile saline to remove any blood contamination, and homogenized. Plasma, lung homogenate, and disks which had absorbed approximately 0.02 ml of pleural fluid were then assayed by large-plate diffusion assay. Disks contaminated with blood were discarded. *Bacillus subtilis* ATCC 6633 was the assay organism for amoxicillin, and clavulanic acid was measured by a β-lactamase inhibition assay with *Klebsiella pneumoniae* NCTC 11228 (13). Zone diameters were measured, and the concentrations were derived from standards prepared in 0.9% phosphate-buffered saline, a diluent previously shown not to differ from pooled rat plasma or lung tissue homogenate (unpublished data). All therapy and distribution studies were performed in triplicate.

RESULTS

Therapy. No bacteremia was detected during any of the experiments, and the mortality rate was between 3 and 6% for the nontreated and amoxicillin-treated animals. Macroscopic examination of the lungs of rats infected with *S. aureus* as a monoculture showed a localized infection of the lower left lung only, which resolved rapidly. The lungs of rats infected with *S. pneumoniae* as either a pure or a mixed inoculum revealed a spread of the infection from the lower left lung to the entire left lung by 24 h. Right lung involvement was noted by 48 h, with marked consolidation and enlargement, particularly in animals which received both organisms. Hematoxylin- and eosin-stained sections of lung tissue showed an almost confluent pneumonia at 48 h post-infection. The inflammatory exudate comprised mainly polymorphonuclear leukocytes and foamy macrophages. Cocci were very common as free organisms in the alveoli, where they appeared to be multiplying rapidly, and in cytoplasmic vesicles of polymorphonuclear leukocytes. Necrosis of polymorphonuclear leukocytes and macrophages was evident. Phagocytosed bacteria in general appeared intact.

Figure 1 shows the growth in rat lungs of *S. pneumoniae* 1629 and *S. aureus* MB9, as monocultures or mixed cultures following intrabronchial instillation. Following the inoculation of 3.0 $\log_{10}$ CFU per rat, *S. pneumoniae* grew rapidly, reaching 4.9 ± 0.6 $\log_{10}$ CFU per pair of lungs by 48 h and

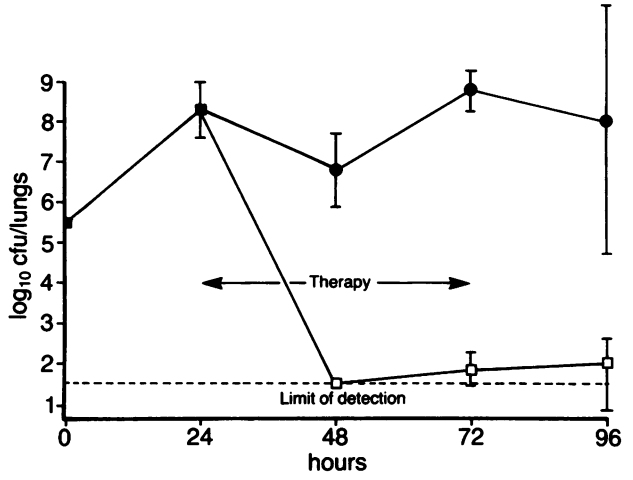

FIG. 2. Efficacy of amoxicillin (200 mg/kg) (□) in the treatment of a *S. pneumoniae* 1629 lung infection in the rat, compared with untreated controls (●).

6.3 ± 1.4 $\log_{10}$ CFU by 120 h. Similar numbers of pneumococci were recovered from the lungs of rats subsequently infected with 5.3 $\log_{10}$ CFU of *S. aureus* per rat. In monoculture, the numbers of *S. aureus* fell to 1.7 ± 0.4 $\log_{10}$ CFU per pair of lungs by 48 h and remained there for the duration of the experiment. In rats previously infected with *S. pneumoniae*, however, staphylococcal numbers persisted at 5.2 ± 1.34 $\log_{10}$ CFU per pair of lungs by 48 h, with 4.9 ± 1.2 $\log_{10}$ CFU per pair of lungs detected at the end of the experiment (120 h).

