Quantitative and Qualitative Analysis of Bacteria in Er(III) Solution by Thin-Film Magnetophoresis

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Magnetic deposition, quantitation, and identification of bacteria reacting with the paramagnetic trivalent lanthanide ion, Er3+, was evaluated. The magnetic deposition method was dubbed thin-film magnetophoresis. The optimization of the magnetic deposition protocol was accomplished with Escherichia coli as a model organism in 150 mM NaCl and 5 mM ErCl3 solution. Three gram-positive bacteria, Staphylococcus epidermidis, Staphylococcus saprophyticus, and Enterococcus faecalis, and four gram-negative bacteria, E. coli, Pseudomonas aeruginosa, Proteus mirabilis, and Klebsiella pneumoniae, were subsequently investigated. Quantitative analysis consisted of the microscopic cell count and a scattered-light scanning of the magnetically deposited material aided by the computer data acquisition system. Qualitative analysis consisted of Gram stain differentiation and fluorescein isothiocyanate staining in combination with selected antisera against specific types of bacteria on the solid substrate. The magnetic deposition protocol allowed quantitative detection of E. coli down to the concentration of 103 CFU ml--1, significant in clinical diagnosis applications such as urinary tract infections. Er3+ did not interfere with the typical appearance of the Gram-stained bacteria nor with the antigen recognition by the antibody in the immunohistological evaluations. Indirect antiserum-fluorescein isothiocyanate labelling correctly revealed the presence of E. faecalis and P. aeruginosa in the magnetically deposited material obtained from the mixture of these two bacterial species. On average, the reaction of gram-positive organisms was significantly stronger to the magnetic field in the presence of Er3+ than the reaction of gram-negative organisms. The thin-film magnetophoresis offers promise as a rapid method for quantitative and qualitative analysis of bacteria in solutions such as urine or environmental water.

Bacterial growth in culture has long been established as a reference method in bacteria quantitation and identification. Typically, bacterial growth in culture may take several days and requires substantial investment in equipment and personnel training (2). The demand for quicker and possibly less accurate tests than cell culture has generated interest in rapid bacterial screens. At present, various rapid screens are available commercially, from a relatively simple dip stick method based on the detection of bacterial metabolites to methods based on computerized image recognition (10). In this paper, we describe a method of a magnetic deposition of bacteria which concentrates cells from solution directly on the microscopic slide, in a manner suitable for rapid cell count and staining. This method was evaluated for its potential use in the clinical microbiology laboratory, in particular for urine analysis.

Previous studies have demonstrated that the presence of a trivalent lanthanide ion, Er3+, as 5 mM ErCl3 in 150 mM NaCl solutions containing bacterial cells, renders these cells susceptible to the external magnetic field (1, 12, 16, 17). The cells acquire the magnetic susceptibility by ionic binding of Er3+ to the bacterial wall, especially at an elevated pH of 8 to 10 (16). These cells, when exposed to a strongly divergent magnetic field acting on a thin film, 210 μm in height, of a flowing cell suspension, formed a solid deposition mass on the wall in contact with the source of the magnetic field (16). This process, which henceforth we propose to name the thin-film magnetophoresis, was shown to be amenable to a quantitative analysis. The reaction time of Er3+ with bacterial cells occurs in minutes, and the magnetically sensitized cells respond almost instantly to the magnetic field in the thin-film arrangement (16).

This study is focused on calibration of the magnetic cell deposition in the presence of Er3+ at various cell concentrations in the initial suspension and on evaluation of this method in combination with histological staining. The direct cell count in the magnetic deposition was complemented by the scattered-light analysis of the solid deposition of the cellular material. The calibration of the magnetic separation was performed on Escherichia coli with cell culture and colony counting as a reference method. The magnetic cell deposition at the initial cell concentration of 106 CFU ml--1 was verified for seven bacterial species important in urine analysis; gram-positive Staphylococcus epidermidis, Staphylococcus saprophyticus, and Enterococcus faecalis and gram-negative E. coli, Pseudomonas aeruginosa, Proteus mirabilis, and Klebsiella pneumoniae. The immunofluorescence staining was performed with rabbit anti-bacterium antisera and fluorescein isothiocyanate (FITC) against E. faecalis and P. aeruginosa.

