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Abu Al-Soud, Waleed; Ouis, Ibn-Sina; Li, Dai-Qing; Ljungh, Åsa; Wadström, Torkel

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LUND UNIVERSITY

PO Box 117
221 00 Lund
+46 46-222 00 00

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Characterization of the PCR inhibitory effect of bile to optimize real-time PCR detection of *Helicobacter* species

Waleed Abu Al-Soud*, Ibn-Sina Ouis, Dai-Qing Li, Åsa Ljungh and Torkel Wadström.

Department of Medical Microbiology, Dermatology and Infection, Lund University, Sölvegatan 23, SE-223 62 Lund, Sweden

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*Corresponding author: Mailing address

Department of Medical Microbiology, Dermatology and Infection

Lund University,

Sölvegatan 23,

SE-223 62 Lund, Sweden

Tel: +46 46 173298

Fax: +46 46 189117

E-mail: abu.al-soud@mmb.lu.se

Abstract

The inhibitory effect of human and porcine bile samples to detect Helicobacter DNA was studied by adding different concentrations of bile samples to PCR mixtures of six thermostable DNA polymerases containing *cagA* specific primers and *H. pylori* DNA. PCR products were amplified by using the Rotorgene system and SYBR Green I. Among the six DNA polymerases tested, r*Tth* had the lowest sensitivity to bile inhibitors, whereas *Taq* and *Tfl* had the highest sensitivity. Bile proteins did not inhibit Ampli*Taq* DNA polymerase, whereas the fraction containing mainly bile acids and their salts inhibited the amplification capacity of Ampli*Taq*. Heating human bile at 98ºC and adding casein and formamide to the reaction mixture reduced the PCR inhibitory effect of bile. Therefore, a pre-PCR treatment based on dilution and heating of bile, adding casein and formamide to the reaction mixture of r*Tth* DNA polymerase was found efficient to amplify DNA directly in bile.

1. Introduction

Normal human bile consists of 70% bile salts, 22% phospholipids, 4% cholesterol, 3% proteins and 0.3 % bilirubin [7]. A number of components were reported to inhibit PCR, therefore various pre-PCR treatment methods were developed to generate PCR-compatible samples (for review see [13]). Characterization of PCR inhibitors is an important step in the development of an efficient pre-PCR treatment method. PCR inhibitors may act by interfering with cell lysis, degrading or capture of the nucleic acids or inactivating the thermostable DNA polymerase [25]. One strategy to overcome PCR inhibition and to enhance the efficiency of PCR in the presence of complex biological samples is by using an alternative thermostable DNA polymerase more resistant to inhibitors and adding amplification facilitators [3; 4; 6; 12; 24]. Amplification facilitators enhance the amplification capacity of DNA polymerases through increasing thermal stability, relieving PCR inhibition, and increasing PCR specificity and fidelity.

The aim of this study was to evaluate the inhibitory effect of human and porcine bile on real-time PCR of six thermostable DNA polymerases, to evaluate the ability of amplification facilitators to reduce PCR inhibition, to detect and to identify major PCR inhibitors in bile and to optimize a pre-PCR treatment for bile to reduce the effect of PCR inhibitors and allow DNA amplification in clinical bile samples.

2. Materials and Methods

2.1. Bile samples and bile inhibition of six DNA polymerases

Normal Human bile without bile stones was obtained from a male patient at laparoscopy. Porcine bile samples were obtained postmortem from three porcine males. All bile samples were stored at -20ºC. Frozen bile samples were thawed at room temperature and different

dilutions of bile in water prepared (0, 2.5, 5, 10, 25, and 50 times) and added to a PCR mixture to evaluate the PCR inhibitory effect of bile.