Amoxicillin, given twice daily for 2 days, eliminated the penicillin-susceptible strain of *S. pneumoniae* from the lungs of rats (Fig. 2), with a count of 1.5 ± 0.4 $\log_{10}$ CFU per pair of lungs detected within 24 h of dose 1, compared with 6.8 ± 0.9 $\log_{10}$ CFU per pair of lungs in the control animals (P = <0.01). The count remained at around the limit of detection (1.5 $\log_{10}$ CFU per pair of lungs) for the duration of the experiment (96 h).

The lung bacterial counts from rats with a mixed respiratory infection caused by 5.0 $\log_{10}$ CFU of *S. pneumoniae* per rat and 6.9 $\log_{10}$ CFU of *S. aureus* per rat are shown in Fig.

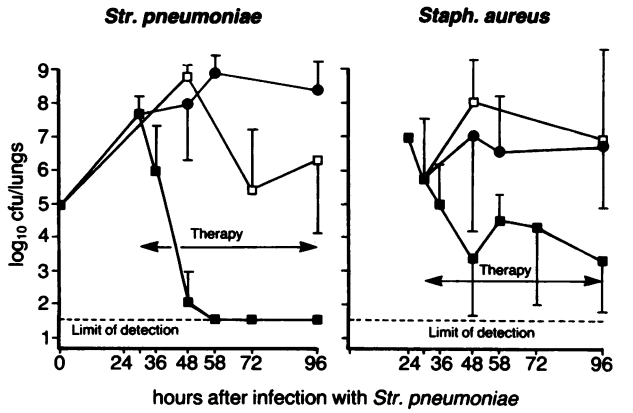

FIG. 3. Efficacy of amoxicillin (200 mg/kg) (□) and amoxicillin-clavulanic acid (200/20 mg/kg) (■) in the treatment of a mixed *S. pneumoniae* 1629-*S. aureus* MB9 lung infection in the rat, compared with untreated controls (●).

3. Under these conditions, amoxicillin given twice daily for 4 days failed to eliminate the penicillin-susceptible strain of *S. pneumoniae* from the lungs of rats infected also with the β-lactamase-producing strain of *S. aureus*. By 48 h after infection with *S. pneumoniae*, the mean pneumococcal count from the amoxicillin-treated rats was 8.6 ± 0.4 $\log_{10}$ CFU per pair of lungs. In contrast, therapy with amoxicillin-clavulanic acid reduced the pneumococcal count to 2.0 ± 1.0 $\log_{10}$ CFU per pair of lungs in the same time (P = <0.01). By 58 h, *S. pneumoniae* could no longer be detected in the lungs of amoxicillin-clavulanic acid-treated rats. It was noted at 36 h, during the administration of dose 2, that rats which had received amoxicillin-clavulanic acid 6 h previously were already much improved in general appearance over the amoxicillin-treated animals and especially over the non-treated rats.

Amoxicillin had little effect on the growth of the β-lactamase-producing strain *S. aureus* MB9, with lung counts of >7.0 $\log_{10}$ CFU per rat measured throughout the experiment. Amoxicillin-clavulanic acid reduced the staphylococcal count, but less rapidly than it reduced the pneumococcal count. After two doses, the numbers of *S. aureus* fell to 3.2 ± 1.5 $\log_{10}$ CFU per pair of lungs by 48 h, compared with 8.0 ± 1.3 $\log_{10}$ CFU per pair of lungs in the amoxicillin-treated animals (P = <0.01), and remained at 3 to 4 $\log_{10}$ CFU per pair of lungs for the duration of the experiment.

