REVIEW

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False-Positive Results and Contamination in Nucleic Acid Amplification Assays: Suggestions for a Prevent and Destroy Strategy

Abstract Contamination of samples with DNA is still a major problem in microbiology laboratories, despite the wide acceptance of PCR and other amplification techniques for the detection of frequently low amounts of target DNA. This review focuses on the implications of contamination in the diagnosis and research of infectious diseases, possible sources of contaminants, strategies for prevention and destruction, and quality control. Contamination of samples in diagnostic PCR can have far-reaching consequences for patients, as illustrated by several examples in this review. Furthermore, it appears that the (sometimes very unexpected) sources of contaminants are diverse (including water, reagents, disposables, sample carry over, and amplicon), and contaminants can also be introduced by unrelated activities in neighboring laboratories. Therefore, lack of communication between researchers using the same laboratory space can be considered a risk factor. Only a very limited number of multicenter quality control studies have been published so far, but these showed false-positive rates of 9–57%. The overall conclusion is that although nucleic acid amplification assays are basically useful both in research and in the clinic, their accuracy depends on awareness of risk factors and the proper use of procedures for the prevention of nucleic acid contamination. The discussion of prevention and destruction strategies included in this review may serve as a guide to help improve laboratory practices and reduce the number of false-positive amplification results.

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Introduction

Since the first publication on primer-mediated enzymatic amplification of DNA sequences, better known as the polymerase chain reaction (PCR), in 1985, the number of reports describing the use of this technique increased exponentially until 1999, after which a more or less stable level of about 30,000 publications each year seems to have been reached (PubMed bibliographic database search using the term 'polymerase chain reaction') [1]. Within a few years, other nucleic acid amplification methods were developed, e.g. nucleic acid sequence-based amplification [2], ligase chain reaction [3], transcription-mediated amplification [4], branched DNA [5], and strand displacement amplification [6].

Soon after the introduction of PCR, people realized that the advantage of this nucleic acid amplification assay, i.e., its great sensitivity, is also a drawback, since even the smallest amount of contaminating DNA can be amplified. In 1988, Lo et al. [7] reported the first false-positive results, with PCR primers directed against hepatitis B virus (HBV) being contaminated with plasmid DNA containing a full-length HBV insert. This observation led to the publication of numerous reports on how to recognize and avoid false-positive results caused by contamination, and how to eliminate contaminating DNA. Most of these reports were published between 1990 and 1993, but, as illustrated in 2002 by Millar et al. [8], this does not mean we have solved this problem. Of all reports on PCR published between 1990 and 2002, the percentage dealing with contamination or false-positive results has been about 2% (range: 1.8–2.1%), and it is not declining (PubMed bibliographic database search using the terms 'polymerase chain reaction' and 'contamination or false positive').

In this review, we focus on the implications of contamination in the diagnosis and research of infectious diseases. Although most researchers using nucleic acid amplification methods will be familiar with carry-over contamination, whereby DNA fragments from previous experiments are re-amplified, other sources of contami-

nants can be very unexpected. In addition, we review the literature on different methods for the prevention and destruction of contaminating DNA. We discuss the functionality and drawbacks of these methods, and give recommendations on how to improve laboratory practices. Although not included in the subject of this review, we would like to remark that false-negative results (e.g., due to the presence of inhibitors, component failure or omission) and methods for their control (e.g., using positive controls and internal process controls) are also important in determining the outcome of amplification assays.

Because terms like 'false-positive' and 'contamination' will be used frequently in this review, it is necessary to define our interpretation of these and related words. (i) False-positive results caused by a 'general contaminant'. This type of contamination will generally affect every sample in the assay. It occurs when unwanted target DNA is introduced to the assay through such means as reagents, laboratory disposables, equipment, or the environment (including carry-over contamination between tests). (ii) False-positive results caused by a 'sample contaminant'. This type of contamination generally only affects a limited number of samples in an assay. It occurs when unwanted target DNA is introduced to certain samples due to events such as sample-to-sample contamination or leakage between samples on agarose gels. (iii) Other false-positive results. These are false-positive results that are not caused by the presence of target DNA, but rather by nonspecific products due to suboptimal assay conditions or PCR reactions in which specificity may not be 100%, even in optimal conditions.

Stated concisely, contamination will always lead to a false-positive result (although this may not be noted when the intended target is amplified), but a false-positive result is not always caused by contamination.

False-Positive Results and Their Implications

False-positive results of nucleic acid amplification assays can have several causes, including contamination, and can have considerable implications, both in research and in the clinic. The following examples show that amplification assays are not always as reliable as is sometimes believed.

In search of causes of infectious diseases, PCR has been used as a tool to demonstrate an association between infectious disease and the presence of microbial DNA. Boyd et al. [9] used PCR and in situ hybridization to study the involvement of human papilloma virus (HPV) in cutaneous lichen planus. Initial results on archival paraffin-embedded biopsy material were encouraging. However, more in-depth evaluation revealed nucleic acid contamination, probably due to sample contamination from HPV-positive material or adjacent wells, and a correlation between cutaneous lichen planus and HPV could not be verified.

