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A Novel Method to Capture Methylated Human DNA from Stool: Implications for Colorectal Cancer Screening

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Background: **Assay of methylated DNA markers in stool is a promising approach for colorectal cancer (CRC) screening. A method to capture hypermethylated CpG islands from stool would enrich target analyte and allow optimal assay sensitivity.**

Methods: **Methyl-binding domain (MBD) protein was produced using a pET6HMBD plasmid with MBD DNA sequence cloned from rat MeCP2 gene and bound to a column of nickel-agarose resin. We first established the feasibility of using the MBD column to extract methylated human DNA in a high background of fecal bacterial DNA. To explore the impact of MBD enrichment on detection sensitivity, the tumor-associated methylated vimentin gene was assayed with methylation-specific PCR from stools to which low amounts of cancer cell DNA (0–50 ng) were added and from stools from CRC patients and healthy individuals. Stools from cancer patients were selected with low amounts of human DNA (median 7 ng, range 0.5–832 ng).**

Results: **With MBD enrichment, methylated vimentin was detected in stools enriched with ≥10 ng of cancer cell DNA and in CRC stool with a range of native human DNA amounts from 4 to 832 ng. Without MBD enrichment, methylated vimentin was not detected in the enriched stools and was detected in only 1 cancer stool with high human DNA (832 ng). In stools from healthy individuals methylated vimentin was not detected, with or without MBD enrichment.**

Conclusions: **MBD capture increases assay sensitivity for detecting methylated DNA markers in stool. Applied clinical studies for stool cancer screening are indicated.**

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Colorectal cancer (CRC)[1] is one of the most common malignancies in the world. In the US, CRC was diagnosed in an estimated 148 610 patients, 37% of whom will die of this disease *(1)*. If detected at early stages, however, CRC is curable. Conventional methods for screening CRC are either invasive or lack accuracy, so there is a need to develop more user-friendly and accurate approaches. Detection of molecular markers in stool is an emerging noninvasive screening approach *(2)*.

Several groups have detected methylated DNA markers in stool, including markers located on p16, MGMT, MLH1, SFRP2, HIC1, and vimentin genes *(3–9)*. Methylated markers are attractive for screening because they occur with high frequency in early-stage neoplasia *(10)* and are predictable assay targets on gene promoter regions. DNA marker assay in stool is compromised, however, when crude stool DNA is used directly as a PCR template, because stool DNA composition is overwhelmingly bacterial and dietary, with human DNA making up <0.1% *(2)*. Analyte enrichment would logically enhance detection of methylated DNA markers from stool.

Methyl-binding domain (MBD) is a functional region of MeCP2 protein that specifically binds symmetrically methylated CpGs in any sequence context and is involved in mediating methylation-dependent repression *(11)*. Cross et al. *(12)* described use of MBD protein bound to a nickel-agarose resin column to purify methylated CpG islands, and such a column has been used to enrich methylated CpG islands in lung cancer tissues *(13)*. However, this method has not been applied to capture the extremely low amounts of methylated human DNA found within a complicated medium such as stool. Furthermore, the affinity of MBD binding to bacterial DNA, normally methylated at adenine and cytosine residues through Dam and Dcm transmethylases *(14)*, is untested. Because of the large excess of bacterial DNA in stool

[1] Nonstandard abbreviations: CRC, colorectal cancer; MBD, methyl-binding domain; MSP, methylation-specific PCR.

compared with the minute amounts of exfoliated human DNA, even loose binding of MBD to methylated DNA sequences in bacteria could hamper this approach.

We describe a method using MBD to universally capture methylated human DNA from stool and thereby increase the sensitivity of this marker class for the detection of CRC.

