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METHOD EFFICIENCY AND SIGNAL QUANTIFICATION OF BACTERIA FOR A GROUNDWATER TRANSPORT EXPERIMENT

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Introduction

Bacterial transport is a key process in delivery of microbes to contaminated sites for bioremediation of chemicals. However, relatively little is known about the geochemical and hydrologic factors controlling the mobility of bacteria and viruses within subsurface systems. Laboratory-scale column studies have provided useful information (Harvey et al, 1989, 1993). However, successful application to *in situ* remediation will require that we identify and understand properties relevant to transport in aquifers. At the field scale, heterogeneities in hydrological and geochemical properties can significantly retard or enhance transport relative to that in homogeneous media.. Field experiments are needed to quantify the transport of biocolloids over a range of spatial scales, and to test the significance and predictive importance of microbial properties that influence bacterial mobility (e.g., the size, surface charge and hydrophobicity, and motility of the microorganisms, as well as relevant physiological functions such as production of exocellular biopolymers). Only through field experiments can we evaluate the scales of physical and chemical heterogeneity in natural aquifers that affect the transport of microbiota in ways not predicted from experiments conducted at the laboratory-scale.

Bacterial transport field experiments cannot be replicated as can column experiments. Rigorous testing of experimental hypotheses will require comparisons of the mobility of multiple strains with contrasting transport properties under identical field conditions. Consequently, a technique is needed to permit the transport of multiple strains of bacteria to be monitored simultaneously in a single field experiment. Molecular techniques can identify virtually any strain, without requiring a coincidence of a selectable phenotype and interesting transport properties. Other approaches are possible, such as the use of selective agar and colony-forming units (CFU) to detect bacterial breakthrough. However, even a range of resistances will not provide the broad flexibility provided by detection of unique DNA sequences.

DISCLAIMER

Molecular techniques can also detect very low levels of injected bacteria. Bacteria are often attenuated by several orders-of-magnitude during transport, and the transport of less-mobile strains is as important as highly mobile strains. The technique of DNA probe hybridization can be used to provide both identification and quantitation of the number of bacteria in a sample, and has been used effectively for many studies in microbial ecology (Sayler and Layton, 1990). The limits of detection with this method, while suitable for laboratory experiments, are often inadequate for field experiments. Polymerase chain reaction (PCR) has also been used successfully for the detection of microorganisms. The strength of PCR for analysis of environmental samples was evident from the work of Steffan and Atlas (1988), who used PCR to detect small populations of a specific *Pseudomonas* species in a high background of soil microorganisms. There have been many subsequent examples of the use of PCR to detect specific strains or groups of microorganisms, either as a screen for certain species in environmental samples or after addition of microorganisms to an ecosystem. This paper explores the use of PCR for identifying and enumerating the arrival of several individual strains of bacteria at monitoring wells downgradient of an experimental tracer injection well.

Materials and Methods

Microbial Strains

Pseudomonas fluorescens 5R was obtained from G. Sayler (University of Tennessee). This strain utilizes naphthalene as a carbon and energy source, and was isolated from a Manufactured Gas Plant soil. It contains a large catabolic plasmid that has demonstrated homology to the well-described NAH7 plasmid. Strain AO500, a Gram positive bacillus, was obtained from R. Bales (University of Arizona). Two strains were obtained from A. Mills (University of Virginia). Strain S139 is a Gram positive bacillus and Strain W5 is a Gram negative bacillus. A strain used as a control for DNA extraction and PCR amplification, *Pseudomonas putida* mt-2, was obtained from ATCC. This strain carries the large archetypal TOL plasmid.

Site Description/Environmental Samples

The field site is a well-characterized, unconfined, sandy coastal-plain aquifer at Clemson University's Baruch Forest Science Institute in Georgetown, SC that was the focus of a series of reactive and non-reactive tracer studies. The thickness of the aquifer is approximately 3 m, with a clay layer at the bottom that is assumed to be an impervious boundary of the aquifer. Injection and withdrawal wells were constructed of 5 cm slotted polyvinyl chloride pipe and separated by a distance of 5 m; sampling wells were spaced at 1 m intervals between injection and withdrawal wells in a highly conductive coarse sand layer (Figure 1). Groundwater was sampled (at a rate of 50 ml min^{-1}) using peristaltic pumps. The bacteria were captured on 0.22 um polycarbonate filters from 300-1000 ml samples, with addition of a fixed amount of control strain mt-2 spiked into the sample. The filters were immediately stored on dry ice.