A case where a false-positive result almost led to the assumption that an HIV-1 vaccine-induced immune response led to an abortive infection with abrogation of seroreactivity (a very tempting theory) was described by Schwartz et al. [10]. A plasma-sample of an HIV-1 seronegative patient who had participated in an HIV-1 vaccine trial tested positive in an RT-PCR assay. Although this result was not confirmed by other assays, retrospective analysis of serum RNA samples obtained from earlier occasions in the vaccine trial showed a cluster of positive results over a limited period, convincing some investigators of the validity of the original positive result and leading to the hypothesis mentioned above. Eventually, all previously reactive samples were retested by RT-PCR in a quality-controlled laboratory. All samples resulted negative for HIV-1 RNA, including the cluster that had previously been reported as positive and the original positive plasma sample. It is not clear what caused the false-positive results in the first RT-PCR assays.

A number of reports have described cases in which contamination had far-reaching consequences for the patients involved. In one case, PCR analyses of pleural fluid of a patient diagnosed with chronic lymphocytic leukemia were positive for *Mycobacterium tuberculosis* on two different occasions. Therefore, antituberculosis therapy was commenced, while treatment for chronic lymphocytic leukemia was postponed. Staining and cultures for *Mycobacterium tuberculosis* were negative. After 9 months, PCR for *Mycobacterium tuberculosis* was still positive, even though there was no evidence of tuberculosis with standard diagnostic tests. Antituberculous treatment was discontinued and high-dose chemotherapy was begun. Active tuberculosis was never ascertained, and the postponement of chemotherapy was apparently based on false-positive results. Again, the source of this contaminant was not clarified [11].

One well-known case of false-positive results in diagnostic tests even led to a patient's death [12]. A 30-year-old woman was diagnosed with chronic Lyme disease based on one PCR assay of blood positive for *Borrelia burgdorferi*. Magnetic resonance imaging of the brain and cerebrospinal fluid examination were unremarkable, and several enzyme immunoassays, Western blot assays, and PCR assays on blood, urine and cerebrospinal fluid were negative or indeterminate. A Groshong catheter was placed and the patient was treated with intravenous antibiotic drugs for 27 months. This therapy was discontinued when impaired liver function and thrombocytopenia were observed. Enzyme immunoassays, Western blot, and PCR assays performed at another hospital were all negative for *Borrelia burgdorferi*. One month later, the patient died as a result of a large *Candida parapsilosis* septic thrombus located on the tip of the catheter, obstructing the tricuspid valve. At autopsy, there were no indications of Lyme disease. The one positive PCR assay on which the whole therapy was based was probably the result of DNA contamination. Interestingly, the laboratory that reported this false-positive result reported another positive *Borrelia burgdorferi* PCR result, which proved to be false-positive as well. Luckily, in that

case the patient was referred for a second opinion, and did not receive unnecessary therapy [13].

The examples described above have one remarkable similarity: in all cases there were results of several other (non-amplification) tests contradicting the (false-) positive results on which the theory or diagnosis was based. From these examples, and from the poor results of quality control studies as described in the last section of this review, clinicians should be aware that PCR test results should be interpreted with great care. In some cases, even though the amplification assay is truly positive, the result may not be of clinical significance, since the sensitivity of nucleic acid amplification assays may lead to the detection of microorganisms that have no clinical consequences, and DNA derived from dead or degrading microorganisms may yield positive results. And of course, there is always a chance that false-positive results may be caused by contaminating DNA. Therefore, PCR results should always be validated by comparison with conventional diagnostic methods as well as clinical data, although the gold standard is sometimes hard to define.

Several authors have reported situations in which extensive retesting was performed because clinical findings and the results of standard diagnostic methods did not agree with PCR results [10, 13, 14, 15]. Needless to say, besides the discomforting uncertainty caused for patients and clinicians, this results in a considerable increase in costs. Therefore, all possible efforts should be made to improve the reliability of amplification assays.

Although we unequivocally recommend that PCR results be compared with other available data, we would like to make one clarifying comment. As was observed by Jehuda-Cohen [16] for HIV-1, it is sometimes claimed that all positive PCR results that are not matched by positive enzyme-linked immunosorbent assay serology are false-positive. Although the gold standard is always the best diagnostic method available, that does not mean there is no room for improvement. For example, blood culture is considered the gold standard for the detection of disseminated yeast infections. However, in many cases (up to 65%) automated blood cultures fail to detect yeasts [17]. We have shown that we were able to improve the detection rate of yeasts in blood cultures by using nucleic acid sequence-based amplification [18]. It is therefore possible that a positive amplification result, which is not confirmed by other tests, is in fact of clinical significance. In summary, all available test results and clinical data should be considered, but one should also be aware of the limitations of the tests used.

Prevent and Destroy

Preventing Contamination

The strategy for preventing false-positive results in nucleic acid amplification assays can be divided into two parts. (i) The risks of contamination should be kept as low as possible. (ii) If contamination occurs, the contaminant should be destroyed.

To reduce the risk of contamination, an inventory of the risk factors associated with contamination should be made. Several factors pose a risk of causing false-positive results, some of which may be very unexpected. Particular attention should be paid to reagents, disposables, laboratory equipment, and the environment (Table 1).

