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Application of Counterimmunoelectrophoresis in the Identification of *Streptococcus pneumoniae* in Clinical Isolates

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The use of counterimmunoelectrophoresis (CIE) as a diagnostic tool in infectious diseases is becoming more widespread. This study was undertaken to determine the possible use of CIE in the more rapid identification of *Streptococcus pneumoniae* in clinical isolates. Typing sera were obtained from the Statens Seruminstitut, Denmark. Sixty-seven out of 68 pneumococcal isolates that were optochin sensitive and bile soluble were typed by CIE. One isolate was nontypable even after mouse passage. An additional three isolates that were optochin resistant were considered to be pneumococci on the basis of the bile solubility test and their typability by CIE. Seventy-seven α-streptococci were tested for the presence of cross-reacting capsular antigens. Fifty-three α-streptococci showed no cross-reactions using the omniserum. Precipitin bands were obtained with the omniserum with 10 of the isolates, but these did not react with type-specific antisera. However, 14 isolates did react with the type-specific pneumococcal antisera. The sensitivity of the test was increased by sonicating whole-cell suspensions before electrophoresis was carried out. Mueller-Hinton broths were inoculated with presumptive pneumococcal colonies that formed the predominant or only colony type on primary blood agar plates. These cultures required a 4-h incubation period with an initial inoculum of 10^6 viable organisms/ml before a precipitin band could be detected. Precipitin bands were observed in 54 out of 56 (97%) broth cultures of pneumococci that had been incubated for 4 h at 37 C. These data suggest that CIE could be a useful tool in the identification of *S. pneumoniae* isolated from clinical specimens.

The development of more rapid diagnostic techniques has been felt necessary in medicine, particularly in the area of infectious diseases where rapid institution of appropriate therapy is essential. In recent years, the application of counterimmunoelectrophoresis (CIE) has become increasingly widespread in the detection of bacterial antigens, both in clinical specimens and in clinical isolates (6). Rytel recently reviewed the literature on the application of CIE in rapid diagnosis of etiological agents of infectious diseases (6). This is a sensitive procedure that can detect very small amounts of either antigens or antibodies. Coonrod and Rytel (3) described the methodology for the detection of pneumococcal capsular polysaccharide (PCP) antigens in body fluids. The purpose of this study was to determine the possible use of CIE as an aid to the more rapid identification of *Streptococcus pneumoniae* isolated from clinical specimens.

MATERIALS AND METHODS

Source and characteristics of isolates. Pneumococci and α-streptococci were obtained from clinical specimens that had been processed by the microbiology laboratory of the Department of Pathology at Milwaukee County General Hospital. Isolates were maintained on 5% sheep blood agar. Sixty-eight pneumococcal isolates were optochin sensitive and bile soluble. Inulin fermentation was variable in those isolates tested. The 13 isolates, chosen randomly, that were tested were found to be highly virulent for mice. Three other pneumococcal isolates were bile soluble but optochin resistant, did ferment inulin, and were not virulent for mice. The 77 α-hemolytic streptococci were optochin resistant and bile insoluble. Two strains fermented inulin but 15 did not. None of the 29 isolates tested was virulent for mice.

Biochemical tests. Optochin sensitivity was determined by placing an optochin (ethylhydrocupreine hydrochloride; BBL, Cockeysville, Md.) disk on an inoculated blood plate, which was incubated for 24 h. A zone of inhibition of growth around the

disk (14 mm or greater) indicated optochin sensitivity.

Bile solubility was determined by growing the pneumococcal culture in Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) until it was slightly turbid. The culture was divided into two tubes, A and B; 2 drops of 10% solution of sodium deoxycholate (Difco) was added to tube A and nothing was added to control tube B. Both tubes were incubated for 30 min at 37 C. A decrease in turbidity and increase in viscosity in tube A indicated that the strain was bile soluble.

Cultures were inoculated into purple broth base (Difco) containing 1% inulin and incubated for 5 days at 37 C. Acid production indicated inulin fermentation.

Mouse virulence test. A volume of 0.5 ml of an overnight broth culture of the organism was injected intraperitoneally into each mouse. The following morning the livers and spleens of the dead mice were aseptically removed, emulsified in saline, streaked onto blood agar plates, and incubated in a candle jar overnight at 37 C. The remaining mice were sacrificed 48 h after inoculation and processed as described above. All plates were checked for the presence of pneumococci or α-hemolytic streptococci. The plates with presumptive pneumococcal or α-streptococcal colonies were checked for bile solubility, optochin sensitivity, and the presence of PCP antigens by CIE.

