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Effects of Amplification Facilitators on Diagnostic PCR in the Presence of Blood, Feces, and Meat

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The full potential of diagnostic PCR is limited, in part, by the presence of inhibitors in complex biological samples that reduce the amplification efficiency. Therefore, different pre-PCR treatments are being used to reduce the effects of PCR inhibitors. The aim of the present study was to investigate the effects of 16 amplification facilitators to enhance DNA amplification in the presence of blood, feces, or meat. Different concentrations of amplification facilitators and inhibitory samples were added to PCR mixtures containing *rTth* or *Taq* DNA polymerase. The addition of 0.6% (wt/vol) bovine serum albumin to reaction mixtures containing *Taq* DNA polymerase reduced the inhibitory effect of blood and allowed DNA amplification in the presence of 2% instead of 0.2% (vol/vol) blood. Furthermore, the addition of bovine serum albumin (BSA) to reaction mixtures containing feces or meat enhanced the amplification capacities of both polymerases. *Taq* DNA polymerase was able to amplify DNA in the presence of 4% instead of 0.4% (vol/vol) feces and 4% instead of 0.2% (vol/vol) meat, and *rTth* was able to amplify DNA in the presence of 4% instead of 0.4% (vol/vol) feces and 20% instead of 2% (vol/vol) meat. The single-stranded DNA binding T4 gene 32 protein (gp32) had a relieving effect similar to that of BSA, except when it was added to PCR mixtures of *rTth* containing meat and of *Taq* DNA polymerase containing feces. The relieving effects of betaine and a cocktail of proteinase inhibitors were more sample specific. The addition of 11.7% (wt/vol) betaine allowed *Taq* DNA polymerase to amplify DNA in the presence of 2% (vol/vol) blood, while the addition of proteinase inhibitors allowed DNA amplification by both polymerases in the presence of 4% (vol/vol) feces. When various combinations of betaine, BSA, gp32, and proteinase inhibitors were tested, no synergistic or additive effects were observed. The effects of facilitators on real-time DNA synthesis instead of conventional PCR were also studied.

Diagnostic PCR is limited, in part, by the presence of inhibitory substances in complex biological samples, which may interfere with the cell lysis step, inactivate the thermostable DNA polymerase, and/or interfere with nucleic acids (1, 4, 9, 15, 19, 28). Much effort is being devoted to the development of various sample pretreatments to generate PCR-compatible samples (for a review, see reference 11). However, sample preparation techniques are at present complicated, require experience, are difficult to handle for large numbers of samples, and are time-consuming. An alternative strategy that can be used to overcome PCR inhibition is to enhance the efficiency of PCR in the presence of complex biological samples. This can be done by using an alternative thermostable DNA polymerase more resistant to inhibitors (3, 9, 13, 27) and by using amplification facilitators such as bovine serum albumin (BSA), single-stranded DNA binding T4 gene 32 protein (gp32), organic solvents, and proteinase inhibitors (4, 5, 10, 13–15). The addition of amplification facilitators has also been found to improve the specificity of PCR and allow the amplification of GC-rich DNA sequences (8, 18, 22, 26), and/or increase the fidelity of DNA synthesis (29).

The aim of this study was to investigate the abilities of 16 amplification facilitators to enhance the amplification efficiencies of *rTth* and *Taq* DNA polymerases in the presence of blood, feces, and meat in conventional PCR. The abilities of the amplification facilitators to mediate DNA synthesis in reaction mixtures containing *rTth* and inhibitory samples with a single-stranded poly(dA) template with an oligo(dT) primer annealed to the 3′ end were also investigated. The reason for using this simplified system instead of conventional PCR was to avoid the interference of primer dimers and nonspecific amplicons.

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MATERIALS AND METHODS

Template DNA. The DNA of *Listeria monocytogenes* 167 vet, which was obtained from Swedish Meats R&D (Kävlinge, Sweden), was used as the target DNA in this study. The DNA extraction was performed in accordance with a standard technique described by Sambrook et al. (20), modified by the addition of 30 U of mutanolysin (Sigma Chemical Co., St. Louis, Mo.) per ml to the lysis solution. The concentration of DNA was determined spectrophotometrically (20).

PCR-inhibitory samples. The blood sample was drawn from a healthy person and placed into 5-ml evacuated blood collection tubes containing 0.1 ml (0.47 mol/liter) of EDTA (Terumo Europe N. V., Leuven, Belgium). A fecal sample (henceforth referred to as feces) was obtained from a healthy person and was diluted 10-fold in physiological saline solution and homogenized for 2 min in a stomacher (Lab-Blender 400; Steward Laboratory, London, United Kingdom). The minced pork meat (henceforth referred to as meat) was diluted 10-fold in physiological saline solution and was homogenized for 2 min in a stomacher. Each PCR-inhibitory sample was poured into sterile 1.5-ml Eppendorf tubes, and the tubes were stored at −20°C. The frozen blood, feces, and meat homogenates were thawed at room temperature, mixed with a vortex mixer, and left for 5 min to allow the large particles to settle before they were diluted and/or added to the PCR mixtures.

