Arch Lebensmittelhyg 63,
47–52 (2012)
DOI 10.2376/0003-925X-63-47

ISSN 0003-925X

Korrespondenzadresse:
langkabel.nina@vetmed.fu-berlin.de

[1]Panel VPH, Institute of Meat Hygiene and Technology, Veterinary Medicine FU Berlin; [2]Institute of Veterinary Anatomy, Veterinary Medicine FU Berlin

Decontamination of steel and reusable tungsten carbide beads for prevention of DNA deportation in a PCR-laboratory

Dekontamination von Stahl- und wiederverwendbaren Wolframcarbidkugeln zur Prävention einer DNA-Verschleppung im PCR-Labor

Nina Langkabel[1], Stephanie Meyer[1], Robin Großpietsch[1], Lieselotte Bräutigam[1], Verena Eckert-Funke[2], Johanna Plendl[2] and Reinhard Fries[1]

Zusammenfassung

Bei der Untersuchung von Lymphknotenproben mittels MAIC-PCR traten falsch positive Leerwerte auf, eine Verschleppung von DNA über die für die Zerkleinerung des Probenmaterials verwendeten Wolframcarbidkugeln konnte nicht ausgeschlossen werden, obwohl diese Kugeln vom Hersteller als Mehrwegartikel angeboten werden. Daraufhin wurden in der Literatur beschriebene Dekontaminationsmethoden auf ihre Wirksamkeit auf die Oberflächendekontamination von Stahl- und Wolframcarbidkugeln untersucht. Die physikalischen Methoden (dreimaliges Waschen mit Aqua dest., Autoklavieren und UV-Lichtbestrahlung) erwiesen sich bei beiden Kugeloberflächen als unwirksam. Die Anwendung von Exonuklease III und dem kommerziellen Kit DNAaway® führte ebenfalls nicht zu einer sicheren Dekontamination und wurde lediglich bei Stahlkugeln angewandt. Die chemischen Verfahren Peressigsäure (PES) 0,25 % (bei pH 5 und pH 7) und Natriumhypochlorit (NaClO) (aktives Chlor 4,7 % bzw. 5,4 %) zeigten eine hundertprozentige Wirksamkeit. Allerdings wurden dabei die Oberflächen der Wolframcarbidkugeln angegriffen, sodass diese für eine weitere Verwendung unbrauchbar wurden.

Auch beim Einsatz von Mahlkugeln im PCR-Labor ist die Verwendung von Einwegmaterialien zu empfehlen, so wie dies für diesen Bereich schon für Reagenzien und andere Geräte der Fall ist.

Schlüsselwörter: PCR, DNA-Kontamination, Dekontamination, Natriumhypochlorid, Peressigsäure, Wolframcarbid

Summary

In order to mince lymph nodes for MAIC PCR–analysis, the Mixer Mill MM200 (RETSCH GmbH, Haan, Germany) was used with reusable tungsten carbide grinding beads. Some of unexpected PCR results indicated the carryover of DNA contamination among different samples. Hence, several decontamination procedures were used and the surface of steel and tungsten carbide beads was examined for remaining and intact DNA. Physical methods (three washes with distilled water, autoclaving and UV treatment) did not eliminate the DNA from both type of surface; this was also true for a procedure with Exonuclease III and the commercial DNA-removing kit DNAaway® (only used for steel beads). Chemical methods 0.25 % peracetic acid (PAA) (pH 5 and pH 7) and sodium hypochlorite (NaClO) (concentraction of active chlorine 4.7% or 5.4 %) removed DNA from tungsten carbide, but caused cracking of the surface of the beads.

In conclusion, for grinding beads, the use of disposable material is suggested, as also employed for reagents and equipment in the PCR laboratory.

Keywords: PCR, DNA contamination, decontamination, sodium hypochlorite, peracetic acid, tungsten carbide

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Introduction

For decontamination, physical, enzymatic and chemical techniques have been suggested and are in use; in addition, commercial kits such as Exonuclease III or DNAaway® are available. Physical techniques include washing (Schmidt et al. 1995), autoclaving (Prince and Andrus 1992, Schmidt et al. 1995) and UV irradiation (Sakar and Sommer 1990, Ou et al. 1991, Sakar and Sommer 1991, Schmidt et al. 1995).

Zhu et al. (1991) have used the commercially available exonuclease III for the sterilization of presumptive DNA/RNA. This enzyme catalyses the destruction of DNA (Weiss 1976).

Chemical techniques, e. g. peracetic acid (PAA) (Wallhäußer 1988) and sodium hypoclorite (NaClO) (Hayatsu et al. 1971, Prince and Andrus 1992, Kemp and Smith 2005) have been employed for this purpose.