Preparation of isolates for CIE. Isolates were inoculated onto 5% sheep blood agar plates and incubated at 37 C in a candle jar for 24 h. The confluent growth was scraped aseptically into 0.5 ml of sterile physiological saline. The resulting cellular suspension was used as the antigen source.

Preparation of broth cultures for CIE. Presumptive pneumococcal colonies were picked from the primary blood agar plates into Mueller-Hinton broth containing 1% human serum, if possible until the suspension was faintly turbid. Broths were incubated for 24 h. Samples for CIE were taken at 0 (no incubation), 4, and 24 h.

Sonication procedure. Overnight growth from a sheep blood agar plate was scraped aseptically into 1 ml of sterile physiological saline. The cellular suspension was sonicated at output control setting no. 6 (30 W), using the microtip of the sonicator (Heat Systems-Ultrasonics, Inc., model W185D, Plainview, N.Y.) for 5 min.

Preparation of CIE plates. A 1% agarose (Marine Colloids, Inc., Rockland, Me.) solution was prepared in 0.05 M veronal buffer (3.44 g of barbital and 7.57 g of sodium barbital [Fisher Scientific Co., St. Louis, Mo.] in 1 liter of distilled water) at pH 8.2 and dispensed in 14-ml volumes. The agarose was melted as required and poured into a plexiglass mold on a glass slide (3.5 by 4 inches [about 8.8 by 10 cm]; Eastman Kodak, Rochester, N.Y.). The solidified agarose plates were removed from the mold and stored in a moist chamber at 4 C. Only plates between 24 and 96 h old were used.

CIE technique (continuous). Two rows of parallel wells, 3 mm in diameter and 3 mm apart, were cut into the agarose plate. The wells on the cathode side were filled with the material to be tested for the presence of the antigen. The wells on the anode side were filled with the appropriate pneumococcal antisera (Statens Seruminstitut, Copenhagen, Denmark). The Danish system of numbering pneumococcal types was used. The antisera comprised the omniserum, which is a pool of all 83 types, pools A through I, which consist of 83 individual types or closely related groups, and 46 individual type or group antisera. The plate was placed in an electrophoresis chamber (Gelman Instrument Co., Ann Arbor, Mich.), and 200 ml of 0.05 M veronal buffer was added to each half of the chamber. Filter paper (Whatman no. 3) wicks connected the agar to the buffer. The distance between the wicks was not more than 5 cm. The power-pack (Gelman) was set at approximately 400 V, which resulted in a current of 10 to 12 mA across the plate for 1 h. The plate was then examined for precipitin bands by using an immunoilluminator (Hyland Division, Costa Mesa, Calif.). The plates were read again after washing in physiological saline overnight at 4 C.

Discontinuous CIE. A 1% agarose solution in 0.01 M veronal buffer, pH 8.2, was prepared. The plates were made as described above. Similar conditions for electrophoresis were used in both the continuous and discontinuous systems, with the exception that 350 rather than 400 V was delivered by the power source and electrophoresis was conducted for 45 min (7).

RESULTS

The types isolated and their frequency and source are listed in Table 1. Seventy of the 71 *S. pneumoniae* isolates were readily typed by CIE. Two of these, a type 8 and a type 23, required passage through mice before detectable PCP antigen levels were obtained. On further subculture, they reverted and became nontypable again. One isolate from a blood specimen was not typable by CIE. It was passaged through a mouse to increase capsule production; however, it remained nontypable. All three isolates caused death of the mice within 24 h. Only 16 of the 48 isolates from sputa were types 1 through 9, the numerically lower types; this is in contrast to the blood isolates, in which

TABLE 1. *Typing of S. pneumoniae isolates by CIE*

Source of isolate	No. of isolates	Types (frequency of isolation)
Sputum ..	51	3 (6), 4 (2), 8 (2), 9 (6), 11 (4), 13 (2), 15 (2), 16 (1), 17 (1), 19 (2), 20 (2), 22 (3), 23 (4), 25 (1), 29 (1), 31 (2), 33 (2), 35 (3), 36 (1), 39 (1), 41 (2), 44 (1)
Blood	12	Nontypable (1), 3 (4), 6 (1), 8 (2), 11 (1), 25 (3)
Throat ...	7	6 (3), 22 (1), 23 (2), 39 (1)
Eye	1	4 (1)

the majority of isolates were the numerically lower types.