PCR assay and incubation conditions. The total volume of the PCR mixtures was 25 μl. The PCR assay was carried out as previously described by Lantz et al. (12). The PCR mixtures contained each of the primers rU8 and LM2 at a concentration of 0.5 μM (12, 16) and each of the deoxyribonucleoside triphosphates at a concentration of 0.2 mM. Reaction buffers for the DNA polymerases, as specified by the manufacturers, were as follows. The PCR buffer for *rTth* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) contained 5% (vol/vol) glycerol, 10 mM Tris-HCl (pH 8.3), 0.1 M KCl, 0.05% (wt/vol) Tween 20, 0.75 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid], 2.5 mM $MgCl_2$, and 1.25 U of *rTth* DNA polymerase. The PCR buffer for *Taq* DNA

TABLE 1. Effects of 16 PCR facilitators on amplification capacity of *Taq* DNA polymerase in the presence of blood, feces, and meat

Facilitator and facilitator concn[c]	PCR results[a] with the following at the indicated concn (% [vol/vol])[b]:															
	Blood					Feces						Meat				
	20	2	0.2	0.04	0.02	20	4	2	0.4	0.27	0.2	20	4	2	0.4	0.2
Acetamide																
0	−, −	−, −	+, −	+, +	+, ±	−, −	−	−, −	−, −	+	−, +	−, −	−, −	−, −	−, −	+, +
1	−, −	+, −	+, −	+, −	+, −	−, −	−	−, −	−, −	+	−, +	−, −	−, −	−, −	−, −	+, ±
2.5	−, −	−, −	±, −	+, −	+, −	−, −	−	−, −	−, −	+	−, +	−, −	−, −	−, −	−, −	±, −
4	−, −	−, −	−, −	−, −	±, −	−, −	−	−, −	−, −	+	−, +	−, −	−, −	−, −	−, −	−, −
Betaine																
0	−, −	−, −	−, ±	−, ±	−, −	−, −	−	−, −	−, +	+	−, +	−, −	−, −	−, −	−, −	+, ±
5.9	−, −	+, +	+, +	+, +	+, +	−, −	−	−, −	−, +	+	−, +	−, −	−, −	−, −	±, ±	+, +
8.8	−, −	±, +	+, +	+, +	+, +	−, −	−	−, −	−, +	+	−, +	−, −	−, −	−, −	−, +	+, +
11.7	−, −	+, +	+, +	+, +	+, +	−, −	−	−, −	−, +	+	−, +	−, −	−, −	−, −	±, +	+, +
BSA																
0	−, −	−, −	−, −	−, −	±, −	−, −	−	−, −	−, +	+	−, +	−, −	−, −	−, −	−, −	±, −
0.2	−, −	−, −	−, ±	±, +	+, +	−, −	−	+, +	+, +	+	+, +	−, −	±, +	+, +	+, +	+, +
0.4	−, −	−, +	−, +	+, +	+, +	−, −	±	+, +	+, +	+	+, +	−, −	−, +	+, +	+, +	+, +
0.6	−, −	−, +	±, +	+, +	+, +	−, −	+	+, +	+, +	+	+, +	−, −	−, +	+, +	+, +	+, +
Dextran 40																
0	−, −	±, −	±, −	±, ±	±, +	−, −	−	−, −	−, −	+	−, +	−, −	−, −	−, −	±, −	±, ±
1	−, −	±, −	−, −	−, +	−, +	−, −	−	−, −	−, −	+	−, +	−, −	−, −	−, −	+, −	+, +
2.5	−, −	−, −	−, ±	−, +	−, +	−, −	−	−, −	−, −	+	−, +	−, −	−, −	−, −	±, −	+, +
4	−, −	−, −	−, ±	−, +	−, +	−, −	−	−, −	−, −	+	−, +	−, −	−, −	±, −	±, −	+, +
Dextran 500																
0	−, −	−, −	−, ±	−, +	−, ±	−, −	−	−, −	−, −	+	−, +	−, −	−, −	−, −	±, −	±, +
1	−, −	−, −	−, −	−, +	−, +	−, −	−	−, −	−, −	+	−, +	−, −	−, −	−, −	+, +	+, +
2.5	−, −	−, −	−, −	−, ±	−, ±	−, −	−	−, −	−, ±	+	−, +	−, −	−, −	−, −	+, −	±, +
4	−, −	−, ±	−, ±	−, ±	−, ±	−, −	−	−, −	−, +	+	−, +	−, −	+, −	+, −	+, ±	+, ±
Dimethyl sulfoxide																
0	−, −	−, −	−, −	−, −	−, −	−, −	−	−, −	−, −	+	−, +	−, −	−, −	−, −	−, −	+, −
2.5	−, −	−, −	−, −	−, −	−, −	−, −	−	−, −	−, −	+	−, +	−, −	−, −	−, −	−, −	+, −
5	−, −	−, −	−, −	−, −	−, −	−, −	−	−, −	−, −	+	−, +	−, −	−, −	−, −	−, −	+, −
7.5	−, −	−, −	−, −	−, −	−, −	−, −	−	−, −	−, −	+	−, +	−, −	−, −	−, −	−, −	+, −
Formamide																
0	−, −	−, −	−, −	−, −	−, −	−, −	−	−, −	−, +	+	−, +	−, −	−, −	−, −	±, −	+, −
0.25	−, −	−, −	−, −	−, −	−, −	−, −	−	−, −	−, +	+	−, +	−, −	−, −	−, −	±, −	+, +
0.5	−, −	−, −	−, −	−, −	−, −	−, −	−	−, −	−, ±	+	−, +	−, −	−, −	−, −	±, −	+, −
1	−, −	−, −	−, −	−, −	−, −	−, −	−	−, −	−, −	−	−, −	−, −	−, −	−, −	±, −	+, −
Glycerol																
0	−, −	−, ±	±, ±	−, −	−, −	−, −	−	−, −	−, −	+	−, +	−, −	−, −	−, −	±, −	+, ±
5	−, −	−, ±	−, ±	−, −	−, −	−, −	−	−, −	−, −	+	−, +	−, −	−, −	−, −	−, −	+, −
7.5	−, −	−, −	−, −	−, −	−, −	−, −	−	−, −	−, −	+	−, +	−, −	−, −	−, −	−, −	±, −
10	−, −	−, −	−, −	−, −	−, −	−, −	−	−, −	−, −	+	−, +	−, −	−, −	−, −	−, −	+, −
gp32																
0	−, −	+, +	+, +	−, −	−, −	−, −	−, −	−, −	−, −	±, −	−, +	−, −	−, −	−, −	−, −	+, ±
0.01	−, −	+, +	+, +	+, +	+, +	−, −	−, −	−, ±	−, +	−, ±	−, +	−, −	+, +	+, +	+, +	+, +
Nonidet P-40																
0	−, −	−, −	+, −	+, −	+, −	−, −	−	−, −	−, −	+	−, +	−, −	−, −	−, −	±, −	+, −
0.1	−, −	−, −	−, −	+, −	+, −	−, −	−	−, −	−, −	+	−, +	−, −	−, −	−, −	−, −	+, ±
0.25	−, −	−, −	−, −	±, −	±, −	−, −	−	−, −	−, −	+	−, +	−, −	−, −	−, −	−, −	+, −
0.5	−, −	−, −	+, −	±, −	±, −	−, −	−	−, −	−, −	+	−, +	−, −	−, −	−, −	−, −	+, −
Polyethylene glycol 400																
0	−, −	−, −	−, −	−, −	−, −	−, −	−	−, −	−, −	+	−, +	−, −	−, −	−, −	−, −	+, −
1	−, −	−, −	±, −	−, −	+, −	−, −	−	−, −	−, ±	+	−, +	−, −	−, −	−, −	−, −	+, −
2	−, −	−, −	±, −	±, −	±, −	−, −	−	−, −	−, +	+	−, +	−, −	−, −	−, −	−, −	+, −
4	−, −	−, −	±, −	+, −	+, −	−, −	−	−, −	−, +	+	−, +	−, −	−, −	−, −	−, −	−, −

