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Exploring Biomarkers for Point of Care Bladder Cancer Detection

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Exploring Biomarkers for Point of Care Bladder Cancer Detection

A thesis submitted in partial fulfillment
of the requirements for the degree of
Bachelor of Science in Biomedical Engineering

by

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Abstract

Bladder cancer is the 5th most common non-cutaneous human cancer in the United States. While effective methods of detecting bladder cancer are currently practiced, they are often expensive and invasive. There is a need for a noninvasive detection method that can be used in areas with few medical resources. Cell free DNA in urine is normally present only in very low concentrations. Abnormally high levels of cell free DNA in urine could be indicative of disease. This study tests the hypothesis that DNA present in urine can be used as a biomarker for bladder cancer before hematuria is seen in vivo. First, different DNA probes were compared, and SYBR green was selected as an ideal probe due to factors such as cost, safety concerns, and specificity. Second, a detection threshold of cells was determined using MB49 and MBT2 bladder cancer cell lines. Cells were lysed with either tap or DI water to determine which kind of water was more effective at creating a hypotonic solution for the cells. Two cell lines were used to determine if there was a statistical difference in the DNA detection threshold. A detection limit of between 600 cells/200μL and 300 cells/200μL was seen in both MB49 and MBT2 cells. Lastly, an in vivo study was done in which a group of mice was implanted with 75,000 MB49 cells. Urine samples were obtained for five days before implantation, as well as for seven days after implantation. Evidence of excess DNA in the urine was seen as early as four days before hematuria was observed. This study provides evidence that quantifying levels of cell free DNA is an effective method of detecting bladder cancer before hematuria is present. Future studies will determine if the DNA being detected is mammalian, and eventually, a low cost, quantitative home test will be developed to aid in early detection of bladder cancer.

1. Introduction

The direct medical costs relating to cancer in the United States were estimated to be $125 billion in 2010. By the end of 2020, bladder cancer is expected to account for more than 3% of those costs [9]. While effective methods of detecting bladder cancer are currently practiced, they are often expensive and invasive. If bladder cancer can be detected earlier, treatment can be administered before the cancer progresses too much. This study proposes a noninvasive bladder cancer test that uses biomarkers to detect the presence of abnormality within the bladder.

Current invasive detection methods for bladder cancer can include a pelvic exam, cystoscopy, and transurethral resection [10]. These methods can involve inserting a camera endoscopically into the bladder or surgically removing a piece of the bladder to check for cancerous cells. All of these methods can be costly and are uncomfortable for the patient. A noninvasive detection test would increase patient comfort and could decrease the cost to the patient.

Bladder cancer recurrence is also a major issue with regards to cost. Cystoscopy is currently the gold standard in detection recurrences. However, this method can currently only detect about 80% of recurrences [9]. The detection test proposed in this study could help to increase the percentage of detectable recurrences while operating at a much lower cost than cystoscopy.

Detection of DNA presents an easy way to check for abnormality within the bladder. As cells naturally die, they are sloughed off the walls of the bladder and are removed from the body through the urine. Thus, there is a consistent amount of detectable DNA within the urine at any given time. Elevated levels of DNA in the urine indicate that there is an abnormality within the bladder. This abnormality could be as simple as a urinary tract infection, or as serious as muscle invasive bladder cancer. By detecting elevated levels of DNA, a physician can begin to explore what might be causing this signal elevation. The methods outlined in this study can be used in

the future to develop a low cost point of care test. This test would qualify as point of care in that it could be performed at the time and place of patient care [8].

The aim of this study is to develop a method to detect cell free DNA in the urine before other indicators of bladder cancer (such as hematuria) are present. The specific objectives to meet this goal involved identifying a biomarker that could detect low levels of DNA and determining the limit of detection of this biomarker. An in vivo test was performed to test whether or not elevated levels of DNA could be seen in the urine before other indicators were present. Future studies will determine if the DNA being detected is mammalian, and eventually, a low cost point of care test will be developed to aid in early detection of bladder cancer.

2. Materials and Methods

2.1 Reagents. Trypsin (1x, 0.25%) was purchased from Mediatech Inc. (Manassas, VA). DPBS (1x, +Ca, +Mg) was purchased from Thermo Scientific (Logan, UT). Trypan blue was purchased from Thermo Scientific (Logan, UT). SYBR green was purchased from Invitrogen (Carlsbad, CA). DMEM was purchased from GE Healthcare Life Sciences (Logan, UT).

