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Interfering Effect of Incubation in Carbon Dioxide on the Identification of Pneumococci by Optochin Discs

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Incubation of pneumococci in a CO_2 environment decreases the zone sizes produced by optochin discs, with the result that pneumococcal-like organisms require further study.

Although serological methods provide the most accurate means for differentiating pneumococci from other alpha-hemolytic, catalase-negative, gram-positive cocci, there are several tests which are more feasible in the hospital diagnostic microbiology laboratory. The basic test for this differentiation is the so-called "bile solubility" test in which bile or the bile salt, sodium deoxycholate, or other surface-active agents such as sodium dodecyl sulfate lead to dissolution of the pneumococcal cell through activation of its autolytic enzymes. Because of the simplicity and accuracy of the test, in the United States susceptibility to 5 μg (0.02 ml of 1:4,000 solution) of ethyl hydrocuprein hydrochloride (optochin) as originally described by Bowers and Jeffries has become the usual screening test employed for the differentiation between pneumococci and alpha-hemolytic streptococci (3). Austrian and Collins demonstrated that 8% of initial clinical isolates of pneumococci require CO_2 if growth is to be detected on the surface of solid media (2). It subsequently has been recommended that optochin susceptibility tests be performed in a candle jar or CO_2 incubator (1).

The present report records data which demonstrate that the performance of optochin susceptibility tests in a CO_2 environment results in a diminution in the size of the zones of inhibition with the result that alpha-hemolytic, pneumococcal-like organisms are considered either as alpha-hemolytic streptococci or are submitted for unnecessary bile solubility testing.

MATERIALS AND METHODS

Specimens of sputum, cerebrospinal fluid and throat swabs initially were streaked on Trypticase soy agar (BBL) plates containing 5% defibrinated sheep blood and incubated at 35 C for 18 hr in a CO_2 incubator which maintained an atmosphere of 5% CO_2. After incubation, single alpha-hemolytic colonies with morphology typical of *Diplococcus pneumoniae* and consisting of catalase-negative, gram-positive cocci were restreaked onto two similar blood-agar plates, and a 6-mm disc containing 5 μg of ethyl hydrocuprein hydrochloride (Taxo P; BBL) was placed on each plate. One plate was incubated at 35 C in a room-air incubator; the other was incubated at 35 C in a 5% CO_2 incubator. Confirmation as *D. pneumoniae* was based upon bile solubility. After 18 to 24 hr of incubation, the diameters of the zones of inhibition were measured.

RESULTS

The susceptibilities of 27 consecutive pneumococcal isolates to 5-μg discs of ethyl hydrocuprein hydrochloride were compared simultaneously under parallel conditions of room air and 5% CO_2 incubation. The mean inhibitory zone diameter and standard deviation for the conditions of room-air incubation were 21.9 ± 2.46 mm, whereas, for the same isolates incubated simultaneously under 5% CO_2, the mean and standard deviation of the inhibition zone diameter was 16.3 ± 1.67 mm. This difference is critical to the interpretation of the procedure. Bowers and Jeffries defined "full sensitivity" as a zone of inhibition of more than 5 mm from the edge of the disc, i.e., a diameter of >16 mm with 6-mm discs, whereas Updyke states that "alpha-hemolytic pneumococcal-like organisms showing more than 18 mm of inhibition may be reported as *Streptococcus pneumoniae*" (1, 4). With the minimal criterion (>16 mm), which is minimal since some discs measure up to 7 mm in diameter, 16 of the 27 isolates (59%) incubated under CO_2 would not have been reported as pneumococci; all isolates incubated in room

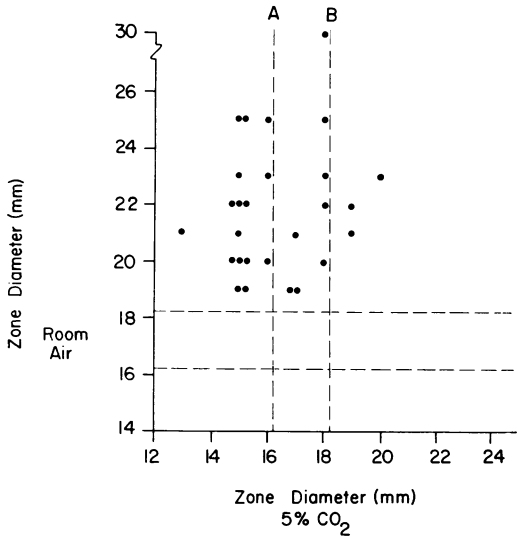

FIG. 1. *Comparison of diameters of zones of inhibition around 5-μg optochin disc (6 mm) for 27 strains of pneumococci incubated in room air and 5% CO_2. Line A is the >16-mm zone diameter of Bowers and Jeffries and line B is the >18-mm zone suggested by Updyke.*

air had zones of >18 mm and would have been reported therefore as pneumococci (Fig. 1). With the criterion of a zone of >18 mm, only 3 of the 27 isolates (11%) incubated under CO_2 would not have required further testing.

For comparative purposes, the susceptibilities of 41 strains of Viridans streptococci to 5-μg discs of ethyl hydrocuprein hydrochloride were similarly compared under parallel conditions of room air and 5% CO_2 incubation. Growth of 40 of the strains was not inhibited by the optochin disc with incubation under either room air or 5% CO_2. For the single remaining strain, the diameter of the zone of inhibition when incubated in 5% CO_2 was 7 mm, whereas it was 12 mm when incubated in room air.

DISCUSSION

Because of its simplicity in performance, the optochin test for differentiating pneumococci from alpha-hemolytic streptococci is widely employed in place of the bile solubility test which requires neutralization of the culture before addition of the sodium deoxycholate. The criteria for interpretation have been based upon zone diameters determined after incubation in room air. Although Bowers and Jeffries state, "nor does the addition of 10% carbon dioxide to the atmosphere affect the phenomenon," they do not present data on comparative zone diameters and our data clearly show a significant influence. Updyke states that "any organism showing a zone of 15 to 18 mm of inhibition should be checked by the standard solubility test." With the application of this criterion, any advantage to the optochin disc is lost by incubation under CO_2. This rather profound influence of the gaseous environment on the susceptibility of a microorganism to an antimicrobial agent when tested by the disc diffusion method has been emphasized by Sherris, Kirby, and Bauer; hence, it is not a phenomenon unique to the optochin test (5).

From our observations, we suggest that after the primary incubation of cultures under 5% CO_2 to assure isolation of the 8% of pneumococci which require CO_2, plates which are to be inoculated to test for susceptibility to the 5-μg content optochin disc should be incubated in a room-air incubator, not in a 5% CO_2 atmosphere. If the subcultured strain does not grow on the agar in room air, a solubility test in liquid medium would have to be performed. However, this would be less frequent than the misinterpretation of current recommendations. As an alternative, standard zone sizes for pneumococci incubated in 5% CO_2 could be determined. We do not have sufficient data to establish such standards, but, from our limited observations, zones observed with pneumococci were $\geq$13 mm in diameter, whereas those observed with Viridans streptococci were $\leq$7 mm in diameter.

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