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MINIREVIEW

Inhibition and Facilitation of Nucleic Acid Amplification

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Factors that inhibit the amplification of nucleic acids by PCR are present with target DNAs from many sources. The inhibitors generally act at one or more of three essential points in the reaction in the following ways: they interfere with the cell lysis necessary for extraction of DNA, they interfere by nucleic acid degradation or capture, and they inhibit polymerase activity for amplification of target DNA. Although a wide range of inhibitors is reported, the identities and modes of action of many remain unclear. These effects may have important implications for clinical and public health investigations, especially if the investigations involve food and environmental screening. Common inhibitors include various components of body fluids and reagents encountered in clinical and forensic science (e.g., hemoglobin, urea, and heparin), food constituents (e.g., organic and phenolic compounds, glycogen, fats, and Ca^{2+}), and environmental compounds (e.g., phenolic compounds, humic acids, and heavy metals). Other, more widespread inhibitors include constituents of bacterial cells, nontarget DNA and contaminants, and laboratory items such as pollen, glove powder, laboratory plasticware, and cellulose. This review discusses the findings of many studies related to clinical, food, and environmental microbiology, including approaches that have been used to overcome inhibition and facilitate amplification for detection and typing.

Few areas of biological science remain untouched by the invention of PCR (34, 81, 99). Other methods for amplifying nucleic acids (72, 123), such as Qβ replicase (18), ligase chain reaction (13, 128), single-stranded sequence replication (17, 47), strand displacement amplification (126, 127), and nucleic acid sequence-based amplification (23, 122), have been described, but these methods have received less attention.

Problems sometimes occur with PCR, however (124). Despite early indications of great sensitivity, the sensitivity of PCR may be a negative aspect of the procedure, since the most commonly reported problem is false-positive results due to cross-contamination (98, 124). This problem can be overcome by UV irradiation (100), with sodium hypochlorite (92), and by photochemical or enzymic methods (25, 36, 40, 78).

One problem that is less discussed is reaction inhibition. This may be total or partial and can manifest itself as complete reaction failure or as reduced sensitivity of detection. In some cases, inhibition may be the cause of false-negative reactions, since few workers incorporate internal controls in each reaction tube. Early evidence of exquisite sensitivity with mammalian cells (53) involving detection of a single molecule of DNA from a hair was not reproduced when PCR was applied to many microbial (and some mammalian) situations, where poor sensitivity, specificity, and reproducibility have been reported (16, 82, 86, 129, 132, 134). There may also be potentially important effects in PCR typing reactions (121), and difficulties can occur in post-PCR manipulation (61). Although systematic study of inhibition has seldom been the focus of published investigations, many workers have reported these effects in the course of other studies (12, 19, 21, 124, 129, 132, 133). Considering the prevalence of this problem, it is surprising that few systematic and mechanistic studies of PCR inhibition have been reported. Rossen et al. (97) contributed the most comprehensive study of PCR inhibition, identifying inhibitory factors in foods, bacterial culture media, and various chemical compounds. These inhibitory factors included organic and inorganic chemicals, detergents, antibiotics, buffers, enzymes, polysaccharides, fats, and proteins.

This review lists and discusses inhibitors and methods that can overcome the attenuation of amplification in clinical, food, and environmental microbiology. It is beyond the scope of this paper to discuss in detail the various physical, enzymic, and chemical methods used in the extraction, purification, and quantitation of nucleic acids. Those methods are presented and discussed in commercial literature and elsewhere (14, 95, 96, 106, 134).

FACTORS AFFECTING DETECTION LEVEL

Cultural detection methods allow quantification of microorganisms with considerable precision. This precision may be of importance in clinical, food, and environmental investigations when commensal organisms are distinguished from those causing disease. Quantitation of a PCR product cannot necessarily be equated with the number of organisms present in the original sample (124).

The minimum number of cells containing a single-copy target gene theoretically detectable in a 10-μl sample aliquot added to a 90-μl reaction mix is one. This figure corresponds to 100 CFU ml^{-1}, a sensitivity similar to that of plate counts, and in practice may be reduced through sampling error. The random distribution of cells near the detection limit means that a single cell or gene copy may not actually be present in some aliquots that have been calculated to contain such numbers. Food, clinical, and environmental samples commonly reduce sensitivity through a wide range of inhibiting substances. Complete failure and false-negative amplification reactions are reported in many cases (27, 113), but often sensitivity is merely reduced (52). Typically, a variety of mechanisms reduce sensitivity by several orders of magnitude. Wernars et al. (129) found that the sensitivity of detection was at least 10-fold poorer than the theoretical minimum, varying between 10^3 and $>10^8$ CFU 0.5 g^{-1} in different brands of soft cheeses. Sensitivity was reduced 1,000-fold in milk powder (132), where 10^5 CFU ml^{-1} was required for detection of *Staphylococcus aureus*

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despite fewer than 10 cells being detectable in diluent. Protein-breakdown products in aged cheeses caused sensitivity problems in the detection of lactic acid bacteria (33). Ten PFU per gram of enteroviruses in diluent was detectable directly in a reverse transcriptase PCR (RT-PCR) assay (59), but only 10^3 PFU g^{-1} was detectable when it was extracted from oyster meat. Such sensitivity is typical of the lower limits of detection that are achieved in practice for clinical and environmental samples and foods.