Distribution. The concentrations of amoxicillin and clavulanic acid in the plasma, pleural fluid, and lungs of infected rats are shown in Fig. 4. Amoxicillin produced levels in plasma of 253 ± 59 μg/ml at 15 min when given alone and 294 ± 59 μg/ml when given with clavulanic acid (Fig. 4). Amoxicillin concentrations of 2.0 ± 0.3 μg/ml (alone) and 1.7 ± 0.4 μg/ml (with clavulanic acid) were still detectable at 180 min after dosing, with elimination half-lives ($t_{1/2\beta}$) of 23.0 min for amoxicillin and 22.4 min for amoxicillin (with clavulanic acid). The concentration of clavulanic acid in the plasma at 15 min was 48.7 ± 28.7 μg/ml but was not detectable (<0.3 μg/ml) after 120 min ($t_{1/2\beta}$, 15.5 min).

Amoxicillin was present at high concentrations in the pleural fluid (Fig. 4). There was little difference between amoxicillin concentrations when the drug was given alone or with clavulanic acid. The concentrations at 15 min were 143.0 ± 67.2 μg/ml and 183.4 ± 79.3 μg/ml, respectively, with 1.0 ± 0.4 μg/ml and 0.8 ± 0.2 μg/ml still present at 180 min ($t_{1/2\beta}$s, 23.3 and 20.8 min, respectively). The concentration of clavulanic acid at 15 min was 65.5 ± 44.6 μg/ml but was short lived, since the drug was undetectable in the pleural fluid after 60 min ($t_{1/2\beta}$, 9 min).

Amoxicillin and clavulanic acid levels in the lung tissue homogenate were considerably lower than levels in the pleural fluid (Fig. 4). Amoxicillin produced concentrations of 43.4 ± 19.7 μg/ml (alone) and 34.1 ± 5.6 μg/ml (with clavulanic acid) at 15 min ($t_{1/2\beta}$s, 18.0 and 21.1 min, respectively). The clavulanic acid concentration at 15 min was 14.7 ± 10.4 μg/ml, and the drug was undetectable after 60 min ($t_{1/2\beta}$, 11.0 min).

Overall, there was little difference between amoxicillin concentrations following dosage alone or with clavulanic acid.

DISCUSSION

The technique for intrabronchial instillation described here is novel, simple, and relatively nontraumatic and avoids the need for surgery, making the inoculation of adequate numbers of animals for infection studies in a relatively short