Continued on following page

TABLE 1—*Continued*

Facilitator and facilitator concn[c]	PCR results[a] with the following at the indicated concn (% [vol/vol])[b]:															
	Blood					Feces						Meat				
	20	2	0.2	0.04	0.02	20	4	2	0.4	0.27	0.2	20	4	2	0.4	0.2
Polyethylene glycol 4000																
0	−, −	−, −	−, −	−, −	−, −	−, −	−	−, −	−, −	+	−, +	−, −	−, −	−, −	+, −	+, −
1	−, −	−, −	−, −	−, −	−, −	−, −	−	−, −	−, ±	+	−, +	−, −	−, −	−, −	±, −	+, −
2.5	−, −	−, −	−, −	−, −	−, −	−, −	−	−, −	−, ±	+	−, +	−, −	−, −	−, −	−, −	+, −
4	−, −	−, −	−, −	−, −	−, −	−, −	−	−, −	−, +	+	−, +	−, −	−, −	−, −	−, −	+, −
Proteinase inhibitor[d]																
0	−, −	−, −	+, −	+, −	+, −	−, −	−	−, −	−, −	+	−, +	−, −	−, −	−, −	+, −	+, −
0.25×	−, −	−, −	±, −	+, −	+, −	−, −	−	−, +	+, +	+	+, +	−, −	−, −	−, −	±, −	+, +
0.5×	−, −	−, −	−, −	±, −	+, −	−, −	−	+, +	+, +	+	+, +	−, −	−, −	−, −	±, −	+, −
1×	−, −	−, −	−, −	−, −	±, −	−, −	+	+, +	+, +	+	+, +	−, −	−, −	−, −	+, −	+, −
TMAC[e]																
0	−, −	−, −	−, −	−, −	−, −	−, −	−	−, −	−, ±	+	−, +	−, −	−, −	−, −	+, −	+, −
2.8×10^{-4}	−, −	−, −	−, −	−, −	−, −	−, −	−	−, −	−, ±	+	−, +	−, −	−, −	−, −	+, −	+, −
5.5×10^{-4}	−, −	−, −	−, −	−, −	−, −	−, −	−	−, −	−, ±	+	−, +	−, −	−, −	−, −	+, −	+, −
1.1×10^{-3}	−, −	−, −	−, −	−, −	−, −	−, −	−	−, −	−, ±	+	−, +	−, −	−, −	−, −	+, −	+, −
Tween 20																
0	−, −	−, −	±, −	+, −	+, −	−, −	−	−, −	−, −	+	−, +	−, −	−, −	−, −	−, −	±, −
0.1	−, −	−, −	±, −	+, −	+, −	−, −	−	−, −	−, −	+	−, +	−, −	−, −	−, −	−, −	±, −
0.25	−, −	−, −	−, −	+, −	+, −	−, −	−	−, −	−, +	+	−, +	−, −	−, −	−, −	−, −	±, −
0.5	−, −	−, −	−, −	±, −	+, −	−, −	−	−, −	−, +	+	−, +	−, −	−, −	−, −	−, −	±, −
Tween 80																
0	−, −	−, −	−, −	−, −	−, ±	−, −	−	−, −	−, +	+	−, +	−, −	−, −	−, −	+, −	+, ±
0.1	−, −	−, −	−, −	−, ±	−, +	−, −	−	−, −	−, +	+	−, +	−, −	−, −	−, −	±, −	+, ±
0.2	−, −	−, −	−, −	−, −	−, ±	−, −	−	−, −	−, +	+	−, +	−, −	−, −	−, −	±, −	+, ±
0.5	−, −	−, −	−, −	−, −	−, ±	−, −	−	−, −	−, +	+	−, +	−, −	±, −	−, −	−, −	+, ±

[a] Results are of one or two independent PCRs; +, PCR product of high yield; ±, PCR product of low yield; −, no PCR product. The results were recorded in the order in which the PCRs were done.