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MATERIALS AND METHODS

Bacterial strains. Three gram-positive bacteria, S. epidermidis, S. saprophyticus, and E. faecalis, and four gram-
negative bacteria, *E. coli*, *P. aeruginosa*, *P. mirabilis*, and *K. pneumoniae*, were evaluated in this study. They were procured from the Quality Control depository of the Department of Microbiology at the Cleveland Clinic Foundation, Cleveland, Ohio. Cells were transferred from -60°C onto agar plates (Prepared Culture Media; Scott Laboratories Inc., West Warwick, R.I.) and allowed to grow for 24 h at 35°C prior to the experiment. Cell colonies were collected from the agar plates and resuspended in sterile 150 mM NaCl (0.9% sodium chloride for irrigation USP, Travenol Laboratories, Deerfield, Ill.) prefiltered with a 0.2-µm-pore-size filter (Gelman Sciences, Ann Arbor, Mich.). The turbidity of cell suspension was adjusted to approximately 10⁷ CFU ml⁻¹ with a number 0.5 McFarland standard of BaCl₂ (aqueous). The stock suspension was sequentially diluted 10-fold in sterile 150 mM NaCl until the desired nominal concentration was reached. The concentration of *E. coli* was varied from 10⁶ to 10⁸ CFU ml⁻¹; the concentration of other strains was held constant at approximately 10⁷ CFU ml⁻¹.

The actual cell concentration in samples used in the experiment was verified by placing approximately 100 µl of cell suspension on the agar plate and by colony counting following 24 h of incubation at 35°C. To inhibit cell growth in suspension during the time of the experiment, the suspensions were kept on ice. Prior to addition of ErCl₃ to the cell suspension, the pH of the suspension was adjusted to 8.5 by dropping 10 µl of 50 mM NaOH.

Thin-film magnetophoresis. The experimental arrangement is presented in Fig. 1A, and the close-up view of the thin-film flow chamber at the high-gradient magnetic field area is shown in Fig. 1B. The permanent magnet assembly consisted of two pairs of the neodymium-iron-boron magnets with the maximum energy product of 28 MG Oersted and the dimensions 25 by 25 by 13 mm. The magnets were connected by soft iron pieces concentrating the magnetic flux lines at the interpolar gap, 1.4 mm wide, filled with aluminum alloy 6061 spacer. The flat-top surface of the magnet block was held inclined at 7.5° to the horizontal plane to aid the flow of solution across the interpolar gap. The thin-film flow chamber consisted of two macroscopic coverslips (dimensions, 60 by 24 by 0.142 mm; Fisher Finest Premium Cover Glass No. 1; Fisher Scientific, Pittsburgh, Pa.), separated by a three layers of double-side adhesive tape (Scotch Brand; 3M Co., St. Paul, Minn.). The cover slides were pretreated with acid ethanol (1% HCl in 70% ethanol solution) and then rinsed in the double-deionized water and air dried. The width of the thin-film flow between the two coverslips was defined by hydrophobic barriers drawn on the inside surfaces of the coverslips with a wax pencil (Berol Blaisdell China Marker; Berol Co., Danbury, Conn.). The width of the following film of solution was 5.5 mm; the height was 210 µm. The solution was collected at the end of the channel with a wick formed from three pieces of filter paper (FisherBrand, Qualitative P5; Fisher Scientific). Three milliliters of the bacterial cell suspension with the cell concentration adjusted to the preset value and pH adjusted to pH 8.5 was mixed with 100 µl of 150 mM ErCl₃ stock solution, to a final concentration of 5 mM ErCl₃. The suspension was immediately transferred to the thin-film flow channel by means of a chromatographic roller pump (Atto, Tokyo, Japan), at a rate of 1 ml min⁻¹, corresponding to the average flow velocity in the thin film of 1.4 cm s⁻¹.

Quantitative analysis of the magnetic cell deposition. The number of cells removed from the film of flowing suspension by the magnetic field and deposited on the bottom coverslip was evaluated directly by transmitted light microscopy and indirectly by measuring the scattered light intensity with a dark-background microscopy. In order to acquire the most representative cell count sample, the cells were viewed at evenly spaced positions along the entire length of the interpolar gap. The total surface area used for cell counting was relatively large for the low and intermediate cell concentration (A = 31.1 × 10⁻² and 1.23 × 10⁻² mm², respectively) and relatively small (A = 0.123 × 10⁻² mm²) for the high cell concentration. The total number of cells, n, divided by the total surface area, A, characterized the cell deposition density, σ, in millimeters⁻². The relationship between σ and cell concentration in suspension, c, subjected to the magnetic field was examined with calibration plots σ = f(c). The microscopic observation of the magnetic deposition has shown that it extends to approximately half the width of the interpolar gap on both sides of the gap, and therefore the total number of cells deposited in the magnetic field was estimated by multiplying σ by twice the surface area in contact with the interpolar gap, 2 × L × W = 2 × 1.4 mm × 5.5 mm = 15.4 mm². The magnetic cell capture efficiency, e, was calculated as a ratio of the total cell number deposited on the slide, σ × 15.4, to the total cell number in the solution.
prior to the magnetic separation, $N$ (equal to the product of the sample volume, 3 ml, and cell concentration, $c$, in the sample). The value of $e$ was calculated for all seven bacterial strains, which were processed at the same initial cell concentration of $c = 10^6$ CFU ml$^{-1}$. The difference in the average $e$ calculated separately for gram-positive and gram-negative organisms was analyzed for statistical significance with the nonpaired Student $t$ test. The reduced data distribution around the mean is given by the standard error.

