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Assessment of Complement-Mediated Killing of *Moraxella* (*Branhamella*) *catarrhalis* Isolates by a Simple Method

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Recently, we showed that complement resistance is an important virulence factor of *Moraxella* (*Branhamella*) *catarrhalis*. Our study used a serum bactericidal assay to determine complement resistance in *M. catarrhalis*. Although the serum bactericidal assay is considered the "gold standard" for determining complement resistance, it is laborious and time-consuming and therefore not well suited for large-scale studies. Using a large number ($n = 324$) of *M. catarrhalis* isolates obtained from the sputa of patients with lower respiratory tract infections ($n = 200$) and young carriers ($n = 124$), we assessed the value of a simple "culture-and-spot" test as an alternative to the serum bactericidal assay. For both groups of isolates, the degree of concordance between the two tests used was very significant ($P < 0.0001$). The agreement between the two assays was estimated to be "excellent beyond chance" (as determined by Cohen's kappa test). The culture-and-spot assay is a valuable alternative to the serum bactericidal assay, not only for screening purposes as shown here but also for studying the mechanism of complement resistance in *M. catarrhalis* at the molecular level.

Moraxella (*Branhamella*) *catarrhalis* is a gram-negative diplococcus frequently found as a commensal in the human upper respiratory tract (6, 8, 11, 13, 25). *M. catarrhalis* also causes infections, including otitis media, sinusitis, and conjunctivitis, in otherwise-healthy children and elderly people (4, 5, 11, 22). In addition, this bacterium is an important cause of lower respiratory tract infections in patients with chronic obstructive pulmonary disease (3, 17, 22). In other immunocompromised adults, *M. catarrhalis* can cause a variety of severe infections, including pneumonia, endocarditis, septicemia, and meningitis (3, 5, 11). Hospital outbreaks of respiratory disease due to *M. catarrhalis* have been described (19, 21), establishing the bacterium as a nosocomial pathogen.

In a recent paper (13), we reported that resistance to complement-mediated killing is a virulence factor of *M. catarrhalis*; that is, 89% of the strains isolated from adults with lower respiratory tract infections were found to be fully or intermediately resistant to human complement, whereas *M. catarrhalis* strains isolated from the upper respiratory tracts of healthy children were, for the most part (58%), complement sensitive. Complement resistance was assessed by a fluid-phase serum bactericidal assay in which killing was measured over a period of 3 h. This is considered the state-of-the-art test (24) for this type of study; the major drawback of this assay, however, is that it is laborious and time-consuming.

The purpose of the present study was to investigate whether the serum bactericidal assay could be replaced with a less laborious "culture-and-spot" test (1, 7, 9). The latter is based on the survival of bacteria on a blood agar plate after the application of a drop of 50% serum. A large number of sputum and carrier isolates of *M. catarrhalis* were used to compare the two methods.

MATERIALS AND METHODS

Reagents. Phosphate (10 mM)-buffered saline (pH 7.3) (PBS) was used to wash microtiter plates precoated with PBS containing 5% bovine serum albumin (BSA) (PBS-BSA). Veronal (5 mM)-buffered saline (pH 7.4) containing 0.15 mM Ca^{2+} and 0.5 mM Mg^{2+} (VBS^{2+}) was used as the buffer for complement-mediated-killing experiments. Human pooled serum (HPS) obtained from 30 healthy volunteers was used as the complement source.

***M. catarrhalis* strains.** *M. catarrhalis* ATCC 252310 was used as a reference strain. This strain is complement resistant in both the serum bactericidal assay and the culture-and-spot test. Patient isolates ($n = 200$) were cultured from 1,047 sputum samples from patients with lower respiratory tract infections. These samples were submitted to the Department of Medical Microbiology, Laboratory of Public Health, Leeuwarden, Friesland, The Netherlands. Gram stains showed leukocytes (>20 per low-power field [16]), and cultures of samples yielded pure cultures of *M. catarrhalis* (about 75%) or *M. catarrhalis* mixed with *Streptococcus pneumoniae* or *Haemophilus influenzae*. All *M. catarrhalis* strains were frozen in skim milk and stored in small aliquots at −70°C. Carrier strains ($n = 124$) were obtained from schoolchildren ($n = 303$; ages, 4 to 13 years) without symptoms of respiratory tract infections. Throat and nose cultures were taken and immediately plated onto 5% sheep blood agar plates.

Patient isolates and carrier strains were the same as those used in our previous study of complement resistance in *M. catarrhalis* (13).