[b] Percentage (vol/vol) of blood and homogenates of feces and meat in PCR mixture for *Taq* DNA polymerase containing 1 ng of *L. monocytogenes* DNA.

[c] Percentage (wt/vol) of 16 amplification facilitators in the reaction mixtures. The PCR sensitivity in the presence of the concentrations of these facilitators was the same as that with a solution of pure water with DNA.

[d] One tablet of Proteinase Inhibitor Complete Mini cocktail (EDTA-free; Roche Molecular Biochemicals) was dissolved in 2 ml of deionized water. This concentration was five times greater than that recommended by the supplier.

[e] Tetramethylammonium chloride.

polymerase (Roche Molecular Biochemicals, Basel, Switzerland) contained 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl (pH 8.3; 20°C), and 0.75 U of *Taq* DNA polymerase. The 0.55-kb PCR product was visualized by 1.3% agarose gel electrophoresis containing ethidium bromide (20). The gel was analyzed with a gel documentation system (Bio-Rad Laboratories, Hercules, Calif.). The results were recorded as + (PCR product of high yield), ± (PCR product of low yield), or − (no PCR product).

Real-time DNA synthesis conditions. The reaction volumes were 25 μl. All mixtures contained 0.2 mM dTTP, a 1:10,000-diluted stock solution of SYBR Green I (Roche Molecular Biochemicals), 4 mM $MgCl_2$, and 10 ng of poly(dA) with oligo$(dT)_{12–18}$ (Amersham Pharmacia Biotech, Uppsala, Sweden). The reaction mixtures for *rTth* DNA polymerase contained 1× chelating buffer and 1.25 U of *rTth* (Perkin-Elmer Cetus). Different concentrations of PCR inhibitors (1, 0.2, and 0.04% [vol/vol] blood; 20, 4, and 2% [vol/vol] feces or meat), with or without PCR facilitators (11.7% [wt/vol] betaine, 0.4% [wt/vol] BSA, 0.01% [wt/vol] gp32, 1× proteinase inhibitor cocktail), were added to glass capillary tubes, and the tubes were incubated at 65°C. The background fluorescence for each sample was measured by measuring the fluorescence of a reaction mixture containing all the sample components except *rTth*. Ninety fluorescence measurements were taken at 20-s intervals. The fluorescence of the samples was monitored online with a LightCycler Instrument (Roche Molecular Biochemicals). The increase in fluorescence due to DNA synthesis was considered the difference between the sample fluorescence and the background fluorescence. The mean fluorescence level of three independent experiments was calculated.

RESULTS

Abilities of amplification facilitators to relieve inhibition of *rTth* and *Taq* DNA polymerases. The effects of 16 PCR facilitators on the amplification capacities of *rTth* and *Taq* DNA polymerases were tested in the presence of different concentrations of blood, feces, and meat (Tables 1 and 2). Except for gp32, three different concentrations that had no positive or negative effect on the detection limit in the absence of added PCR-inhibitory samples were investigated. Among the 16 facilitators tested, only BSA reduced the inhibition of both *rTth* and *Taq* DNA polymerases in the presence of all types of inhibitory samples. The addition of 0.4% (wt/vol) BSA allowed DNA amplification by *Taq* DNA polymerase in the presence of 2% instead of 0.02% (vol/vol) blood, 4% instead of 0.4% (vol/vol) feces, and 4% instead of 0.2% (vol/vol) meat. The corresponding values for *rTth* were 4% instead of 0.4% (vol/vol) feces and 20% instead of 0.4% (vol/vol) meat. *rTth* was, as observed earlier (3), able to amplify DNA in the presence of 20% (vol/vol) blood without the addition of any facilitators. When 0.01% (wt/vol) gp32 was added to the PCR mixtures, the inhibitory effects of blood and meat on *Taq* DNA polymerase were reduced by the same level as the addition of 0.4% (wt/vol) BSA. A similar effect was also observed when gp32 was added to reaction mixtures of *rTth* containing feces or meat. However, the ability of gp32 to reduce the inhibition of *Taq* DNA polymerase by feces was not reproducible when different batches of *Taq* DNA polymerase and buffers were used. For

TABLE 2. Effects of 16 PCR facilitators on amplification capacity of *rTth* DNA polymerase in the presence of feces and meat