The indirect evaluation of the magnetic cell deposition consisted of scanning the length of the slide by dark-background microscopy and recording the intensity of the (visible) light scattered by the cells on the slide as a function of distance down the slide. This method has been modified from previous studies (16). In brief, a photographic exposure meter attached to the microscope (BH-2; Olympus, Tokyo, Japan) was used as the light intensity sensor. In a configuration with a 10× objective (DPPlan 10, 0.25, 160/0.17; Olympus) and a 10× ocular (WK 10×20L; Olympus), the sensor collected light from an area of about 50 $\mu$m in diameter. A constant-speed, motorized longitudinal displacement of the microscopic stage was recorded with a 10-revolution potentiometer coupled to the stage knob. The dark background was produced by offsetting the microscopic condenser. The light exposure readings, in seconds, were made at the film speed setting of 50 ASA. The differences in electric potentials representing the microscopic stage position ($X$ signal) and the light intensity ($Y$ signal) were fed to the computer data acquisition system (CODAS; Data Instruments, Akron, Ohio). The average intensity of light scattered by the magnetic deposition of cells was calculated as the area under the curve $Y = f(X)$ and cut by a baseline recorded in the area of the slide away from the contact with the interpolar gap. The average intensity of the scattered light is denoted by $ASL$. The relationship between $ASL$ and the cell deposition density, $\sigma$, was analyzed with plots $ASL = f(\sigma)$.

**Qualitative analysis of the magnetic cell deposition.** Prior to histological staining of the magnetic cell deposition, the top slide forming the thin-film flow chamber was removed. The Gram stain was applied by a standard protocol utilized for differentiation of cells in smears (2). All the reagents, i.e., crystal violet, iodine, acetone, and safranin solutions, were prefiltered with a 0.2-$\mu$m-pore-size filter (Gelman Sciences). The stained slides were examined microscopically at 1,000× magnification with an immersion oil. The fluorescein-antibody staining was carried out according to a protocol developed for tissue staining (Department of Immunopathology, The Cleveland Clinic Foundation). The primary antibodies used in this study were rabbit antisera directed against $P$. aeruginosa (anti-$P$. aeruginosa antiserum, Bacto-$P$. aeruginosa; Difco Laboratories, Detroit, Mich.), and against Streptococcus (anti-Streptococcus antiserum, Bacto-Streptococcus; Difco). The latter antiserum is also specific to Enterococcus spp. (previously classified as the genus Streptococcus). The antisera were diluted in phosphate-buffered saline and applied to the cellular deposition on the slide, and then incubated for 20 min at room temperature. Slides were rinsed in the double-deionized water and the secondary antibody conjugated to FITC was applied (anti-rabbit immunoglobulin G-FITC conjugate; Sigma Chemical Co., St. Louis, Mo.). Slides were incubated for 20 min at room temperature and rinsed with double-deionized water. Appropriate working concentrations of the antisera ensuring minimum nonspecific binding were determined by chessboard titration with serial dilutions of the antiserum. The cell fluorescence was observed by fluorescence microscopy (UV Microscope, IV FL Epifluorescence condenser, and Microscope Illuminator 100; Zeiss, Oberkochen, Germany). The specificities of the antisera towards the target cell in the magnetic deposition were tested by using mixtures of $P$. aeruginosa and $E$. faecalis and comparing cell fluorescence in samples stained with anti-$P$. aeruginosa with that in samples stained with the anti-$E$. faecalis. The presence of both types of cells in the magnetic deposition was verified by comparing the appearances of cells in the immunostained material with that in the Gram-stained one ($P$. aeruginosa is gram negative; $E$. faecalis is gram positive).