Molecular Techniques

Total chromosomal DNA was obtained from all bacteria on the filters using a modified extraction procedure. PCR samples were run on 1% agarose gels in TAE buffer (Sambrook et al., 1989) at 3 volts cm^{-1}. Gels were stained with 1% ethidium bromide prior to fluorescence photography using a Fotodyne Polaroid camera mount. DNA markers included Lambda phage cut with *Hin*dIII and a DNA ladder marker (Gibco-BRL).

Polymerase Chain Reaction Conditions

A Perkin Elmer Cetus DNA Thermal Cycler was used for the Polymerase Chain Reaction. PCR was carried out for 40 cycles consisting of 94° C (1 minute), 50° C (1 minute), and 72° C (2 minutes). Taq DNA polymerase (Gibco-BRL) was used according to the manufacturer's specifications. Each of four nucleotides was available at an initial concentration of 200 μM. Final reaction volume was 25 microliters. Mineral oil was placed over the reaction solution to prevent evaporation. All DNA were obtained from Operon Technologies (Alameda, CA) and used at a final concentration of 1 μM each. Sequences for these primers are shown in Table 1.

After PCR amplification, 2 ul from the first PCR amplification was removed for use with the nested primers. The remainder was combined with 2.5 ul of loading dye (0.25% bromophenol blue in 40%

sucrose) in each tube and the contents were electrophoresed on 1 % agarose gels. Gels were treated as described above.

Results and Discussion

During the transport experiment DNA from bacteria collected by filtration of groundwater from monitoring wells was extracted and a small amount (1% of total DNA) was used in PCR reactions. PCR amplification of DNA extractions from groundwater samples collected from the injection and monitoring wells before the experiment revealed no background for any of the four test strains or for the mt-2 control strain. Each of the sampling sites was examined over a period of 10 days, although breakthrough was observed after a few hours. Under the conditions of these PCR analyses, a positive signal should result only from the detection of bacteria transported to the sampling site during the experiment.

Evidence indicates that the threshold for detection of a signal from 1 microliter of extracted DNA is 1000 bacteria when one round of PCR amplification is used. Reamplification is needed when the bacterial concentration is below 1000 bacteria in these experiments. This is equivalent to a detection limit of 10^5 bacteria per liter for one round of amplification, since only 1% of the total DNA is used for each PCR reaction. The limit of detection is more difficult to estimate when reamplification with the nested primers is used, although a lower limit of 10^2 bacteria per liter is likely. This is higher than the limits reported in the literature, probably because environmental samples are more difficult to work with than laboratory samples. Although humic acid contamination has been cited as a problem for PCR reactions, it is more likely that the presence of iron oxides in the groundwater was the deleterious factor. The DNA extraction protocol was modified by increasing the EDTA concentration to remedy this problem. In addition, there is the possibility that the DNA sample might be lost during sample processing. Either of these errors could result in a false negative response. In order to avoid false negative results, a known amount of the mt-2 strain was spiked into each 1 liter sample prior to filtering. Unless PCR amplification of this strain resulted in a positive response, the sample is suspect, and is marked in black in Table 2.

Results indicated that extraction efficiency for the mt-2 spike varied over a range of approximately two orders of magnitude among the samples examined. However, the extraction efficiency of the spiked DNA on each filter can be used as a correction factor for estimating concentration of

bacteria recovered from the groundwater. In some instances the signal for the mt-2 was missing, even after reamplification with nested primers. These results were discarded. In most instances, the absence of a signal was due to the loss of DNA from the sample, rather than the presence of contaminating substances. This was shown by spiking the sample with purified DNA and performing PCR amplification.