2.2 DNA Probe Comparison. Multiple DNA probes were compared qualitatively to determine which would be the best for this study. The DNA probes considered for this study were ethidium bromide, GelRed/GelGreen, SYBR green, SYBR gold, acridine orange, and DAPI. Criteria of cost, safety concerns, and specificity were considered.

2.3 In Vitro Study

2.3.1 Cell Culturing. MB49 cells were seeded in a T75 flask containing DMEM with 10% FBS and 1x penicillin streptomycin. In a cell culture hood, cell culture media was aspirated and cells were rinsed with 3mL phosphate buffered saline (PBS) twice to wash off excess media. 2mL

trypsin was added to the flask and the flask was placed in a 37°C incubator for 3-5 minutes, or until the cells detached from the wall of the flask. 4mL of cell culture media was introduced to the flask to neutralize the trypsin. The contents of the flask were placed into a 15mL centrifuge tube. The tube was centrifuged at 1400rpm for 5 minutes. The centrifuge tube was then transferred back into the cell culture hood and liquid was aspirated, leaving a pellet of cells at the bottom of the tube. 5mL of cell culture media was added to the tube and the cells were resuspended in the media. 12mL of fresh media was placed into a new T75 flask. A portion of the resuspended cell solution was pipetted into the new flask to obtain a 1/20 ratio of cells to media. Cells were passaged when the wall of the flask was 80% confluent. The same procedure was followed for MBT2 cells.

2.3.2 Isolating DNA from Cells. After passaging the MB49 cells, the leftover resuspended cell solution was divided into four equal parts and placed into microcentrifuge tubes. Each tube contained approximately 1.25mL of cell solution once divided. The four tubes were centrifuged at 1400rpm for 5 minutes. The supernatant solution was aspirated, leaving a small cell pellet at the bottom of the tubes. Three experimental groups and one control group were set up as follows. 3mL of deionized water was added to tube 1. 3mL of tap water was added to tube 2. 1.5mL of deionized water and 1.5mL of tap water was added to tube 3. 3mL of PBS was added to tube 4. Cells were resuspended in solution. After 5 minutes, the number of cells in each flask were counted using a hemocytometer. 20µL of cell solution, 10µL trypan blue, and 170µL of water or PBS (deionized water for tube 1, tap water for tube 2, 85µL deionized water/85µL tap water for tube 3, and PBS for tube 4) were mixed together. 20µL of this solution was placed onto each half of the hemocytometer (dilution factor of 10). Equation 1 was used to determine how many cells were present in the original solution:

$$\# \; of \; cells = avg \; \# \; counted \; per \; square * 10^4 * dilution \; factor \qquad \text{(Equation 1)}$$

This procedure was repeated in triplicate, and was also performed for MBT2 cells.

A separate experiment was performed in which leftover MB49 and MBT2 cells after passaging were divided equally into a six-well plate and allowed to grow to 90% confluence. Media was aspirated and the cells were washed with PBS 2 times. The PBS was aspirated and the plate was placed onto a phase contrast microscope. Images were taken in real time as either tap or deionized water was added to each well under 10x magnification.

2.3.3 Cell Staining. MB49 cells were cultured and lysed using methods stated previously. Cells were resuspended in both deionized and tap water at 100,000 cells/mL and sat for 5 minutes to allow total lysis. This was repeated with the MBT2 cells. SYBR green staining solution was prepared using 10µL concentrated SYBR green in 50mL TAE buffer (1:5000 dilution). In a black walled 96-well plate, 100µL of tap water was added to wells B-H 1-3 and 10-12. 100µL of deionized water was added to wells B-H 4-9. An additional 100µL of tap water was added to wells H1-3 and an additional 100µL of deionized water was added to wells H4-6. 200µL of MBT2 cells in tap water was added to wells A 1-3. 200µL of MBT2 cells in deionized water was added to wells A 4-6. 200µL of MB49 cells in deionized water was added to wells A 7-9. 200µL of MB49 cells in tap water was added to wells A 10-12. Serial dilutions were performed down each column of the well plate for rows A-G, so that each well in these rows had 100µL of solution. 100µL of diluted SYBR green was added to wells A-G 1-12 and H 7-12. The final volume in every well on the plate was 200µL. A summary of the contents of each well is shown in Table 1.

