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Bacterial Tracking Using Ferrographic Separation

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An innovative technique for tracking bacteria at low concentrations in water was used to capture bacteria from natural waters. The technique utilizes an immunomagnetic method to confer magnetic susceptibility specifically to the bacteria of interest and uses a Bio-Ferrograph to concentrate the deposition of magnetically tagged bacteria onto an exceedingly small area on a glass cover slip. Complete recovery of low concentrations of bacteria spiked into natural waters was achieved using the method after a blocking agent and a complexing agent were added to mitigate nonspecific interaction of antibodies with mineral colloids in the water and to prevent cation-induced antibody coagulation. The ability to visually identify bacterial aggregation and shape using this method provides additional information potentially useful in studying bacterial transport.

Introduction

Bacterial transport in the subsurface is studied for a number of reasons including bioaugmentation, which is the addition of bacteria to the subsurface for the purpose of enhanced bioremediation. It is also an important concern in the protection of groundwater supplies from contamination by pathogens. Bacterial transport studies have been performed in the laboratory and in the field using various techniques to monitor the transport of added bacteria (*1, 2*). Tracking techniques used to monitor the concentration of a specific bacterium must allow selective identification of the particular microbe added to the system. In addition, high-resolution counting (e.g., quantitation down to 100 cells/mL or less) is required due to the dramatic decrease in suspended cell concentration with distance from the injection point. Furthermore, significant information regarding the kinetics of bacterial attachment and detachment can be determined from examination of cell concentrations many orders of magnitude below the injected concentration (*3*).

One of the most successful bacterial tracking techniques used in the laboratory utilizes radiolabeled cells (*4, 2*). However, the impracticability of introducing radiolabel to the environment has required the use of other bacterial tracking techniques in field studies of bacterial transport. Techniques conventionally used in tracking bacterial transport in the field have drawbacks that limit their use. Culturing samples using growth media provides the ability to detect low concentrations of bacteria under ideal conditions. However, this technique may be complicated by growth of strains indigenous to the subsurface and by the fact that culturing typically underestimates cell count (*2*). Methods using the polymerase chain reaction (PCR) allow genetic identification of bacteria in natural systems; however, these techniques do not presently provide high-resolution enumeration due to the requirement of true exponential amplification for accurate quantification (*5, 6*). Fluorescence staining of bacteria potentially allows photometric quantification of cells with high resolution; however, interference from dissolved organic matter is a significant concern in groundwater. Filtering the sample to capture fluorescent-stained bacteria and to visually identify them under an epifluorescence microscope can eliminate some of the interference from natural organic matter. However, filtering provides low resolution when only small sample volumes are available, e.g., 10^4 cells/mL is the limit of quantitation for a 1-mL suspension. In addition, some natural particles are autofluorescent; hence, nonselective capture of all suspended particles on the filter surface may interfere with the analysis.

Techniques involving stable carbon isotope analysis (*7*), flow cytometry (*8*), ice nucleation (*9*), and immunomagnetic separation (*8*), to name a few, may potentially be employed by researchers studying bacterial transport. This paper describes one innovative technique recently developed for tracking bacterial concentrations. The technique employs immunomagnetic tagging and ferrographic separation. The technique provides selectivity due to reliance on antibody−antigen recognition to magnetically tag the bacteria of interest, and it provides high-resolution enumeration due to deposition of the magnetically tagged bacteria onto an exceedingly small area on a glass slide for visual identification under an epifluorescence microscope. Visual identification of the bacteria provides information on the relative shapes, sizes, and possible aggregation of bacteria that may be important characteristics in their transport behavior.

Conventional immunomagnetic separation techniques have been applied to the enumeration of low concentrations of bacteria (*10, 11*) and protozoan oocysts (*12*−*14*) in environmental waters. These investigations have shown that the accuracy of enumeration depends strongly on the recovery of the microbes from the paramagnetic beads used in their capture. This separation step is required before the concentrated microbial suspension is added to antibody-labeled well slides for enumeration by fluorescence microscopy, and its efficiency depends chiefly on the acid dissociation procedure used in bead−microbe separation (*12*). In contrast, the technique described in this paper utilizes extremely small paramagnetic beads that remain attached during enumeration, negating the need for an acid dissociation step. In addition to requiring less sample manipulation relative to conventional immunomagnetic separation techniques, the method described here may preserve structures that might otherwise not be observed were an acid dissociation step required.

