Use of the RapID-ANA System and Sodium Polyanetholesulfonate Disk Susceptibility Testing in Identifying \emph{Haemophilus ducreyi}

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\emph{Haemophilus ducreyi} has traditionally been difficult to identify. We have utilized simple test methods to identify 19 fresh isolates obtained during a recent outbreak of chancroid in Houston and six strains of \emph{H. ducreyi} from other outbreaks. Tests were performed from growth on chocolate agar after 48 h of incubation at 35°C with increased humidity and CO\textsubscript{2}. All isolates exhibited typical colonial morphology and Gram stain. Isolates were catalase negative and oxidase and nitrate positive (in enriched broth). The RapID NH system failed to identify these strains because of negative reactions with alkaline phosphatase and nitrate reductase. However, by using the RapID-ANA system, all strains were positive for alkaline phosphatase and arginine, glycine, and serine aminopeptidases. Their biochemical profiles were distinct from those obtained with 66 strains representing 13 species similar to \emph{H. ducreyi}. We also investigated the use of sodium polyanetholesulfonate (SPS) disk susceptibility to identify and differentiate \emph{H. ducreyi} from other species. All \emph{H. ducreyi} isolates were susceptible, as evidenced by the presence of a zone of inhibition with an average size of 15 mm around the SPS disk. With the exceptions of \emph{Neisseria gonorrhoeae}, \emph{Gardnerella vaginalis}, and \emph{Capnocytophaga} spp., no other strain showed any evidence of inhibition. The latter three organisms can be easily differentiated from \emph{H. ducreyi} by various features including reactions in the RapID-ANA. We conclude that, by considering simple growth and biochemical characteristics, SPS susceptibilities, and reactions in RapID-ANA, it is possible for more clinical laboratories to definitively identify this organism.

\emph{Haemophilus ducreyi} is the causative agent of chancroid, an endemic disease in several developing countries (4, 15, 24). Throughout the world, including the United States, chancroid occurs in epidemics. In the United States, the reported incidence of chancroid seems to be increasing (12, 17). However, because the organism is extremely labile and fastidious, the recovery of \emph{H. ducreyi} is a difficult task, discouraging serious attempts to demonstrate the organism in culture. As a result, the laboratory diagnosis of chancroid was commonly based on microscopic appearance of stained films of ulcer exudate, despite the dubious reliability and poor predictive value of this method (12, 20). In the past 10 years, however, several culture methods and media which could enhance the ability of clinical laboratories to recover this organism have been described (3, 6, 7, 19, 21).

Once the organism has been isolated, \emph{H. ducreyi} has few dependable biochemical characteristics, making it difficult to identify except on the basis of morphological and cultural characteristics. Discrepant results of key identification tests such as oxidase, catalase, and nitrate reduction have been reported (13, 14, 22). Recent studies on the enzymatic profile of \emph{H. ducreyi} provided new information on the biochemical activity of this organism by demonstrating the presence of aminopeptidases and esterases with various activities (2, 22, 25). Commercially available systems such as Mintek (BBL Microbiology Systems, Cockeysville, Md.) (14), API ZYM (Analytab Products, Plainview, N.Y.) (2), RapID NH (Innovative Diagnostics Systems, Atlanta, Ga.) (8), and Micro-ID (General Diagnostics, Div. of Warner-Lambert Co., Morris Plains, N.J.) (18) have been used to detect enzymes produced by \emph{H. ducreyi}, but these systems did not give identical results for some key tests. The RapID NH is a qualitative micromethod that employs conventional and chromogenic substrates for the identification of medically important species of \emph{Neisseria}, \emph{Haemophilus}, and a few other related genera. The principle of the RapID-ANA system is the same, but it tests for 18 preformed enzymes, which include alkaline phosphatase and seven different aminopeptidases, and it has been designed primarily to identify anaerobic organisms. In this report, we evaluated the ability of the RapID-ANA system to biochemically distinguish \emph{H. ducreyi} from other organisms. In addition, since susceptibility to a sodium polyanetholesulfonate (SPS)-containing disk is commonly used as a means of identifying certain organisms (5, 16, 26), we assessed the value of this test as a screening method to identify and differentiate \emph{H. ducreyi} from similar organisms.