Table 1. Plate layout for DNA detection. Every well contained a final volume of 200µL. The numbers represent the number of cells from which DNA is being detected.

	Lysate											
	MBT2 Cells						MB49 Cells					
	Tap Water			DI Water			DI Water			Tap Water		
	1	2	3	4	5	6	7	8	9	10	11	12
A	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000
B	5000	5000	5000	5000	5000	5000	5000	5000	5000	5000	5000	5000
C	2500	2500	2500	2500	2500	2500	2500	2500	2500	2500	2500	2500
D	1250	1250	1250	1250	1250	1250	1250	1250	1250	1250	1250	1250
E	625	625	625	625	625	625	625	625	625	625	625	625
F	312.5	312.5	312.5	312.5	312.5	312.5	312.5	312.5	312.5	312.5	312.5	312.5
G	156.25	156.25	156.25	156.25	156.25	156.25	156.25	156.25	156.25	156.25	156.25	156.25
H	tap water			DI water			SYBR + DI water			SYBR + tap water		

The plate was covered and kept in a dark environment for 30 minutes to allow time for the SYBR green to bind to DNA.

2.3.4 Plate Reading. The 96-well plate was quantified on a Synergy HT plate reader at an excitation of 480nm and an emission of 530nm. Fluorescence levels were recorded and a standard curve was generated for each cell type, as well as the type of water the cells were lysed in.

2.4 In Vivo Study

2.4.1 Sample Collection. Samples were collected from four groups of five mice for a total of 12 days. Implantation of 75,000 MB49 cells occurred on day 5, and the samples taken for that day occurred before implantation. Urine samples of at least 20µL were obtained from each mouse and frozen at -80°C until all samples were collected.

2.4.2 Cell Staining. Urine samples were warmed to room temperature on the lab bench. 5µL of urine and 95µL of deionized water were placed in each well of a black walled 96-well plate. Each sample was performed in triplicate (so a total of 15µL of urine from each mouse was used). The well plates were allowed to sit for 5 minutes to allow total lysis of cells. 100µL of diluted SYBR green staining solution was added to each well. The final volume in every well on the

plate was 200µL. A total of four well plates were used to read the samples from days N1-5 (naïve mice), and a total of four well plates were used to read the samples from days I1-7 (implanted mice). All eight plates contained a negative control of 100µL of SYBR green/100µL of deionized water. The four plates with samples from the implanted mice also contained a positive control with MB49 cells seeded from 10,000 cells to 156.25 cells. The plates were covered and kept in a dark environment for 30 minutes to allow time for the SYBR green to bind to DNA.

2.4.3 Plate Reading. The 96-well plates were quantified on a Synergy HT plate reader at an excitation of 480nm and an emission of 530nm. Fluorescence levels were recorded and a quantification of number of cells in urine per day was generated. Statistical analysis of this data was performed using a t-test.

3. Results

3.1 DNA Probe Comparison. The DNA probes listed in Table 2 were evaluated qualitatively based on cost, safety concerns, and specificity. From these criteria, SYBR green was chosen to be the DNA probe for this study.

Table 2. An evaluation of DNA stains in regards to cost effectiveness, safety concerns, and other measures.

	Cost	Safety Concerns	Potential Problems
Ethidium Bromide [3]	$6/mL	Highly mutagenic	Used mostly in agarose gels and CsCl gradients
GelRed/GelGreen [4]	$200/mL	N/A	Can't penetrate intact cell membranes
SYBR Green [6][7]	$453/mL	Can be irritating to the skin	Prolonged exposure to light decreases effectiveness
SYBR Gold [5][7]	$298/mL	Can be irritating to the skin	Typically, only used for gel electrophoresis
Acridine Orange [1]	$99/g	Can be irritating to the skin	Binds to acidic vacuoles and RNA as well as DNA
DAPI [2]	$89/10mg	N/A	Only binds to A-T rich regions in DNA