Dilution of samples, as with enrichment culture in the presence of potential growth inhibitors such as spices and disinfectants, provides a simple method that can facilitate amplification, albeit with reduced sensitivity. The sensitivity of PCR can be exploited to amplify target DNA that is still present when inhibitors have been diluted out (5, 119, 120, 137).

Conversely, PCR detection of intracellular bacteria may be more sensitive than expected because of multiple bacterial cells in each infected blood cell (73). This may provide the opportunity to achieve greater sensitivity than that suggested above. Amplification of short-lived multiple-copy RNA species may provide increased sensitivity relative to the number of DNA targets in some situations.

Authors sometimes are unclear about whether the number of cells detectable is per milliliter or per reaction volume (normally 100 μl). There are apparent contradictions in the published works that discuss inhibition. One substance may be reported as being both an inhibitor and a facilitator in different systems. At different concentrations, the same compound has been reported to act as both facilitator and attenuator in the same system (37, 139). Both bacterial cells and nontarget DNA have been shown to be both inhibitory and noninhibitory in different systems. Steffan and Atlas (112) were able to detect a 15- to 20-copy target sequence in *Pseudomonas cepacia* cells at 1 CFU g^{-1} against 10^{11} nontarget organisms in samples of sediment. This represents detection 10^3-fold more sensitive than that derived by probing nonamplified samples despite high numbers of potentially inhibitory nontarget organisms. Andersen and Omiecinski (8) found that $>10^5$ CFU of indigenous bacteria did not inhibit specific amplification of target DNA from enteroinvasive *Escherichia coli*. Dickinson et al. (30) found that high levels of background bacterial DNA were not inhibitory to specific amplification and that 10^2 to 10^4 CFU ml^{-1} could be detected in several food types. Sensitivity was lower for cheese than for coleslaw or chicken. Those authors speculated that nontarget DNA may have improved sensitivity by acting as a carrier for target DNA during precipitation. Nevertheless, bacterial cells can have an inhibitory effect on PCR (6, 70, 116, 133). A study of the efficiency of DNA extraction from soil and PCR inhibition by humic compounds (116) showed that high levels of nontarget DNA could inhibit PCR. Sensitivity may vary with the microbial species detected, by identical extraction protocols, since rates of recovery may be different between species (112, 116). Alvarez et al. (6) found that 10^3 to 10^4 CFU m^{-3} of environmental organisms interfered with detection of *E. coli* during bioaerosol sampling. Freeze-thaw lysis and sample dilution allowed rapid and sensitive detection of a single cell.

Where nonspecific bacterial effects inhibited an antigen-antibody reaction, skim milk was found to overcome this inhibition (125). It also acts as a blocking agent that prevents the nonspecific adhesion of bacteria to immunomagnetic beads (125) and is used to similar effect for blocking nonspecific reactions during membrane hybridization. Skim and full-cream milk have also been found to be inhibitory to PCR (56, 94, 132), partly because of DNA loss during extraction procedures and partly because of amplification inhibition. In PCR, milk components may block DNA and shield it from access by polymerase.

Reaction conditions have long been recognized as important to sensitive and reproducible amplification. Contamination must also be considered as a possible cause of reaction failure.

REACTION CONDITIONS

Suboptimal reaction conditions may arise for a number of reasons. The primary ones are inappropriate primers, improper time or temperature conditions, variable polymerase quality, and incorrect Mg^{2+} concentration. These factors should be optimized by running several temperature profiles based on calculated primer melting points and reactions to the Mg^{2+} dilution series before PCR is relied on for sensitive and specific detection from a given medium.

Temperature inconsistencies across the block of a thermal cycler have been found to be responsible for poor amplification (22, 121), and this result has been overcome by the addition of formamide, which reduces the melting temperature of DNA (22). This addition is not an ideal solution, and further studies may reveal that the specificity of the reaction may suffer from this treatment. Excellent thermal consistency is important for sensitive and specific testing and should be considered when one chooses between the many models of thermal cycler available. The reproducibility of cycle time and temperature should be checked, particularly when a different machine or tubes with different thermal transfer properties are used, and the sample block should be cleaned to ensure consistent performance.

Physical modifications to the PCR such as hot start may be helpful for improving the yields and specificities of reactions. Adding reactants when the reaction has reached denaturation temperature is particularly useful when one is detecting low-copy-number molecules amid a high background of nontarget DNA. The addition of a key reactant after denaturation reduces the formation of primer-oligomers and misprimed reactions that compete with the specific amplification of the targeted sequence.

Dimethyl sulfoxide (DMSO) has been shown to improve reaction yield during RT-PCR (107). Sensitivity was improved up to 25-fold for some viral target RNAs. That study involved the use of recombinant *Thermus thermophilus* polymerase, and no inhibitors were mentioned. The optimal concentration of DMSO was dependent on the template and facilitated both the reverse transcriptase and PCRs. It was suggested that DMSO may enhance PCR by eliminating nonspecific amplification or altering the thermal activity of the polymerase or that it may improve the annealing efficiencies of primers by destabilizing secondary structures within the template.