**RESULTS**

The thin-film magnetophoresis produced tracings of cellular deposition on slides in the area in contact with the interpolar gap. The macroscopic appearance of the cell deposition ($E$. coli) was presented in Fig. 1A (cf. Fig. 1B). Cell deposition, indicated by an arrow, was concentrated in the area twice the width of the interpolar gap. In Fig. 2B to G, a sequence of microphotographs presenting microscopic appearance of cells ($E$. coli) against dark background, with the increasing cell concentration (B, control; C through G, $10^0$, $10^1$, $10^2$, $10^3$, and $10^6$ CFU ml$^{-1}$, respectively), is shown. Particles observed on the control slide (Fig. 2B) were identified as debris produced in the manufacturing process of the thin-film flow channel. The presence of such debris can also be observed in slides with low cell deposition density, $\sigma$, corresponding to $10^0$ CFU/ml in the initial suspension. The high-intensity optical signal coming from the light scattered by the cell, as well as the increase in the magnetic deposition density with the increasing concentration of cell in suspension, is evident from these microphotographs.

The results of the quantitative thin-film magnetophoresis are presented in Table 1 and in Fig. 3. In Table 1, cell counts of $E$. coli in the magnetic deposition are presented. The presence of bacteria was detected in all slides produced with $10^2$ CFU ml$^{-1}$ and higher cell concentrations but was not detected in some slides produced with $10^3$ CFU ml$^{-1}$ and lower cell concentrations. This result suggests limitations of the direct cell counting method, which excludes those cells that are outside of the observation area, and also indicates that not all of the cells may be trapped by the magnetic field. The dependence of the magnetic cell deposition density, $\sigma$, on cell concentration in suspension, $c$, is presented in Fig. 3 ($E$. coli). The average cell capture efficiency, $e$, varied from $1.3 \pm 0.5 (n = 3)$ to $0.6 \pm 0.3 (n = 3)$, with $c$ varying from $10^0$ to $10^6$ CFU ml$^{-1}$, respectively.

The scattered-light scan across the magnetic cell deposition permitted a continuous analysis of the cellular material along a narrow pathway parallel to the flow channel. The correlation between the $ASL$ and the cell deposition density, $\sigma$, is presented in Fig. 4 ($E$. coli).

The magnetic cell capture efficiency, $e$, measured at the same cell concentration in solution, $c = 10^6$ CFU ml$^{-1}$, varied for different bacterial strains. The differences in $e$ were not significant within the same Gram class of organisms, viz., $E$. coli, $1.14 \pm 0.28$; $P$. aeruginosa, $3.28 \pm 1.39$; $P$. mirabilis, $1.23 \pm 0.27$; and $K$. pneumoniae, $0.46 \pm 0.07$ (gram negatives) and $S$. epidermidis, $8.06 \pm 0.61$; $S$. saprophyticus, $4.85 \pm 0.15$; and $E$. faecalis, $3.00 \pm 0.82$ (gram positives) (all strains, $n = 4$). The difference in $e$ was significant, however, between the gram-positive and gram-negative classes of the organisms, $5.31 \pm 2.52 (n = 12)$ and $1.52 \pm 1.85 (n = 16)$, respectively ($P < 0.001$).
FIG. 2. Photographs of the magnetic deposition of *E. coli* in 150 mM NaCl plus 5 mM ErCl₃ solution, in the dark-background mode. (A) Macrophotograph of the thin-film flow channel, with the magnetic deposition marked by an arrow (cell concentration, 10⁸ CFU/ml). (B through G) Microphotographs of the magnetic deposition in the increasing order of the cell concentration in the solution; 0 (control), 10⁵, 10⁶, 10⁷, and 10⁸ CFU/ml, respectively.

The results of the qualitative thin-film magnetopheresis are summarized in Fig. 5. In Fig. 5A, the mixture of *P. aeruginosa* and *E. faecalis* after Gram staining is shown. Differentiation between gram-positive and gram-negative organisms is clearly visible. In Fig. 5B, the same mixture of the organisms was subjected to immunohistological staining with anti-*P. aeruginosa* antibody-FITC and showed the highly visible gram-negative organisms in the fluorescence microscope and the absence of the gram-positive organisms. This result indicates that the anti-*P. aeruginosa* antibody retains its specificity against the bacterial cells treated with Er³⁺. In the same mixture of cells, when treated with anti-*E. faecalis* antibody-FITC and observed under the fluorescence microscope (Fig. 5C), only typical gram-positive cells are visible. Thus, one may also conclude that the binding of Er³⁺ does not interfere with the specificity of the anti-*E. faecalis* antibody.

**DISCUSSION**

The magnetic removal of bacteria treated with Er³⁺ was accomplished with the use of the thin-film magnetopheresis, a term proposed here for a technique in which the magnetically labelled cells are removed from the thin film of the flowing cell suspension exposed to a strongly divergent magnetic field. The bacteria acquired a magnetic susceptibility because of the high atomic magnetic moment of erbium bound to the bacterial cell (5, 6). The magnetic susceptibility of bacteria was higher than that of the surrounding solution because of the concentration of Er³⁺ on the bacterial cell (3).
TABLE 1. E. coli counts in the magnetic deposition

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* n₁₄₆ E. coli counts 1 to 6; Avg n, average cell count; A (in mm²), area of the observation field; σ, magnetic cell deposition density (σ = Avg n/A [in mm⁻²]).