It is known that some reagents may be contaminated with DNA. For many applications involving yeasts or fungi, a pretreatment protocol is necessary to lyse the cells before DNA extraction. In most cases, lyticase, lysing enzymes and/or zymolyase are used. Rimek et al. [19] found that different batches of Zymolyase-20T from two different companies contained fungal DNA. The fragment that was amplified in the negative controls of their panfungal PCR assay showed 100% sequence identity to the *Saccharomyces* sensu stricto complex (*Saccharomyces*

Table 1 Examples of risk factors and sources of contamination

Risk factor	Examples	Source of contamination	References
Reagents	zymolyase	*Saccharomyces* spp., *Kluyveromyces* spp.	[19, 20]
	lyticase	fungal DNA	[20]
	lysing enzymes	fungal DNA	[20]
	10x PCR buffer	*Acremonium* spp.	[20]
	primer preparations	unknown, plasmid with HBV-insert	[7, 21]
	Taq DNA polymerase	unknown	[22, 23, 24, 25, 26, 27]
	DNA extraction columns	*Legionella* spp.	[28]
Disposables/equipment	sterilized bronchoscopes	*Mycobacterium tuberculosis*	[29]
	reaction tubes	mostly human	[30]
Environment	air, laboratory benches,	*Aspergillus* spp., CMV,	[15, 20, 31]
	surface of equipment, clothing, skin of hands, etc.	*Bordetella pertussis*, plasmid with TEM β-lactamase-gene	[PR]
Insufficient communication	environment (see above)	CMV, *Bordetella pertussis*,	[15, 31]
		plasmid with TEM β-lactamase gene	[PR]

HBV, hepatitis B virus; CMV, cytomegalovirus; PR, present review

cerevisiae, *Saccharomyces pastorianus*, *Saccharomyces paradoxus* and *Saccharomyces bayanus*) and to *Kluyver-omyces lodderae*. The same contamination was described by Loeffler et al. [20]. They found fungal DNA in specific lot numbers of zymolyase powder, but also in batches of lyticase and lysing enzymes. The zymolyase appeared to be contaminated with DNA from *Saccharomyces cerevisiae*. The origin of the DNA found in lyticase and lysing enzymes could not be specified.

Several components of PCR mixtures have also been shown to contain contaminating DNA. Different tubes of one lot number of 10× PCR buffer were contaminated with DNA from *Acremonium* spp. [20]. Furthermore, commercial primer preparations used by Goldenberger and Altwegg [21] were the source of contaminants in an assay using broad-range primers directed against 16S rDNA. The origin of this contamination was not further specified. Another example of contaminated primers was reported by Lo et al. [7]. Their primers were contaminated by plasmid containing a full-length HBV insert. Although it was not mentioned, it is most likely that this contamination originated from their own laboratory, as opposed to the other examples, which could all be traced back to the manufacturers.

Contamination of *Taq* DNA polymerase with bacterial DNA has been reported several times [22, 23, 24, 25, 26, 27]. All of the researchers used several polymerases from different suppliers, often including low-DNA *Taq* DNA polymerase, and although quantitative differences between the products from different companies were observed, all preparations yielded false-positive results. In three of the studies, universal primer systems directed against rDNA sequences were used. It is important to note, however, that false-positive results were also obtained when more specific primer systems were used (i.e., 16S rDNA sequences of mycobacteria [24], 5S rDNA sequences of *Legionella* [25], and rDNA sequences of *Escherichia coli* [26]). When an attempt was made to specify the identity of the contaminating DNA, all authors agreed that the bacteria in which the *Taq* polymerase was produced (*Escherichia coli* and *Thermus aquaticus*) could be ruled out as a source. However, the exact identity could not be elucidated. It is generally believed that more than one strain or species is responsible for the occurrence of false-positive results when using *Taq* DNA polymerases, which is most likely the main reason why identification is difficult. Even though it has not been unequivocally proven, all findings point to the involvement of either the buffers, the chromatography columns, or the water used in the purification of the enzyme. Thus, it is likely that other biological products are also contaminated; indeed, the purification process is also assumed to be the source of the contamination in some primer preparations [21].

Commercially obtained columns used to isolate DNA from samples may also be contaminated. Two laboratories in The Netherlands discovered that 10–70% of some batches of Qiagen nucleic acid extraction columns were contaminated with DNA from *Legionella* spp. other than *Legionella pneumophila*, but full details of the tests were not given. The *Legionella* DNA was probably introduced during production, when column material is continuously flushed with water [28].

Besides the reagents, laboratory disposables and equipment can also be the source of contaminating DNA. An obvious example is the need to disinfect the rubber septum of evacuated sample tubes or blood culture bottles before drawing blood. Besides leading to false-positive culture results, this can also cause problems when blood is used for diagnostic amplification methods [32].

Another problem can occur when people disregard the fact that 'sterile' does not necessarily mean 'DNA-free'. In a study performed by Kaul et al. [29], it was shown that 3.6% of sterilized bronchoscopes used to obtain broncho-alveolar lavage samples contained amplifiable *Mycobacterium tuberculosis* DNA. When looking at 277 *Mycobacterium tuberculosis* PCR results in retrospect (validation and clinical samples), five false-positive samples were detected, four of which were from bronchoalveolar lavage samples.

