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BACTERIAL INFECTIONS

Antibiotic Degradation by Commensal Microbes Shields Pathogens

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ABSTRACT The complex bacterial populations that constitute the gut microbiota can harbor antibiotic resistance genes (ARGs), including those encoding β-lactamase enzymes (BLA), which degrade commonly prescribed antibiotics such as ampicillin. The prevalence of such genes in commensal bacteria has been increased in recent years by the wide use of antibiotics in human populations and in livestock. While transfer of ARGs between bacterial species has well-established dramatic public health implications, these genes can also function in *trans* within bacterial consortia, where antibiotic-resistant bacteria can provide antibiotic-sensitive neighbors with leaky protection from drugs, as shown both *in vitro* and *in vivo*, in models of lung and subcutaneous coinfection. However, whether the expression of ARGs by harmless commensal bacterial species can destroy antibiotics in the intestinal lumen and shield antibiotic-sensitive pathogens is unknown. To address this question, we colonized germfree or wild-type mice with a model intestinal commensal strain of *Escherichia coli* that produces either functional or defective BLA. Mice were subsequently infected with *Listeria monocytogenes* or *Clostridioides difficile*, followed by treatment with oral ampicillin. The production of functional BLA by commensal *E. coli* markedly reduced clearance of these pathogens and enhanced systemic dissemination during ampicillin treatment. Pathogen resistance was independent of ARG acquisition via horizontal gene transfer but instead relied on antibiotic degradation in the intestinal lumen by BLA. We conclude that commensal bacteria that have acquired ARGs can mediate shielding of pathogens from the bactericidal effects of antibiotics.

KEYWORDS antibiotic resistance, gut microbiota, infection

Antibiotic administration has markedly reduced the morbidity and mortality associated with bacterial infections in the preantibiotic era. Increasing antibiotic-resistance in pathogenic microbes, mediated in part by acquired genes that encode antibiotic-degrading enzymes, represents a major threat to human health (1).

The gut microbiota contains trillions of commensal bacteria that can also harbor antibiotic resistance genes (ARGs) (2). Notably, antibiotic exposure can increase ARG gene representation and expression by the gut microbiota (3). Horizontal ARG transfer represents a mechanism by which drug-sensitive microbes can acquire resistance, e.g., by acquisition of genes encoding antibiotic-degrading hydrolases (4, 5). Thus, it is possible that commensal bacterial species transfer ARGs to intestinal pathogens upon antibiotic exposure in the gut lumen. However, another possibility is that production of antibiotic-degrading enzymes by the resident intestinal microbiota protects otherwise drug-sensitive pathogens in *trans*, thereby facilitating their replication and spread in the host. In fact, within microbial communities leaky protection from β-lactams and chloramphenicol can be provided to antibiotic-sensitive bacteria by antibiotic-

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degrading microbes, as shown both *in vitro* and *in vivo*, using mouse models of pneumonia and subcutaneous abscesses (6–12). Importantly, antibiotic degradation could be operated by bacteria belonging to the *Enterobacteriaceae* family (11) or *Bacteroides* genus (10, 12), which are highly represented in the human and mouse intestinal tract, suggesting that pathogen-shielding antibiotic degradation might occur also in the gut. Based on these considerations, we set out to investigate whether harmless autochthonous bacteria might degrade orally administered antibiotics in the intestinal lumen, thereby impairing their ability to combat intestinal pathogens.

RESULTS

To test this hypothesis in a controlled system, we reconstituted germfree (GF) mice with an *E. coli* strain, utilized here as a model commensal, that expresses either a wild-type (WT) form of β-lactamase (TEM-1) or an inactive point mutant (here referred to as *WT* β-lactamase enzyme [BLA] or *mut* BLA, respectively) (Fig. 1A) (13). This approach yielded cohorts of mice that, with the exception of one codon, harbor identical metagenomes, thus excluding differences in microbiota functions (e.g., immune activation, colonization resistance, etc.) that are not related to the β-lactam degradation.