In this study, model solutions of bacteria typically found in bacteriuria, i.e., three gram-positive bacteria, S. epidermidis, S. saprophyticus, and E. faecalis, and four gram-negative bacteria, E. coli, P. aeruginosa, P. mirabilis, and K. pneumoniae, were analyzed. The solution contained 150 mM NaCl and 5 mM ErCl₃, with the pH adjusted to pH 8.5 with 50 mM NaOH. It was possible to avoid interference from potentially competitive reactions between Er³⁺ and noncellular components because of the lack (or trace amounts) of P, and other solutes, such as proteins, in the solution.

The high affinity of bacterial cells to heavy metals, to

FIG. 3. Log-log correlation between cell concentration in solution, c, and the magnetic cell deposition density, σ (E. coli). The log-log regression equation is log σ = 0.871 log c - 1.918 (n = 13); correlation coefficient is r = 0.976.

FIG. 4. Log-log correlation between average scattered-light density, ASL, and the cell density in the magnetic deposition, σ (E. coli). The log-log regression equation is log ASL = 0.882 log σ - 3.489 (n = 13); correlation coefficient is r = 0.896.
which erbium also belongs as a lanthanide, is well documented (9). The reported mechanism of the lanthanide binding to the bacterial cell is predominantly ionic and involves the negatively charged function groups on the bacterial cell wall (3). The characteristic properties of the Er$^{3+}$ reaction with the bacterial cells indicating its ionic character, such as high reaction rate and dependence on the pH of the solution, were reported earlier (13, 16). The saturation of the negatively charged functional groups on the cell wall in the reaction with Er$^{3+}$ is indicated by the linear log-log dependence between the magnetic cell deposition density, $\sigma$, and the cell concentration in suspension, $c$, over a wide range of log $c$, from 4 to 8. The saturation of the Er$^{3+}$ binding sites on the bacterial cell was also verified in earlier studies by evaluating $\sigma$ at increasing Er$^{3+}$ concentrations, from 0.2 to 10 mM (unpublished data). These findings compare well with other studies in which bacterial sorption of heavy metals was shown to plateau at the metal ion concentration of approximately 5 mM (9).

Saturation of the negatively charged function groups on the cell wall by 5 mM Er$^{3+}$ is also related to the observed difference in the magnetic cell deposition density, $\sigma$, between gram-negative and gram-positive species. In this study we found a statistically higher $\sigma$ for the gram-positive than for gram-negative bacteria, which is in accord with other studies on the metal sorption by bacteria and indicates generally higher affinity of gram-positive bacteria to the heavy metal ions than that of the gram-negative bacteria (9). The variability in the magnetic cell capture efficiency within each Gram class of the organisms was not significant and was as low as 0.46% (gram-negative K. pneumoniae) and as high as 8.06% (gram-positive S. epidermidis). The cell capture efficiency could be increased by decreasing the flow velocity of the cell suspension, set in this study at 1.4 cm s$^{-1}$, and thus reducing the hydrodynamic drag competing with the magnetic force (4, 15).

The magnetic cell deposition density, $\sigma$, correlated well with the average scattered-light intensity, ASL, analyzed with the use of the computer-assisted data acquisition system. Such an analysis suggests possible uses of the thin-film magnetophoresis in the automated, rapid screening of solutions suspected of bacterial contamination, especially those which are of a relatively simple composition compared with whole blood, such as urine, dialysis fluid, or environmental water samples (7, 8, 11). Further studies will be needed to evaluate the interferes with the magnetic cell deposition in solutions containing other components competing for binding with Er, such as P, and proteins.

The ionic and labile character of Er$^{3+}$ bonding to bacterial cells played a significant role in the results of the histological and immunohistological staining of the magnetic cellular deposit. Both types of staining revealed a typical appearance of the bacterial cells on a solid substrate, indicating lack of interference from Er$^{3+}$. This lack of interference could be explained by removal of Er$^{3+}$ from the bacterial cell by numerous washings in solutions deficient in erbium ion and of slightly acidic pH water rinses (14). The sensitivity of the erbium-binding bacteria concentrated in the magnetic deposition to Gram and immunofluorescence staining may increase the resolution of this method in samples containing other components in the magnetic deposition, such as proteins.

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