PCR inhibitory effect_of bile was studied by adding different dilutions of bile to PCR mixtures of six thermostable DNA polymerases (25µl) containing Helicobacter DNA. Amplification and detection of PCR product was done by real-time PCR. All reaction mixtures contained 0.2 mM of each of the dNTP's, 5 mM $MgCl_2$, 0.5 µM of each of the previously published primers *cagA*-F and *cagA*-R [16], and 100,000 times diluted SYBR Green I solution (Roche Diagnostics Scandinavia AB, Bromma, Sweden). The reaction mixtures of DNA polymerases were according to manufactures' recommendations. The DNA polymerases and their concentrations were Ampli*Taq* (1 U; Applied Biosystems), r*Tth* (1.25 U; Applied Biosystems), *Tth* (1.25 U; Roche Diagnostics Scandinavia AB), *Tfl* (1 U; Promega), *Taq* (1 U, Promega), and *Pfu* Ultra (1 U; Stratagene). *H. pylori* DNA (0.1 ng/reaction tube) and 5 µl of the different human or porcine bile dilutions were added to the PCR mixtures of different DNA polymerases. PCR mixtures without bile containing *H. pylori* DNA was used as a positive control, whereas 5 µl of sterile Millipore-filtered deionized water was used as a negative control. Amplification and detection of PCR products was done in a Rotorgene system (Corbett Research, Mortlake, Australia). The incubation conditions were: pre-denaturation at 95°C for 120 s, followed by 40 cycles of 95°C for 15 s, 55°C for 20 s and 72°C for 25 s. The specificity of the amplification was checked using Melt curve analysis and agarose gel electrophoresis. Melt curve analysis of the amplified PCR products was done immediately after the PCR by increasing the temperature linearly from 60 to 99°C at a rate of 1°C per s, with the signal acquisition mode set to continuous. The noise band was set manually to exclude data points in the earlier part of the amplification reactions that cannot be distinguished from the background fluorescence. The threshold cycle (C_T), which is the intersection between each fluorescence curve and a threshold line, was calculated using the

Rotorgene software. For the agarose gel electrophoresis 1.5% agarose gels containing ethidium bromide were used [19]. Analysis of the gel was performed using a GelFotoStation (Techtum Lab, Umeå, Sweden).

2.2. Evaluation of heating and amplification facilitators on the ability of bile to inhibit the amplification capacity of AmpliTaq and rTth DNA polymerases

Different concentrations of human bile (0, 2.5, 5, 10 and 25 times) were prepared in water, and were heated at 98ºC for 10 min before the addition of 5µl to the PCR mixtures of Ampli*Taq* and r*Tth* DNA polymerases (the least sensitive DNA polymerases to inhibitory substances in bile) containing *H. pylori* DNA and *cagA* primers. Amplification and detection of PCR products were done as described above.

The ability of 11 amplification facilitators to reduce PCR inhibitory effect of bile was investigated by adding amplification facilitators shown in Table 1 to PCR mixtures of Ampli*Taq* and r*Tth* DNA polymerases containing *H. pylori* DNA, *cagA* primers, and 5 µl of the different dilutions of human bile (0, 2.5, 5, 10 and 25 times). The effect of adding a mixture of BSA and formamide or mixture of casein and formamide to reaction mixtures of Ampli*Taq* and r*Tth* DNA polymerases was also investigated. Amplification and detection of PCR products were done as described above.

A pre-PCR treatment based on dilution, heating and addition of amplification facilitators was evaluated. Human bile diluted 2.5 times in water, spiked with *H. pylori* (10^8, 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 cfu/ml), and heated at 98ºC for 10 min before it was added (5 µl) to PCR mixtures of Ampli*Taq* and r*Tth* DNA polymerases containing *cagA* primers, and casein and formamide. Amplification and detection of PCR products were done as described above.