DNAaway® (effective component: sodium hydroxide; Carl Roth GmbH & Co. KG, Kahrlsruhe, Germany) is a commercial DNA-removing kit (Champlot et al. 2010).

PCR examination is a sensitive technique and contamination can lead to false positive results (Kwok and Higuchi 1989, Sakar and Sommer 1990), as originally reported in 1988 by Lo et al. (see also Borst et al. 2004). Since then, this phenomenon has been observed frequently. According to Borst (2004), false positive results appear in as many cases as 2 % and disposable materials (pipette tips, gloves and vessels) should be used or decontamination should be conducted (Kwok and Higuchi 1989).

The aim of this study was to compare decontamination procedures of steel and tungsten carbide grinding beads after grinding of samples in order to determine a decontamination method that excludes DNA-carry over when tungsten carbide beads are reused.

Material and Methods

In 2006 various decontamination methods were tested on steel beads for practicability and efficiency. Procedures that resulted in complete DNA removal were used on tungsten carbide beads in 2006 and 2007.

Suspension of mycobacteria

A pure culture of *M. avium* ssp. *avium* DSM 44156T (DSMZ, Braunschweig, Germany) was grown on Middlebrook 7H10-agar with OACD-enrichment (oleic acid, albumin, dextrose, catalase) (Becton, Dickinson and Company, Sparks, Maryland, USA) for 20 days at 37 °C; 10 subcultures were then kept in Middlebrook 7H9-bouillon with 10 % OACD-enrichment and 25 ml / l PANTA (Polymyxin B (AppliChem GmbH, Darmstadt), Amphotericin B (AppliChem GmbH, Darmstadt), Nalidixin acid (AppliChem GmbH, Darmstadt), Trimethoprim (SIGMA, Steinheim), Azlocilin (SIGMA, Steinheim)) at 37 °C for 30 days. Subcultures were shaken daily.

Bacterial growth was determined via optical density according to McFarland (1907) with the ELISA-Reader EL 800 Universal Microplate Reader (BIO-TEK INSTRUMENTS, INC., Winooski, Vermont, USA). This technique was performed for workers protection.

For this, 10 µl subculture was added to a well of a Microtiter Plate (MTP) and measured at a wavelength of 630 nm on days 0, 5, 10, 15, 20, 25 and 30. All subcultures were then brought together and the final suspension was used for all experiments.

The beads

Steel and tungsten carbide beads were used.

Steel beads (RETSCH GmbH, Haan, Germany; Ø 5 mm; Art.-No.: 22.455.0003) for one way use were examined. Tungsten carbide beads (RETSCH GmbH, Haan, Germany; Ø 5 mm; Art.-No.: 05.368.0038) were claimed by the manufacturer to be reusable but no decontamination procedure is mentioned.

Experimental procedure

For each decontamination technique, 5 beads pre-cleaned with water and organic solvent (ethanol) were used in 5 parallel experiments. Additionally to each experiment one uncontaminated bead was used as negative (bead) control.

Preparation

1 ml Mycobacteria suspension was placed in a 2 ml reaction vessel (Eppendorf AG, Hamburg, Germany; 2 ml; Art.-Nr. 0030 120.086), a bead was added and ground in the Mixer Mill MM200 (RETSCH GmbH, Haan, Germany) for 5 minutes at 30 rps.

Removal of the culture

Culture liquid was removed with a Pasteur pipette (Carl Roth GmbH & Co. KG, Karlsruhe, Germany, Art.-No. 4518) and the beads were transferred to a new reaction vessel in which the decontamination technique was performed.

Decontamination

After the decontamination procedure, each bead was washed with 1 ml distilled water (in a Mixer Mill MM200 for 1 minute with 30 rps); 250 µl of this liquid was taken and DNA extraction was conducted with a DNA extraction kit (High Pure PCR Template Preparation Kit, Roche Diagnostics GmbH, Mannheim, Germany). After decontamination with PAA and NaClO, beads were washed with distilled water (removal of reagents) to minimise chemical influence and placed into a new reaction vessel. Tissue Lysis Buffer (TBL, High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim)) was added and a simulated grinding step was performed for 5 minutes at 30 rps. Afterwards lysis took place. Subsequently, 250 µl was taken and DNA extraction was performed as described above.