MATERIALS AND METHODS

Bacterial strains. Nineteen clinical isolates of \emph{H. ducreyi} recovered from patients with chancroid during a recent outbreak in Houston were evaluated in this study. In addition, \emph{H. ducreyi} strains obtained from the following sources (number of strains) were tested: Centers for Disease Control, Atlanta, Ga. (n = 3); Dallas outbreak (n = 2); and ATCC 33921. For comparison of biochemical activity and SPS susceptibility, the following gram-negative organisms were also tested: \emph{H. influenzae} (n = 5), \emph{H. parainfluenzae} (n = 5), \emph{H. aphrophilus} (n = 1), \emph{H. haemolyticus} (n = 3), \emph{H. (Actinobacillus) actinomycetemcomitans} (n = 8), \emph{Eikenella corrodens} (n = 8), \emph{Cardiobacterium hominis} (n = 4), \emph{Pasteurella multocida} (n = 5), \emph{Capnocytophaga ochracea} (n = 4), \emph{Capnocytophaga canimorsus} (formerly CDC group DF-2) (n = 1), \emph{Gardnerella vaginalis} (n = 10), \emph{Neisseria gonorrhoeae} (n = 5), and \emph{Moraxella} spp. (n = 7). All strains were

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grown on chocolate agar at 35°C in the presence of 7 to 8% CO₂ and 95% humidity. 

**Identification.** Colonies were presumptively identified as *H. ducreyi* on the basis of their cohesiveness, which made them able to be moved intact across the agar plate (14). Whole colonies were transferred to a microscope slide, fixed, Gram stained, and examined for typical arrangement of cells. Colonies were then tested for their X-factor requirement, catalase, oxidase, and nitrate reductase. The porphyrin test was performed according to the method of Kilian (9, 10). Briefly, a heavy suspension of a 48-h growth was added to 0.5 ml of the enzyme substrate δ-aminolevulinic acid hydrochloride (2 mM δ-aminolevulinic acid and 0.08 mM MgSO₄ in 100 ml of 0.1 M phosphate buffer, pH 6.9). Tubes were incubated at 35°C and examined for red fluorescence with a Wood lamp after 4 and 24 h of incubation. *H. influenzae* and *H. parainfluenzae* were the positive and negative control organisms, respectively. The absence of red fluorescence, which indicated that δ-aminolevulinic acid had not been converted to porphyrins, was considered positive for the growth requirement of X-factor. The standard slide method was used to test for the enzyme catalase. The presence of cytochrome oxidase was determined by adding a few drops of tetramethyl-p-phenylenediamine dihydrochloride on well-isolated colonies on chocolate agar and observing for 2 min for the appearance of a violet color. Testing for nitrate reductase was done by growing the organism for 48 h in 0.2% KNO₃ heart infusion broth at 37°C and then adding suitable reagents at the end of incubation.

The RapID NH and RapID-ANA systems (Innovative Diagnostics) were used. Additionally, an API ZYM 20-test kit (Analytab), was evaluated in the first part of this study. All tests were used according to the recommendations of the manufacturer. Heavy inoculum was prepared by harvesting growth from 18- to 24-h plate cultures (40 to 48 h for *H. ducreyi* and *G. vaginalis*) into a tube containing the inoculation fluid for each kit. The inoculum was then transferred to the test panel and incubated, and the reactions were interpreted according to the instructions of the manufacturer.

The SPS disk susceptibility test for *Peptostreptococcus anaerobius* and *G. vaginalis* was performed as described previously (16, 26). A turbid suspension of the organism was prepared by harvesting growth from 18- to 24-h plate cultures (40 to 48 h for *H. ducreyi* and *G. vaginalis*) in phosphate-buffered saline plus 0.002% Tween 80 to help disperse the cells and produce a homogenous suspension. This suspension was then used to prepare an inoculum equivalent in turbidity to a 0.5 McFarland standard. A cotton swab was used to swab the surface of fresh chocolate agar plates. A single disk containing 0.001 g of SPS (Remel, Lenexa, Kans.) was placed on the surface of each test plate, which was then incubated for 24 to 48 h at 35°C in a humidified incubator. Plates were examined for the presence of zones of inhibition around the disk. A zone of >12 mm was considered to indicate susceptibility.