2.3. Mucin effect on amplification capacity of AmpliTaq and rTth DNA polymerases

The ability of bovine (Sigma chemical, St.Louis. USA), murine (Worthington Biochemical, New Jersey, USA) and porcine (Sigma chemical, St.Louis. USA) gastric mucins to inhibit amplification capacity of Ampli*Taq* and r*Tth* DNA polymerases was studied by the addition of different concentrations of mucin (50, 25, 12.5, 6.25 and 3.125 µg/reaction tube) to PCR mixtures of Ampli*Taq* and r*Tth* DNA polymerases containing *H. pylori* DNA and *cagA* primers. Amplification and detection of PCR products were done as described above.

3. Results

3.1. Inhibitory effect of bile on the amplification capacity of thermostable DNA polymerases

Human and porcine bile samples tested in this study inhibited DNA amplification capacity of all thermostable DNA polymerases (Ampli*Taq*, r*Tth*, *Tth*, *Tfl*, *Taq* and *Pfu* Ultra) (Fig. 1). Human bile and porcine bile-2 were found more inhibitory than the other two porcine bile samples. In addition, the DNA polymerases tested varied concerning sensitivity to PCR inhibitory substances in bile, for example the r*Tth* DNA polymerase had the lowest sensitivity to both human and porcine bile samples and 2.5 times dilution was enough to remove the inhibitory effect of all bile samples except porcine bile-2, which required to be diluted 10 times. Ampli*Taq* was the second least sensitive polymerase to bile, so that the bile had to be diluted 5 or 10 times to remove the inhibitory effect of bile. On the other hand, bile of human and porcine-2 bile sample required more than a 25 time dilution to remove inhibition of *Taq* and *Tfl* DNA polymerases. It was necessary to dilute human bile and porcine bile 25 and 50 times to remove inhibitory effect against *Pfu* Ultra and *Tth* DNA polymerases, respectively.

3.2. Effect of pre-PCR treatment methods on PCR inhibition by bile

The amplification capacities of the DNA polymerases Ampli*Taq* and r*Tth* were tested in the presence of different dilutions of heated and unheated human bile. Heating was efficient in reducing the PCR inhibitory effect of bile, which allowed Ampli*Taq* and r*Tth* DNA polymerases to amplify *H. pylori* DNA in presence of undiluted human bile. However, PCR inhibitors in porcine bile-2 were more resistance to heat treatment, so that porcine bile-2 had to be diluted 10 times to allow DNA amplification of both polymerases.

Among the 11 amplification facilitators tested in this study for their ability to reduce the PCR inhibitory effect of human bile, only BSA, casein and formamide were able to reduce the PCR inhibitory effect of human bile (Table 1). The addition of 0.04% (wt/vol) BSA, 0.01% (wt/vol) casein or 0.01% (wt/vol) formamide reduced the inhibitory effect of bile and allowed Ampli*Taq* polymerase to amplify *H. pylori* DNA in presence of 5 times diluted bile, whereas the amplification capacity of r*Tth* DNA polymerase was not affected (Fig. 2). On the other hand, adding a mixture of formamide and BSA or casein further reduced the inhibitory effect to Ampli*Taq* and r*Tth*, which amplified DNA in presence of 2.5 and non-diluted human bile, respectively.

The effect of different pre-PCR treatments (dilution, boiling and addition of formamide and casein) on the PCR inhibitory effect of human bile spiked with different concentrations of *H. pylori* was investigated. The addition of 2.5 times diluted spiked human bile to reaction mixtures of Ampli*Taq* and r*Tth* DNA polymerases reduced the sensitivity of both polymerases by three log units compared to sensitivity in water (Fig. 3). Heating was found to reduce the effect of PCR inhibitors and to increase the sensitivity by one log unit compared to sensitivity of heating water containing *H. pylori*. Addition of 0.01% (wt/vol) Casein and 0.01% % (wt/vol) formamide reduced the PCR inhibitory effect of bile by one log unit compared to reactions without amplification facilitators. When the 2.5 times diluted spiked human bile was heat treated and added to reaction mixtures of both polymerases containing

casein and formamide the sensitivity of Ampli*Taq* and r*Tth* DNA polymerases increased by 5 and 4 log units, respectively.