Mg^{2+} ions are a vital cofactor for *Taq* polymerase, and their concentration will affect the success and specificity of amplification. The sequestration of Mg^{2+} ions by various compounds and interference by Ca^{2+} ions may inhibit amplification (12, 101). The Ca^{2+} ions in milk may be a cause of its inhibitory properties.

A number of DNA polymerases are now commercially available. These polymerases originate from several extreme thermophiles and exhibit differences in processivity and fidelity, making some more suitable than others for specific tasks. Batches of polymerase may be pooled to overcome variability in enzyme quality. Production of PCR product may be improved in some situations if mixtures of different polymerases are used. This may allow improved yield, since some enzymes may be less susceptible than others to specific inhibitors and will permit amplification that would not otherwise occur.

Inhibitory effects may be minimized by optimizing the reac-

tion conditions and ensuring that appropriate quantities of all reactants are freely available within the reaction mixture by minimizing the presence of any contaminants and known inhibitors.

CONTAMINATION AND OTHER FACTORS

Contamination and other factors intimate to the reaction, even apparently inert components, may cause inhibition at any of the points of molecular interaction discussed below. Their modes of action are not yet understood, but there may be chemical or physical interference with the availability or activity of an essential reaction component. Contamination may be endogenous (e.g., sample, enzyme, and tubes) or exogenous (e.g., bacteria, dust, and pollen) to the reaction components.

The most obvious origin of PCR inhibitors in endogenous contamination is compounds present in insufficiently purified target DNA. Inhibition can also arise from other endogenous sources, including reaction components. Commercial preparations of *Taq* polymerase, including a low-level-DNA product, have been shown to be contaminated with eubacterial DNA that originates from neither *E. coli* nor *Thermus aquaticus* (55). In particular, this contamination may reduce the effective application of PCR with broad-range eubacterial primers because of the production of false-positive reactions. For more specifically targeted reactions, such contamination generally is of little importance. The study of Hughes et al. (55) demonstrated that enzyme-contaminating sequences can be destroyed by UV irradiation. It also showed that microcentrifuge tubes from different manufacturers gave greatly different results. Strong inhibition resulting from the use of some brands and batches of PCR tubes has also been identified by other workers, but the cause could not be discovered (20). With respect to *Taq* polymerase, considerable variability in its performance based on batch, concentration, and supplier has been documented in a commentary that details many parameters affecting multiple arbitrary amplicon profiling (121). In addition to reaction failure, enzyme contamination may be manifested as spurious background bands during amplification-based DNA fingerprinting by methods such as randomly amplified polymorphic DNA. By nature of their low stringency, such typing techniques rely on both specific and nonspecific priming and combine the detection of artifactual variation with true polymorphism. Contamination, concentration-dependent effects, spurious bands, and inhibition may complicate the interpretation of banding patterns and give rise to misleading results.

Like reaction tubes, other apparently inert components may be inhibitory. Cellulose and nitrocellulose filters were found to inhibit PCR (11). In the study of Bej et al. (11) polycarbonate filters proved not to be inhibitory, perhaps because of differences in binding properties for DNA or its contaminants relative to those of cellulose-based filters. Mineral oil has been shown to have an inhibitory effect on PCR when it is irradiated with UV light (31). The inhibition is dependent on the UV dose. Unirradiated mineral oil has been reported as a facilitator in oil-free reactions containing high concentrations of nonionic detergents by Katzman (62). That author suggested that components of detergent preparations (monomers, micelles, or impurities) may be responsible for adverse effects on the specificity of annealing. It is likely that detergents allow the greater solubilization of inhibitors that might otherwise aggregate and precipitate during preparation or in the reaction tube. Oil overlays may facilitate amplification by segregating inhibitors at the oil-water interface and remain an option in thermal cyclers designed for oil-free reactions.

Reaction failure may also be caused by exogenous contamination. This source of inhibition differs from the other sources of inhibition discussed above which result from compounds present in insufficiently purified DNA or from contaminants in reaction components. However, it may involve the same mechanisms of inhibition. Inhibition may be due to even <10 grains of pollen that may enzymically digest an essential reaction component (113), glove powder (27), which may nonspecifically bind DNA, or other factors that enter reaction tubes when hygienic conditions are not sufficiently controlled.

Contamination can be prevented by good laboratory practice and scrupulous attention to aseptic technique, which also serves to prevent cross-contamination of target sequences, although this problem can be dealt with by UV irradiation or with uracil *n*-glycosylase (89). Restriction enzymes have been shown to be inhibited, or their specificities have been shown to be altered, by multiple uracil substitutions in restriction sites (43). This may also have implications for the fidelity of PCR typing methods. Laminar-flow cabinets may be of use in preventing airborne contamination, and cabinets equipped with a UV light source are available specifically to minimize contamination during PCR work. Ironically, the good practice of changing gloves may in some cases actually predispose reactions to failure. Powders from gloves were shown to have inhibitory effects on PCR which vary depending on the manufacturer (27). If it is suspected that glove powder is the source of problems, washing gloves and the selection of nonpowdered brands may be helpful.