Results from this experiment demonstrate that transport followed a complex pattern. As summarized in Table 2, during the first hours after the start of the transport experiment the bacteria moved quickly through the aquifer. The S139, S15, and 5R strains are detected at well 3 initially, then wells 1, 5, and 6, and then the withdrawal well several hours later. The absence of signal from well 1 after the first few hours is due to the presence of substances that inhibit the PCR reaction. The AO500 strain was found at the Injection well during this time period, and appeared infrequently during the remainder of the experiment. There is a sharp contrast between the transport behavior of the bacteria and that of a nonreactive tracer. The nonreactive tracer moved as expected from the classical advection/dispersion theory. However, transport of the bacteria was much more erratic. It is difficult to ascribe a hydrologic cause for the irregular behavior. The coarse sand is well-sorted and the existence of discrete preferred flow paths is unlikely. The erratic breakthrough of the bacteria may reflect the discrete behavior of small numbers of cells attaching to--and detaching from--the porous media. Although there do not appear to be clear patterns in the arrival times of the different bacterial strains, there do appear to be systematic differences in the persistence of the bacteria over the 10 days of that the wells were monitored. The reasons for this extended tailing of some species is being examined.

Bacterial strains can be tracked during field experiments by utilizing PCR to amplify a specific fragment of DNA that is unique to that strain. DNA sequence information is required for this technique, although methods to obtain this information are well-established and do not present a major obstacle. The ability of PCR analysis to amplify weak signals is especially valuable for transport experiments, in which attenuation of bacteria may be significant. Although signal strength of the injected strain versus the control strain gives a useful approximation qualitative measure of breakthrough, more rigorous quantification of specific strains remains a labor-intensive activity.

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References

Harvey, R.W., L.H. George, R.L. Smith, and D.R. Leblanc. 1989. Transport of microspheres and indigenous bacteria through a sandy aquifer: results of natural- and forced-gradient tracer experiments. Environ. Sci. Technol. 23: 51-56.

Harvey, R.W., N.E. Kinner, D. MacDonald, D.W. Metge, and A. Bunn. 1993. Role of physical heterogeneity in the interpretation of small-scale laboratory and field observations of bacteria, microbial-sized microsphere, and bromide transport through aquifer sediments. Water Resources Res. 29: 2713-2721.

Sambrook, J., E.F. Fritsch, and T. Maniatis. Molecular Cloning - A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989.

Sayler, G.S. and A.C. Layton. 1990. Environmental application of nucleic acid hybridization. Ann. Rev. Microbiol. 44: 625-648.

Steffan, R.J. and R.M. Atlas. 1988. DNA amplification to enhance detection of genetically engineered bacteria in environmental samples. Appl. Environ. Microbiol. 54: 2185-2191.

Figure Legends

Figure 1: Schematic of the bacterial transport site.

Figure 2: Results from the transport experiment. In each box the detected strain(s) are listed, with those strains required reamplification with nested primers listed in parenthesis. Timescale is hours after start of injection. A gray box denotes no sample taken; a black box denotes a suspect sample. AO = AO500.