3.3. Inhibitory effect of mucins

The PCR inhibitory effect of bovine, porcine and murine gastric mucins was evaluated in this study (Table 2). The bovine and porcine mucins were more PCR inhibitory than murine mucins. The minimum bovine and porcine inhibitory concentration to Ampli*Taq* than r*Tth* DNA polymerases was 25 and 50 µg/reaction tube, respectively. Whereas, addition of 50 µg per reaction tube murine mucins was not inhibitory to the amplification capacity of both polymerases.

4. Discussion

The association of enteric and gastric *Helicobacter* species to chronic infections of the liver and bile tree is disputed, and conflicting studies reported presence or absence of Helicobacter DNA in the hepatobiliary samples [21]. This might be in part due to the presence of PCR inhibitory substances in bile, and highlights the necessity to optimize a pre-PCR treatment that will allow DNA amplification. Bilirubin and bile salts, which are major components of bile, have been found PCR inhibitory [5; 12; 14]. Widjojoatmodjo et al. [23] have investigated the inhibitory effects of the bile salts and found that 50 µg per ml of sodium glycocholate and sodium taurocholate inhibited PCR assay. In this study, high molecular weight proteins precipitated by acetone and fractionated by a Sepharyl-S300 column, was not inhibitory to Ampli*Taq* DNA polymerase based on the C_T-values obtained by real-time PCR and agarose gel electrophoresis. Therefore, we decided to cancel further purification and identification of bile proteins in the different fractions (data not shown). On the other hand, the acetone unprecipitated substances (including bile acids and their salts) were found

inhibitory to amplification capacity of Ampli*Taq* DNA polymerase and the inhibition was relived by dilution, which was similar to results of total bile (10 times) (data not shown). Heating the acetone unprecipitated fraction containing the bile salts did not reduce inhibition of Ampli*Taq* DNA polymerase, which demonstrate that heating ability to reduce the inhibitory effect of bile requires the presence of bile proteins. The complex nature of clinical bile samples due to the presence of varying concentrations of bile stones, bacterial cells, degraded tissues, immunoglobulins and blood among different bile samples will make it impossible to obtain a representative bile sample.

A previous study [2] has reported differences in sensitivity of thermostable DNA polymerases to PCR inhibitory samples, and it was observed that *Pwo* and r*Tth* were the most resistant DNA polymerases to fecal samples, which suggests that the inhibitory components in feces, such as bile salts, have a direct effect on the DNA polymerase. Our study confirmed the inhibitory effect of various bile samples tested. r*Tth* DNA polymerase was the least sensitive to inhibitors in bile, whereas *Taq* and *Tfl* were the most sensitive. In addition, some of the polymerases were more sensitive to one bile sample than the other ones tested e.g. *Pfu* Ultra was much more sensitive to bile from human, whereas *Tth* was more sensitive to bile from porcine-2. These results demonstrate the complexity of bile sample and highlight the importance of optimization of diagnostic PCR by the selection of less sensitive DNA polymerase to inhibitors.

One of the simple pre-PCR treatments is dilution, which increases the physical distance between the inhibitors and the target molecule [13]. However, to dilute some strongly PCR-inhibitory samples is not a feasible way to circumvent PCR inhibition, since it will dilute the target DNA or cells and increase the risk of false negative results. Heating is routinely used as a pre-PCR treatment to break the cell wall of microorganisms and to inactivate heat-labile PCR inhibitors [13]. However, prolonged heating at a high temperature of samples containing

high concentration of proteins such as serum causes gelling and may lead to formation of complexes between DNA and protein molecules making the DNA inaccessible for the DNA polymerase [1]. In the present study, heating was efficient in reducing the inhibitory effect of bile and no gelling or blocking of target DNA was noticed.