Even the single-use plasticware, which has replaced washable glassware in our laboratories, is not always free of contaminating DNA. For example, according to Schmidt et al. [30] reaction tubes show contamination rates ranging from 20% up to 80–90%, depending on the supplier. The vast majority of these contaminants was found to be of human origin; in one case the sample did not show any similarity with a human reference sequence, but the identity of this sequence was not given.

Microorganisms and their DNA are present everywhere around us. For example, fungal spores like conidia from *Aspergillus* spp. can be present in the air. This can lead to false-positive results due to airborne spore inoculation during DNA extraction, as was detected by Loeffler et al. [20]. Environmental contaminants can also be a problem when testing fixed formaldehyde/paraffin-embedded samples by PCR. It is important to decontaminate the surface and only use samples obtained from below the surface for DNA extraction.

The following examples show that it is also very important to be aware of all activities that take place in your laboratory, even in your building. Situations that are routine for one person can turn out to be an unexpected and huge problem for another.

Porter-Jordan and Garrett [31] found false-positive results when using a PCR for the detection of human cytomegalovirus. Upon further investigation, they realized that this contamination could originate from a laboratory situated one floor below theirs, where cytomegalovirus culture material was autoclaved before disposal. It turned out that the autoclaved positive material included small DNA fragments that were contaminating the environment, which may have produced positive signals in their PCR assays.

A second example was described by Taranger et al. [15]. They found a discrepancy of 57% between PCR (91% positive) and culture results (34% positive) for *Bordetella pertussis* in one pediatric outpatient clinic, while in samples from another clinic no corresponding

PCR-positive and culture-negative results were seen. All surfaces tested in the two rooms where vaccinations and diagnostic work-ups were done (e.g., laboratory benches, steel tables for equipment, the staff members' clothes and the skin of the staff members' hands) were contaminated with *Bordetella pertussis*-positive material. However, even though the same environmental findings were made in the vaccination room of the second clinic, there, the vaccinations were given in rooms located far away from the examination rooms where patient samples were taken for diagnostic purposes. This environmental contamination was caused by droplets from a whole-cell pertussis vaccine.

In our own laboratory we recently encountered a problem with contamination of a diagnostic test in which PCR was used to detect TEM β-lactamases in clinical isolates (unpublished observations). At a certain time, all negative controls started to become false-positive, and this problem could not be resolved by extensive cleaning of the working areas and the use of new reagents. However, a research group sharing the same laboratory areas used cloning- and expression systems for the production of proteins. It became clear that the vector applied in their cloning- and expression systems contained a commonly used ampicillin-resistance selection marker, which is a TEM β-lactamase gene. Since the focus of their research was to study proteins, the work was done in a regular laboratory room. The researchers were not restricted from entering areas where PCR-premixes were prepared, and often, after purification and analysis of expressed proteins, non-related PCRs were performed. No one realized that the protein preparations were highly contaminated with vector-associated DNA, resulting in high-copy TEM β-lactamase-gene contamination of the environment.

From these examples we can conclude that communication is very important, especially in larger laboratories where several research groups make use of the same rooms and equipment. Even non-molecular biologists may be working with large amounts of DNA (culturing, plasmids, etc.), thereby forming an unexpected risk of contamination. The same holds true for researchers using species-specific amplification assays: these tests may not be very sensitive for contamination, but other tests performed in the same area may be. All researchers using the same laboratory space and equipment should therefore use the precautions necessary for the assay that is most sensitive to contamination, without any exceptions. It is advisable to have regular meetings about the work being performed by all personnel using the same laboratory space, even though the main subject of the different research groups may be unrelated (which usually is a reason for having separate meetings). A regular technical laboratory meeting about the proper use of labware and devices and current problems in the lab may also be helpful.

The difficulty with contaminated reagents is that it is impossible for a laboratory to prevent such contamination. It is therefore very important to communicate such problems to the manufacturer. In our experience, compa- nies are not always aware of the very diverse range of sources that can cause contamination of their products. It is often believed that a room (or manufacturing hall) is 'clean', unless it is used by people working with DNA. However, microorganisms, cells and DNA are every- where. Even though it may be difficult to implement in a production process, it may be sensible for companies to apply the concept that a room is 'dirty', except for small areas that can easily be cleaned.

The amount of contaminating DNA/RNA that is present varies: in general, during sample preparation only low amounts of nucleic acids are generated (so-called 'low copy', corresponding with low risk), whereas the handling of recombinant plasmids or phages and amplification techniques involves high amounts of DNA (so called 'high copy', corresponding with high risk). Therefore, attention must be paid to the workflow. Laboratories should be subdivided into 'no copy', 'low copy' and 'high copy' working areas, and, if possible, equipped with over- pressure (no copy, i.e. clean areas) or underpressure (high copy, i.e. contaminated areas). These working areas can be delineated by separate rooms, or the use of biosafety hoods or cabinets designed for this purpose [33, 34, 35]. Also, flow cabinets can be used, in which the direction of flow ensures that aerosols are pushed down to the base of the cabinet, away from the top of the reaction tubes [36]. Activities in which no DNA is involved (e.g., preparation of PCR-mixes) should be performed first, after which activities can proceed via low copy (e.g., extraction of low-copy nucleic acids, adding template to premixes) to high-copy operations (e.g., analysis of amplification products or other sources of high-copy DNA). Subse- quently, the no- and low-copy work area should not be entered again on that particular day. Furthermore, it is recommended that reagent aliquots be prepared in an area that is free of nucleic acids, and that different freezers and refrigerators be used for the storage of reagents and samples.