MECHANISMS OF INHIBITION

The inhibition of amplification may be due to a number of factors, none of which has been investigated thoroughly. Details of the inhibitory effects of some compounds that have been reported in clinical, food, and environmental systems may be found in Tables 1 to 3. Inhibition may originate from poorly controlled reaction conditions; from the sample itself; from contaminants in reagents, containers, or disposables; or from unintentional contamination during reaction preparation. Some sources of inhibition are well-known to the biomedical community, but there is a dearth of systematic and biomechanistic studies that offer greater insight into the physical causes of the problem. Such studies are essential for the advancement of the science and the improved application of this powerful technology. Inhibition may involve multiple causes and complex interactions that are difficult to distinguish.

Some compounds have been reported as both inhibitors and facilitators in different studies and even in the same system (37, 139). It is essential that these problems are understood and resolved. An automated high-throughput filtration assay for the identification of RNA polymerase inhibitors has recently been described (136) and might be applied to inhibitors in RT-PCR. Other drug-discovery assays might be employed for rapid and quantitative screening of many classes of inhibitor. The mechanisms of inhibition may be grouped into three broad categories by their point of action in the reaction. These mechanisms are discussed below. The categories are by no means absolute, since an inhibitor may act in more than one way and the relationships between chemical, enzymic, and physical factors often cannot be distinguished given the poor current knowledge on the subject. It is likely that many inhibitors act through various physical and chemical means by interfering with the interaction between DNA and polymerase. This functional framework may serve as a focus for future systematic studies of the biomechanical origins of inhibition and lead to simple technological improvements to facilitate the use of rapid and sensitive DNA diagnostics.

TABLE 1. Inhibitors and facilitators in clinical samples[a]

Substrate(s)	Target organism(s)	Inhibitor(s)	Facilitator(s)	Reference
Feces	*Escherichia coli*	$>10^3$ bacterial cells	Ion-exchange column	84
CSF	*Treponema pallidum*	Cellular debris causing nonspecific amplification	Nested primers	50
Whole blood	Mammalian tissue	≥4 μl of blood/100-μl reaction mixture (hemoglobin)	<1–2% blood per reaction	79
Feces	Rotavirus	Unknown	Dilution, cellulose fiber paper	131
Clinical specimens	Cytomegalovirus	Unidentified components	Glass bead extraction	15
Human blood and tissue	Human genes	DNA-binding proteins	Thermophilic protease from *Thermus* strain rt41A	77
	Mammalian tissue genetics		Organic solvents, DMSO, PEG, glycerol	90
	Mammalian tissue genetics	Thermal cycler inconsistencies	Formamide	22
Clinical specimens	*Treponema pallidum*	Various unknown factors	Various substrate-specific physicochemical methods	46
	Many	Many	Many	97
Forensic semen samples	Interference of vaginal microflora with sperm genotyping	Genotyping errors and selective or total PCR inhibition by vaginal microorganisms		70
Feces	*Salmonella enterica*	Various body fluids	Immunomagnetic separation	130
Feces	Various enteric viruses	Unknown	Size-exclusion chromatography, physicochemical extraction	26
Clinical specimens	Herpes simplex virus	Endogenous inhibitors, random effects	Repurification, coamplified positive control	24
Feces	*Escherichia coli*	Nonspecific inhibitors, urea, hemoglobin, heparin, phenol, SDS	Additional primers and reaction cycles, booster PCR	102
Tissue culture	Cytomegalovirus, human immunodeficiency virus	Glove powder		27
Suspensions, skin biopsies	*Mycobacterium leprae*	Mercury-based fixatives, neutral buffered formalin	Reduced fixation times, ethanol fixation	38
Clinical specimens	*Mycobacterium tuberculosis*	Unknown inhibitors in pus, tissue biopsies, sputum, pleural fluid	Physicochemical extraction	64
	Mammalian tissue genetics	Unknown contaminant of reverse transcriptase	Additional DNA	37
Formalin-fixed paraffin tissue	Hepatitis C virus	Ribonucleotide vanadyl complexes	Phenol-chloroform extraction	67
Nasopharyngeal aspirates and swabs	*Bordetella pertussis*	Unknown inhibitors	Phenol-chloroform extraction	103
Human mononuclear blood cells	Human immunodeficiency virus type 1	Detergents	Mineral oil	62
Bloodstain	Human mitochondrial DNA	Unidentified heme compound, hemin	BSA	3
Blood	Various	Heparin	Alternative polymerases and buffers, Chelex, spermine, $[Mg^{2+}]$, glycerol, BSA, heparinase	101
Sputa	*Mycoplasma pneumonia*	*N*-Acetyl-L-cysteine, dithiothreitol, mucolytic agents		29
Human tissue	HLA-DRB1 genotyping	Pollen (<10 grains), glove powder, impure DNA, heparin, hemoglobin		113
Clinical specimens	*Mycobacterium tuberculosis*	Unknown	Competitive internal control	63
Bovine semen	Bovine herpesvirus 1	Unknown	Dilution, adjustment of ionic conditions	137
Feces	*Salmonella enterica*	Hemoglobin degradation products, bilirubin, bile acids, feces	Immunomagnetic separation	39
Dental plaque	Many	Unknown	Diatomaceous earth, guanidium isothiocyanate, ethanol, acetone	85
Ancient mammalian tissues	Cytochrome *b* gene	Unknown	Ethidium bromide, ammonium acetate	48
Sputum	*Mycobacterium tuberculosis*	Hemoglobin and others	Anion-binding resin	7
Bovine feces	*Cryptosporidium* spp.	Unknown	Spin columns	69
Solution	*Bacteriodes* spp.	Heme; bilirubin; bile salts; humic substances (complex polyphenolics); EDTA; SDS; Triton X-100; hemin; tannic, fulvic, and humic acids; bacterial extracts; proteases	BSA, gp32	65