3.2 In Vitro Study. Results from counting cells after placing them in tap or deionized water were inconclusive. Figures 1 and 2 show real time cell death progression in deionized water and tap water.
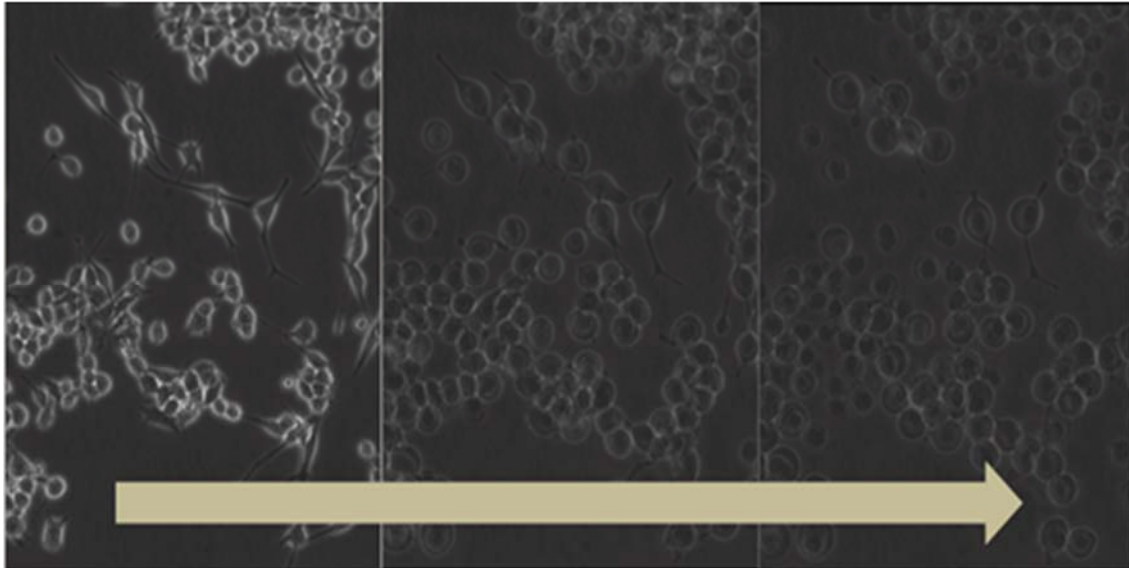

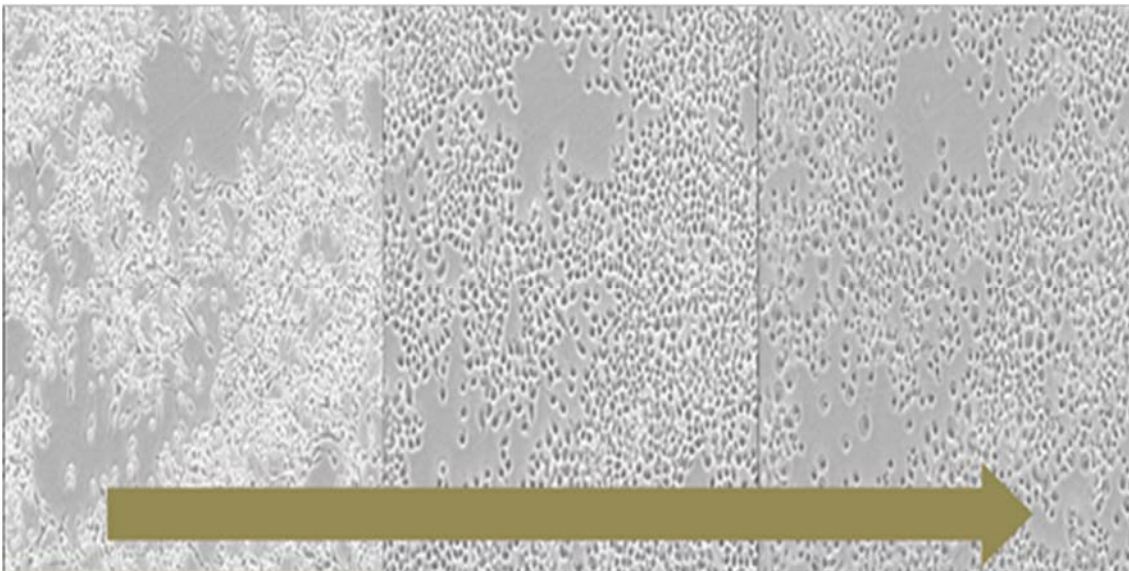Figure 1. Phase contrast image (10x) of cell death progression in deionized water.

Figure 2. Phase contrast image (10x) of cell death progression in tap water.