Providing each work area with separate sets of supplies and equipment, like racks, pipettes, test-tube holders, centrifuges, etc., substantially reduces the risk of carry- over contamination. To prevent contamination by aerosols containing nucleic acids, the use of positive displacement pipettes [33, 34] or disposable filtertips/plugged pipette tips [33, 37, 38] is advised. However, differences in the quality of filtertips from different suppliers have been observed [38, personal observation]. To avoid aerosols, reaction tubes should be opened with caution [33, 34]. Furthermore, it is advisable to use the minimum number of cycles during amplification reactions, to produce only as much amplicon as is needed to obtain the desired results. Of course, any changes to a protocol need to be validated, especially in assays for diagnostic purposes.

Additionally, the risk of contamination can be reduced by the careful handling of waste disposal in order to prevent aerosol formation. Disposables, which contain RNA/DNA, like pipette tips and reaction tubes, but also electrophoresis buffer, are important potential sources of contaminants. It is recommended that nucleic-acid con-

taminated plasticware be collected in disposable bags and that the bags be closed directly after activities are finished. Electrophoresis buffer can be decontaminated with sodium hypochlorite directly after use (see below).

Appropriate disposable clothing should be present in each room, and these should be changed frequently (e.g. gloves, masks, coats, mob caps, goggles) [34, 39, 40]. Additionally, it is recommended that the working areas, equipment, and literally everything that is routinely touched by hands, like doorknobs, handles of freezers, telephones etc., be cleaned on a regular basis. Cleaning agents, like HNO_3, ethanol, and commercial cleaners such as *Extran*$^©$ (cleaner for laboratory use) are not effective in the elimination of contaminating DNA [30]. Better decontamination methods are described below.

DNA contamination should be monitored by routine wipe tests, as described by Cone et al. [41]. However, since the wipe test can be false-negative due to inhibitory substances originating from laboratory surfaces, a duplicate of each wipe-test sample should be tested routinely with an amplification-positive control [42].

Destroying Contaminants

Prevention of DNA contamination is part of the job, but if contamination occurs, one has to try to eliminate this problem. Many suggestions have already been published in this regard. The conditions for using a protocol should be established and evaluated for each target system. In general, destruction of contaminating DNA can be performed before amplification, to avoid false-positive results in the experiment that is performed, and/or after amplification, to reduce the risk of contaminating following experiments.

Procedures that achieve elimination of contaminating DNA in general are irradiation, enzymatic treatment, and the use of sodium hypochlorite, HCl or hydroxylamine hydrochloride. Other methods specifically destroy amplification products by the use of modified primers, the uracil-DNA-glycosylase/dUTP approach and irradiation with the addition of (iso)psoralen. In addition there are specific decontamination methods for reagents and disposables.

Several kinds of enzymes have been described for destroying DNA in PCR-reagent mixtures like DNase I, exonuclease III, and restriction enzymes [20, 43, 44, 45, 46, 47, 48, 49]. DNA is degraded whereas other components like the *Taq* polymerase remain unaffected. After inactivation of the nucleases by heating, target DNA must be added. This implies that tubes have to be reopened, which increases the risk of environmental contamination. Another disadvantage is that an enzyme combination is used (i.e., *Taq* polymerase and a nuclease). Therefore, reaction conditions have to be optimal for both enzymes used. But, other methods have also been described to eliminate contaminating DNA from buffers, primers, and disposables. These include ultrafiltration [30, 50] and autoclaving under conditions that enable bacterial

decontamination [34, 37]. However, the effectiveness of these methods is controversial [30, 51]. Several preparative methods have been described to eliminate contaminating DNA from the *Taq* polymerase as anion exchange chromatography [52], polyethyleneimine precipitation followed by centrifugation and dialysis [53], and an aqueous/organic biphasic system [54].

Ultraviolet-irradiation is mainly used to treat PCR premixes and to decontaminate work areas or equipment, and it has been described extensively. Both single wavelength (254, 300 or 365 nm) and double wavelength (254 and 300 nm) treatments have been examined [21, 37, 51, 55, 56, 57, 58, 59, 60, 61]. The mechanism is based on oxidation of bases, induction of single- and double-strand breaks, and formation of cyclobutane rings between neighboring pyrimidine bases. The cyclobutane rings form intrastrand pyrimidine dimers that inhibit polymerase-mediated chain elongation [62, 63]. Ultraviolet-treatment is not always effective [36, 37, 51, 55, 64]. Its efficiency depends on the distance, intensity, wavelength, exposure-time, and UV absorption characteristics of water and the tubes used [36, 51, 58, 59]. In the treatment of PCR premixes the presence of nucleotides influences the elimination of contaminating DNA in a dose-dependent manner [36]. Furthermore, the size and internal sequence of the contaminating DNA fragment are important [37, 55, 57, 58, 59, 60, 61, 63, 65]. However, these findings have been contradicted [56, 60, 61].