**RESULTS**

All *H. ducreyi* isolates were catalase negative and oxidase and nitrate positive and required X-factor for growth. In a brief evaluation of an API ZYM 20-test panel, only alkaline and acid phosphatase and leucine aminopeptidase were positive for the 10 strains of *H. ducreyi* examined by this system. No biochemical activity could be demonstrated by using the RapID NH system, including tests that are classically reported positive for *H. ducreyi* (i.e., alkaline phosphatase and nitrate reductase). These negative reactions distinguished *H. ducreyi* from all the other organisms, which consistently gave various positive reactions with this system. The RapID-ANA system was more useful in identification, with *H. ducreyi* giving a distinct biochemical profile (Table 1). Positive results for *H. ducreyi* were limited to a maximum of 7 reactions on this panel of 18 tests. All other organisms consistently gave positive results in at least one additional test that was negative for *H. ducreyi*. With this system, all *H. ducreyi* isolates were positive for phosphatase and the following aminopeptidases: glycine, arginine, and serine. In addition, 2, 4, and 18 strains were positive for proline, phenylalanine, and leucylglycine aminopeptidases, respectively. All *H. ducreyi* isolates showed zones of inhibition around SPS disks after 48 h of incubation (Table 1). The zone sizes ranged between 14 and 17 mm, with a mean of 15 mm. With the exceptions of *G. vaginalis*, *N. gonorrhoeae*, and *Capnocytophaga* spp., no other strains tested showed any evidence of inhibition.

**DISCUSSION**

In our laboratory, all *H. ducreyi* isolates were presumptively identified on the basis of colony morphology, Gram stain, and catalase and oxidase reactions. As previously reported by others (1, 11, 14), we valued the diagnostic importance of simple observations such as colonial cohesiveness and the fingerprint or railroad track arrangement of cells in gram-stained smears of these colonies. Recent electron microscopy studies suggest that these typical features of *H. ducreyi* may be related to the presence of areas of intracellular adhesions (12).

Although *H. ducreyi* is described as catalase negative and oxidase positive (10), conflicting reports concerning these enzymes exist, and the results have been shown to depend on the methods and reagents used. For example, Sturm and Zanen (22) were unable to detect catalase activity after dropping 5% H₂O₂ on colonies of *H. ducreyi* growing on an enriched agar medium, but observed a positive catalase reaction with the tube test. The detection of cytochrome oxidase appears to depend on the substrate used in the test. Positive results have been observed with tetramethyl-p-phenylenediamine dihydrochloride (13, 24); both positive (22) and negative (2, 13) test results have been observed with *N,N*-dimethyl-p-phenylenediamine dihydrochloride. We have used only the slide method to test for catalase production and were unable to detect gas production, even by microscopic examination of the slide. *H. ducreyi* consistently gave a positive reaction for oxidase, a finding that is in agreement with previous studies using the same substrate (tetramethyl-p-phenylenediamine dihydrochloride). However, we found that the age of culture is extremely critical because of the tendency of cultures older than 72 h to give a negative oxidase reaction (Ribhi Shawar, unpublished observations).

*H. ducreyi* is in the most traditional biochemical tests and is considered to be asaccharolytic (10). Weak positive reactions for glucose, mannose, fructose, and urea metabolism have been reported (18, 19), but these findings have not been confirmed by other investigators. Besides its hemin requirement, the classic biochemical activities of *H. ducreyi* are reduction of nitrate and production of alkaline phosphatase. However, several investigators have reported that some strains fail to reduce nitrate (7, 22). This failure has been attributed either to incubation time or to the test used.
### TABLE 1. Results of diagnostic tests: comparison of *H. ducreyi* with other gram-negative organisms