Although *WT* BLA and *mut* BLA *E. coli* reached identical luminal bacterial densities in reconstituted mice, a colorimetric assay confirmed that only the intestinal content of mice reconstituted with *WT* BLA *E. coli* retained the capacity to hydrolyze β-lactams (Fig. 1B and C). At 1 week after reconstitution, mice were orally infected with the foodborne pathogen *Listeria monocytogenes* 10403s. *L. monocytogenes* is highly sensitive to β-lactam antibiotics and can expand in the gut lumens of mice that lack colonization resistance (14). Mice were then administered ampicillin on days +1 and +2 after *L. monocytogenes* infection and sacrificed on day +3. As expected, *L. monocytogenes* reached identical densities in the intestines of *WT* BLA or *mut* BLA *E. coli* reconstituted mice on day +1, indicating that the two *E. coli* strains did not differ in their inability to provide colonization resistance against *L. monocytogenes* (Fig. 1D). However, we found significantly higher *L. monocytogenes* burdens in multiple organs in mice harboring *WT* BLA *E. coli* on day +3, consistent with the notion that β-lactamase-dependent ampicillin degradation shielded *L. monocytogenes* from the therapeutic antibiotic's action (Fig. 1E).

To exclude the possibility that *L. monocytogenes* might have acquired resistance to ampicillin via horizontal gene transfer, we inoculated single *L. monocytogenes* colonies recovered from the cecal contents of *WT* BLA *E. coli*-reconstituted, *L. monocytogenes*-infected mice into liquid medium either in the presence or in the absence of ampicillin. Notably, none of the inoculated *L. monocytogenes* colonies grew in the presence of ampicillin, in contrast to *WT* BLA-expressing *E. coli* colonies recovered from the same mice (Fig. 1F). Furthermore, none of the *L. monocytogenes* colonies tested positive for the presence of the β-lactamase gene, which was uniformly detected in colonies of *WT* BLA *E. coli* by PCR (Fig. 1G).

To confirm that the increased *L. monocytogenes* burden observed above was due to antibiotic degradation by resident *E. coli*, we collected the cecal contents of mice reconstituted with either *WT* BLA or *mut* BLA *E. coli* and treated with ampicillin in the drinking water for two consecutive days to allow for luminal accumulation of the antibiotic. Inoculation of *L. monocytogenes* into serial dilutions of the cecal content supernatants revealed that the cecal contents from GF mice reconstituted with *mut* BLA *E. coli* had a higher inhibitory capacity than cecal contents recovered from mice reconstituted with *WT* BLA *E. coli* (Fig. 1H). These results were replicated using WT mice reconstituted with the model *E. coli* strains following antibiotic treatment (see Fig. S1 in the supplemental material). Since the presence of active β-lactamase was the only *bona fide* difference between the cecal contents of the two cohorts of mice used in the above-described experiments, we conclude that the microbiota-encoded enzymatic activity curtailed the efficacy of ampicillin treatment against *L. monocytogenes*.