After cells were plated and read, a standard curve was generated, as shown in Figure 3. The

detection threshold for all four experimental groups was determined to be between 300 and 600
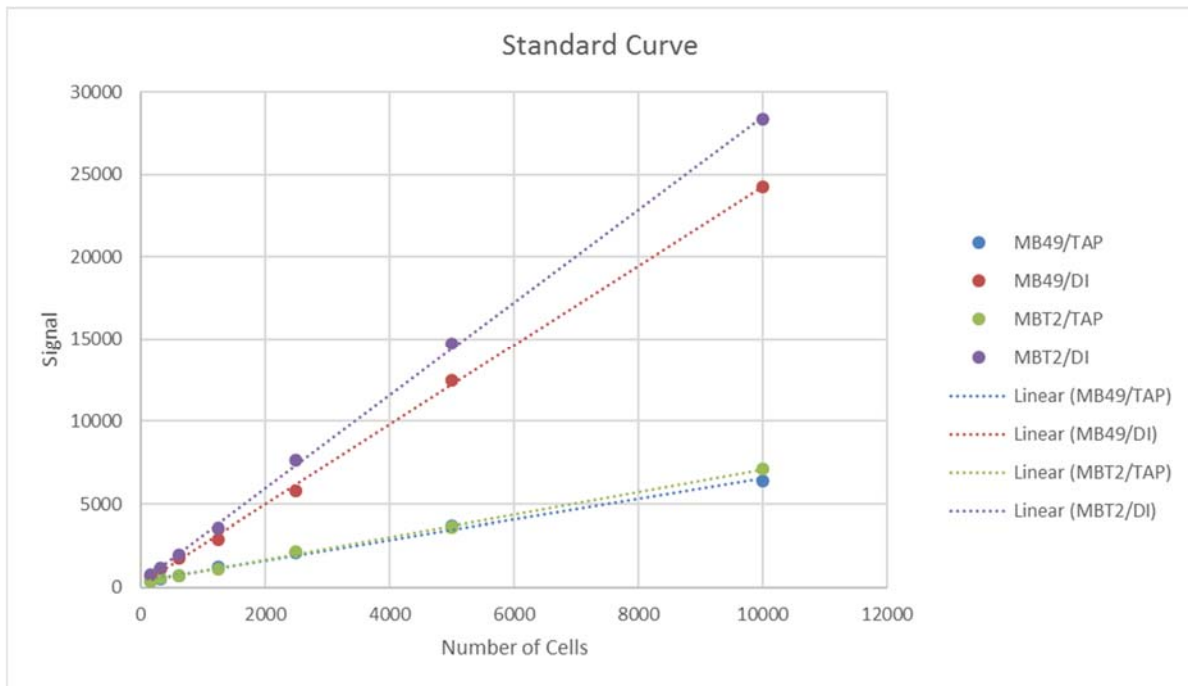
cells.

Figure 3. Standard curve showing fluorescent signal vs. number of cells for MB49 and MBT2 cell lines. As shown in the graph, this is a roughly linear relationship. Cells lysed in deionized water produced higher signals than cells lysed in tap water, although both cases produce a strong signal. Limit of detection was determined to be between 300 and 600 cells for both cell lines.

3.3 In Vivo Study. Hematuria was seen in 100% of mice by day I7. However, elevated levels of

DNA in the urine was seen as early as day I3. A standard curve was generated from cultured

MB49 cells, as seen in Figure 4. Figure 5 shows the average number of cells in the urine per day

of this study, based on fluorescent signals obtained from reading the plate, and a cross