Facilitator and facilitator concn[c]	PCR results[a] with the following at the indicated concn (% [vol/vol])[b]:									
	Feces					Meat				
	20	4	2	0.4	0.2	20	4	2	0.4	0.2
Acetamide										
0	−, −	−, −	−, −	+, ±	+, +	−, −	−, −	±, −	+, ±	+, ±
1	−, −	−, −	−, −	+, −	+, ±	−, −	−, −	−, −	+, ±	+, +
2.5	−, −	−, −	−, −	+, −	+, ±	−, −	−, −	−, −	+, −	+, ±
4	−, −	−, −	−, −	±, −	+, −	−, −	−, −	−, −	±, −	±, −
Betaine										
0	−, −	−, −	−, −	+, −	+, +	−, −	−, −	−, −	±, +	±, +
5.9	−, −	−, −	−, −	+, −	+, +	−, −	±, −	+, −	±, +	+, +
8.8	−, −	−, −	−, −	+, ±	+, +	−, −	−, ±	±, ±	+, +	+, +
11.7	−, −	−, −	−, −	+, +	+, +	−, −	−, −	+, −	+, +	+, +
BSA										
0	−, −	−, −	−, −	−, +	+, +	−, −	−, −	±, −	+, +	+, +
0.2	−, −	+, −	±, ±	−, +	+, +	+, +	+, +	+, +	+, +	+, +
0.4	−, −	+, ±	+, +	+, +	+, +	+, +	+, +	+, +	+, +	+, +
0.6	−, −	−, ±	−, +	+, +	+, +	+, +	+, +	+, +	+, +	+, +
Dextran 40										
0	−, −	−, −	−, −	+, +	+, +	−, −	−, −	±, −	+, +	+, +
1	−, −	−, −	−, −	+, +	+, +	−, −	+, −	±, +	+, +	+, +
2.5	−, −	−, −	+, −	+, +	+, +	−, −	−, −	−, −	+, +	+, +
4	−, −	−, −	−, −	+, +	+, +	−, −	−, −	+, +	+, +	+, +
Dextran 500										
0	−, −	−, −	−, +	+, +	+, +	−, −	−, −	+, ±	+, +	+, +
1	−, −	−, −	−, −	+, +	+, +	−, −	−, −	±, −	+, +	+, +
2.5	−, −	−, −	−, −	+, +	+, +	−, −	−, −	±, −	+, +	+, +
4	−, −	−, −	−, −	+, +	+, +	−, −	−, −	−, −	±, +	+, +
Dimethyl sulfoxide										
0	−, −	−, −	−, −	+, +	+, +	−, −	−, −	±, −	±, +	+, +
2.5	−, −	−, −	−, −	+, +	+, +	−, −	−, −	−, −	±, +	+, +
5	−, −	−, −	−, −	+, +	+, +	−, −	−, −	−, −	−, ±	+, +
7.5	−, −	−, −	−, −	+, −	+, −	−, −	−, −	−, −	−, −	±, −
Formamide										
0	−, −	−, −	−, −	+, +	+, +	−, −	−, −	±, ±	±, +	+, +
0.25	−, −	−, −	−, −	+, −	+, +	−, −	±, −	±, −	±, +	+, +
0.5	−, −	−, −	−, −	+, −	+, +	−, −	−, −	±, −	+, −	+, +
1	−, −	−, −	−, −	+, −	+, −	−, −	−, −	±, −	±, −	+, −
Glycerol										
0	−, −	−, −	−, −	+, +	+, +	−, −	−, −	−, −	+, +	+, +
5	−, −	−, −	−, −	+, +	+, +	−, −	−, ±	−, ±	+, +	+, +
7.5	−, −	−, −	−, −	+, +	+, +	−, −	−, ±	+, ±	+, +	+, +
10	−, −	−, −	−, −	+, +	+, +	−, −	−, ±	−, +	+, +	+, +
gp32										
0	−, −	−, −	+, +	+, +	+, +	−, −	−, −	−, −	+, +	+, +
0.01	−, −	+, +	+, +	+, +	+, +	−, −	+, +	+, +	+, +	+, +
Nonidet P-40										
0	−, −	−, −	−, −	+, +	+, +	−, −	−, −	−, ±	+, +	+, +
0.1	−, −	−, −	−, −	+, +	+, +	−, −	−, −	−, −	+, +	+, +
0.25	−, −	−, −	−, −	+, +	+, +	−, −	−, −	−, −	+, +	+, +
0.5	−, −	−, −	−, ±	+, +	+, +	−, −	−, −	−, −	+, +	+, +
Polyethylene glycol 400										
0	−, −	−, −	−, −	+, +	+, +	−, −	−, −	−, −	+, +	+, +
1	−, −	−, −	−, ±	+, +	+, +	−, −	−, −	−, −	−, +	±, +
2	−, −	−, −	−, ±	+, +	+, +	−, −	−, −	−, −	−, +	±, +
4	−, −	−, −	−, ±	+, +	+, +	−, −	−, −	−, −	−, ±	−, +