To expand our observations beyond the *Listeria* model and to assess whether

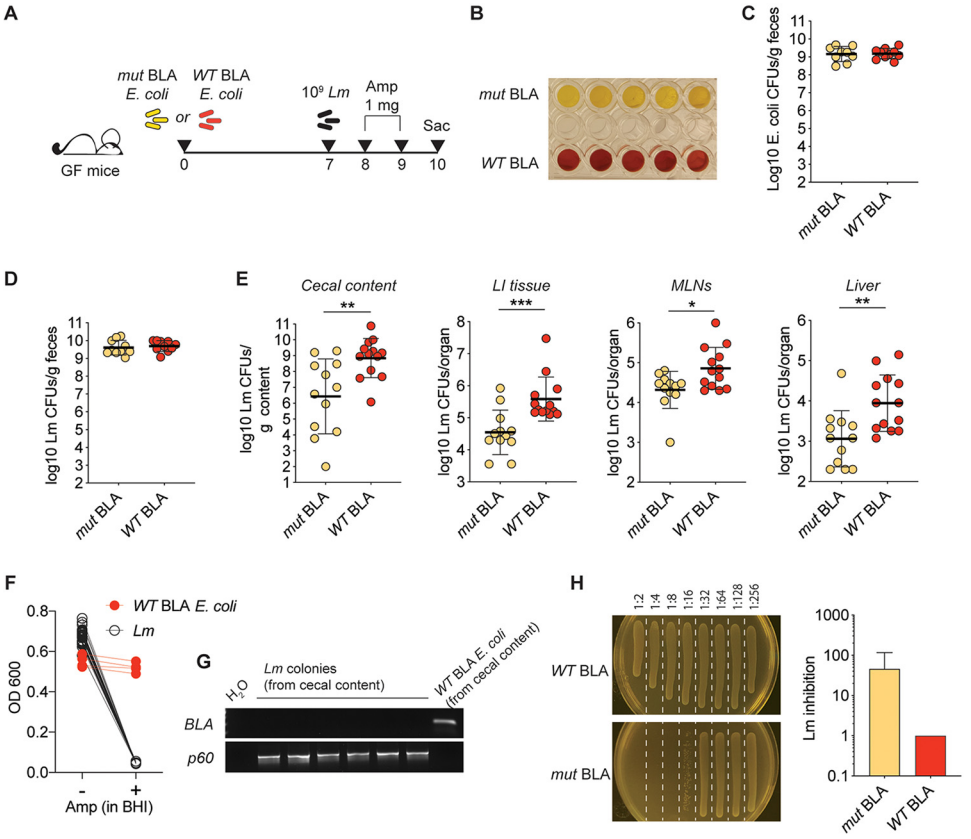

FIG 1 β-Lactamase production by a model commensal curtails the efficacy of ampicillin against *L. monocytogenes*. (A) Schematic representation of the experimental design. (B) A nitrocefin assay was performed on resuspended fecal pellets obtained from the depicted groups of mice. Each well represents a different mouse. The results of one representative experiment of three performed are shown. (C) Reconstitution levels for the depicted *E. coli* strains, as measured by plating of fecal pellets on day 7 after reconstitution (day of infection), onto selective plates ($n = 8$ to 10; data are pooled from two independent experiments; shown are individual data points and geometric means). (D) Luminal *L. monocytogenes* burdens in the depicted mice at 1 day postinfection, measured by plating fecal pellets onto selective plates. (E) *L. monocytogenes* burdens in the depicted compartments at day 3 postinfection. In panels D and E, data are pooled from three independent experiments ($n = 12$) and shown as individual data points and geometric means (Mann-Whitney test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). (F) Individual *L. monocytogenes* colonies ($n = 28$) or *WT* BLA *E. coli* colonies ($n = 4$) from four to five different mice were inoculated into BHI with or without ampicillin. The OD was measured after overnight culture. (G) Colonies utilized for the experiment depicted in panel F were also subjected to PCR with primers specific for the TME-1 β-lactamase gene or p60 (*L. monocytogenes* positive control). Shown are results for six *L. monocytogenes* colonies and one *E. coli* colony. Identical results were obtained for all tested colonies. (H) The cecal contents of WT mice reconstituted with either *WT* or *mut* BLA *E. coli* and administered ampicillin in drinking water for 2 days were serially diluted and inoculated with *L. monocytogenes*. *L. monocytogenes* growth was assessed after overnight culture by measuring the OD and direct plating (one representative plate per condition shown on the left). Plotted values correspond to the first dilution allowing for detectable *L. monocytogenes* growth (with 1 indicating *L. monocytogenes* growth at all dilutions; $n = 3$, means ± standard deviations are shown). Similar results were obtained utilizing antibiotic-treated, *E. coli*-reconstituted animals (see Fig. S1 in the supplemental material).

commensal-mediated antibiotic degradation may represent a mechanism that is relevant to other infectious agents, we adapted our experimental strategy to an established infection model using *C. difficile* (15) (Fig. 2A), an important intestinal pathogen that is also sensitive to ampicillin (Fig. S2). Of note, this model allowed us to investigate the relevance of our findings in a setting where expansion of an antibiotic-resistant microbe takes place following antibiotic-mediated depletion of the intestinal microbiota, a common occurrence in hospitalized patients (16). Importantly, expansion of the utilized *E. coli* strains in this setting was restrained by competition with the residual autochthonous flora (Fig. 2B), which allowed us to verify the effects of BLA-mediated ampicillin degradation in a context of *bona fide* reduced enzymatic concentration.