[a] Abbreviations: CSF, cerebrospinal fluid; PEG, polyethylene glycol; SDS, sodium dodecyl sulfate.

TABLE 2. Inhibitors and facilitators in food samples[a]

Substrate(s)	Target organism(s)	Inhibitor(s)	Facilitator(s)	Reference
Milk	*Listeria monocytogenes*	Unknown	Enzymatic digestion, membrane solubilization	110
Skim milk	*Staphylococcus aureus*	Thermonuclease, proteins, bacterial debris	NaOH, NaI, physicochemical extraction, nested PCR	132
Soft cheeses	*Listeria monocytogenes*	Brand-specific inhibitors, denatured protein	Phenol extraction, Qiagen column	129
Various foods	*Escherichia coli*	Bean sprouts, oyster meat	Magic Minipreps	8
Foods and cultures	*Staphylococcus aureus*, *Escherichia coli*, *Salmonella* spp.		Lectin-affinity chromatography	88
Various	*Listeria monocytogenes*	Various		97
Skim milk	*Staphylococcus aureus*	Thermonuclease, proteins, bacterial debris	NaOH, NaI, physicochemical extraction, nested PCR	133
Solution	*Staphylococcus aureus*	Thermonuclease, proteins, bacterial debris	NaOH, NaI, physicochemical extraction, nested PCR	134
Oyster meat	Poliovirus 1, Norwalk virus, hepatitis A virus	Polysaccharides, glycogen	Organic flocculation, CTAB, PEG extraction	10
Meat	*Brochothrix thermosphacta*	Fetuin, meat components	Lectin binding	45
Milk	*Listeria monocytogenes*	Proteinase	BSA, proteinase inhibitors	91
Soft cheese	*Listeria monocytogenes*	Unknown	PEG-dextran extraction	66
Various foods	*Listeria monocytogenes*	Unknown	Enrichment, NASBA	122
Various foods	*Listeria monocytogenes*	Unknown	NaI-alcohol precipitation	74
Oysters	Enteroviruses	Unknown	Freon, PEG, chloroform, CTAB	58
Raw milk	*Clostridium tyrobutyricum*	Unknown	Chemical extraction, centrifugation	51
Shellfish	SRSVs	Unknown	PEG, Freon, ultracentrifugation	68
Various foods	Various bacteria	Unknown	Enzymic and physicochemical extraction	30
Oyster meat	Poliovirus, hepatitis A virus, Norwalk virus	Acidic polysaccharides, glycogen	Freon, DMSO, glycerol, PEG, Pro-Cipitate	59
Drinking water	Enteroviruses, hepatitis A virus	Humic acid-organic compounds	Pro-Cipitate, PEG, antibody capture	104
Raw milk	*Brucella* spp.	Milk proteins	Physicochemical extraction, nested PCR	94
Cold-smoked salmon	*Listeria monocytogenes*	Food components, sucrose, ovalbumin, phenolic compounds	Ether and column extraction, Tween 20	108
Ground beef	*Escherichia coli*	Food components	Enrichment culture, Taqman	135
Milk	*Listeria monocytogenes*	Ca^{2+}	Chelation, $[Mg^{2+}]$	12

[a] Abbreviations: CTAB, cetyltrimethylammonium bromide; NASBA, nucleic acid sequence-based amplification; PEG, polyethylene glycol; SRSVs, small round-structured viruses.

Failure of lysis. An elementary aspect of DNA amplification is that the failure to expose nucleic acids as targets for amplification will result in reaction failure. Loss of cell wall integrity may not be enough to permit amplification of DNA, and enzymic degradation of cellular debris will often be necessary (134). Protocols which rely on some or all of the physical, chemical, and enzymic methods for cell lysis exist. Inadequate lysis may result from inadequate lysis reaction conditions, enzyme inactivation, or lytic enzymes of poor quality or consistency.