A previous study examining enumeration of dilute concentrations of spiked bacteria in pure water (Milli-Q) resulted in determination of the optimal concentrations of magnetic beads, antibodies, and saline as well as maximum flow rates to be used in the ferrographic separation method (*15*). The purpose of the present study was to determine modifications to the method that were necessary to achieve complete recovery of spiked bacteria in natural waters. Specifically, the addition of blocking agents to minimize nonspecific interaction of antibodies with naturally occurring mineral colloids and the addition of complexing agents to minimize suspected cation-induced antibody coagulation were examined. This study also described the shape and aggregation of indigenous bacteria captured by the ferrographic separa-

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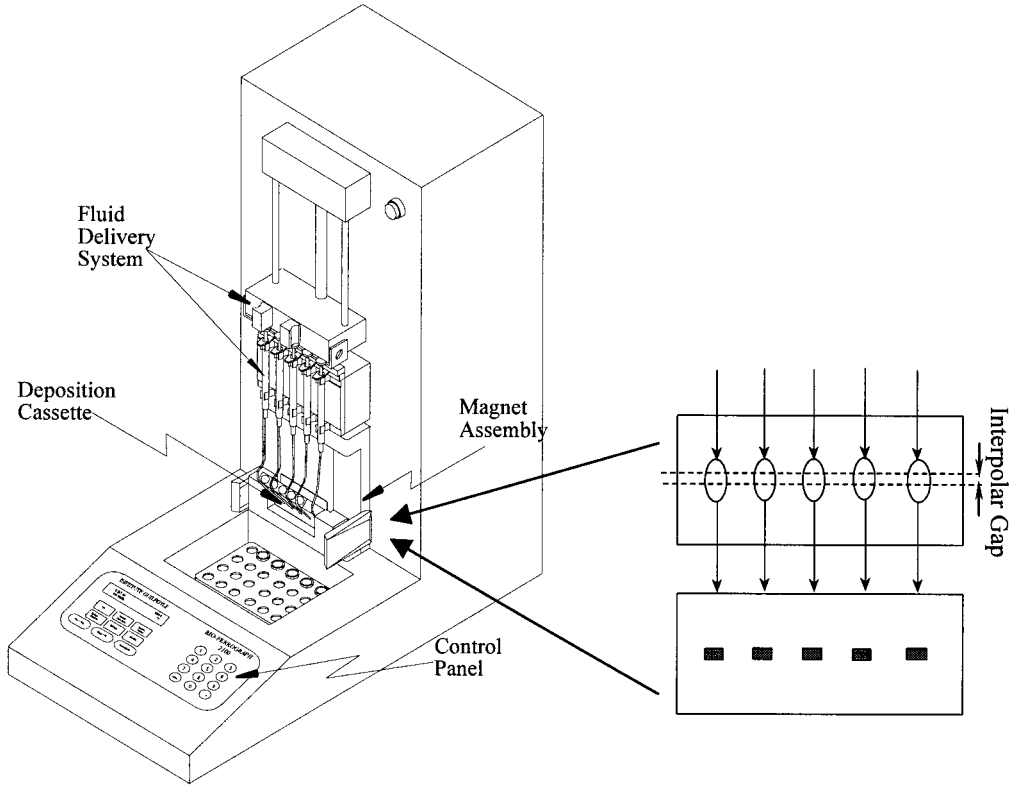

FIGURE 1. Bio-Ferrograph (Institute Guilfoyle), the device used to deposit magnetically tagged bacteria onto the glass cover slip.

tion method and examined the ability to distinguish autochthonous from allocthonous bacteria.

Methods

Bio-Ferrograph. Ferrographic enumeration employs a Bio-Ferrograph (*16*) to remove immunomagnetically tagged bacteria from suspension. The Bio-Ferrograph utilizes a magnetic field that has a maximum field strength across an interpolar gap where the collection of magnetically susceptible particles occurs (Figure 1). The maximum magnetic field strength across the gap is 1.8 T. However, the gradient of that field is at a maximum at the edges of the gap, thereby concentrating deposition at the gap edges. The Bio-Ferrograph directs sample volumes through a small chamber over a glass substratum on the magnetic gap, allowing efficient collection of magnetically susceptible particles. Minimal sample−apparatus contact, involving transport through about 7 cm of Teflon tubing prior to deposition onto the glass slide, minimizes loss of bacteria from suspension upstream of the deposition chamber. The configuration employed allows simultaneous processing of five samples, allowing blanks to be run along with replicates or other samples.