Materials and Methods

EXPERIMENTAL DESIGN

This study included 4 related experiments: The 1st experiment was designed to test whether the MBD column could separate methylated DNA from a high background of bacterial DNA and unmethylated DNA. The 2nd experiment optimized a buffer panel for enriching methylated DNA with the MBD column. The 3rd experiment tested the effect of MBD enrichment on assay sensitivity in detecting trace amounts of methylated cancer cell DNA added to stool DNA. The 4th experiment explored whether MBD enrichment could increase assay sensitivity for detecting tumor-specific methylated markers in patient stools, which contained low amounts of human DNA. Approval of this study was obtained from the Institutional Review Board of Mayo Foundation.

CELL LINES

Four cell lines derived from human digestive cancer were used, including 2 colon cancer cell lines (RKO and SW480), 1 gastric cancer cell line (RF-1), and 1 pancreatic cancer cell line (Capan2). RKO was grown in RPMI 1640 medium, SW480 and RF-1 in Leibovitz's L-15 medium, and Capan2 in DMEM. Media were supplemented with 10% fetal bovine serum, 100 000 U/L of penicillin, 100 g/L of streptomycin, and 2 mmol/L of L-glutamine. Cells were incubated at 37 °C in the presence of 5% CO_2.

TISSUE AND STOOL SAMPLES

Fourteen paraffin-embedded colon cancer tissues were used to check the methylation status of vimentin in tumor. Stools from 8 patients with a corresponding methylated tumor were selected to test the MBD column. Stools from 6 individuals with normal colonoscopy findings were used as controls. All stools were collected before colonoscopy or surgery. None of the CRC patients had undergone chemotherapy or radiotherapy before stool collection. Any previous instrumentation or cathartic preparation had occurred more than 2 weeks before stool collection. A plastic bucket device was used to collect whole stool. Stools in sealed buckets were immediately transported to our laboratory and stored at −80 °C.

DNA EXTRACTION

Tissue sections were examined by a pathologist who circled histologically distinct lesions to direct careful microdissection. Genomic DNA from both microdissected tissues and cell lines was extracted by use of a Qiagen DNA Mini Kit (Qiagen). Stool was homogenized in ASL buffer (1 g stool:10 mL buffer) and extracted with a QIAamp® DNA Stool Mini Kit (Qiagen).

BISULFITE TREATMENT

Sodium bisulfite converts unmethylated, but not methylated, cytosine residues to uracil. DNA from tissue, cell line, and total stool was bisulfite modified using the EZ DNA Methylation Kit (Zymo Research). Because stool DNA samples after capture with MBD column typically contain <1 μg DNA, whole DNA purified from each MBD elute was bisulfite modified (see below). We used 30 μL of buffer to elute bisulfite-modified tissue and cell-line DNA and 10 μL for stool DNA.

METHYLATION-SPECIFIC PCR

Bisulfite-modified DNA (1 μL for tissue and cell DNA and 4 μL for stool DNA) was amplified in a total volume of 25 μL containing 1× PCR buffer, 1.5 mmol/L $MgCl_2$, 200 μmol/L of each dNTP, 400 nmol/L of each primer, and 1.25 unit of AmpliTaq Gold polymerase (Applied Biosystems). Amplification included hot start at 95 °C for 12 min, 35 cycles for tissue and cell DNA or 40 cycles for stool DNA at 95 °C for 45 s, annealing temperatures for 45 s, 72 °C for 45 s, and a final 10-min extension step at 72 °C. The methylation-specific primers for vimentin were 5′-TCG TTT CGA GGT TTT CGC GTT AGA GAC-3′ (sense) and 5′-CGA CTA AAA CTC GAC CGA CTC GCG A-3′ (antisense), and the annealing temperature was 68 °C *(4)*. The unmethylation-specific primers for vimentin were 5′-TTG GTG GAT TTT TTG TTG GTT GAT G-3′ (sense) and 5′-CAC AAC TTA CCT TAA CCC TTA AAC TAC TCA-3′ (antisense), and the annealing temperature was 60 °C. The methylation-specific primers for TPEF were 5′-CGG TAA AGA TTC GAG TAA GGA ACG T-3′ (sense) and 5′-AAA ACA TCG ACC GAA CAA CGA CGT C-3′ (antisense), and the annealing temperature was 65 °C. The unmethylation-specific primers for TPEF were 5′-GTT ATT TGG TAA AGA TTT GAG TAA GGA ATG-3′ (sense) and 5′-AAA ACA TCA ACC AAA CAA CAA CAT C-3′ (antisense), and the annealing temperature was 60 °C. Bisulfite-treated human genomic DNA and CpGenome™ Universal Methylated DNA (Chemicon) were used as positive controls for unmethylation and methylation, respectively. The methylation-specific PCR (MSP) amplicons for both vimentin and TPEF were located in CpG island regions without *Mse*I cutting sites (TTAA).