Similar to the results obtained with *L. monocytogenes*, we observed indistinguishable

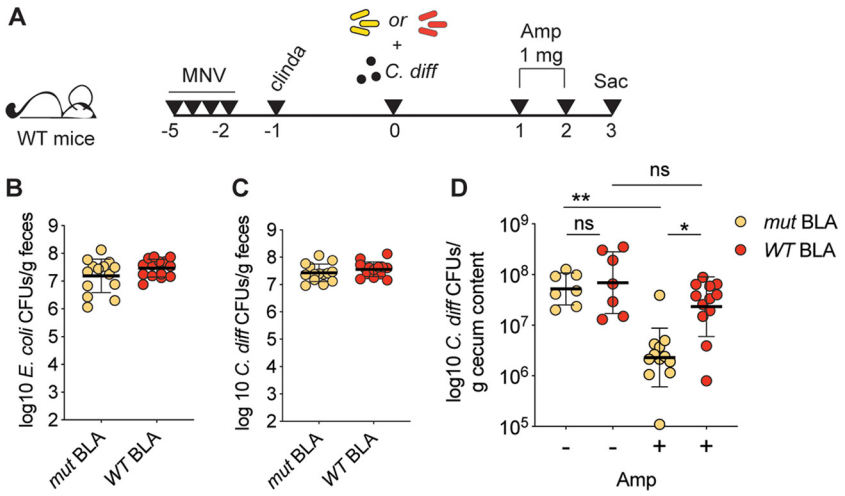

FIG 2 Endogenous antibiotic degradation impacts treatment of *C. difficile* infection. (A) Schematic representation of the experimental design. (B) Reconstitution levels for mice reconstituted with either *WT* or *mut* BLA *E. coli* (as depicted in panel A) at 1 day after oral gavage, as assessed by selective plating of fecal pellets ($n = 14$; data are pooled from two independent experiments; individual data points and geometric means are shown). (C) *C. difficile* burdens in mice treated as depicted in panel A at 1 day after oral gavage, as assessed by selective plating of fecal pellets ($n = 14$; data are pooled from two independent experiments; individual data points and geometric means are shown). (D) *C. difficile* burden in the cecal content of mice treated as depicted in panel A at day 3 postinfection ($n = 7$ for controls; $n = 12$ for ampicillin treated). Data are pooled from three independent experiments. Individual data points and geometric means are shown (Kruskal-Wallis test with multiple comparisons: *, $P < 0.05$; **, $P < 0.01$).

levels of expansion for both *E. coli* and *C. difficile* on day +1 after reconstitution or infection, respectively, in all groups of mice (Fig. 2B and C). In agreement with our previous findings, the *C. difficile* burden was significantly reduced by ampicillin treatment in mice reconstituted with *mut* BLA *E. coli* but not in mice reconstituted with *WT* BLA *E. coli* (Fig. 2D). Direct comparison of the ampicillin-treated mice confirmed a significantly higher burden in mice whose intestinal flora had the capacity to hydrolyze β-lactams (Fig. 2D).

DISCUSSION

The ability of antibiotic-resistant organisms to degrade antibiotics, thus facilitating growth of antibiotic-sensitive bacteria within a microbial community, is well established and has been demonstrated using different antimicrobial compounds (7–9).

In particular, earlier studies *in vivo* (6, 10–12, 17) revealed that β-lactamase- or chloramphenicol-acetyltransferase-producing bacteria inoculated in conjunction with antibiotic-sensitive pathogens in the hypodermis or lungs could hinder the efficacy of β-lactams or chloramphenicol, respectively. Clinical data also suggested that the presence of one β-lactamase-producing bacterial strain at the site of infection could enhance persistence of a pathogen upon antibiotic treatment (18). In these settings, members of the *Bacteroides* genus, among the most highly represented genera in the human intestine (19), were also identified as BLA carriers.

Our findings expand on the studies described above by suggesting that ARGs expressed by commensal bacteria can shape the chemical niche of the intestine and confer an apparent antibiotic-resistant phenotype to pathogens in *trans*, without direct acquisition of ARGs by the pathogenic microbe. We refer to this activity as commensal-mediated pathogen shielding.

Using two different infection models, we show that the production of β-lactamases, a prototypical antibiotic resistance factor, by resident intestinal microbes can significantly reduce the effectiveness of ampicillin treatment, thereby generating a safe environment in which otherwise sensitive pathogens are shielded from this drug.