The advantage of the ferrographic enumeration technique is the extremely small area of deposition in the interpolar gap. This interpolar area comprises about 0.011 cm^2, an area of deposition that is nearly 1000 times smaller than the area of deposition achieved when filtering through a typical filter. For a given sample volume, this decrease in deposition area alone results in an increase in sensitivity by 3 orders of magnitude over filtering.

Bacteria. The studies described in this paper examine enumeration of *Escherichia coli* strain XL1-Blue, obtained from Dr. Joseph Holden of the Department of Pathology at the University of Utah Medical Center. The bacteria were grown in 10% tryptic soy broth to a stationary phase of about 10^9 cells/mL. At the beginning of stationary phase, the cells were diluted into suspensions ranging from 10^3 to 10^4 cells/mL with Milli-Q water containing 150 mM NaCl. These suspensions were added to streamwater samples to provide spiked cell concentrations ranging from 200 to 800 cells/mL.

Immunomagnetic Separation. Fluorescein isothiocyanate (FITC)-conjugated polyclonal rabbit antibodies raised to whole and ruptured cells of *Escherichia coli* (Virostat, Inc.) were attached to anti-FITC-coated paramagnetic beads (50 nm diameter, Miltenyi Biotec) by addition of 10 μL of stock antibody serum (5 mg/mL) and 10 μL of stock bead solution to 1 mL of Milli-Q water. The solution was vortexed at low speed for 15 min at room temperature. Removal of antibodies unattached to beads was performed with MACS MS^+ separation columns (Miltenyi Biotec) using the manufacturer's protocol to give 0.5 mL of bead−antibody suspension. The bead−antibody suspension (100 μL) was added to 1 mL of aqueous bacterial suspension and was also vortexed at lowest speed for 15 min at room temperature.

The bacteria−bead suspension was introduced to the Bio-Ferrograph at a 0.008 mL/min flow rate, the flow rate determined to be the maximum that produced 100% recovery for the combination of magnetic beads and antibodies used in this study (*15*). DAPI (4′,6-diamidino-2-phenylindole), which nonselectively stains cells by interacting with the DNA, was introduced to the Bio-Ferrograph at the end of each run to stain the captured cells and to allow visual identification under an epifluorescence microscope.

Source Waters. The ability of the ferrographic enumeration technique to quantitatively retrieve a low spike of bacterial cells (ranging from 200 to 800 cells/mL) in water from Mill Creek, Salt Lake County, UT, was examined. Retrieval of spiked bacteria from Milli-Q water containing kaolin colloids (10 mg/L) was examined to determine the effect of mineral colloids on spike recovery. Sewage treatment effluent (secondary treated) from Salt Lake City, UT, was also examined in terms of the qualitative ability of the ferrographic enumeration technique to capture nonculturable bacteria in the presence of elevated concentrations of dissolved organic matter. Retrieval of spiked bacteria from

TABLE 1. Percent Recovery of Spike from the Various Waters Examined in This Study[a]

	Mill Creek	Milli-Q + kaolin (10 mg/L)			Mill Creek		
amendment	none	none	skim milk (1%)	BSA (1 mg/mL)	skim milk (1%)	skim milk (1%) EDTA (2, 10 mM)	BSA (1 mg/mL) EDTA (2, 10 mM)
% recovery	0, 0	0	>80	>80	32, 12, 8	85, 81	90, 97
spike concn (cells/mL)	~500	~750*	~750*	~375*	~500	375	187
comments	possible nonspecific antibody loss to colloids	nonspecific antibody loss to clays	nonspecific association effectively blocked by skim milk	nonspecific association effectively blocked by BSA	antibodies appeared to have coagulated	good recovery in presence of skim milk and EDTA	complete recovery in presence of BSA and EDTA

[a] Commas separate replicate results or results from experiments with differing EDTA contents. An asterisk (*) means estimated based on concentration at stationary phase.