M. catarrhalis strains were identified by colony morphology; Gram staining; the production of DNase, catalase, and/or oxidase; and tributyrin conversion (Rosco Diagnostics, Taastrup, Denmark).

Complement resistance assays. The serum bactericidal and culture-and-spot assays were performed in parallel for all strains.

(i) Serum bactericidal assay. *M. catarrhalis* was grown overnight in a shaking incubator at 37°C in Mueller-Hinton broth (Difco, Detroit, Mich.) containing 0.5% BSA to prevent clumping of the bacteria during growth. The bacteria were spun (3,000 × *g*) at room temperature; resuspended in VBS^{2+} to an optical density at 660 nm of 0.1 (as determined with a Dr. Lange photometer; Dr. Bruno Lange GmbH, Berlin, Germany), which corresponds to approximately 1.5×10^8 CFU/ml; and diluted 1/1,250 in VBS^{2+} (approximately 1.2×10^5 CFU/ml). Sterile U-well microtiter plates (catalog no. 1-663320; Nunc, Roskilde, Denmark) were precoated with PBS-BSA (at room temperature for 1 h) to avoid loss of viable bacterial counts due to adherence to plastic and were stored overnight at 4°C. The plates were washed three times with sterile PBS and used to incubate 125 μl of bacterial suspension with an equal volume of HPS. The mixtures were incubated at 37°C in a water bath for 3 h, and samples (20 μl) for determining viable counts were taken at 0, 15, 30, 60, 120, and 180 min. Viable counts were determined by culturing the samples on 5% sheep blood agar plates at 37°C for 18 h. After incubation, the number of colonies per sample was counted. Bacteria

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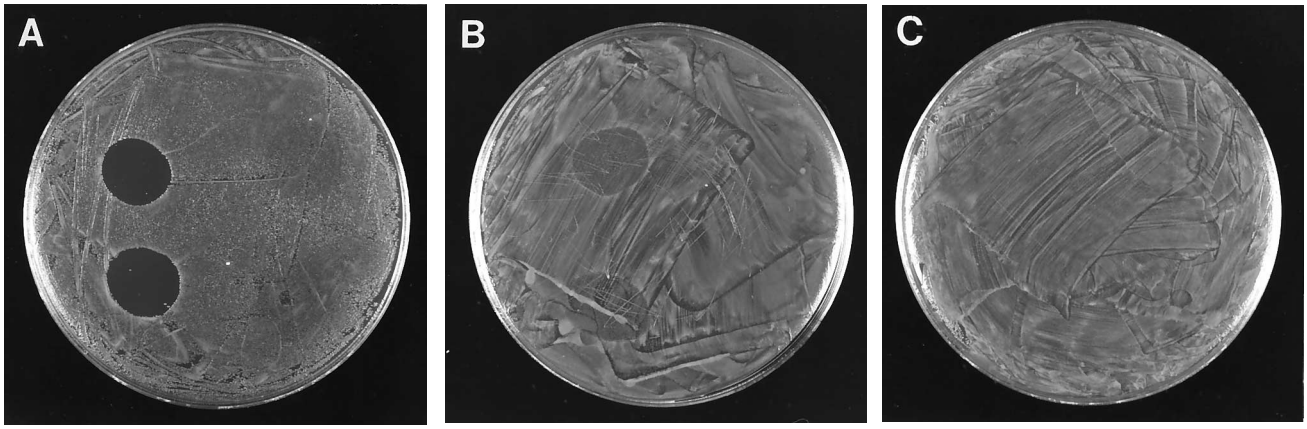

FIG. 1. Pictures of the three *M. catarrhalis* phenotypes (complement sensitive [A], intermediate [B], and complement resistant [C]) as observed in the culture-and-spot assay. Two drops of active human serum were dropped onto the left side of each blood agar plate, whereas two drops of heat-inactivated human serum were dropped onto the right side of each plate. The droplet area of active human serum applied on a blood agar plate is easily visible as a hemolytic zone upon transillumination (not shown).

incubated in 50% heat-inactivated (56°C; 30 min) HPS instead of in native HPS served as a growth control. Survival in HPS was expressed as a percentage of that of the growth control. Upon testing of the 324 *M. catarrhalis* strains, three populations could be distinguished: sensitive, resistant, and intermediate. Most sensitive strains were 100% killed within 15 min; none of the strains survived incubation lasting longer than 1 h. Most resistant strains showed 100% survival or even growth during incubation for 3 h. Intermediate strains showed a gradual decline in survival during the 3-h incubation period; at least 10% of the bacteria survived for 1 h, and less than 50% of the bacteria were recovered after 3 h of incubation. On the basis of these results, the strains were discriminated as follows: resistant strains were those showing >50% survival after 3 h of incubation, sensitive strains were those showing <3% survival after 1 h of incubation, and intermediate strains were those showing intermediate survival. Eighty-four percent of the strains gave reproducible results upon duplicate testing. The other strains were tested five times and could all be classified.