From these data, therefore, the percentage of false negatives is approximately 1.5%. The precipitin bands formed by using the A-I pools or the individual type antisera were usually stronger than those obtained by using the omniserum. There was no instance, however, in which isolates were negative with the omniserum but positive with the pool or type antisera. Occasionally faint cross-reacting bands were observed, particularly with pools C, D, F, and I. No cross-reactions were observed with the type-specific antisera.

The results of the biochemical and virulence tests are shown in Table 2. Sixty-eight *S. pneumoniae* isolates (group i) were bile soluble and optochin sensitive. Ten of the 14 isolates tested fermented inulin. The 13 isolates tested were virulent for mice. Three other pneumococcal isolates (group ii) were bile soluble but optochin resistant. All three fermented inulin but were not virulent for mice. Seventy-seven strains of α-streptococci were bile insoluble and optochin resistant. Thirty-nine of the 41 strains tested did not ferment inulin, but two isolates did. None of the 29 isolates tested was virulent for mice.

The PCP antigens of 70 out of 71 *S. pneumoniae* isolates, including the three optochin-resistant isolates, were readily detectable after 1 h of CIE (Table 3). Two of the optochin-resistant pneumococci were type 11 and one was type 44.

The detection of cross-reacting antigens by CIE in α-streptococci is described in Table 4. Cross-reactions with the omniserum were not observed in 53 isolates (68.8%). A further 10 isolates (13.0%) showed a precipitin band, sometimes faint, with the omniserum but did not react with the type-specific antisera. However, 14 isolates (18.2%) did react with the type-specific antisera. These comprised types 17, 19, 34, 37, 38, 45, and 47.

Samples of the broth cultures prepared from the primary blood plates were tested against the omniserum by discontinuous CIE (Table 5). All these isolates were typed after the experiment was completed. An initial inoculum of 10^6 viable organisms/ml was necessary to obtain a detectable precipitin band after 4 h of incubation. Thirty-nine broth cultures gave a precipitin band without any incubation. A further 15 became positive after 4 h of incubation, making a total of 54 cultures (97.0%) positive by CIE. The remaining two cultures were still negative after 24 h of incubation. The remaining two cultures grew poorly; possibly insufficient antigen was produced to be detected. The 11 isolates of the greatest clinical significance, those iso-

TABLE 3. *Detection of capsular antigen by CIE in S. pneumoniae*

S. pneumoniae group	Optochin sensitivity	Antigen detection		
		No. positive	No. tested	% Positive
i	+	67	68	98.8
ii	−	3	3	100.0

TABLE 4. *Detection of cross-reacting antigen in α-streptococci*

Isolates	No.	Percent	Antigen type detected (frequency of isolation)
Tested	77		
Negative by CIE	53	68.8	−
Positive with omniserum	10	13.0	−
Typed	14	18.2	+ 17 (1), 19 (5), 34 (2), 37 (2), 38 (2), 45 (1), 47 (1)

TABLE 2. *Determination of some biochemical and virulence tests for S. pneumoniae and α-streptococci isolates*

Organism	No. of isolates	Bile solubility	Optochin sensitivity	Inulin fermentation	Mouse virulence
S. pneumoniae					
Group i[a]	68	+	+	+ (10)[b] − (4)	+ (13)
Group ii	3	+	−	+ (3)	− (3)
α-Streptococci	77	−	−	− (39) + (2)	− (29)

[a] *S. pneumoniae* isolates were divided into groups i and ii on the basis of differing optochin sensitivity.
[b] Number of isolates tested.

TABLE 5. *Detection of pneumococcal capsular antigen in broth cultures by CIE*

Organisms present on primary blood plate	Source of specimen	No. with detectable antigen levels			Total percent	No. with no detectable antigen levels after 24-h incubation	Total percent
		0 h	4 h	Total			
Predominantly pneumococci	Sputum	27	+12	39		2	
	Throat	1	+2	3		0	
	Spinal fluid	1	0	1		0	
	Blood	10	+1	11		0	
	Total	39	+15	54	97	2	3
Mixed population, some pneumococci	Sputum	0	0	0		5	
	Throat	0	0	0		5	
	Total	0	0	0	0	10	100

lated from blood and spinal fluid, were all positive after 4 h of incubation. The broth cultures prepared from the 10 primary plates that had only a few presumptive pneumococcal colonies present in a mixed population did not give any detectable antigen levels even after 24 h of incubation.

The sensitivity of the test can be increased by sonication of the whole cell culture. Precipitin bands were detected at a consistently higher dilution of the PCP antigen which had been sonicated than that which had not (Table 6).