Continued on following page

TABLE 2—*Continued*

Facilitator and facilitator concn[c]	PCR results[a] with the following at the indicated concn (% [vol/vol])[b]:									
	Feces					Meat				
	20	4	2	0.4	0.2	20	4	2	0.4	0.2
Polyethylene glycol 4000										
0	−, −	−, −	−, −	+, +	+, +	−, −	−, −	−, −	+, +	+, +
1	−, −	−, −	−, ±	+, +	+, +	−, −	−, −	−, −	+, +	+, +
2.5	−, −	−, −	−, +	+, +	+, +	−, −	−, −	−, −	−, +	+, +
4	−, −	−, −	−, ±	+, +	+, +	−, −	−, −	−, −	±, ±	±, +
Proteinase inhibitor[d]										
0	−, −	−, −	−, −	+, +	+, +	−, −	−, −	±, −	+, +	+, +
0.25×	−, −	−, −	+, ±	+, +	+, +	−, −	−, −	−, −	+, +	+, +
0.5×	−, −	−, −	+, ±	+, +	+, +	−, −	−, −	±, −	+, −	+, +
1×	−, −	+, ±	+, ±	+, +	+, +	−, −	−, −	−, −	±, −	+, +
TMAC										
0	−, −	−, −	−, −	+, ±	+, +	−, −	−, −	−, −	±, +	+, +
2.8×10^{-4}	−, −	−, −	−, −	+, ±	+, ±	−, −	−, −	−, −	+, +	+, +
5.5×10^{-4}	−, −	−, −	−, −	+, ±	+, +	−, −	−, −	−, −	±, +	+, +
1.1×10^{-3}	−, −	−, −	−, −	+, ±	+, +	−, −	−, −	−, −	±, +	+, +
Tween 20										
0	−, −	−, −	−, −	+, +	+, +	−, −	−, −	−, −	±, ±	+, +
0.1	−, −	−, −	−, −	+, +	+, +	−, −	−, −	±, −	±, ±	+, +
0.25	−, −	−, −	−, −	+, +	+, +	−, −	−, −	−, −	+, ±	+, +
0.5	−, −	−, −	−, −	+, +	+, +	−, −	−, −	−, −	±, +	+, +
Tween 80										
0	−, −	−, −	−, −	+, +	+, +	−, −	−, −	−, −	+, +	+, +
0.1	−, −	−, −	−, −	+, +	+, +	−, −	−, −	±, −	+, ±	+, +
0.25	−, −	−, −	−, ±	+, +	+, +	−, −	−, −	±, −	+, +	+, +
0.5	−, −	−, −	−, +	+, +	+, +	−, −	−, −	±, −	+, +	+, +

[a] Results are of two independent PCR results; +, PCR product of high yield; ±, PCR product of low yield; −, no PCR product. The results were recorded in the order in which the PCRs were done.

[b] Percentage (vol/vol) of homogenates of feces and meat in PCR mixture for *rTth* containing 1 ng of *L. monocytogenes* DNA.

[c] Percentage (wt/vol) of 16 amplification facilitators in the reaction mixtures. The PCR sensitivity in the presence of the concentrations of these facilitators was the same as that with a solution of pure water with DNA.

[d] One tablet of Proteinase Inhibitor Complete Mini cocktail (EDTA-free; Roche Molecular Biochemicals) was dissolved in 2 ml of deionized water. This concentration was five times greater than that recommended by the supplier.

example, in the first run of experiments, *Taq* DNA polymerase amplified DNA in the presence of 2% (vol/vol) feces. However, in the second run of an experiment with a new *Taq* DNA polymerase and buffer, the addition of gp32 could not overcome the inhibitory effect of feces. The addition of 11.7% (wt/vol) betaine allowed the *Taq* DNA polymerase to amplify the specific product in the presence of 2% (vol/vol) blood and 0.4% (vol/vol) meat, in comparison to weak amplification in the presence of 0.2 or 0.04% (vol/vol) blood and 0.2% (vol/vol) meat without any facilitator. Betaine was also found to relieve inhibition of *rTth* by meat and to allow DNA amplification in the presence of 4% instead of 0.4% (vol/vol) meat. Without proteinase inhibitors, *rTth* and *Taq* DNA polymerase were able to amplify DNA in the presence of 0.4% and 0.27% (vol/vol) feces, respectively. The addition of proteinase inhibitors, however, reduced the inhibition of both polymerases by feces and allowed DNA amplification in the presence of 4% (vol/vol) feces. No enhanced efficiency of PCR was observed for the other 12 facilitators.

Four facilitators (11.7% betaine, 0.4% BSA, 0.01% gp32, and 1× proteinase inhibitor mixture) which had the highest relieving effects were selected to study the effects of their combinations on the amplification capacities of *rTth* and *Taq* DNA polymerases in the presence of blood, feces, and meat (Table 3). No synergistic or additive effects were observed by the different combinations of the four facilitators. However, the PCR product yield was increased when betaine was combined with BSA or gp32 and when BSA was combined with gp32 in reaction mixtures of *Taq* DNA polymerase containing feces.

Quantitative effects of amplification facilitators. The capacity of *rTth* to synthesize DNA was monitored by the increase in fluorescence levels as a result of SYBR Green I binding to the double-stranded DNA formed. The effects of betaine, BSA, gp32, and proteinase inhibitors on real-time DNA synthesis of *rTth* in the presence of blood, feces, and meat are shown in Fig. 1. These fluorescence measurements were taken at the end of the incubation period. The maximum fluorescence was 100, even though the fluorescence for 21 of a total of 144 samples exceeded this value. The background fluorescence of reaction mixtures without inhibitory samples or facilitators was less than 1.5, which excludes the interference of poly(dA) with oligo(dT) on the fluorescence level. The highest background fluorescence (39%) was in the presence of 20% (vol/vol) feces, while the highest background fluorescence signals in the presence of 1% (vol/vol) blood and 20% (vol/vol) meat were 2 and 24%, respectively. The standard deviation values of the mean fluorescence showed large variations between the three runs of

TABLE 3. Effects of different combinations of four amplification facilitators on amplification capacities of *rTth* and *Taq* DNA polymerases in the presence of blood, feces, and meat