Amplification facilitators are substances used to increase PCR specificity and fidelity and to enhance thermal stability of DNA polymerases, as well as to relieve PCR inhibition. In this study, BSA, casein and formamide were able to reduce the PCR inhibitory effect of human bile. Furthermore, addition of BSA and formamide or casein and formamide enhanced the amplification capacity of Ampli*Taq* and r*Tth* DNA polymerases and allowed r*Tth* to amplify 0.1 ng *Helicobacter* DNA in reaction mixture containing 5µl undiluted human bile. Serum albumin, the most abundant protein in the circulatory system, is the major transport protein for unesterified fatty acids, diverse range of metabolites, drugs and organic compounds [9]. The remarkable binding properties of albumin may explain its ability to reduce amplification inhibition. Casein and BSA are used to prevent non-specific binding of proteins and nucleic acids to nitrocellulose in Northern, Western and Southern blotting procedures [18]. Organic, non-ionic detergents, and polymers amplification facilitators were found to enhance DNA amplification by increasing thermal stability of the polymerase [11], increasing specificity [17; 20] and relieving PCR inhibition [15]. In this study, addition of formamide reduced the inhibitory effect of human bile and the inhibition was farther decreased when it was added to reaction mixture together with BSA or casein.

Mucins are high-molecular weight epithelial glycoproteins with a high content of clustered oligosaccharides O-glycosidically linked to tandem repeat peptides rich in threonine, serine, and proline [8]. Mucins are characterized by a high content of oligosaccharide chains (up to 80% in weight), which vary in length and composition (neutral, sialylated, sulfated) [22]. In this study, we found that gastric mucins (bovine and porcine)

inhibited the activity of Ampli*Taq* DNA polymerase more than of r*Tth*, whereas murine mucin did not inhibit the activity of both polymerases at the tested concentrations. The differences in PCR inhibition among the tested mucins might be related to structural differences between the tested mucins for example the level of sulfation. Previously, it has been shown that neutral polysaccharides, such as arabinogalactan, carrageenan, dextran, gum guar, pectin, starch and xylane, were not inhibitory to amplification capacity of *Taq* DNA polymerase, whereas acidic polysaccharides such as dextran sulfate and gum ghatti were inhibitory [10].

In conclusion, bile salts, but not the high molecular weight proteins, most probably are responsible for the inhibitory effect of bile. The use of DNA polymerases less sensitive to PCR-inhibitory components in bile, heat treatment of bile and the use of appropriate amplification facilitators (casein and formamide) can, to some extent, eliminate the need for extensive sample processing of bile samples prior to diagnostic PCR.