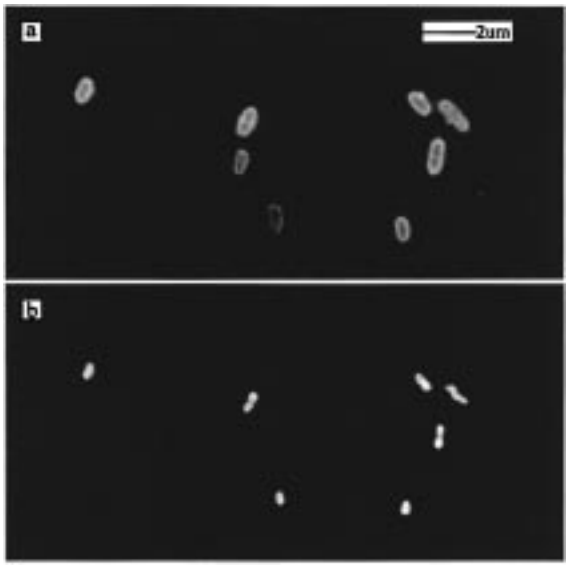

FIGURE 2. Spiked *Escherichia coli* recovered from Milli-Q. Illuminated inder (a) FITC epifluorescence and (b) DAPI epifluorescence.

Milli-Q water gave complete recovery under the conditions of these experiments (*15*). Hence, experiments examining spike recovery from Milli-Q were run simultaneously with those examining Mill Creek water to determine the spiked cell concentrations. Otherwise the spiked concentration was determined from the estimated cell count at stationary phase, which averaged 10^9 cells/mL, but varied by a factor of 2 between cultures. Prior to analysis, all solutions (except sewage effluent) were ammended to give 150 mM NaCl, an optimal salinity for immunomagnetic tagging (*15*). Skim milk (1%) and bovine serum albumin (BSA) (1 mg/mL) were examined as blocking agents in this study. EDTA (ethylenediaminetetraacetic acid) (2−10 mM) was utilized as a complexing agent. These agents were added to the bacteria bead suspension prior to vortexing.

Results and Discussion

Complete recovery of spiked bacteria was achieved from Milli-Q due to lack of mineral colloids, dissolved cations, or organic macromolecules, which can interact nonspecifically with the antibodies as described below. Images of bacteria captured from Milli-Q water were clear and free of mineral and organic debris (Figure 2). Bacteria illuminated under FITC epifluorescence (Figure 2a) show larger size relative to bacteria illuminated under DAPI epifluorescence (Figure 2b).

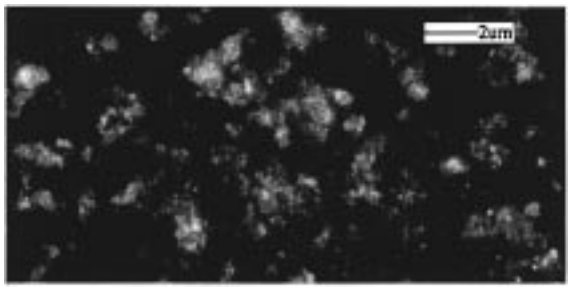

FIGURE 3. Mineral colloids recovered from unmodified Mill Creek water. Illuminated under FITC epifluorescence.

The apparent size difference occurs due to the FITC fluorophore being contained in the linkage between the bacterial antibody and the magnetic bead, thereby outlining the bacteria with hundreds of minute fluorescent points, whereas DAPI was taken up internally by the cell. In contrast to the excellent spike recovery from Milli-Q water, the recovery of spiked bacteria from unmodified Mill Creek water was negligible (Table 1). Here the term unmodified means no addition of blocking or complexing agents. Deposits ferrographically recovered from unmodified Mill Creek water showed no bacteria but many mineral colloids (Figure 3). Deposition of mineral colloids from unmodified Mill Creek water presumably occurred due to nonspecific interaction with antibody−bead complexes, as evidenced by the FITC coatings on the deposited colloids. The apparent lack of bacterial deposition may have been due to lack of available antibodies for attachment due to nonspecific association with mineral colloids or possibly due to burial of deposited bacteria beneath mineral colloids.