Ultraviolet-irradiation may influence the activity of PCR reagents. Although nucleotides and primers are relatively resistant [21], the activity of primers [55, 56, 57] and undoubtedly of *Taq* polymerase [21, 37, 55, 57, 61] and uracil-N-glycosylase [21] is affected. Thus, it may be wise to add these components after UV-irradiation of the premixes. This, however, illustrates an important drawback of this decontamination technique: after sterilization, tubes have to be reopened to add reagents and template DNA, which increases the risk of contamination. Besides, reagents like *Taq* polymerase can contain contaminants themselves (as described above), which will remain unaffected. Furthermore, Niederhauser et al. [66] showed that degraded amplification products and primer artifacts accounted for decreased amplification sensitivity. Another point of concern was raised by Linquist et al. [67] who reported that UV-irradiation of polystyrene pipettes released PCR inhibitors.

Ultraviolet-irradiation is also recommended for eliminating contaminating DNA/RNA from surfaces, like laboratory benches, floors, instruments, microcentrifuge tubes and racks [34, 37], but it is much less effective in eliminating dried DNA [60, 61, 64]. Guidelines for eliminating dried DNA by UV-irradiation are clearly described by Cone et al. [68]. A point of consideration for eliminating dried DNA is that the surface must be perpendicular to the light source to achieve maximal light intensity. Additionally, other materials dried with the target DNA, such as irrelevant DNA and nucleotides, can shield the target, making inactivation less efficient. It is

also important to note that UV lamps will still look blue even though their UV output has decreased.

Several publications have described the combination of (iso)psoralen and long-wavelength UV photoactivation (320–400 nm) for sterilization of PCR amplicons [35, 37, 40, 65, 69, 70, 71]. (Iso)psoralens are known to intercalate between base pairs of nucleic acids. This results in the formation of cyclobutane adducts with pyrimidine bases and cross-links when excited by 320–400 nm light [69, 70], which inhibit the extension by polymerases [72, 73, 74]. In general, (iso)psoralens are added prior to amplification, while photoactivation by UV takes place after amplification. Isopsoralen-modified PCR-products can be probed by hybridization and are therefore favored above psoralen [65, 69]. However, lower hybridization stringencies may be required to compensate for the presence of isopsoralen in the amplified DNA [35, 65, 69], and a significant loss of sensitivity is observed at high concentrations [58]. This loss of sensitivity can be corrected by adding glycerol or DMSO [35, 65, 69]. Isopsoralen inactivation of contaminating DNA depends on the length and nucleotide base composition of the amplicon [35].

In summary, the efficacy of UV-irradiation has not been uniform. UV-irradiation should be seen as an additional precaution rather than a replacement for careful laboratory practice. Intensity, wavelength, duration of exposure and effects on the sensitivity of the PCR have to be determined empirically. However, (iso)psoralen treatment in combination with long-wavelength UV irradiation is an effective method for sterilizing PCR amplicons. Since each amplicon has its own base sequence and length, optimal sterilization conditions must be evaluated on a test-by-test basis. Note that (iso)psoralen must be handled with care due to their mutagenic properties.

Inactivation of DNA templates by γ-irradiation was described by Deragon et al. [75]. The efficiency of this method depends on several factors including the length of the DNA fragment and the precise composition of the PCR mixture. Irradiation conditions (like dose) have to be established for each amplification system. Unfortunately, laboratories routinely have no γ sources available.

Sodium hypochlorite has been mentioned as a cleaning agent for work areas and equipment. Prince et al. [40] recommended a concentration of 0.08% sodium hypochlorite (w/v, 5 min) for fragments as small as 76 bp. This concentration should be stable for 1 week. However, our own observations revealed that a 1-week-old solution of 0.4% (w/v) hypochlorite needed an incubation time of 30 min before an RNA target of 200 bases was no longer detectable by nucleic acid sequence-based amplification. Better results were obtained after a 5 min incubation of a 0.4% (w/v) solution prepared daily (unpublished data).

Results for decontamination by depurination with 1 M HCl [34, 76, 41], possibly in combination with detergent to reduce surface tension and/or UV treatment [30], are controversial. Schmidt et al. [30] described that long-term treatment (2.5 h) with HCl did not yield complete destruction, and Prince and Andrus [76] noted that even 2 M HCl did not completely destroy DNA detectable by PCR.