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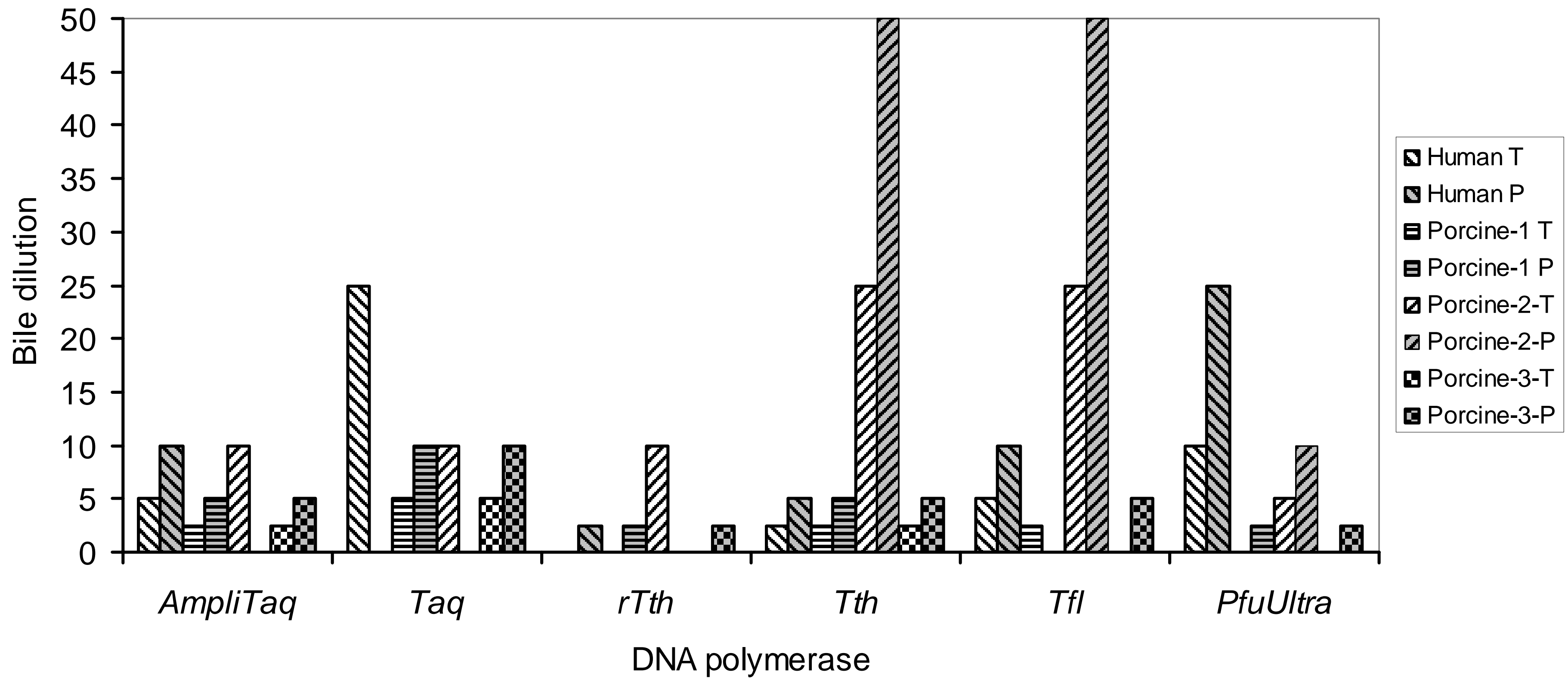
Table 1

PCR facilitators and their concentration tested in this study

PCR facilitator	Concentration per reaction tube % (wt/vol)			
BSA (Roche Diagnostics, Mannheim, Germany)	0	0.01	0.02	0.04
Casein (Bovine milk; Sigma chemical, St.Louis, USA)	0	0.0025	0.005	0.01
Dex-500 (Pharmacia Fine Chemicals, Uppsala, Sweden)	0	0.05	0.125	0.25
DMSO (Prolabo, Fontenay sous bois, France)	0	0.25	0.5	0.75
Formamide (Aldrich Chemicals, Milwaukee, USA)	0	0.0025	0.005	0.01
Gelatin (Type B bovine; Sigma chemical Co, St.Louis. USA)	0	0.001	0.005	0.01
Glycerol (Merck, Darmstaldt, Germany)	0	0.25	0.5	0.1
Nonidet P-40 (Roche Diagnostics, Manheim, Germany)	0	0.01	0.025	0.05
Triton X-100 (Sigma chemical Co, St.Louis. USA)	0	0.005	0.025	0.05
Tween 20 (Prolabo, Fontenay sous bois, France)	0	0.005	0.025	0.05
Tween 80 (Merck, Darmstaldt, Germany)	0	0.005	0.025	0.05