Although our findings were partly obtained using a model of GF mouse monocolonization, previous studies in healthy volunteers demonstrated that upon treatment with cephalosporins, subjects harboring BLA-producing commensal strains, unlike

BLA-negative subjects, had undetectable concentrations of the drug in the feces and maintained a rich microbiota, providing evidence that BLA concentrations sufficient to inactivate antibiotics are commonly achieved in humans (20, 21). Consistent with these observations, our laboratory recently showed that a few bacterial strains, out of the dozens composing the microbiota of a mouse colony treated with ampicillin for over 8 years, had the capacity to hydrolyze ampicillin, while the other bacterial strains, in isolation, remained sensitive to ampicillin and thus were protected in *trans* by a minor subset of the microbiota (7; see Fig. 4A in reference 22).

Whether or not ARG enrichment within the gut microbiota is detrimental to host health is a complex question, and the answer is likely to be context dependent. For instance, oral administration of recombinant beta-lactamase or BLA-producing bacteria was shown to preserve the integrity of the microbiota following parenteral administration of β-lactam antibiotics in animal models, without affecting drug concentration in the serum (23–28). These approaches were shown to be advantageous in that they preserved colonization resistance against pathogens (23–28).

However, our study suggests that the presence or absence of commensal bacterial strains that inactivate β-lactam antibiotics is likely to impact clinical responses to antibiotic treatment, possibly contributing to interindividual variability in therapy outcomes.

In conclusion, we propose that commensal-mediated pathogen shielding in the intestine can impair the effectiveness of some antibiotic treatments during infection. Although pharmacokinetic studies have generally focused on antibiotic absorption, distribution, enzymatic modification, protein binding, and biliary/renal clearance, the role of microbiota-mediated antibiotic degradation in the gut lumen and its potential for dramatically impacting responses to antibiotic treatment have received less attention. Our findings extend the recently uncovered broad capacity of the gut microbiota to metabolize drugs, affecting their efficacy (29, 30). Within this model, antibiotics represent an additional class of xenobiotics that commensals can metabolize.

We propose that occurrence of pathogen shielding might be a relevant element to consider in the engineering of probiotic bacterial strains to be employed in clinical practice.

MATERIALS AND METHODS

Mouse husbandry. All experiments using WT mice were performed with C57BL/6J female mice that were 6 to 8 weeks old; the mice were purchased from Jackson Laboratories. Germfree (GF) mice were bred in-house in germfree isolators. After reconstitution, the mice were housed in sterile, autoclaved cages with irradiated food and acidified, autoclaved water. All animals were maintained in a specific-pathogen-free facility at Memorial Sloan Kettering Cancer Center Animal Resource Center. Experiments were performed in compliance with Memorial Sloan-Kettering Cancer Center institutional guidelines and approved by the institution's Institutional Animal Care and Use Committee.

Generation of *E. coli* strains. Plasmids encoding WT TEM-1 β-lactamase (pDIMC8-TEM1) or mutated TEM-1 β-lactamase (pDIMC8-TEM1 W208G) were extracted from the RH06 and RH09 *E. coli* strains, published elsewhere (13), gel purified, and utilized for the transformation of Stellar competent cells (TaKaRa Bio) according to the manufacturer's instructions. The resulting strains were utilized for experiments throughout this study. Of note, the plasmids conferred resistance to chloramphenicol, and although the expression of the TEM-1 gene was placed under the regulation of a *tac* promoter, we did not induce it by IPTG (isopropyl-β-D-thiogalactopyranoside) treatment but rather exclusively relied on leaky transcription of the gene to produce more physiologically relevant conditions.

Antibiotic treatment, reconstitution, and infections. GF mice were gavaged with either of two strains of *E. coli*, encoding a functional or a point-mutated version of TEM-1 β-lactamase, respectively. At 1 week after reconstitution, mice were gavaged with 10^9 CFU of *L. monocytogenes* strain 10403s and administered 1 mg of ampicillin (Fisher) by oral gavage daily for 2 consecutive days. Animals were euthanized at day 3 postinfection. Upon reconstitution of WT mice with *E. coli* strains for *in vitro* experiments involving dilution of cecal content, the mice were treated for 3 days with metronidazole and vancomycin in drinking water (0.5 g/liter), left on regular water for 1 day, and then gavaged with the appropriate *E. coli* strain. At 1 week after reconstitution, the mice were treated with ampicillin in drinking water (0.5 g/liter) for 2 days prior to being euthanized.