A DNA destruction method that only affects amplification products is the use of primers containing a 3′-terminal ribose residue. *Taq* DNA polymerase is able to both extend and copy a single ribose residue efficiently, which generates a cleavable ribonucleotide linkage within the amplified product [77]. Cleavage can be established either by RNase A treatment (pre-amplification) or NaOH treatment (post-amplification). In the case of RNase treatment, addition of a sulfhydryl reducing agent and thermal denaturation is necessary to inactivate the enzyme. This does not affect the activity of *Taq* DNA polymerase. Efficiency of treatment with NaOH varies, depending on the number and position of the 3′-ribose residues [58]. Also, tubes have to be opened after amplification to add the base, risking the possibility of aerosol formation. Amplicons generated with 3′-ribose primers can be used for sequencing, cloning, and all other research applications of PCR-products.

Use of the uracil-DNA glycosylase (UDG) or uracil-N-glycosylase (UNG)-dUTP approach is another method to combat carry-over contamination of amplification products. The method was introduced by Longo et al. [78], and commercial diagnostic tests using this system are currently available [33]. UDG removes the uracil residues from the sugar moiety of either single- or double-stranded DNA, creating abasic sites in the phosphodiester backbone [79]. This method involves substituting dUTP for dTTP in all PCRs to ensure that all DNA arising from these amplifications will contain dUTP. Since UDG does not function on dT-containing DNA, dUTP, UTP or RNA, amplification of natural target RNA or DNA is not affected. If all amplification products contain dUTP, contaminants can be eliminated prior to amplification without tubes being reopened to add polymerase or template. However, the fidelity of the incorporation of dUTP in place of dTTP is not known for all polymerases. In some cases, PCR does not proceed with quite the same efficiency when dTTP is completely replaced with dUTP [79]. This inefficiency appears to be sequence-specific and is not necessarily related to the length of the fragment to be amplified [33]. Poor reaction efficiency is probably due to lower incorporation efficiency of dUTP by *Taq* polymerase or to changes in primer annealing on dUTP substituted templates. Higher concentrations of dUTP with compensating magnesium concentrations can increase product yields [33, 35, 78, 79]. Although there is no significant activity during typical PCR thermal cycling [80], UNG is not completely inactivated at the elevated temperatures in the amplification procedure. So, following thermal cycling, prolonged incubation at either 4°C or 25°C increases the risk of degradation. Therefore, it is recommended that soak files be set at 72°C to protect amplified dUTP-containing products, or to use the UDG inhibitor protein [80]. According to other authors, however, dUTP sites are heat labile and break during temperature cycling [31].

DNA containing dUTP is normal in most respects (e.g., it is cut by many restriction enzymes and hybridizes to oligonucleotide probes) [81], but Beebe et al. [82] found that restriction endonuclease cleavage was dependent on the specific endonuclease used as well as the sequences flanking the endonuclease recognition site. For cloning, amplification products must be introduced into a UDG-deficient *Escherichia coli* host to avoid destruction of the amplified DNA. Last but not least, high levels of contaminants cannot be destroyed completely by this system, which results in false-positive signals [79].

Aslanzadeh [83] described the use of hydroxylamine hydrochloride as an effective alternative for the destruction of amplicons after amplification. Hydroxylamine is a mutagenic agent, which disrupts normal nucleic acid pairing. Hydroxylamine hydrochloride-modified PCR products do not appear to bind to and modify other PCR reagents such as *Taq* polymerase.

A comparison of three different methods for the elimination of amplification products (pre-PCR treatment of a dU-containing PCR product with UNG, post-PCR UV-irradiation in combination with isopsoralen, and post-PCR alkaline primer hydrolysis) showed that all three methods were effective and were able to eliminate up to 10^9 copies of the product [58]. Also, the combination of different protocols like treatment with UV (amplification reactions excluding polymerase, primers, and template) followed by DNase I treatment of polymerase and primers, appeared to be practicable [46]. Methods that sterilize the whole PCR-mixture directly before amplification starts are preferable, because reopening of tubes increases the risk of cross-contamination. For this, Udaykumar et al. [84] have introduced the use of a wax-barrier.

Controls

In the end, it is worthwhile to check whether precautions and sterilizing protocols have functioned. Therefore, it is recommended that negative sample- and assay-controls be run with every test. Negative sample controls should be similar to the tested samples, but they should not contain any target DNA. For example, blood from healthy individuals or culture medium can be used. These control samples should be subjected to all preparation steps in parallel with the extracted samples. Assay-controls should consist of all PCR components except template DNA. Negative controls should be added for every batch of 10 samples analyzed [40]. It is desirable to place one negative control at the beginning of a series of samples (to check whether the sample itself, the reagents, and the environment are free of contaminants), and other negative controls in-between and at the end of a series of samples (to check whether cross-contamination between samples has occurred). However, although the presence of contaminated reagents or gross contamination of the environment should be observed by using these controls, sporadic contamination can occur and will be more difficult to recognize.

When there is suspicion that the environment may be contaminated, negative controls prepared in the different laboratory spaces can be used. These controls are similar to sample- or assay-controls, but they differ with respect to the preparation area. At least one control should be prepared in each area used for the assay: i.e., sample controls where DNA is extracted, assay controls in the areas where premixes are made (no-copy area) and where DNA is added to the premixes (low-copy area) [85]. If a contaminated room or area is located, wipe tests can be used to check whether the source can be localized to specific benches or other surfaces, equipment, or even laboratory coats [41].