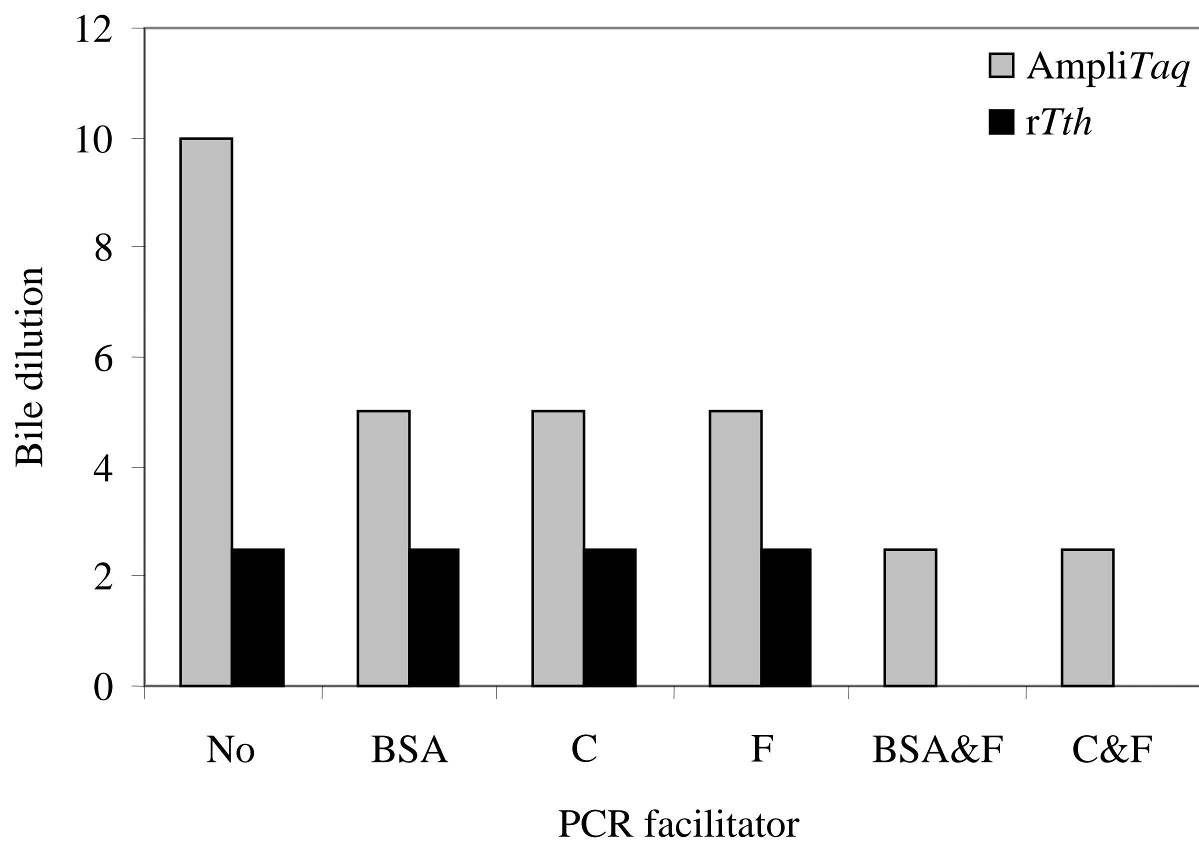


1

2 Fig. 1. The inhibitory effect of different dilutions of human and three porcine bile samples on the amplification capacity of a six thermostable

3 DNA polymerases using *cagA*-primers. T, the DNA polymerase was totally inhibited; P, the DNA polymerase was partially inhibited when the