PREPARATION OF MBD COLUMN

A previously reported method of column preparation was followed *(12)*. MBD protein tagged with 6 histidines was expressed from a pET6HMBD plasmid-containing a MBD encoding DNA (a gift from Dr. Adrian Bird, Wellcome Trust Centre for Cell Biology, University of Edinburgh), which was cloned from rat MeCP2 gene *(12)*. Briefly, MBD was produced in BL21 star (DE3) pLysS (Invitrogen), and partially purified with a cation exchange resin,

Fractogel® EMD SO_3^- (M; EMD Chemicals), in a Econo-Pac Column (Bio-Rad). MBD protein was then coupled to an Ni-NTA Superflow (Qiagen), a nickel-agarose gel that specifically binds to protein with proteins tagged with 6 histidines, in a 10-mL Poly-Prep Chromatography Column (Bio-Rad) to generate the MBD column. Approximately 10 mg MBD was coupled per mL Ni-NTA Superflow.

sample preparation

Stool DNA and cancer cell DNA to be loaded on the MBD column for separation were first digested overnight with *Mse*I (4 units/μg), which recognizes the sequence TTAA, so the majority of CpG islands were kept intact, but other genomic regions were cut into short fragments. Digested DNA was then extracted with phenol/chloroform/isoamyl alcohol (25:24:1), precipitated in ethanol, and eluted in nuclease-free water.

testing specificity of mbd column

To test whether the MBD column separates methylated DNA from a high background of bacterial DNA, 5 μg of cancer cell DNA and 50 μg of stool DNA (all *Mse*I cut) were loaded on the MBD column in MBD buffer/0.1 mol/L NaCl (20 mmol/L HEPES, pH 7.9, 10% glycerol, 0.1% Triton X-100, 0.1 mol/L NaCl). The cancer cell DNA, which was used to simulate DNA exfoliated from cancers in digestive tract, was from a methylated cell line and an unmethylated cell line (2.5 μg each; RKO with SW480, or RF-1 with Capan2). Vimentin is methylated in RKO, but not SW480; TPEF is methylated in RF-1, but not Capan2. DNA fragments bound on MBD protein were eluted using MBD buffers with gradient concentrations (0.2–1.0 mol/L) of NaCl.

Each eluate sample (5 mL) from the MBD column was concentrated with Amicon Ultra Centrifugal Filter Devices (30 000 MWCO, Millipore) to 200 μL and then extracted with phenol/chloroform/isoamyl alcohol (25:24:1), precipitated in ethanol, and eluted in nuclease-free water. DNA from each eluate was amplified with primers specific to *Escherichia coli* DNA, or bisulfite-treated for methylation analysis.

E. coli, a common bacterium in human stool, was used to represent fecal bacteria. Two sets of primers were designed to amplify *E. coli* DNA, 1 set targeting the dnaK gene and the other targeting a randomly selected undefined region. The primers specific for dnaK gene were 5′-GTG CCG GAT TAG CCA ACT TA-3′ (sense) and 5′-GTG ACG ATT CCA GCC GTA CT-3′ (antisense), and the primers for the undefined *E. coli* DNA region were 5′-ACT CCT GCG AAA CAT CAT CC-3′ (sense) and 5′-CGG CAC CTT GCT AAG TCT TC-3′ (antisense). We amplified 1 μL of stool DNA in a total volume of 25 μL containing 1× iQ™ Supermix (Bio-Rad), 200 nmol/L of each primer under the following conditions: 95 °C for 3 min, followed by 28 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 40 s, and a final 10-min extension step at 72 °C.