For *C. difficile* infection experiments, WT C57BL/6 mice were administered a combination of metronidazole, neomycin, and vancomycin (0.25 g/liter each) in drinking water for 3 days, and at 24 h after antibiotic regimen cessation they were injected intraperitoneally with clindamycin (200 μg). On the following day, the mice were reconstituted with either *WT* or *mut* BLA *E. coli* (5×10^4 CFU) and 200 to 500 spores of *C. difficile* strain VPI 10463 (ATCC 43255).

β-Lactamase detection assay. Fecal pellets from animals were collected and resuspended in phosphate-buffered saline (PBS) at 100 mg/ml. Samples were left undisturbed for 5 min to allow particulate matter to sediment. Next, 50 μl of the suspension was pipetted into a 96-well plate with 50 μl of nitrocefin (Oxoid), followed by incubation for 30 min at room temperature while protected from light.

CFU enumeration and selective plating. *L. monocytogenes* was identified through plating of serial dilutions of homogenized organs (prepared as described elsewhere [14]) or fecal material (resuspended 100 mg/ml in PBS) onto brain heart infusion (BHI) plates supplemented with streptomycin (100 μg/ml) and nalidixic acid (50 μg/ml).

E. coli CFU were enumerated after plating of serial dilution of fecal material onto Luria-Bertani (LB) plates supplemented with chloramphenicol (50 μg/ml). *E. coli* CFU numbers obtained from plating of ex-GF mice at day of infection onto LB plates (not supplemented with antibiotics) yielded identical numbers, indicating that plasmids carrying the chloramphenicol (CM) resistance cassette, as well as the *WT/mut* BLA gene, were maintained even in the absence of any selective pressure.

For detection of *C. difficile*, fecal pellets or cecal content was resuspended in deoxygenated PBS, and 10-fold dilutions were plated on BHI agar supplemented with yeast extract, taurocholate, L-cysteine, cycloserine, and cefoxitin at 37°C in an anaerobic chamber (Coylabs) overnight.

***L. monocytogenes* culture in cecal content.** Cecal contents were recovered from *E. coli* reconstituted WT or GF animals, resuspended in PBS at 300 mg/ml (WT), and spun down at 3,000 rpm for 10 s. Serial 1:2 dilutions of the resulting supernatant were generated using PBS, and 100 μl of each dilution was plated in replicate in flat-bottom 96-well plates. An equal volume of BHI medium supplemented with streptomycin (200 μg/ml) and nalidixic acid (100 μg/ml) acid (to prevent growth of residual *E. coli*) containing 100 to 1,000 CFU of *L. monocytogenes* 10403s was added on top. *L. monocytogenes* for this assay was prepared by reinoculating an overnight culture in liquid BHI at 37°C on shaker until the logarithmic phase of growth was reached (optical density [OD] = 0.1 to 0.4). After an overnight incubation at 37°C, the plate was assayed by OD_{600} reading, and individual dilutions were plated onto BHI-Strep-NA plates to assess *L. monocytogenes* growth. Normalized inhibition index was calculated as 1/first dilution allowing for *L. monocytogenes* growth, with the initial dilution being 1:2 to take into account the addition of a volume of BHI equivalent to that of the medium. For example, if the first dilution where *L. monocytogenes* was detected was 1:16, the resulting inhibition index would be 16. Within each experiment samples were then normalized to the baseline, obtained by averaging the values obtained in the control group, represented by mice reconstituted with *mut* BLA *E. coli*.

PCR. PCR was carried out with specific primers for β-lactamase (forward, 5′-GCTATGTGGCGCGGTATTAT-3′; reverse, 5′-AAGTAAGTTGGCCGCAGTGT-3′; product, 191 bp) and p60 (forward, 5′-GCGCAACAAACTGAAGCAAAGGATGC-3′; reverse, 5′-CTCGCGTTACCAGGCAAATAGATGGACG-3′; product, 1,300 bp) using SapphireAMp Fast PCR master mix (TaKaRa Bio) and the following conditions: 94°C for 1 min, followed by 30 cycles at 98°C for 5 min, 58°C for 5 min, and 72°C for 15 min.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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