Early evidence that PCR could successfully take place on unpurified DNA released from cells by boiling was presented (111). Such an approach saves considerable time compared with that needed by more elaborate extraction protocols. Nevertheless, if whole cells are loaded into the reaction tube and released DNA fails to be sufficiently separated from structural and DNA-binding proteins by boiling, PCR inhibition may result. Extraction by boiling alone has been noted to reduce sensitivity, due to the mechanism described above or poor lysis efficiency, and may give rise to spurious bands in some cases (41) or prevent amplification altogether (117). The high concentration of salt in *Listeria* selective media was found to be the reason for unlysed cells and false-negative reactions by the Accuprobe DNA probe test (86), and PCR may be similarly affected.

In work with *S. aureus*, I have found that lysis with lysostaphin may be inconsistent. Subsequent results in my laboratory showed that a genetically engineered version of this enzyme that had become available worked more reliably. Proteolytic enzymes and denaturants may degrade enzymes used for lysis. Phenolic compounds (108) from the sample or carried over from organic DNA purification procedures can inhibit the reaction by denaturing the lytic enzymes (57) and failing to expose the DNA. It may be that the stage of the growth cycle and nutrient conditions are important for the susceptibility to lysis of some cells. These factors have been little studied.

Nucleic acid degradation and capture. Degradation or sequestration of target or primer DNA can also be a cause of failed reactions. Amplified DNA may also be degraded, and

TABLE 3. Inhibitors and facilitators in environmental samples[a]

Substrate(s)	Target organism(s)	Inhibitor(s)	Facilitator(s)	Reference
River sediment	*Pseudomonas cepacia*	None	Physicochemical extraction	112
Suspension	*Salmonella typhimurium*, *Listeria monocytogenes*	Unknown	Lectin-affinity chromatography	87
Water	Various bacteria	Cellulose and nitrocellulose filters	PTFE filters	11
Sediments	*Escherichia coli*	Humic substances	Gel filtration	120
Water	*Escherichia coli*	Low sensitivity	Membrane filtration, 75 solid-phase PCR cycles and radiometric detection	118
Soils and sediments	*Escherichia coli*	Humic substances, iron	Dilution	119
Plant tissue	Plant tissue	Acidic plant polysaccharides and buffer additives, SDS	Tween 20, DMSO, PEG 400	28
Soil	*Pseudomonas cepacia*	Humic compounds that interfere with lytic enzymes	Physicochemical extraction, cation-exchange resin	57
Soil	Various	Humic compounds, nontarget DNA	Ion-exchange chromatography, T4 gene 32 protein	116
	Plant material	Concentration-dependent inhibition and facilitation by polyamines (spermine and spermidine); glycerol; formamide	Variable with concentration	2
Soil	*Pseudomonas fluorescens*	Humic substances	Physicochemical extraction	109
Soil	*Escherichia coli*	Phenolic compounds, PVP	Retardation of phenolic migration by PVP agarose gel electrophoresis	139
Sediment	*Pseudomonas putida*	Sediment, bacterial cells	Physicochemical extraction, PVPP	52
	Hypoxylon truncatum	RNA that interferes with amplification of RAPD markers	RNase	138
Sediment, soil, and water	RNA from various species	Humic-like compounds	Lysozyme–hot-phenol extraction and gel filtration	80
Sludge and soil	Enteroviruses	Unknown	Beef extract, size-exclusion chromatography	114
Soil	Enteroviruses	Humic and fulvic acids, heavy metals	Physical separation, solvent extraction, size-exclusion or ion-exchange chromatography, double and seminested PCR	115
Bioaerosols	*Escherichia coli*	Bacterial cells, nontarget DNA	Dilution, filtration	5
Bioaerosols	*Escherichia coli*	$>10^3$ CFU of bacterial cells m^{-3} nontarget DNA	Freeze-thaw lysis, dilution	6
Water	*Cryptosporidium* spp.	Formaldehyde, potassium dichromate, feces	Flow cytometry, magnetic antibody capture	60
Sewage or effluent	*Cryptosporidium* spp., *Giardia* spp.	Humic and fulvic acids	Percoll-sucrose centrifugation, vortex flow filtration, nested PCR	75

[a] Abbreviations: PTFE, polytetrafluoroethylene; RAPD, randomly amplified polymorphic DNA; SDS, sodium dodecyl sulfate.

band smearing is sometimes evident after running amplified products after storage (133). These effects may occur by physical, chemical, or enzymic processes. The primary structure of DNA is susceptible to instability and decay, mainly due to hydrolysis, nonenzymic methylation, oxidative damage, and enzymic degradation (71). Amplification of long DNA sequences may become increasingly difficult as DNA strands fragment after cell death. Nucleases may enter the reaction through careless handling, from the sample material, from various bacteria in the sample, and in some cases from the target organisms themselves (42, 133). The DNA of some gram-negative bacteria is protected in nuclease-resistant vesicles that are involved in the export of genetic material (32). Nonspecific autodegradation of DNA can occur in the presence of restriction endonucleases (4). It was reported (132–134) that staphylococcal thermonuclease was not destroyed during thermal cycling and limited the sensitivity of PCR. Nucleases are produced by many other bacteria, but staphylococcal DNase exhibits uncommon heat stability and was able to hydrolyze genomic and primer DNA during amplification reactions. DNase activity was reported to prevent pulsed-field gel electrophoresis typing of Lior biotype II (DNase positive) *Campylobacter jejuni* isolates. Treatment in formaldehyde solution for 1 h neutralized the DNase activity and allowed typing of these strains to proceed (42).