It is recommended that the lot numbers of the reagents used be recorded so if contamination occurs it can more easily be traced. A possible way to check the reagents for the presence of contaminating DNA is to prepare PCR mixes lacking individual components, and to treat these mixes with UV-light. After UV-treatment, the lacking component is added and a PCR performed. If a product is formed, the component that was not exposed to UV-light is contaminated. Since *Taq* DNA polymerase is sensitive to treatment with UV-light (see above), it may be wise to add this enzyme only after the UV-treatment, together with the other missing component [21].

The enzymes used for cell-lysis can be examined by dissolving them in (clean) water, followed by heat-inactivation at 95°C, DNA extraction and concentration by standard methods, and amplification [19]. Obviously, using DNA extraction and an amplification assay to look for contaminating DNA is risky; besides the risk of introducing contaminating DNA from yet another source, it is possible that the contaminant will remain undetected because the amount of contaminating DNA in the sample is too low, or some of it is lost during sample preparation.

To prevent recurrence of the same problem, it is often important to not only localize but also to identify the source of the contamination. At this point, it is wise to check with other researchers using the same or neighboring laboratory spaces to determine which microorganisms, plasmids or DNA sequences are being used. If plasmids containing target DNA are commonly used, amplification with primers that span the vector-insert junction can help identify the plasmid as the source of the contamination [7]. Otherwise, sequencing of the PCR product, preceded by cloning into a vector if necessary, followed by sequence similarity searches in sequence databanks may be essential.

Besides being caused by contaminating DNA, positive results of negative controls may also be due to suboptimal assay conditions. Suboptimal PCR assays may lead to nonspecific bands after gel analysis. Optimizing the annealing temperature may help avoid this problem. However, instead of using gel analysis, based only on the size of the product obtained, it is advisable to use a more specific detection assay (e.g., hybridization with internal probes), when possible, to circumvent problems with nonspecific products. In some cases, a simple RFLP analysis will be sufficient to distinguish false-positive

results from targets. More complex techniques like SSCP or sequencing may also be helpful [24, 26, 86]. However, this will only work when nonspecific products are formed, or when the primers recognize more species or strains than anticipated. In case of carry-over contamination, these methods will not be of any help.

Another problem occurring during gel analysis is 'leaking', i.e., when a slot contains a large amount of target DNA, this may leak into neighboring slots in the gel and cause a false-positive result in an adjacent lane. Obviously, in such cases blotting of the gel and hybridizing with a specific probe will still lead to a false-positive result. When high amounts of PCR product are expected, it is best to skip lanes between samples [9].

When equipment is re-used, the cleaning method may not always be sufficient. In some cases additional testing may be necessary to distinguish false-positives from true-positives. Upon realizing that sterilized bronchoscopes were the source of contamination, Kaul et al. [29] requested that a sterile prewash be performed on the bronchoscope for analysis along with the actual patient sample when BAL samples were submitted for PCR testing.

Balfe [87] described a statistical method that can be used to determine whether positive results of PCR reactions carried out in a microtiter plate are unlikely to have occurred by chance, and hence whether these results are false. A similar method for tube-based PCRs is also available. These methods are based on expected probability distributions.

It is striking that in many cases the results of amplification assays differ between laboratories [12, 13], sometimes even when the same samples are used [10]. Because false-positive results can have very unfortunate effects on research and especially in the clinic, extensive quality control of amplification tests is essential. Quality control should be executed continuously by the technicians or researchers, for example, by blindly retesting positive and negative as well as equivocal samples. Also, quality assurance through the use of proficiency panels should be conducted by an independent organization at regular intervals. Surprisingly, only a limited number of such independent, multicenter studies have been published, and the results were generally alarming [88, 89, 90, 91]. Four examples of multicenter quality control studies (PCRs on hepatitis B, C and G virus, GB virus C, and *Mycobacterium tuberculosis*) showed false-positive rates of 9% up to 57% [88, 89, 90, 91]. Interestingly, in all cases there was no association between good results and the methods used for nucleic acid extraction, the primers used in the amplification, the use of nested PCR, detection by Southern blot analysis with or without radioactive probes, or the use of standardized commercially available kits. This indicates that the way in which the technique is handled, is more critical than the assay itself. In addition to continuing with and increasing the number of multicenter quality control studies, more attention should be paid to the in-house aspects of quality control, before amplifica-tion assays can be used reliably in the diagnosis of infectious diseases.

Concluding Remark

Contamination of samples in PCR can have far-reaching consequences, and the sources of contaminants are diverse and sometimes very unexpected. For example, contaminants can be introduced by unrelated activities in neighboring laboratories, which implies that lack of communication between researchers using the same laboratory space can be considered a risk factor. The poor results of the few multicenter quality control studies that have been published so far show that better implementation of procedures to prevent and control nucleic acid contamination is needed.

The overall conclusion is that, although most nucleic-acid amplification assays are basically useful both in research as well as in the clinic, their accuracy depends on awareness of risk factors and proper use of procedures for the prevention and control of nucleic-acid contamination. The discussion of prevention and destruction strategies included in this review may serve as a guide to help improve laboratory practices and to reduce the number of false-positive amplification results.

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