4 C_T-value was higher than the C_T-value of the positive control.

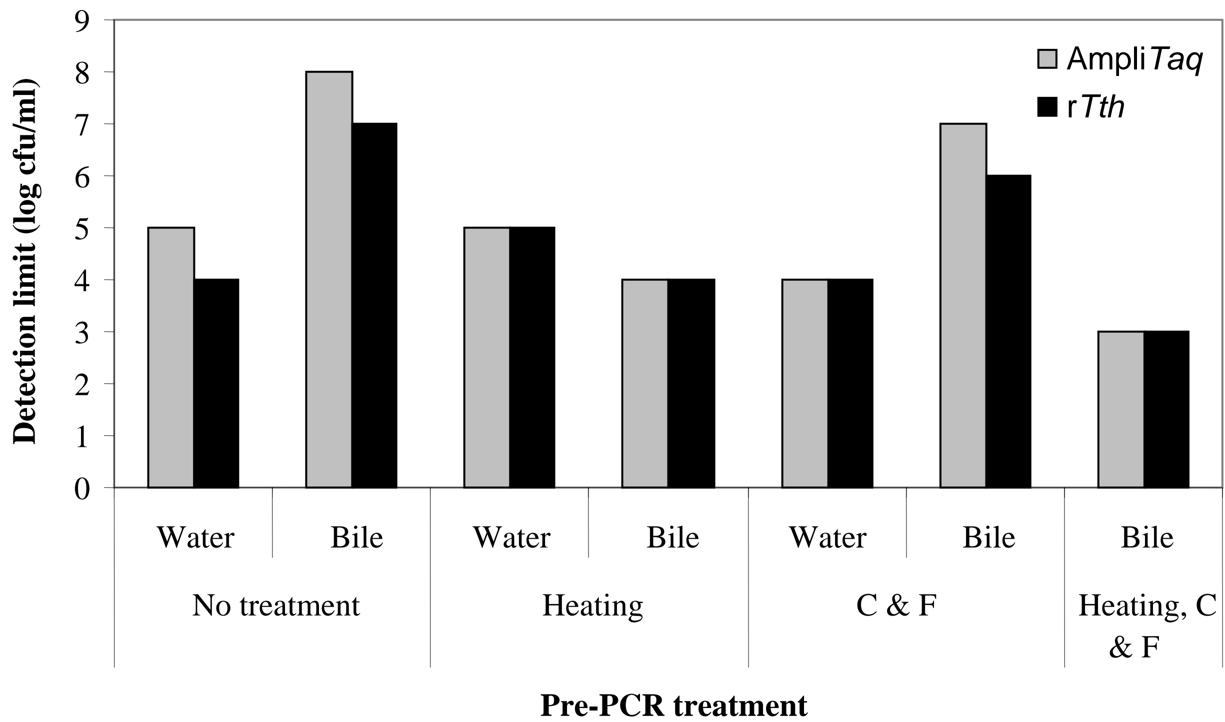


1

2 Fig. 2. The effect of adding 0.04% (wt/vol) BSA, 0.01% (wt/vol) casein (C) or 0.01% (wt/vol)

3 formamide (F)on ability of Ampli*Taq* and r*Tth* DNA polymerases to amplify of *H. pylori* DNA in

4 presence of different concentrations of human bile.



1

2 Fig. 3. Effect of heating and adding 0.01% (wt/vol) casein (C) and 0.01% (wt/vol) formamide (F)
3 on ability of r*Tth* and Ampli*Taq* to amplify DNA of *H. pylori* spiked in water (W) and in 2.5x
4 diluted human bile (B).

Table 2

Inhibitory effect of bovine, porcine and murine mucin on the amplification capacity of Ampli*Taq* and r*Tth* DNA polymerases

Mucin source and conc. (µg/RT)		DNA polymerase			
		Ampli*Taq*		r*Tth*	
		AGE[a]	C_T[b]	AGE	C_T
	0	+	12	+	15.2
Bovine	50	-		-	
	25	-		+	15.0
	12.5	+	14.4	+	13.9
	6.25	+	13.5	+	15.2
	3.125	+	13.1	+	13.7
Porcine	50	-		-	
	25	-		+	15.9
	12.5	+	12.5	+	14.7
	6.25	+	12.9	+	15.6
	3.125	+	12.5	+	15.3
Murine	50	+	13.8	+	18.7
	25	+	11.6	+	19.1
	1.25	+	14.0	+	19.1
	6.25	+	14.3	+	19.9
	3.125	+	15.8	+	20.4

[a] Agarose gel electrophoresis

[b] The threshold cycle