Chemotactic Behavior of Pathogenic and Nonpathogenic Leptospira Species

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We have developed a capillary tube assay in combination with real-time PCR to quantify the number of chemoattracted Leptospira cells. We identified Tween 80, glucose, sucrose, and pyruvate as attractants for Leptospira cells; amino acids and vitamin B12 were found to be nonchemotactic or weakly chemotactic. This assay has the general applicability to further our understanding of leptospiral chemotaxis.

Human leptospirosis is an emerging disease with more than 1,000,000 cases occurring annually, with a case fatality rate exceeding 10% (5). Leptospira spp. belong to the spirochete phylum and consist of both saprophytic and pathogenic species, with the pathogenic species being the etiological agents of leptospirosis. Leptospira species are highly motile spirochetes, and their unique motility likely plays a role in their ability to rapidly disseminate into the host (9, 10, 16). Spirochete motility is unique as it allows these bacteria to swim in highly viscous gel-like media that slow or stop the motility of peritrichous bacteria (3, 12). Leptospira motility depends on the presence of two periplasmic flagella, each arising from one of the subpolar ends of the cell without overlapping at the cell center (6). The direction of flagellar rotation is modulated by chemotaxis, which is defined by the movement of an organism toward or away from a chemical compound. The latter is called an attractant if it induces a movement toward itself (22). Among the spirochetes, chemotaxis has been studied to a limited extent in Borrelia burgdorferi, Brachyspira hydysenteriae, Spirochaeta aurantia, and Treponema dentium (11, 15, 20, 24). In Leptospira spp., hemoglobin was found to be an attractant (27), but this has yet to be verified as most bacteria are attracted to small molecules, including diffusible molecules and small peptides, but not to intact proteins.

In this study, we developed and modified a capillary tube assay previously used to analyze B. burgdorferi chemotaxis (2, 21) that allowed us to compare the chemotactic behavior of the saprophyte Leptospira biflexa serovar Patoc strain Patoc I with the pathogen Leptospira interrogans serovar Manilae strain L495.

Development of a capillary tube assay. To carry out the assay, actively motile exponential-phase Leptospira cells in Ellinghausen-McCullough-Johnson-Harris (EMIH) culture medium (optical density at 420 nm of 0.5, which corresponds to approximately 5 × 10^7 bacteria/ml) were centrifuged at low speed and gently resuspended in motility buffer consisting of 7 mM Na_2HPO_4, 2.2 mM KH_2PO_4, 17.1 mM NaCl, 4.7 mM NH_4Cl, 14.8 μM thiamine, and 0.5% bovine serum albumin (BSA). The resuspended cells were then preincubated overnight at 30°C to allow bacteria to recover motility and to deplete nutrients that were carried over by centrifugation. Approximately 60% of the bacteria were translating (less than 5% were nonmotile, with the remaining cells usually showing gyrating ends without translational movement), which is similar to values obtained for log-phase cells in EMJH medium (data not shown).

The chemotaxis chamber used was based on the one described for B. burgdorferi (2, 21). Briefly, a 96-well plate (1.2-ml square well storage plate; Thermo Scientific) was filled with 200 μl of suspension (2 × 10^7 L. interrogans cells counted using a Petroff-Hausser chamber) per well, and the inverted plate was perforated at the bottom of each well facing the cell suspension to place the capillary tubes (75-mm, 60-μl, 0.95-mm-diameter hematocrít capillary tubes; Hirschmann Laborgeräte) containing hypothetical attractant solutions. After incubation, approximately 40 μl was eluted from the capillary tube, and the total genomic DNA was extracted using a cell DNA purification kit (Maxwell; Promega). Enumeration of Leptospira cells entering in the capillary tubes by viable plate counts is a low-throughput method, especially for L. interrogans, as it takes several weeks to form colonies (10). Whereas the previous study used flow cytometry to count B. burgdorferi cells (2, 21), we developed a SYBR green (SsoFAST EvaGreen Supermix; Bio-Rad) quantitative PCR (qPCR) assay targeting lipL32 or rpoB to enumerate L. interrogans and L. biflexa cells, respectively, in the capillary tubes (18). Standard curves for the quantification of leptospires were constructed using DNA extracts from known numbers of leptospires counted in a Petroff-Hausser chamber. All PCR assays were performed in duplicate, and control reactions without template were included in each assay. For each assay, at least two independent experiments with three to four capillary tubes were performed.

To begin to identify chemoattractants, we first tested specific nutrients present in the EMJH culture medium. EMJH is a complex medium composed of Tween 80, glycerol, pyruvate, BSA, vitamin B12, thiamine, and salts. Pyruvate, which stimulates the
TABLE 1 Chemotactic responses of *L. biflexa* and *L. interrogans* to various compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th><em>L. biflexa</em></th>
<th><em>L. interrogans</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty acids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tween 80</td>
<td>2%</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>100 mM</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>100 mM</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>1.5 mM</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Amino acids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamate</td>
<td>50 mM</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Asparagine</td>
<td>50 mM</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Leucine</td>
<td>50 mM</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>100 mM</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Hemin</td>
<td>0.3 mM</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Sugars</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>100 mM</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td>Glucose</td>
<td>100 mM</td>
<td>8</td>
<td>6</td>
</tr>
</tbody>
</table>

*a* All compounds were tested in a range of 0.1 mM to 100 mM (1 to 2% for Tween 80). Stearic and palmitic acids were first dissolved in chloroform, which by itself had no chemotactic response. The results shown are the concentrations that gave the maximum response.