(ii) Culture-and-spot test. The culture-and-spot test is a modification of an assay previously described by Criado et al. (7). *M. catarrhalis* was grown overnight in a shaking incubator at 37°C in Mueller-Hinton broth (Difco) containing 0.5% BSA to prevent clumping of the bacteria during growth. The bacteria were spun, resuspended in VBS^{2+}, and adjusted to a concentration of approximately 1.5×10^8 bacteria per ml (optical density at 660 nm, 0.1). Samples (100 μl each) were spread over 5% sheep blood agar plates (diameter, 8.2 cm). After the broth had been absorbed into the agar, duplicate 50-μl samples of 50% HPS in VBS^{2+} were dropped onto the plates. Fifty percent heat-inactivated (56°C; 30 min) serum was used as a control on every plate. To allow optimal complement activation, the plates were placed in a 37°C incubator immediately after serum application. After overnight incubation, the number of colonies growing in the droplet area was estimated. Upon transillumination, the droplet area of active HPS on a sheep blood agar plate is easily visible as a hemolytic zone. On the basis of the results obtained with the 324 *M. catarrhalis* strains, three categories could be distinguished: resistant strains (those showing normal growth), sensitive strains (those showing 0 to 50 colonies), and intermediate strains (those showing intermediate growth). Eighty-nine percent of the strains gave reproducible results upon duplicate testing; the other strains could be classified after being retested four times. In Fig. 1, typical examples of sensitive, intermediate, and resistant strains are shown.

Statistical analysis. Results were evaluated by χ^2 analysis (with a 3-by-3 table). Differences with P values of <0.05 were considered significant. To establish agreement between the two complement resistance assays, Cohen's kappa test was used (10). Kappa was expressed as the mean value ± the standard error.

RESULTS

To assess the practical and discriminative value of the culture-and-spot test as a less laborious alternative to the serum bactericidal assay, two different, large groups of *M. catarrhalis* isolates were tested.

Patient isolates. Two hundred isolates obtained from 1,047 sputum samples from patients with bronchopulmonary disease were tested by the two assays for complement-mediated killing (Table 1). A highly significant degree of agreement between the two assays was observed ($P < 0.0001$) and it was considered "excellent beyond chance" (Cohen's kappa [10]). However, the culture-and-spot test yielded a slightly higher percentage of intermediate and resistant strains than did the serum bactericidal assay.

Carrier strains. Similarly, 124 strains obtained from healthy schoolchildren were tested (Table 2). Again, a highly significant degree of agreement was observed ($P < 0.0001$); it was also excellent beyond chance (Cohen's kappa [10]). Here also, a shift in the direction of complement resistance was observed with the culture-and-spot test, although this shift was less pronounced than with the patient isolates.

DISCUSSION

Many different techniques have been used to study complement resistance in gram-negative bacteria (2, 12, 14, 18, 20, 28, 29). As a consequence, studies dealing with complement resistance are difficult to compare. In general, the serum bactericidal assay, which measures time-dependent survival of bacteria incubated in serum, is considered the definitive method for assessment of complement resistance (24). Complement resistance in *M. catarrhalis* has been studied only by different variants of the serum bactericidal assay (4, 6, 13, 15, 23, 30). The serum bactericidal assay described in this report was developed by following the guidelines of Taylor (24). For example, the buffer used in the serum bactericidal assay can have significant and unpredictable effects on complement-mediated killing. VBS^{2+}, which contains the essential divalent cations necessary for optimal complement activity, was used as the buffer in our assay. Other buffers (Eagle's minimal essential medium, Dulbecco's minimal essential medium, Hanks' balanced salt solu-

TABLE 1. Distribution of *M. catarrhalis* strains ($n = 200$) isolated from the sputa of patients with lower respiratory tract infections

Culture-and-spot assay result	No. (%) of strains with serum bactericidal assay result[a]:		
	Sensitive	Intermediate	Resistant
Sensitive	13 (6.5)	0	0
Intermediate	7 (3.5)	45 (22.5)	7 (3.5)
Resistant	2 (1)	9 (4.5)	117 (58.5)

[a] $\chi^2 = 229.3$, $P < 0.0001$. Cohen's kappa = 0.76 ± 0.044 (excellent agreement beyond chance).