capture of cancer cell dna added to stools

To test the sensitivity of the MBD column to capture methylated DNA in a stool model, trace amounts of cancer cell DNA (0, 2, 10, and 50 ng; RKO with SW480) were added to stool aliquots (1 g each) from a homogenized normal stool. Stool DNA was extracted and digested, as described above. Whole DNA from each stool aliquot was loaded on the MBD column in MBD buffer/0.52 mol/L NaCl. Loosely bound DNA was washed away in MBD buffer/0.6 mol/L NaCl. Tightly bound DNA was eluted in MBD buffer/1.0 mol/L NaCl, concentrated, extracted, and bisulfite-treated for methylation analysis targeting tumor-specific methylated vimentin gene *(4)*.

capture of methylated human dna from patient stools

The MBD column was used to capture methylated human DNA in clinical stool samples from 8 CRC patients and 6 control individuals described above. Stool DNA was extracted, digested, and loaded on the MBD column in MBD buffer/0.52 mol/L NaCl, and then washed with MBD buffer/0.6 mol/L NaCl. Methylated DNA bound to the column was retrieved with MBD buffer/1.0 mol/L NaCl and prepared as above for amplifying methylated vimentin.

real-time *Alu* pcr

Human DNA in patient stools was quantified using a real-time *Alu* PCR method, as we first reported *(15)*. Primers specific for the human *Alu* sequences (sense, 5′-ACG CCT GTA ATC CCA GCA CTT-3′; and antisense, 5′-TCG CCC AGG CTG GAG TGC A-3′) were used to amplify sequences approximately 245 bp inside *Alu* repeats *(15, 16)*. Stool DNA was diluted 1:5 with nuclease-free water for PCR amplification. We amplified 1 μL water-diluted stool DNA in a total volume of 25 μL containing 1× iQ™ SYBR® Green Supermix (Bio-Rad) and 200 nmol/L of each primer under the following conditions: 95 °C for 3 min, followed by 23 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 40 s in a real-time iCycler (Bio-Rad). A calibration curve was created for each plate by amplifying 10-fold serially diluted human genomic DNA samples (Novagen). A melting curve was made after each PCR to confirm that only 1 product was amplified for all samples. Amplification was carried out in 96-well plates in an iCycler (Bio-Rad). Each plate consisted of stool DNA samples and multiple positive and negative controls. Each assay was performed in duplicate.

Results

separation of methylated cancer cell dna from high background bacterial dna

This study was performed to determine whether methylated human DNA derived from a cancer cell line could be