Unavailability of target or primer DNA by nonspecific blocking or sequestration may inhibit amplification or cause misleading band variations during typing based on PCR. Bacterial cells or debris, proteins, and polysaccharides that have caused inhibition in many studies may do so by physical effects, such as making the target DNA unavailable to the polymerase. Milk proteins were reported as inhibitory (94) and may also act in this way by restraining DNA in high-molecular-weight complexes. An extraction procedure using benzyl chloride and the differential solubility and precipitation of DNA and polysaccharides was shown effectively to overcome inhibition by the high-molecular-weight polysaccharides from fungi that prevent enzymic DNA manipulation (93). High concentrations of reverse transcriptase or an unidentified compound associated with some preparations of the enzyme have been shown to inhibit RT-PCR of RNA (37). The inhibitory effect was demonstrated to vary with the use of products of different manu-

facturers and of different batches. The addition of DNA overcame inhibition, and Fehlmann et al. (37) believe that a contaminant of some enzyme preparations binds to DNA, thus blocking PCR.

In a study of how forensic sperm genotyping was undermined by inhibition or allele dropout due to the presence of vaginal microorganisms, two possible mechanisms were suggested (70). Efficient primer extension may be prevented by small, sheared, single-stranded lengths of microbial DNA binding to the target sequence. Alternatively, the effective primer concentration may be reduced by nonspecific binding to nontarget microbial DNA. In other studies (112) high numbers of nontarget organisms were not found to be detrimental to PCR. This result may depend on the DNA sequences involved and on fragmentation size during extraction. Also, it has been shown that chemical insults such as the spermicide nonoxinol-9 do not prejudice the forensic examination of DNA (54).

It was speculated (2) that polyamines binding to DNA prevented polymerases from accessing the template. Those workers found that the polyamines spermine and spermidine, along with formamide and glycerol, had a concentration-dependent effect on the yield and specificity of PCR. Spermidine has previously been reported to facilitate amplification by precipitating inhibitors (48). Formamide has also been reported to improve amplification in tissue typing (22).

Glove powder has been reported to inhibit amplification and may do so by nonspecific DNA binding (27). Since the binding of DNA by mineral compounds such as glass (15) and diatomaceous earth (14, 83, 85) is the basis of a number of nucleic acid extraction methods, the unavailability of DNA because of binding to potentially complex sample components such as sorption to sediments in environmental samples is a rational explanation of inhibition.

Nucleic acid sequestration and degradation may be overcome by physicochemical separation of target DNA from destructive compounds as soon as possible after cell lysis. Heat (97) and proteases (77) may be of use for destroying nucleases. If used, proteases must themselves be eliminated before polymerase is added to prevent enzymic inactivation of the polymerase.

Humic compounds are the most commonly reported group of inhibitors in environmental samples (Table 3) and appear to have deleterious effects on several reaction components and their interactions (57). Public health and ecologic investigations that involve environmental sampling may be hampered by inhibition from humic compounds. It was shown that as little as 1 μl of humic-acid-like extract was enough to inhibit a 100-μl reaction mix and that this was unlikely to be due to chelation of Mg^{2+} by humic compounds (119, 120). Sephadex spun columns helped facilitate PCR in that work. In the extraction of DNA from ancient human bone it was found that 5 ng of ancient DNA was inhibitory to the amplification of 1 ng of recent DNA, due to coextraction with humic compounds or Maillard products of reducing sugars (44). These workers found that solvent extraction, ethanol precipitation, the addition of bovine serum albumin (BSA), gelatin, and high concentrations of *Taq* polymerase all failed to facilitate amplification, although ion-exchange chromatography removed inhibitors. Young et al. (139) explained the commonly reported inhibition by soil humic compounds as follows. The phenolic groups of humic compounds denature biological molecules by bonding to amides or oxidize to form a quinone which covalently bonds to DNA or proteins. The addition of polyvinylpolypyrrolidone (PVPP) or polyvinylpyrrolidone (PVP) overcame the inhibition and allowed separation of humic compounds from DNA during agarose gel electrophoresis. PCR yield was reduced by the addition of >0.5% PVP, however.

Recent work on facilitating amplification in the presence of humic acids, and perhaps other inhibitors, in soils involved introducing a component with an affinity for the inhibitor higher than that of the essential reaction component that was inhibited (76). McGregor et al. (76) consider inhibition to be due to interference in the interaction between polymerase and target DNA. Of nine proteins tested, BSA proved the most effective in overcoming inhibition, as had previously been indicated by other workers.

MICs were calculated for effects of humic acids on *Taq* polymerases (116). Up to an eightfold difference in MICs was dependent on the source of the humic acid and the commercial producer of the enzyme. The inhibitory effect was reduced by the addition of T4 gene 32 protein. Humic acids inhibited not only lytic enzymes (57) and polymerase activity during PCR but also DNA-DNA hybridization, restriction enzyme digestion of DNA, and transformation of competent *E. coli* cells. In that study (116) high concentrations of nontarget DNA were also identified as inhibitory to PCR. These high concentrations may prevent specific interaction between polymerase and target DNA.