TABLE 2. Distribution of *M. catarrhalis* strains ($n = 124$) found in nose and/or throat cultures from young carriers

Culture-and-spot assay result	No. (%) of strains with serum bactericidal assay result[a]:		
	Sensitive	Intermediate	Resistant
Sensitive	63 (50.8)	1 (0.8)	0
Intermediate	9 (7.3)	7 (5.6)	0
Resistant	0	2 (1.6)	42 (33.8)

[a] $\chi^2 = 149.2$, $P < 0.0001$. Cohen's kappa = 0.83 ± 0.045 (excellent agreement beyond chance).

tion, Hanks' balanced salt solution plus 0.1% gelatin, and VBS^{2+} plus 0.1% gelatin) were also tested but did not have any advantages over VBS^{2+}. In addition, the growth phase of the bacteria may influence complement susceptibility, with strains grown to early logarithmic phase being more sensitive to killing by serum. In our study, the growth phases of *M. catarrhalis* strains did not influence their complement susceptibilities. This is in accordance with results of others on complement resistance of *M. catarrhalis* (6). The major drawbacks of the serum bactericidal assay are the large amounts of time and effort required. Therefore, the assay is not suited for large-scale screening of complement resistance.

We investigated whether the less laborious culture-and-spot assay (1, 7, 9) could replace the serum bactericidal assay to test large numbers of *M. catarrhalis* strains. In contrast to studies reviewed in reference 24, we did not encounter problems with the anticomplementary activity of 5% sheep blood agar. Results obtained with this agar were comparable to those obtained with Mueller-Hinton agar. However, the droplet area of active human serum was more easily discerned on sheep blood agar plates than on Mueller-Hinton agar plates. Growth in the droplet area of heat-inactivated human serum could not be distinguished from normal growth outside droplet areas on the blood agar plate.

On the basis of our large-scale study, we were able to distinguish a third phenotype in addition to the currently recognized complement-sensitive and complement-resistant strains (15, 23). In our opinion, there is a marked difference between complement-sensitive strains, which survive less than 30 min in human serum (most are killed within 15 min), and intermediate strains, which show a more gradual decline in viable counts and survive, at least in part, incubation for longer than 60 min. A clear difference between intermediate and complement-resistant strains is also present. The latter survive the 3-h incubation period and do not show a significant decline in viable counts. The absence of an intermediate phenotype in previous studies (15, 23) may be ascribed first to the small numbers of strains tested and further to the difference in assay parameters, including incubation time, incubation buffer, and sampling time points. In addition, the geographical origins of strains could play a role.

The culture-and-spot assay allowed us to test up to 100 *M. catarrhalis* strains per day. Furthermore, the results were very easy to interpret. To date, the culture-and-spot test has been used to establish complement resistance in *Escherichia coli* (1) and *Neisseria meningitidis* strains (7). In addition, only limited data on the comparability of the culture-and-spot test and the serum bactericidal assay are available (1, 9). This prompted us to perform the present comparative study with two completely different panels of *M. catarrhalis* strains. The first panel consisted of patient isolates showing a very low frequency (11%) of complement sensitivity as determined by the serum bactericidal assay. The second panel comprised carrier strains, isolated from healthy schoolchildren, with a high proportion (58%) of complement sensitivity.

For both panels, the agreement between the two tests was highly significant ($P < 0.0001$ [Tables 1 and 2]), indicating that, for *M. catarrhalis*, the culture-and-spot test can replace the serum bactericidal assay. The slight shift in the direction of complement resistance with the culture-and-spot test is probably due to the shorter time of exposure of the bacteria to serum. This may suggest enhanced survival and, as a result, a slight overestimation of complement resistance among *M. catarrhalis* isolates.

The good correlation between the results of the culture-and-spot test and the serum bactericidal assay suggests that the latter test is also suited to the study of the mechanism of resistance to human complement in *M. catarrhalis* (26). Using the culture-and-spot assay, we were able to show that complement resistance in *M. catarrhalis* can be abolished by pretreatment of the bacteria with trypsin (data not shown) and is mediated by a surface protein which binds human vitronectin (26, 27). We are currently applying the culture-and-spot assay to study the phenomenon of inducing complement resistance in complement-sensitive *M. catarrhalis* strains by incubation in serum. In these studies also, the culture-and-spot test appears to be a reliable substitute for the serum bactericidal assay.

In conclusion, our study demonstrates that, for *M. catarrhalis*, the culture-and-spot test is in more than one respect a valuable alternative to the fluid-phase serum bactericidal assay. Whether this conclusion holds for other bacterial species remains to be established.

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