Nonspecific interaction of antibodies with mineral colloids was demonstrated by the low recovery of spiked bacteria in Milli-Q water samples containing kaolin colloids (10 mg/L) (Table 1). Addition of skim milk or BSA to the kaolin-ammended Milli-Q water resulted in apparent complete recovery of spiked bacteria (Table 1), although this result is qualitative since a Milli-Q blank was not run simultaneously to determine exact spike concentration. Nevertheless, the beneficial effect of the blocking agent was obvious.

In contrast to the beneficial effect of a blocking agent in Milli-Q water with kaolin, the addition of skim milk to Mill Creek water did not improve bacterial recoveries (Table 1). The majority of the antibodies in these samples coagulated into aggregates of various sizes estimated to be about 0.5 μm^3 and smaller (determined visually by epifluorescence microscopy). Coagulation of macromolecules may have occurred due to the presence of divalent cations such as calcium and magnesium (i.e., cation bridging). Since calcium

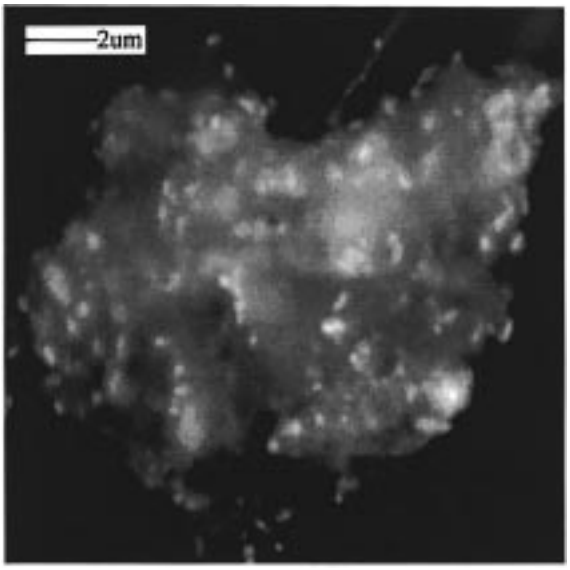

FIGURE 4. Aggregate of indigenous bacteria in Mill Creek water. Illuminated under DAPI epifluorescence.

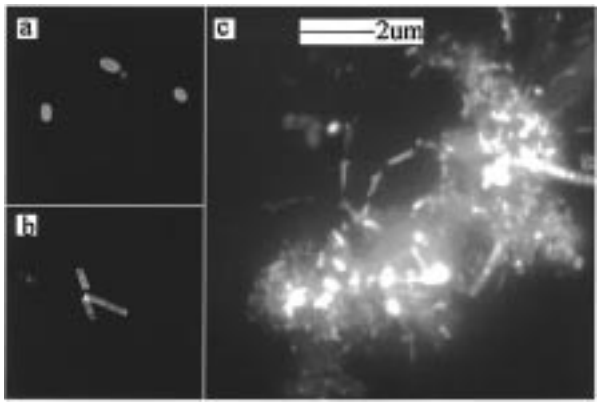

FIGURE 5. Microbes captured from sewage treatment effluent. Three shapes exclusively and consitently observed: (a) oval-shaped, (b) cyclindrical rods, and (c) aggregates of small rod-shaped bacteria associated with unidentified segmented strands. Illumination of panels a and b under FITC epifluorescence. Illumination of panel c under DAPI epifluorescence.

and magnesium are significant in Mill Creek water (typical of the hard waters of the intermontane region of the western United States), it was hypothesized that coagulation might be mitigated by addition of a complexing molecule such as EDTA. Addition of EDTA to Mill Creek water at concentrations ranging from 2 to 10 mM resulted in much improved recoveries that were independent of the two EDTA concentrations examined. However, recoveries in the presence of BSA (1 mg/mL) were slightly higher relative to recoveries in the presence of skim milk (Table 1).