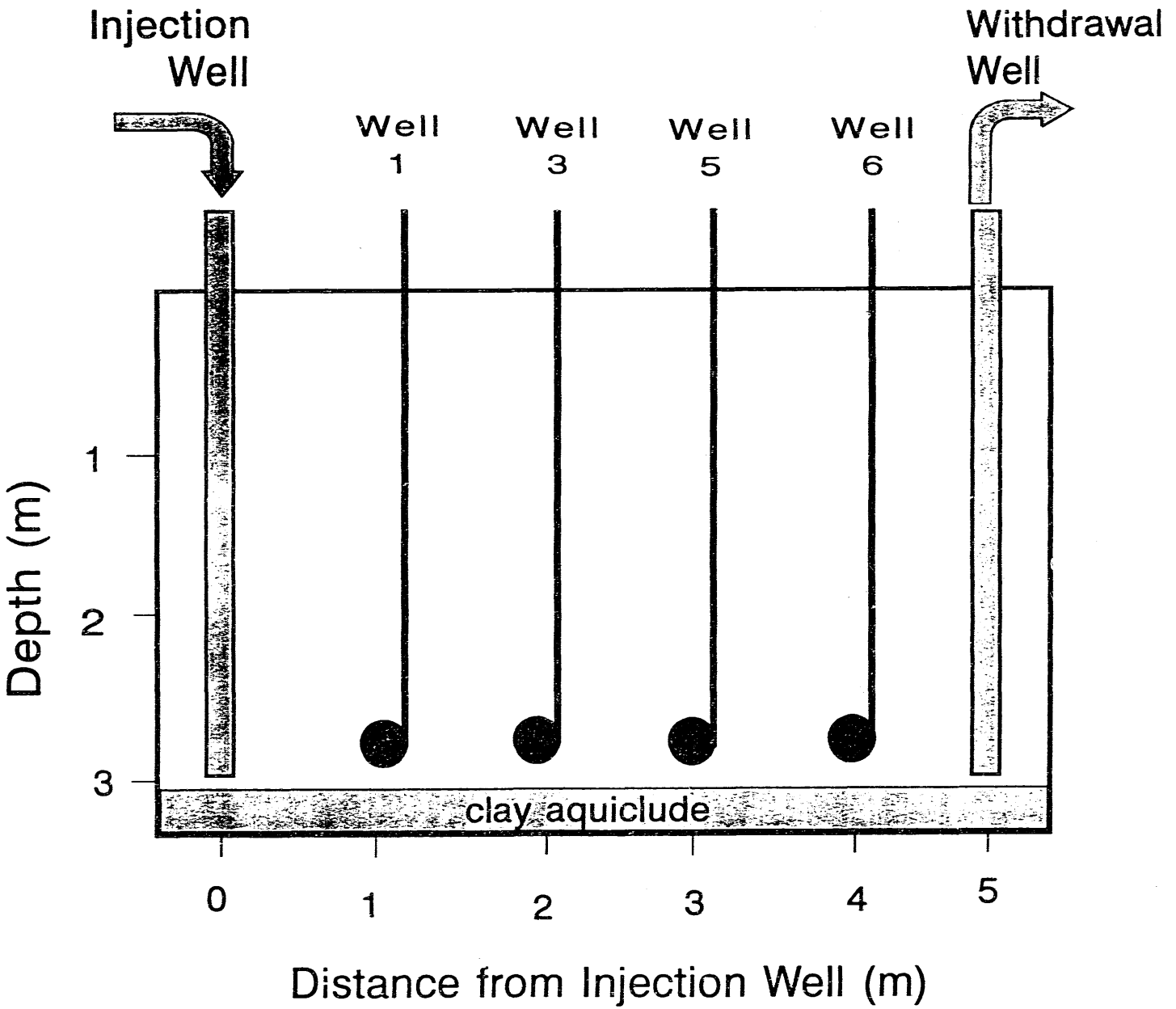
Table 1- PCR primers

Strain	Primers	Nested primers	Size of fragment
mt-2	AAACCCAAGATGCAGCATGA	ATATGCAAGACCTTAGCAGC	600 bp
	CTTGTAGGCTGGCGAATGGC	ATGCCGTAATACTGCCCATC	
AO500	GAGTGGGCATATTTCCCTTC	CAGCGGAACATCTGATTCGT	600 bp
	GATTGTGAGAGATACCGAAG	ACACAACATAAGACTCCCGC	
S139	GAGGATCGCTTTCCAGATGG	CGATGCCGAGGCGTGCGAGG	350 bp
	ATCCCACTCGATTCGATGCC	TTCCAGATGGCGGAGAGGGT	
W5	AGGGACTGGCCCTATGATAC	GCTCGGATGAATCATAAGCG	700 bp
	TAGTGAAGGACTCGTTGTCG	ACTGTGCCAATTCCAGCAAG	
5R	TCTGGGCTGACCCAAAAGCA	TGATTCATGGCGATGAAGAA	700 bp
	AGTCTGCGCTATGCACGCCT	ATATCCGTCCCACAACACAC	

Table 2

Hours	Injection	Well 1	Well 3	Well 5	Well 6	Withdraw
0		[redacted]				
2		S15		S15	[redacted]	(S139)
6		S15	5R S139 S15	(S139) S15	(S139) S15	
7	5R AO S15					
8	5R (S139) S15	(S139) S15	(5R) (S139) S15		(5R) (S139) S15	
10		(5R) S15	(5R) S139 S15	(5R) S15	S15	S139 S15
12	5R AO	5R (AO) S139 S15	(5R) S139 S15	5R (S139) (S15)	5R (AO) S139 S15	
14		[redacted]	S139 S15	(5R) S139 S15	(AO) (S139) S15	
16		(5R) S139 S15	(5R) S139 S15	(5R) S15	S139	
18	(AO) S15	[redacted]	(5R) (AO) (S139) S15	(S139) S15	(AO) S139 S15	5R S139 S15
20		(S139)	5R S15	(5R) (S139) S15	S139 S15	(5R) AO S139 S15
22	5R AO S139 S15	(S139)	5R (S139)	(S139)	AO (S139) (S15)	5R (S139) S15
24	(AO) (S139) S15	[redacted]	S15	(S139) S15	(S139) S15	