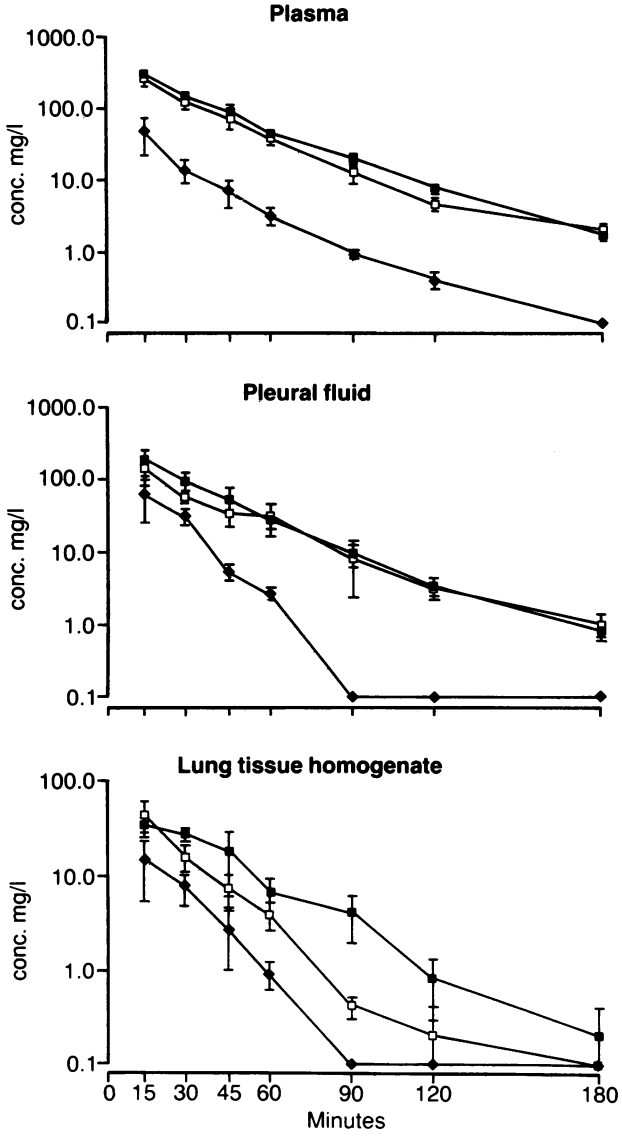

FIG. 4. Concentrations of amoxicillin (■)-clavulanic acid (◆) (200/20 mg/kg), and amoxicillin (200 mg/kg) (□) in the plasma, pleural fluid, and lung tissue homogenate of rats infected with *S. pneumoniae* 1629-*S. aureus* MB9.

period of time possible. The deposition of bacteria directly into the lower respiratory tract ensures that each animal receives a consistent inoculum of known size. In these studies, *S. pneumoniae* 1629 was capsulate, survived sufficiently to cause infection in the lungs of weanling rats, and was not potentiated by the subsequent inoculation of staphylococci. Weanling rats were found to be more susceptible to the infection and had less natural flora than older animals. When inoculated alone, *S. aureus* MB9 was unable to cause an infection suitable for the assessment of therapy because of early resolution, but staphylococcal growth was eventually enhanced in the lungs of rats which had previously been infected with pneumococci. Potentiation of the virulence of one organism by another in mixed infections is a well-studied phenomenon (3, 21). The high counts of pneumococci and staphylococci recovered from the lungs of nontreated rats resembled those from human respiratory infection, which may contain 10^5 or more bacteria per milliliter of exudate or per gram of tissue (1). The inoculation with staphylococci 24 h after infection with *S. pneumoniae* would represent clinical bacterial superinfection of pneumococcal pneumonia.

Rats infected with *S. pneumoniae* alone responded well to therapy with amoxicillin. In contrast, the amoxicillin treatment failed to eradicate pneumococci from the lungs of rats infected with both *S. pneumoniae* and *S. aureus*, demonstrating the role of staphylococcal β-lactamase in diminishing the activity of the β-lactamase-labile penicillin. Hydrolysis of amoxicillin was not detected in pleural fluid or lung tissue homogenates of infected rats, but it is possible that the extracellular staphylococcal β-lactamase was able to destroy only the amoxicillin which was in intimate contact with the bacterial cells, leaving intact the antibiotic distributed throughout the pulmonary tissues which were remote from the foci of infection. The reduction in efficacy of penicillins in mixed infections involving β-lactamase-producing bacteria in other experimental models and in clinical infections has been reported (4, 5, 12).

When amoxicillin was administered in combination with clavulanic acid, the pneumococci were eliminated rapidly from the lung tissue, a result similar to that observed with amoxicillin treatment of the *S. pneumoniae* infection. In addition, the numbers of amoxicillin-resistant *S. aureus* cells declined in response to therapy with amoxicillin-clavulanic acid. These results demonstrate the role of clavulanic acid in inhibiting the staphylococcal β-lactamase, thereby preventing consequent inactivation by β-lactamase-producing *S. aureus* in this mixed infection. These data agree with the findings of other studies on the effects of β-lactamase inhibitors in experimental mixed infections (2, 6, 15).

These results indicate the utility of this model for the study of polymicrobial respiratory infections and illustrate the importance of directing antimicrobial therapy against all components of such mixed bacterial infections. The data also illustrate the potential of amoxicillin-clavulanic acid in the treatment of bacterial respiratory infections in which β-lactamase-producing organisms may be involved.

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