In clinical samples, many substances that cause inhibition by one or more methods that are not understood may be present (3, 15, 24, 38, 39, 46, 50, 64, 130). These substances may come from the body or from sample preparation. Blood was reported as inhibitory if it was present at 4% or more of the reaction volume. Mercier et al. recommended keeping blood below 1 to 2% of the 100-μl reaction volume to enable amplification of sequences in blood (79). Serum proteins may act as blocking agents and prevent access to target DNA by polymerases. Various components of blood may cause inhibition, and the degree of inhibition may possibly vary with their differential production during disease processes.

Polymerase inhibition. As discussed above, humic compounds are widely reported as causing inhibition, and some authors have identified this as being due to their interfering with lytic enzymes (57), binding to DNA and proteins (137), and interfering with the binding between target DNA and polymerase (116). Proteolytic enzymes and denaturants may also inactivate polymerase and must be promptly inactivated if they are used in cell lysis. Urea may cause inhibition by denaturing polymerases (102).

Phenolic compounds (108) from the sample or carried over from organic DNA purification procedures can inhibit the reaction by binding to (139) or denaturing the polymerase. Proteinases and denaturants used for cell lysis may be carried over and inactivate polymerase if DNA purification is not adequate. Proteinase was prevented from inhibiting amplification in one study (91) by the addition of proteinase inhibitors and BSA. Kreader (65) also was able to restrict inhibition by factors, including proteases, through the addition of BSA, which provides an alternative substrate for catalysis by these enzymes. Cheese proteases were found to inactivate *Taq* polymerase but could be removed by hot-NaOH extraction (97). Bile acids and salts may cause problems with both clinical samples and enteric bacteria enriched in culture media containing these compounds. Bacterial proteases and nucleases in feces, as well as cell debris, bile acids, and other factors, may prevent amplification by physicochemical and enzymic effects.

Post-PCR restriction analysis may be inhibited by excess PCR primers (1). Chain extension by polymerase may be interrupted by primer-target noncomplemantarity. Primer extension during PCR may sometimes be terminated by single base mismatches between the primer and target DNA strands. This

inhibitory effect on enzymic extension has been exploited to assess oligonucleotide and target sequence complementarity (9). Careful consideration must be given to primer design to avoid this form of premature termination.

Physicochemical separation or inactivation may be used to overcome factors causing inhibition by these methods (84, 129). Specific inhibitors of inhibitors and competing substrates may also be of use (65). For many situations, dilution of inhibited samples provides a rapid and straightforward way of permitting amplification. This dilution exploits the sensitivity of PCR by reducing the concentration of inhibitors relative to target DNA and is analogous to the dilution of substrates containing antimicrobial compounds prior to culture.

CONCLUSION

Although DNA amplification technologies continue to provide useful tools for the detection and investigation of microorganisms, their promise will not be completely fulfilled until improved and automated amplification and detection systems become available at affordable prices. For such technology to be widely applicable, methods which allow the rapid and efficient removal of inhibitors and attenuators of amplification must exist. Many reports of different nucleic acid extraction methodologies have been made. A range of techniques is being investigated, but it seems unlikely that a single method which is suitable for all sample types will emerge. The separation of cells from samples by many techniques, including multiplexed methods (106), general methods (87, 96), lectins (88), phytohemagglutinin (35), free-flow electrophoresis (49), immunomagnetic separation (130), electroelution (95), and electrofractionation (105), has been discussed.

A large number of commercial rapid-extraction methods are available. These are based mainly on ion-exchange chromatography, size exclusion, and sorption. Such methods are advertised particularly for the purification of nucleic acids from relatively clean sources, such as mammalian tissues, broths, and purified cultures. They do not often feature in studies where DNAs are purified from more complex samples, such as body fluids, food, soil, and surface water. Although rapid extraction methods may be suitable for many purposes, relatively few workers reporting inhibition have used such products as solutions to the inhibition. This probably reflects a lack of confidence in the suitability of such technology for these purposes, as most workers continue to prefer to use methods based on phenol-chloroform extraction. Experimentation may also have shown that the more laborious extraction methods provide higher yields of purer DNA, particularly from complex samples such as sediments and foods. This has been my experience with several commercial systems. Immunomagnetic separation can be used for cells or nucleic acids in cell lysate and perhaps more complex samples. Although impaired by fats, immunomagnetic separation is perhaps the most promising general isolation method.

For some time a confusing variety of methods will continue to be required for investigations of different sample types. This review shows that many studies have demonstrated a range of inhibitory compounds and that they achieve their effects at one or more of three sites. As better understanding of these points of inhibition is achieved, simplified and improved extraction methods may become available for various sample types. Improved understanding of the compounds and mechanisms of inhibition should enable the rationalization of approaches to purification. A smaller range of effective facilitating treatments which can be applied to nucleic acids from different source materials may be developed. This development should improve both the sensitivities and applicability of these methods in every field.

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