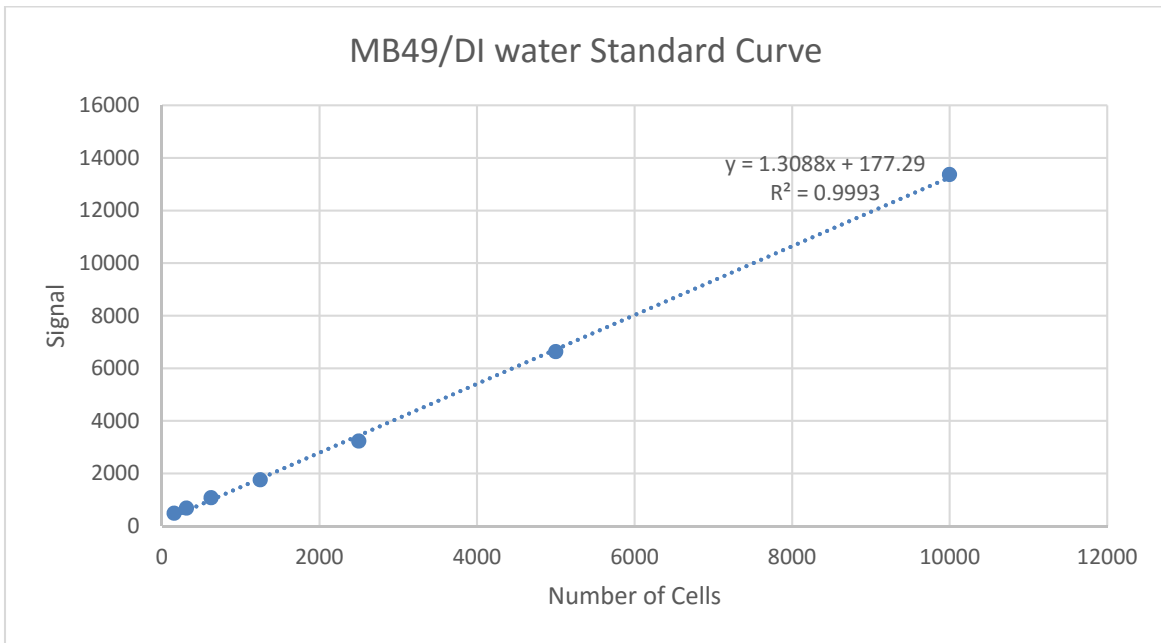comparison of the standard curve generated in Figure 4.

Figure 4. Standard curve showing fluorescent signal vs. number of cells for MB49 cell lines.
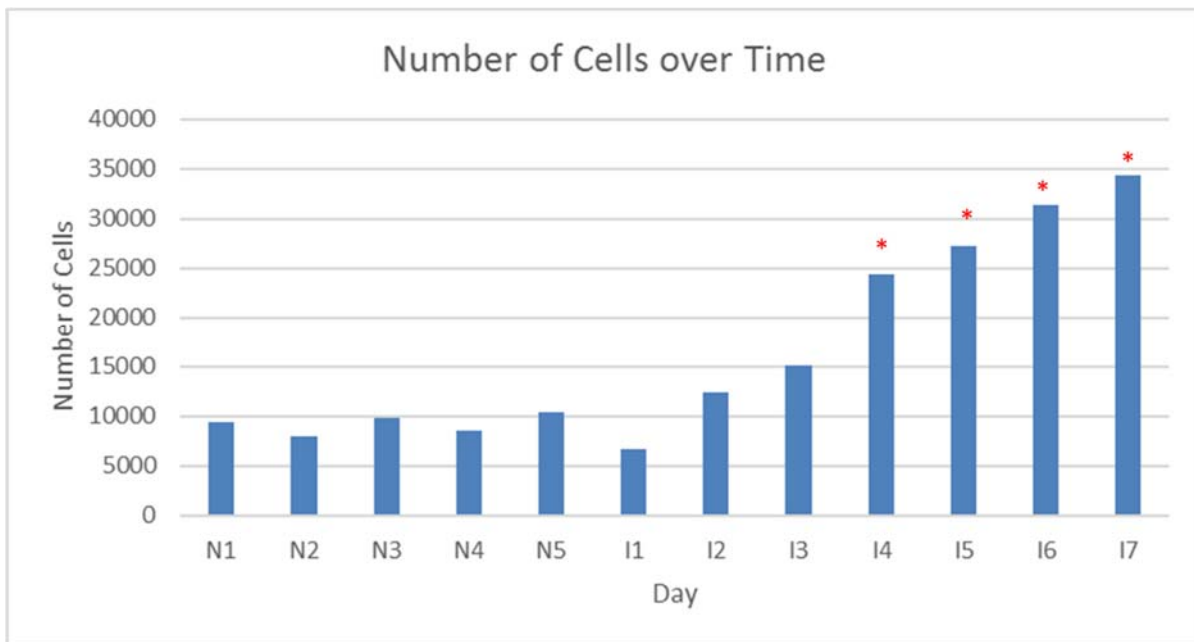

Figure 5. Average number of cells from all 20 mice over a period of 12 days. Implantation of MB49 cells took place on day N5. Hematuria was seen in 100% of mice by day I7. An * denotes a statistical difference between before implantation samples and after implantation samples ($p<0.05$ via t-test).