TABLE 6. *Effect of sonication on detectable capsular antigen levels of suspensions of S. pneumoniae*

Source of isolate	Type	Detectable antigen levels (titers)	
		Sonicated	Nonsonicated
Sputum	22	1:128	1:32
Sputum	35	1:128	1:4
Sputum	35	1:128	1:32
Sputum	11	1:64	1:32
Throat	23	1:128	1:16
Throat	6	1:128	1:32
Sputum	33	1:128	1:64
Sputum	9	1:128	1:32
Throat	6	1:128	1:32

DISCUSSION

From these data, we feel that the percentage of false negatives, i.e., those pneumococcal isolates that are nontypable by this CIE procedure, is low, approximately 1.5%. However, the validity of this percentage would be further substantiated by typing a greater number of isolates. Not all pneumococcal types are readily detected by the CIE method used. Type 7 migrates very slowly towards the anode, whereas type 14 actually migrates slightly towards the cathode (2). It is probable that the nontypable isolate (Table 1) was either type 7 or type 14. Type 14 is known to be a major cause of pneumonia in children; therefore, this technique as presently used may not be suitable for pediatric specimens (1). Type 7 has been reported to cause 6% of pneumonias in a group of patients studied in a New York hospital (1); however, it is known that the prevalence of certain types differs geographically. Thus, the percentage of false negatives by CIE may vary geographically. Other tests presently used in clinical labs such as optochin sensitivity or bile solubility would serve as a check on the CIE results and would reduce the chances of a false-negative report. Studies are in progress to attempt to circumvent the problem of nontypable isolates.

Not all *S. pneumoniae* strains are optochin sensitive. Since this is one of the standard tests used to separate pneumococci from other less pathogenic α-streptococci, CIE would be very useful in preventing the misidentification of pneumococci that are optochin resistant.

In comparing the discontinuous CIE method with the continuous one, we found the precipitin bands were not as sharp with the discontinuous method, but they became visible in a shorter period of time, usually within 30 min after electrophoresis was started. The discontinuous method was found to be more sensitive after 1 h of electrophoresis, although in general the continuous method gave the same results after the plates had been washed in physiological saline overnight at 4 C.

Lund (4) and Yurchak and Austrian (8) have observed that α-streptococci and pneumococci possess some antigens in common that can be detected by the Quellung reaction using the omniserum from the Statens Seruminstitut. Cross-reactions were also observed in 31.2% of the α-streptococci in this study (Table 4). However, the precipitin bands obtained with most α-streptococci were fainter than those obtained with the pneumococci, and 13% of these, almost half, could not be typed. Thus, true cross-reac-

tions, i.e., where the α-streptococcus was typable with the pneumococcal type-specific antisera, occurred in only 18.2% of the isolates. It should also be pointed out that Merrill et al. (5) did not observe any cross-reactions with α-streptococci when using the omniserum to perform the Quellung reaction on sputum smears. They found that the Quelling test and culture results correlated very well and felt that cross-reacting streptococci would not be a practical problem. The colonial morphology of the α-streptococci does differ from that of the pneumococci, and suspected cross-reacting isolates could be distinguished on the basis of the bile solubility and optochin sensitivity tests.

Identification of pneumococcal isolates can be achieved more rapidly with CIE since it does not require an overnight incubation period. This is particularly important in blood or spinal fluid specimens. These usually contain only one pathogen, and the growth from the primary blood plates can be readily used as the antigen source in CIE. The lower numerical types usually found in blood and spinal fluid specimens have not been found to possess any capsular antigens in common with other α-streptococci; therefore, cross-reactivity in this type of specimen would not be a practical problem.

Typing of isolates may be helpful in interpreting the clinical significance of pneumococcal isolates from sputa since it is the lower numerical types that have the greater pathogenic potential for pneumonia. In this study, most of the isolates obtained from blood were of the lower serotypes except for those isolates of type 25 (an uncommon pathogen), which were obtained from documented cases of pneumonia. Typing would also be useful from an epidemiological standpoint in that it would provide an indication of the types prevalent in a given community. This is especially important in view of the current work on the polyvalent pneumococcal vaccine.

In conclusion, CIE as an ancillary procedure for the identification of *S. pneumoniae* isolates has the distinct advantage of rapidity over the current methods used in a clinical laboratory. This procedure would be of particular value in identifying isolates from blood and spinal fluids. Since the serotypes of isolates obtained from these specimens have rarely been observed to share antigens with other α-streptococci, the results of CIE would be quite specific.

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