Fig. 1


Injection Well
Withdrawal Well
Well 1
Well 3
Well 5
Well 6
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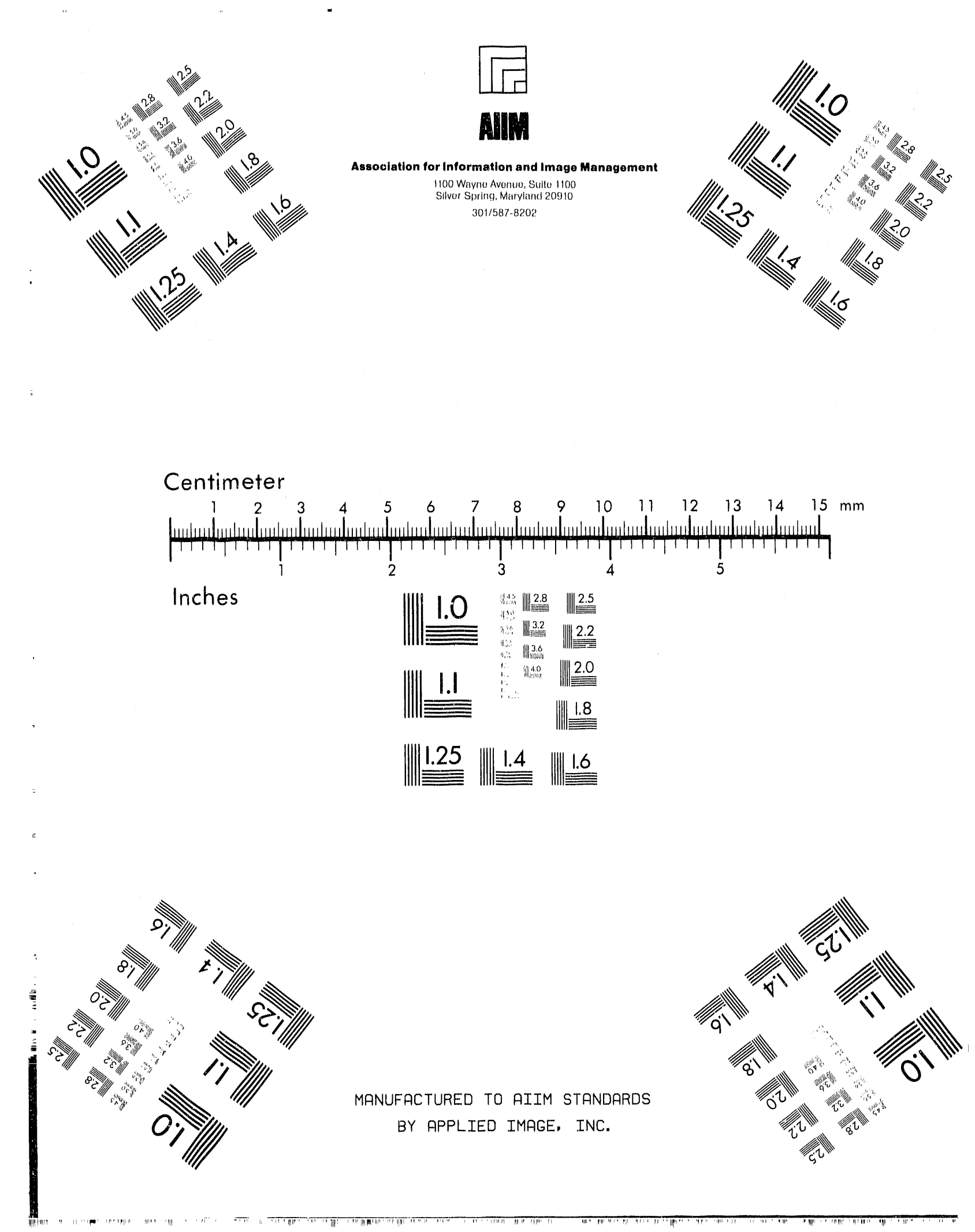
5
Distance from Injection Well (m)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