Polymerase and facilitator (facilitators concn[c])	PCR results[a] with the following at the indicated concn (% [vol/vol])[b]:								
	Blood[d]			Feces			Meat		
	20	4	2	20	4	2	20	4	2
rTth									
BSA (0.4), gp32 (0.01)				−, −	+, +	+, +	+, +	+, +	+, +
Betaine (11.7), gp32 (0.01)				−, −	−, +	+, +	−, −	+, +	+, +
gp32 (0.01), proteinase inhibitor (1×)				−, −	−, −	−, −	−, −	+, +	+, +
BSA (0.4), betaine (11.7)				−, ±	+, +	+, +	+, +	+, +	+, +
BSA (0.4), proteinase inhibitor (1×)				−, −	+, +	+, +	−, −	+, +	+, +
Betaine (11.7), proteinase inhibitor (1×)				−, −	+, +	+, +	−, −	−, −	−, −
Taq									
BSA (0.4), gp32 (0.01)	−,−	+,+	+,+	−, −	+, +	+, +	+, −	+, +	+, +
Betaine (11.7), gp32 (0.01)	−,−	−,−	−,−	−, −	+, +	+, +	−, −	+, +	+, +
gp32 (0.01), proteinase inhibitor (1×)	−,−	−,−	−,−	−, −	−, −	−, −	−, −	+, −	+, −
BSA (0.4), betaine (11.7)	−,−	+,+	+,+	−, −	+, +	+, +	−, −	+, +	+, +
BSA (0.4), proteinase inhibitor (1×)	−,−	+,+	+,+	−, −	+, +	+, +	−, −	+, +	+, +
Betaine (11.7), proteinase inhibitor (1×)	−,−	+,+	+,+	−, −	−, −	−, −	−, −	−, +	−, +

[a] Results are of two independent PCRs; +, PCR product of high yield; ±, PCR product of low yield; −, no PCR product. The results were recorded in the same order in which the PCRs were done.
[b] Percentage (vol/vol) of blood and homogenates of feces and meat in the PCR mixtures for *rTth* and *Taq* DNA polymerase containing 1 ng of *L. monocytogenes* DNA.
[c] Percentage (wt/vol) of amplification facilitators in the reaction mixtures.
[d] The polymerase *rTth* was able to amplify DNA in the presence of all concentrations of blood tested without the addition of any amplification facilitators.

experiments and were found to increase as the mean fluorescence signal increased. The mean fluorescence signal was the lowest in the presence of blood and the highest in the presence of meat. Inclusion of higher concentrations of blood was not possible due to the precipitation of blood proteins, which hindered the detection of the fluorescence at the tip of the glass capillary tubes. Despite the limitation of the assay, it was shown that addition of PCR-inhibitory samples reduced the fluorescence signal, and a linear relation was observed between the decrease in the concentration of inhibitor and the increase in the fluorescence signal, mainly when blood and feces were added to the reaction mixture without amplification facilitators (Fig. 1A and B). Addition of 11.7% (wt/vol) betaine was found to enhance the fluorescence signals in the reaction mixtures containing blood, feces, and meat. The maximum enhancement effect was found in the presence of meat, so that the addition of meat homogenate at ≤4% was no longer inhibitory compared to the inhibitory effects of reaction mixtures without inhibitors or facilitators, as determined from the fluorescence signals. In addition, a linear relation was observed between decreasing the concentration of inhibitors and an increase in the fluorescence signal in the presence of betaine. The fluorescence signals were only slightly affected in the presence of BSA, gp32, and proteinase inhibitors.

DISCUSSION

Betaine, BSA, gp32, and proteinase inhibitors improved the amplification capacities of *rTth* and *Taq* DNA polymerases in the presence of blood, feces, and meat when the effects of 16 amplification facilitators were investigated. BSA and, to a lesser extent, gp32 were the most efficient facilitators in conventional PCR. The presence of 0.4 to 0.6% (wt/vol) BSA was found to partially relieve the inhibitory effects of blood, feces, and meat when *Taq* DNA polymerase was used in conventional PCR. In a recent study (3), it was found that *rTth* was more resistant to biological samples than *Taq* DNA polymerase. In this study, it was shown that the resistance of *rTth* to biological samples was improved by including BSA in the reaction mixture. The ability of albumin to relieve inhibition may be related, in part, to its ability to bind to inhibitors such as heme (4). In a study by Tsutsui and Mueller (25), it was found that addition of heme-binding protein from rabbit serum completely restored the activity of Rauscher murine leukemia virus reverse transcriptase in the presence of 10^{-4} M hemin, whereas addition of ovalbumin, a non-heme-binding protein, had no effect on heme inhibition. In a similar study (23), inhibition of PCR detection of Epstein-Barr virus by paraffin-embedded gastric carcinoma tissue was removed successfully by the addition of BSA and other proteins (plasma α_2-macroglobulin, rabbit muscle phosphorylase *b*, rabbit muscle lactate dehydrogenase, and chicken egg white lysozyme). In a previous study (2), it was found that addition of 0.5 μg of BSA per reaction tube removed the PCR-inhibitory span at a dilution of about 1:500 of the blood culture medium containing 26.8% whole blood, while the undiluted blood culture medium remained PCR inhibitory.

The relieving effect of gp32 was noted previously with DNA polymerases and reverse transcriptases (13, 24). The gp32 protein is a single-stranded DNA-binding protein, which is encoded by gene 32 of bacteriophage T4 (6). The first mechanism by which gp32 may relieve PCR inhibition is through protection of single-stranded DNA from nuclease digestion (29). The second possible mechanism may be by binding to inhibitors, which is similar to the ability of BSA and other proteins to bind to inhibitors. Feces are known to contain different proteinases.