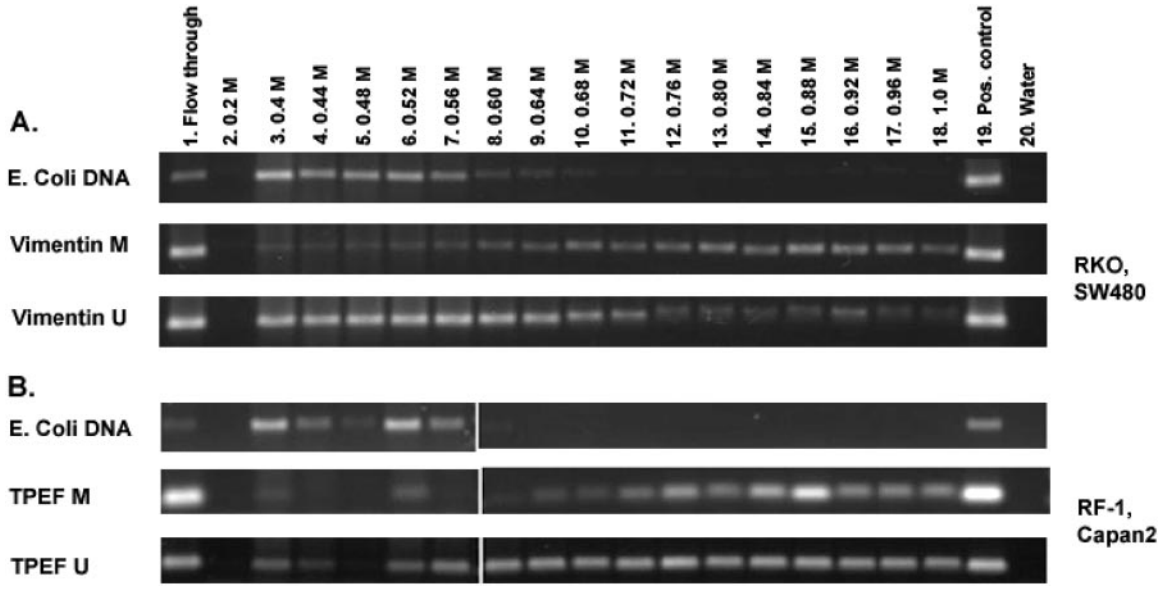

Fig. 1. Separation of methylated DNA added to a high background of bacterial DNA using an MBD column.

After digestion with *Mse*I, 5 μg of cancer cell DNA from a methylated and an unmethylated cell line (2.5 μg each; RKO with SW480, or RF-1 with Capan2) and 50 μg stool DNA were loaded onto an MBD column and eluted in MBD buffers with 0.2 to 1.0 mol/L NaCl (5 mL each). DNA in each eluate was amplified with primers specific to *E. coli* DNA, methylated (*M*) and unmethylated (*U*) DNA sequences. *Lane 1*, flow-through; *lane 2*, MBD buffer/0.2 mol/L NaCl; *lanes 3–18*, MBD buffers with stepwise gradient of NaCl (0.4–1.0 mol/L by 40 mmol/L per step); *lane 19*, positive (*Pos.*) control; *lane 20*, water control. (*A*), RKO and SW480 DNA and stool DNA were MBD separated and eluted in MBD buffers with different concentrations of NaCl. Pure *E. coli*, RKO, and SW480 DNA samples were used as the positive controls for *E. coli* and methylated and unmethylated vimentin PCRs, respectively. (*B*), RF-1 and Capan2 DNA and stool DNA were MBD separated and eluted in MBD buffers with different concentrations of NaCl. Pure *E. coli*, RF-1, and Capan2 DNA samples were used as the positive controls for *E. coli* and methylated and unmethylated TPEF PCRs, respectively.

separated from a background of highly abundant bacterial DNA as would be found in stool. Stool DNA with added cancer cell DNA was loaded on the MBD column and then eluted using MBD buffers with stepwise increases in the concentration of NaCl. More tightly bound DNA requires elution with buffers at higher concentrations of NaCl. By either set of primers for the *E. coli* genome, *E. coli* DNA was amplified mostly in gradient MBD eluates with 0.4–0.6 mol/L NaCl, but less in eluates with >0.6 mol/L NaCl. Methylated vimentin and TPEF were found mostly in MBD eluates with >0.6 mol/L NaCl, and less in eluates with ≤0.6 mol/L NaCl. Unmethylated vimentin was mainly in eluates with 0.4–0.72 mol/L NaCl, and unmethylated TPEF was detected in almost all eluates with 0.4–1.0 mol/L NaCl (Fig. 1).

Optimization of buffer panel for MBD enrichment

This study was performed to optimize a buffer panel for the application of MBD column in enriching methylated DNA in stool. When stool DNA without added cell DNA was loaded to the column in MBD buffer/0.1 mol/L NaCl and then sequentially eluted with MBD buffers with 0.2, 0.52, 0.6, and 1.0 mol/L NaCl, most *E. coli* DNA was eluted into buffers with ≤0.6 NaCl (Fig. 2). Quantification with a photospectrometer revealed that <1% of total stool DNA was left in the 1.0 mol/L NaCl eluate.