<table>
<thead>
<tr>
<th>Organism (no. of strains)</th>
<th>% Susceptible to SPS</th>
<th>% Positive with RapID-ANA substrate:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PO4</td>
<td>LGY</td>
</tr>
<tr>
<td><em>Haemophilus ducreyi</em> (25)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><em>H. influenzae</em> (5)</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td><em>H. parainfluenzae</em> (5)</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td><em>H. aphrophilus</em> (1)</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td><em>H. haemolyticus</em> (3)</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td><em>H. actinomycetemcomitans</em> (8)</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Gardenerella vaginalis (10)</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Cardiobacterium hominis (4)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Eikenella corrodens (8)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pasteurella multocida (5)</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Moraxella sp. (7)</td>
<td>0</td>
<td>57</td>
</tr>
<tr>
<td>Neisseria gonorrhoeae (5)*</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>Capnocytophaga ochracea (4)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Capnocytophaga canimorsus (DF-2) (1)</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

* Reactive ingredients in substrates: PO4, p-nitrophenolphosphate; LGY, leucylglycyl-b-naphthylamide; ONPG, o-nitrophenyl-b-D-galactoside; GLY, glycyll-b-naphthylamide; aGLU, p-nitrophenyl-a-D-glucoside; PRO, prolyl-b-naphthylamide; BGLU, p-nitrophenyl-b-D-glucoside; PAL, phenylalanyl-b-naphthylamide; aGAL, p-nitrophenyl-a-D-galactoside; ARG, arginyl-b-naphthylamide; aFUC, p-nitrophenyl-a-D-Fucoside; SER, seryl-b-naphthylamide; PYR, pyridoxylb-naphthylamide; TTZ, triphenyltetrazolium; ADH, arginine; TRE, trehalose; IND, tryptophane.

* Nineteen clinical isolates, 5 isolates from other chancroid outbreaks, and 1 ATCC strain.

* Three isolates gave 8-, 9-, and 11-mm zones of inhibition.

As seen in Table 1, *H. ducreyi* exhibits distinct biochemical and enzyme activities that are not present in other gram-negative bacteria, allowing for its differentiation from other organisms. The presence of positive reactions for alkaline phosphatase activity in the RapID-ANA system indicates the ability of *H. ducreyi* to hydrolize a variety of substrates, including aminated and unaminated compounds. This property is essential for the organism's survival and interaction with the host. In addition, the presence of positive reactions for other enzymes such as acid phosphatase, esterases, and aminopeptidases further supports the identification of *H. ducreyi* in clinical samples. These results, when combined with the absence of reactions for enzymes not typically associated with *H. ducreyi*, provide strong evidence for the organism's presence. The RapID-ANA system, therefore, serves as a rapid and reliable method for the identification of *H. ducreyi* in clinical samples, facilitating timely diagnosis and appropriate treatment for this sexually transmitted infection.
ducreyi, but additional positive tests for other organisms on the panel separated _H. ducreyi_ from other organisms. Our results indicate that the RapID-ANA system appears to offer a rapid alternative method to confirm the identity of _H. ducreyi_ when used with simple tests such as production of catalase and oxidase and observations for characteristic colony morphology, Gram stain morphology, and growth requirements.

We found SPS disk susceptibility to be extremely useful in identifying and differentiating _H. ducreyi_. To our knowledge, susceptibility to SPS has not been reported for _H. ducreyi_. The mechanism for this susceptibility is not understood by us at this time. However, _H. ducreyi_ is an extremely susceptible organism whose viability is affected by several growth condition factors, and a possible role for autolysins has been proposed (14). Although susceptibility to SPS is not a very specific characteristic of _H. ducreyi_, organisms sharing this property can be easily distinguished from _H. ducreyi_ by features such as colony morphology, Gram stain, or simple biochemical tests. Therefore, the specificity of SPS disk susceptibility testing can be greatly increased when such simple features are taken into consideration. We found this to be a highly sensitive, simple, and relatively inexpensive method. If necessary, further differentiation between SPS-susceptible organisms can be achieved by using the RapID-ANA test panel. On this panel, _N. gonorrhoeae_, _G. vaginalis_, and _Capnocytophaga_ spp. can be differentiated from _H. ducreyi_ by three, five, and seven tests, respectively (Table 1). We propose that presumptively identified _H. ducreyi_ be first subjected to an SPS disk susceptibility test, the results of which are ready after 48 h. The heavy lawn of bacterial growth on the chocolate agar plate away from the SPS disk can then be used to inoculate the 4-h RapID-ANA panel, allowing full identification in only 2 days.

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**LITERATURE CITED**