4. Discussion

4.1 DNA Probe Comparison. Six DNA probes were evaluated based on cost, safety concerns, and potential problems in regards to this study. Ethidium bromide, although very cheap, was quickly eliminated due to the highly mutagenic nature of the probe, as well as the fact that it's typically used in agarose gel staining. Acridine orange was eliminated because it wasn't specific enough to cell free DNA (this probe also binds to RNA and acidic vacuoles). DAPI was eliminated because it only binds to A-T rich regions of DNA and there was concern that the fluorescent signal wouldn't be strong enough to detect at low cell concentrations. SYBR gold typically is used in gel electrophoresis, and did not fit for this type of application in solution. GelRed/GelGreen was ultimately eliminated because the probes couldn't penetrate intact cell membranes. If any cells remained intact in water, these probes wouldn't be able to detect it. SYBR green was ultimately chosen despite its high cost. The amount of SYBR green used in each experiment was 10µL. So, 1mL of SYBR green would last for approximately 100 experiments. Based upon this, it would only cost $4.53 per experiment to use SYBR green. This was determined to be worth the high initial cost.

4.2 In Vitro Study. There were no cells still intact when they were counted after being placed in tap or deionized water for 5 minutes. Based on this, it was impossible to determine whether tap or deionized water was more effective at lysing cells. However, when cell death progression was imaged in real time, the cells in deionized water became more hypotonic and burst more quickly. Figure 1 clearly shows a bigger change in size from left to right than Figure 2 does. However, after 5 minutes, all cells were lysed in both tap and deionized water. Based on these observations, it was determined that either kind of water would be fine for lysing cells as long as

the cells were allowed to sit in water for at least 5 minutes. For the rest of the experiments performed for this study, deionized water was used for consistency.

From the standard curve, it was determined that the relationship between fluorescence and the number of cells plated is consistently linear. The background signal of just SYBR green and water was usually around 300-400. While the data points on the standard curve did not have the background signal subtracted out, it was clear which data points fell below the background threshold. Based on this, the limit of detection of MB49 and MBT2 cells in either tap or deionized water was determined to be between 300 and 600 cells for every replication of this experiment. It is also clear to see that cells lysed in deionized water produced higher signals than cells lysed in tap water for both cell types. It is interesting to note that the limit of detection remained about the same for both types of water, even though the signal levels were drastically different.

4.3 In Vivo Study. Hematuria was visibly seen in some of the urine as early as day I5. However, hematuria was not seen in 100% of mice until day I7. A standard curve from the positive control was generated (Figure 4) and the linear regression equation was used to convert fluorescent signal to number of cells in Figure 5. Figure 5 shows a flat trend from days N1 to approximately I2. From I3 to I7, a steady increase in the number of cells in the urine is seen. It is interesting to note that day I1 had a lower number of cells than the N signals. This is most likely the case because the bladder was washed with poly-L-lysine before implantation on day N5. Therefore, all cells not attached to the bladder wall would have been washed out of the bladder.

There is not a statistical difference between N1-5 readings and I readings until day I4. This statistical difference was noted before hematuria was seen in any mice. This is indicative that infection in the bladder can be detected before hematuria is observed.

5. Conclusion

This study evaluated the ability to detect elevated DNA levels in mouse urine before hematuria is visibly seen. It was demonstrated that deionized water is more effective than tap water at releasing DNA from cells, although both types of water provided strong signals. When DNA from MB49 and MBT2 cells was labeled with SYBR green in vitro, a consistent linear relationship between fluorescence signal and number of cells was seen. This standard curve was used to cross compare fluorescence signal in the in vivo study and determine the approximate number of cells present in urine per day of sampling. Indication of infection is present over a threshold of 300 to 600 cells. This was seen by day I4 of sampling. This indicates the presence of carcinoma in the bladder. In addition, a significant number of cells were present in the urine at least 2 days before hematuria was visibly present.

This evidence suggests that labeling DNA with SYBR green can lead to earlier detection of bladder carcinoma in vivo. Future directions of this research include developing a second urine screen to distinguish bacterial from mammalian DNA in a low cost test for areas with low resources. In addition, micro hematuria levels could be monitored to see if indication of carcinoma can be detected even earlier. This study provides the foundation to fabricate a home kit that can be used as an aid in early screening for bladder cancer.

6. Acknowledgements

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