Because a buffer cutoff at 0.6 mol/L NaCl separated methylated DNA from background bacterial DNA, we were able to use a selected sequence of MBD buffers for the enrichment process. Stool DNA was loaded to the MBD column in MBD buffer/0.52 mol/L NaCl to allow the binding of most methylated human DNA, but little bacterial DNA; an additional MBD buffer/0.6 mol/L NaCl further washed off loosely bound bacterial DNA and part of unmethylated human DNA; and a last MBD buffer/1.0 mol/L NaCl retrieved most methylated human DNA and a portion of unmethylated human DNA.

Increased sensitivity of detecting methylated marker in stool with added DNA

This experiment was designed to test the effect of MBD enrichment on detection of trace amounts of methylated cancer cell DNA added to stool DNA. Trace amounts of cancer cell DNA were captured from stools using the MBD column and then tested with MSP by detecting

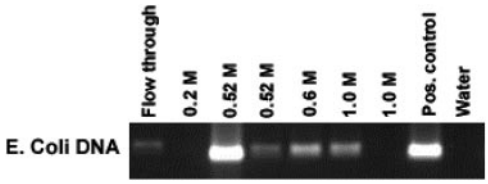

Fig. 2. Simplification of MBD buffer panel for enrichment study.

Stool DNA digested with *Mse*I was loaded to the column in MBD buffer/0.1mol/L NaCl and then sequentially eluted using MBD buffers with 0.2, 0.52 × 2, 0.6, and 1.0 × 2 mol/L NaCl. DNA from each eluate was amplified with primers for *E. coli* DNA.

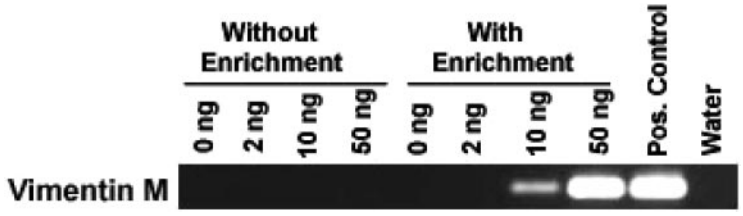

Fig. 3. Capture of low amounts of methylated DNA added to stools.

Cancer cell DNA (0, 2, 10, and 50 ng; RKO with SW480) was added to stool aliquots from a homogenized stool. Methylated vimentin was amplified with MSP in DNA samples from these stool aliquots with and without MBD enrichment.

methylated vimentin. With MBD enrichment, methylated vimentin was detectable in stool aliquots to which 10 and 50 ng RKO and SW480 cancer cell DNA were added, but not in those with 0 and 2 ng cancer cell DNA. Without MBD enrichment, methylated vimentin was not detectable in any stool aliquot with added DNA (Fig. 3).

ENHANCED DETECTION OF METHYLATED MARKER IN PATIENT STOOLS

This study was designed to test whether MBD enrichment increases the sensitivity of detecting methylated markers in patient stools. The effect of MBD enrichment on the clinical detection of CRC detection was evaluated on stools from patients whose matched CRC tissue demonstrated methylated vimentin. Vimentin was methylated in 8 of the 14 paraffin-embedded CRC tissues. The 8 stools with methylated tissues were captured with the MBD column and then tested with MSP by detecting methylated vimentin. With MBD enrichment, methylated vimentin was detected in 4 CRC stool samples with 4, 27, 408, and 832 ng human DNA, but not in the other 4 samples with 0.5, 1, 2, and 10 ng of human DNA; without MBD enrichment, methylated vimentin was detectable in only 1 CRC stool sample with 832 ng human DNA (Fig. 4). Methylated vimentin was not detected in 6 normal stools with or without MBD enrichment (data not shown).