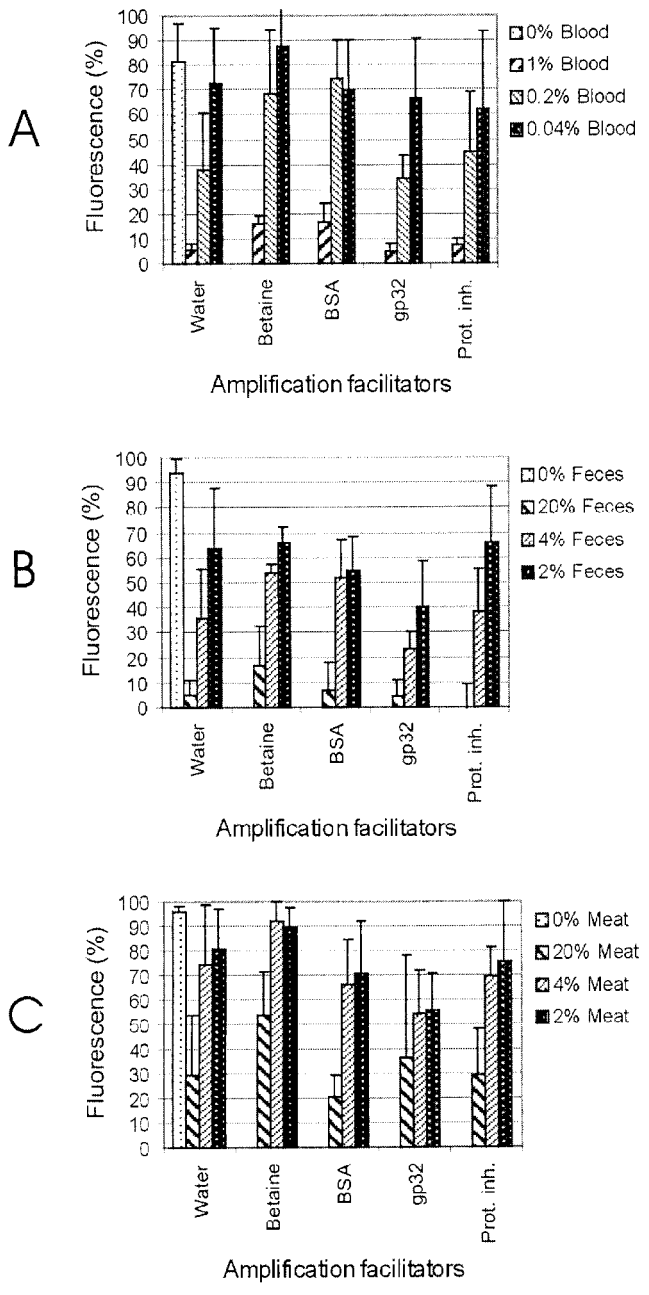

FIG. 1. Effects of amplification facilitators on real-time DNA synthesis of *rTth* in the presence of blood (A), feces (B), and meat (C). prot. inh., proteinase inhibitors.

BSA, gp32, and proteinase inhibitors reduced the level of PCR inhibition by feces (Tables 1 and 2). The mechanism by which BSA and gp32 remove the effects of proteinases may be by being the main targets for these polymerases. Proteinases have been found to be PCR inhibitory in milk (15) and blood (3), and the addition of BSA or proteinase inhibitors (soybean inhibitor or α_2-macroglobulin) was found to overcome the inhibition of *Taq* DNA polymerase by milk, while the addition of lima bean trypsin inhibitor reduced the inhibition of *rTth* by blood.

Betaine was found to reduce the inhibition of *Taq* DNA polymerase by blood. Betaine (*N,N,N*-trimethylglycine) carries both positive and negative charges at pH close to neutrality (17). Betaine has been used to enhance the yields and specificities of PCR amplifications (7), which has been suggested to be due to its ability to destabilize GC-rich DNA sequences, while AT-rich DNA sequences are destabilized much less (17). Betaine has also been found to increase the thermal unfolding transition temperatures of proteins (21).

When the results of the conventional PCR were compared with the results of the real-time DNA synthesis, it was found that feces and meat were less inhibitory than blood. The relieving effects of BSA, gp32, and proteinase inhibitors were not seen. Among the four amplification facilitators tested, only betaine was found to enhance the fluorescence signal in the presence of blood, feces, and meat. These results showed that the effects of amplification inhibitors and facilitators on DNA synthesis by conventional PCR were different from those on real-time DNA synthesis. This could be related to the difference in the principles of the two assays. The real-time DNA synthesis excludes (i) the effects of amplification inhibitors and facilitators on the efficiency of primer annealing and (ii) the effect of high temperature on the different components of the reaction mixtures. In addition, new factors can interfere with DNA synthesis and/or detection of the double-stranded DNA (dsDNA) formed, for example, (i) the ability of glass capillary tubes to bind to different components of the reaction mixture including inhibitors and facilitators, (ii) the interference of inhibitors with SYBR Green I and/or dsDNA can reduce the number of dye molecules that bind to the dsDNA formed, and (iii) the formation of an opaque precipitate can block fluorescence detection. Therefore, further investigations are needed to overcome these limitations and to enhance the reproducibility. This study demonstrates, however, that the PCR-inhibitory effects of biological samples can be reduced or eliminated by the use of an appropriate combination of thermostable DNA polymerase and PCR facilitator. For example, the combination of *rTth* and BSA eliminated the inhibition of 20% (vol/vol) meat. In addition, we showed that it is possible to study quantitatively the effects of inhibitors and facilitators by using real-time DNA synthesis instead of conventional PCR.

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