*b* The relative chemotactic response is the ratio of cells that migrate into attractant-filled versus motility-buffer-filled capillary tubes. Compounds eliciting a relative chemotactic response of ≥2 were considered chemotactants (2). The data shown are representative of at least two independent experiments.

The sugars glucose and sucrose were strong attractants of both pathogenic and saprophytic strains (Table 1). These findings suggest that glucose, which stimulates the growth of some *Leptospira* strains (4, 8), might be sensed by *Leptospira*. Sucrose was also found to be an attractant. It is unlikely that sucrose is metabolized by *Leptospira*, as both strains lack the genes that code for enzymes that break down this sugar. However, sucrose could still serve as an attractant without being metabolized by binding directly or indirectly to a chemoreceptor. Thus, *E. coli* shows strong attraction to the nonmetabolizable galactose analog fucose (1).

Leptospiral attraction toward amino acids was relatively weak. This includes leucine, which has been shown to be readily transported and utilized by the leptospires (7, 26). Interestingly, neither *L. biflexa* nor *L. interrogans* displayed strong attraction toward vitamin B12, which is essential for *Leptospira* growth (23).

Our experiments indicated positive chemotaxis of *L. interrogans*, but not *L. biflexa*, toward hemin, which can be used as a source of both iron and heme (19) (Table 1).

**Conclusion.** Our capillary tube assay in combination with real-time PCR is likely to have several applications, such as the analysis of chemotactic *Leptospira* mutants or the study of the chemotactic response to compounds that could be important fac-

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*Leptospira* (4, 14), was first tested as a putative chemotactant of both pathogenic and saprophytic strains. The capillary tubes were filled with 100 mM pyruvate, and comparisons were made to tubes filled with motility buffer, which served as a negative control. The accumulation of *L. biflexa* and *L. interrogans* in the capillary tubes filled with pyruvate reached a plateau in both strains after 30 min of incubation at 30°C or 37°C. In contrast, no significant increase in spirochete numbers increased in the buffer control (data not shown). Based on these findings, an incubation time of 1 h at 30°C was selected for further experiments.

**Identification of chemotactants.** The relative chemotactic response is defined as the relative number of cells accumulating in the capillary tube with attractant compared to that in tubes without attractant. These responses were 7 and 8 with pyruvlate at 100 mM for *L. biflexa* and *L. interrogans*, respectively (Table 1). To confirm we were assaying for chemotaxis, pyruvate was added in the bacterial suspension prior to the assay, therefore lowering the gradient of the attractant. Under these conditions, the number of bacteria in the capillary tubes decreased, further confirming that pyruvate is a chemotactant (Fig. 1). We also found that for both *Leptospira* species, the number of spirochetes accumulating in the capillary tubes increased with the concentration of pyruvate (Fig. 2).

We then tested numerous compounds present in EMJH medium for their ability to serve as chemotactants (Table 1). Tween 80, which was chosen for its role as a source of long-chain fatty acids, such as oleate, palmitate, and stearate, in *Leptospira* (10, 13), functioned as an attractant for both pathogenic and saprophytic strains. A previous study demonstrated that free fatty acids are often toxic to *Leptospira* (25), and we confirmed this observation when testing chemotaxis toward oleate and instead found it to act as a lysing agent for *Leptospira* (data not shown). In contrast, stearate and palmitate were not found to be toxic under our conditions. Interestingly, pathogenic and saprophytic species showed differential chemotactic behavior to palmitate, as it served as attractant for *L. interrogans* but not *L. biflexa* (relative chemotactic response of 1 versus 16 in *L. biflexa* and *L. interrogans*, respectively). The basis of this specificity to palmitate, which is the most common long-chain fatty acid in *Leptospira* (17), remains unclear. Long-chain fatty acids which are present in the Tween 80 are utilized as the sole carbon source and are metabolized by β-oxidation in *Leptospira* (10).

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FIG 1 Chemotaxis increases with increasing concentration gradient of attractant. Capillary tubes (C) filled with 100 mM pyruvate were dipped into the bacterial suspensions of *L. interrogans* in wells (W) containing 0, 50, and 100 mM pyruvate. Capillary tubes filled with chemotaxis buffer were used as a negative control. Fewer cells enter capillaries containing pyruvate as its concentration in the bacterial suspensions is increased. Data are representative of one experiment and are the means of PCR duplicates of three to four capillary tubes. The error bars represent standard deviations.
tors in the natural host cycle of the bacteria. Our data suggest that the chemotactic behavior of a pathogenic strain is not identical to that of the saprophyte \textit{L. biflexa}. These differences likely reflect the ecology and metabolic requirements of the two species. The annotated chemotaxis proteins in \textit{Leptospira} genomes show that \textit{L. interrogans} has 13 chemoreceptor homologs, whereas the saprophyte \textit{L. biflexa} has twice as many (http://mistdb.com/). Chemoreceptors in \textit{L. biflexa} likely play a role in the adaptation of this aquatic bacterium to a wide range of environmental conditions. Further studies are required to evaluate the chemotactic responses of a larger panel of \textit{Leptospira} strains from distinct sources. Similarly, other spirochetes, including \textit{B. burgdorferi}, \textit{B. hyodysenteriae}, \textit{S. aurantia}, and \textit{T. denticola}, varied significantly in their attraction to a panel of chemical compounds (2, 11, 15, 20, 21, 24).

Further experiments will no doubt continue to expand our knowledge of the mechanisms that allow chemotaxis in these unique bacteria.

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