Discussion

This report describes a new method to capture methylated human DNA from stool samples using MBD protein chelated into a nickel-agarose matrix in a chromatography column. With the enrichment of the MBD column, methylated vimentin could be detected by MSP in a stool model with as little as 10 ng of added human cancer cell DNA and in patient stool with only 4 ng total human DNA. However, when MSP was directly applied to crude stool DNA, methylated vimentin was detectable only in a stool sample that contained a large amount of human DNA (832 ng). These results demonstrate that capturing methylated CpG islands with MBD can increase the detection sensitivity of stool-based methylated marker assays, even in stools with low concentrations of template human DNA.

Recently, we reported that the median concentration of human DNA in fresh CRC stools was 309 ng/g stool, with a range of 5–21 115 ng/g *(15)*. Stools in the present study were selected to include multiple samples with low concentrations of human DNA; the median concentration was 24 ng/g and the range was 3–6027 ng/g. These concentrations corresponded to a range of 0.5–832 ng of human DNA loaded onto the MBD column. Thus, it would appear that the large majority of CRC stools in practice contain human DNA amplifiable with MBD capture. However, considering that human DNA concentrations are <100 ng/g stool in approximately 40% of CRC stools *(15)*, the detection of methylation markers without enrichment may be limited in a substantial proportion of stools. Analyte enrichment by MBD capture or some other approach will likely be necessary to achieve adequately sensitive stool-based methylated marker assays intended for the early detection of colorectal neoplasia.

Another method of stool analyte enrichment has been developed in which specific DNA sequences are captured with complementary oligonucleotide probes *(17–19)*. This method requires separate capture probes for each marker assayed. In contrast, because the methyl-CpG binding domain is a shared functional domain of MBD proteins (MeCP2, MBD1–4) *(20)* and binds to the large majority of methylated CpG islands *(21)*, the MBD column method could theoretically capture most methylated CpG islands and so enrich multiple methylated genes in a single capture. But additional experiments using tumor-specific

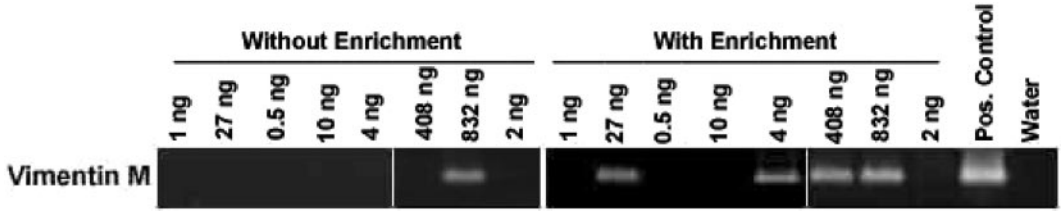

Fig. 4. Improved sensitivity of detecting methylated vimentin in clinical stool samples through MBD enrichment.

Stools from 8 CRC patients with methylated tumor were used. Methylated vimentin was amplified with MSP in stool DNA with and without MBD enrichment. Human DNA amounts in 8 stool DNA samples, quantified with real-time *Alu* PCR, were 1, 27, 0.5, 10, 4, 408, 832, and 2 ng.

methylated genes are needed to fully test the universal capture potential of this method.

Of note, our results show that MBD protein has very low affinity to bacterial DNA, which has a higher CG:AT ratio than the human genome and is densely methylated at cytosine and adenine residues by Dcm and Dam methyltransferase *(14)*. Bacterial DNA fragments could be eluted into buffers at concentrations <0.6 mol/L NaCl. Methylated human DNA was enriched approximately 100-fold by the MBD column without interference by the abundant bacterial DNA in stool.

Although the current study has demonstrated the feasibility of the MBD capture method to increase stool assay sensitivity of methylated vimentin for CRC detection, additional studies are needed to assess the broad use of this method with other methylated genes and its application in clinical practice. Results must be corroborated in large patient studies, and technological refinements to simplify this method would enhance its pragmatic use.

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