Indigenous bacterial strains were also captured by the polyclonal antibodies used in this study. The indigenous strains were easily differentiated from the spiked bacteria by their relatively small size (0.2 μm) and by the fact that indigenous strains occurred in diffuse aggregates (Figure 4), suggestive of possible entrainment in polymeric material such as extracellular polysaccharides or other natural organic material. In contrast, the spiked bacteria were relatively large ($\sim$1 μm in longest dimension) and were always monodisperse. The indigenous bacteria were observed to be present in concentrations of about 50 aggregates/mL, with numbers ranging from about 10 to 100 cells/aggregate. Routine membrane filtration studies of Mill Creek water, performed by the Salt Lake City Public Utilities Laboratory simultaneously with our experiments, indicated total coliform counts of around 50−60 colony forming units (cfu)/mL. Around 80% of these counts represented fecal coliform bacteria (personal communication, Florence Reynolds, Salt Lake City Public Utilities Water Quality Division). Because polyclonal antibodies were used in this study, the method presumably captured bacterial strains closely related to *E. coli* in addition to *E. coli* proper. Although the comparison between our results versus results from membrane filtration is not straightforward, it is nevertheless interesting that the number of clumps recovered by the ferrographic method is on the order of the number of cfu determinined from membrane filtration, indicating that the observed colonies were most likely formed from cell aggregates rather than individual cells.

The number of autochthonous bacteria visually identified in Mill Creek water using ferrography was an order of magnitude higher than the number of cfu detemined from membrane filtration. Hence, the visual identification used in ferrographic enumeration may provide additional information not available from other methods. Such information may be important in understanding characteristics governing bacterial transport, e.g., the use of aggregate diameter rather than individual diameter to predict filtration behavior in the subsurface. Likewise, the surface chemistry of the polymer-entrained aggregate may differ significantly from the surface chemistry of individual bacteria, which is another important consideration in transport.

Capture of autochthonous (not spiked) bacteria from sewage treatment effluent (secondary treated) was performed without the addition of blocking or complexing agents in order to qualitatively examine the effect of elevated dissolved organic matter on ferrographic separation. This experiment provided qualitative information since the spike addition was not performed on these samples. Samples were collected on four different occasions during the summer of 1998, with three replicates run on each sample. Samples were diluted 1:100 due to the extremely high cell concentrations recovered. Cell counts were observed to change insignificantly over time; hence, all results were averaged. Three distinct shapes of microbes were exclusively and consistently observed: ovoid-shapes, cylindrical rods, and large aggregates of very small rod-shaped bacteria associated with segmented strands of unknown material (Figure 5). Cell counts averaged 8900 $\pm$ 4700 cells/mL (n = 12), and these tended to be distributed in aggregates with about 300 $\pm$ 100 aggregates/mL (n = 12). Routine most-probable-number estimates conducted by personnel at the Salt Lake City Wastewater Treatment Plant yielded about 5−10 cells/mL for this same period. Clearly, the ferrographic separation method was effective in capture of nonculturable bacteria even in the presence of elevated concentrations of dissolved organic matter.

Having demonstrated that ferrographic separation can be successfully applied in natural waters of significant mineral and organic colloid content, we are proceeding to utilize the method in studies of bacterial transport in the laboratory and, subsequently, the field. In these studies, the bacteria will be stained (DAPI) prior to transport to allow easy differentiation from autochthonous strains. DAPI is an especially useful stain for this purpose since it is long lasting, and it associates with cell DNA, thereby minimizing alteration of cell surface characteristics and inhibiting cell growth (*2*).

Ferrographic separation may also provide a useful system to separate subsets of bacteria from larger populations for further analyses. Since the method holds captured bacteria in place magnetically in a closed chamber, it is simple to subsequently introduce additional reagents (e.g., growth media, molecular probes) to determine attributes such as organism viability and to differentiate organisms beyond the

degree offered by the antibodies used in capturing the organisms.

An advantage of the ferrographic separation method is the small volume of solution required, which is a concern in field transport studies where limited sample volumes may be retrieved. Additionally, relative to serial dilution and culturing, there is a relatively small amount of waste generated by the method.

The presence of large numbers of magnetically susceptible natural colloids would possibly interfere with ferrographic analysis; however, this problem has not been observed in the relatively limited number of samples examined in our work to date. Furthermore, natural magnetically susceptible colloids can be removed from solution by an initial pass through the Bio-Ferrograph prior to immunomagnetic tagging. The key to success using this approach, of course, is the ability to find or develop antibodies to the organism of interest. If the antibody is available commercially, as was the case in this study, the cost ranges roughly between $1 and $5 per analysis, including both microbeads and antibodies. If the antibody is not available, there are ever-increasing numbers of commercial and university laboratories capable of developing antibodies to organisms, and their fees range from one to several thousands of dollars depending upon the specific antibody desired.

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