PCR

For determination of DNA residues on the bead surface, PCR according to Thierry et al. (1993), slightly modified, was performed. For PCR reactions DyNAzyme II Polymerase Kit (Finnzymes Oy, Espoo, Finland) with Optimized Buffer containing 1.5 mM MgCl2 was used. A 25 µl reaction volume contained 1U Polymerase, 200 µM dNTPs, 2 µM of each Primer (AV6 / AV7, eurofins MWG Operon, Ebersberg) and 2.5 µl of template DNA. The reactions were overlaid with 50 µl mineral oil (Roth, Karlsruhe, Germany Art.-No. HP50.3). Amplification was performed in Trio Thermocycler (Biometra, Göttingen) as follows:

- 10 min 95 °C
- 30 cycles consisting of 1 min DNA denaturing at 94 °C
- 15 sec primer annealing at 70 °C
- 15 sec DNA extension at 72 °C
- final extension step (5 minutes, 72 °C)

Each PCR assay included a positive control (DNA from DSM 44156T) and a negative control (reaction blank).

Electrophoresis of the amplificates was carried out in a 1.5 % agarose gel which was strained with ethidiumbromide and photographed under UV-light.

Decontamination techniques

Decontamination techniques washing, autoclaving, UV irradiation, DNAaway® and Exonuclease III have been used only for steel beads. Peracetic acid and sodium hypochloride were applied for steel and for tungsten carbide beads.

Washing

All 5 steel beads were washed three times together in a 10-ml polypropylene vessel with 10 ml distilled water each. The vessel was shaken for 5 minutes with 70 rpm in a Certomat U Shaker (B. Braun, Melsungen, Germany).

Autoclaving

Beads were autoclaved for 15 minutes with 121 °C.

UV irradiation

The steel beads were irradiated in polystyrene cuvettes (Brand, Wertheim, Germany; Art.-No.: 759005) and in UV cuvettes (Brand, Wertheim, Germany; Art.-No.: 759170).

The cuvette was irradiated in a Transilluminator (Macro-Vue-20, Hoefer Sc., San Fransisco, USA) with λ = 302 nm from below and with an UV hand lamp (desaga UVIS 254 / 366 nm, Desaga, Heidelberg, Germany) with λ = 254 nm from above for 20 minutes. Cuvettes were turned round four times during irradiation in order to guarantee irradiation of the complete surface of the beads.

DNAaway®

The commercial DNA removing kit DNAaway® of Carl Roth GmbH & Co. KG (Karlsruhe, Germany; Art.-No.: X996) was used. Following the manufacturer's instruction, 1 ml DNAaway® was added to the reaction vessel and an overnight incubation at room temperature took place. The liquid was removed with Pasteur pipettes and the beads were placed into a new reaction vessel.

Exonuclease III

Exonuclease III at 100 and 200 Units (Sigma E-1131, Sigma-Aldrich, Taufkirchen, Germany) was used. Each bead was incubated with 500 µl Exonuclease III buffer (50 mM Tris-HCl, pH 8.0, 10 mM Mercaptoethanol, 5 mM $MgCl_2$) containing 100 Units or 200 Units Exonuclease III following the manufacturer's information for 30 minutes at 37°C in a water bath with shaking (GFL Gesellschaft für Labortechnik GmbH, Typ 1083, Burgwedel, Germany). The solution was then removed with a Pasteur pipette and the beads were washed with distilled water and placed into a new reaction vessel.

Peracetic acid (PAA)

This technique was used for steel and tungsten carbide beads with different concentrations of PAA and with different pH between 5 and 7. Steel beads were decontaminated with 0.1 % PAA (pH value 5 and 7) and 0.25 % PAA (pH 5 and 7); tungsten carbide beads were decontaminated with a concentration of 0.25 % PAA (pH value 5 and 7), 0.5 % PAA (pH-value 7) and 0.75 % PAA (pH-value 7).

TABLE 1: *Concentrations of sodium hypochloride used in the experiments.*

steel beads		tungsten carbide beads	
dilution	concentration active chlorine*	dilution	concentration active chlorine*
1:10	1.1 %		
1:5	2.2 %		
1:2.5	3.0 %		
1:1	5.4 %	1:1	5.4 %
1:1 buffered ** (pH 7)	3.7 %–2.2 %***		
1:0.9 buffered** (pH 8.5)	4.7 %	1:0.9 buffered** (pH 8.5)	4.7 %

*: Concentration of active chlorine was determined by iodometrical titration during incubation time in an aliquot; **: diluted with Phosphate Buffered Saline (PBS), pH adjusted with 2M H_2SO_4; ***: decline of chlorine concentration during incubation time (instability of NaClO at pH 7)

For decontamination, 1 ml of the respective concentration was used. Treatment took place for 1 hour in a waterbath (Julabo F 26, JULABO, Seelbach, Germany) at 20 °C; 1 ml phosphate-buffered saline (PBS) with 2 % Na_2SO_3 was then added and the reaction vessels were shaken several times. After a short washing step with 1 ml distilled water the beads were placed in new reaction vessels and ground with TBL for 5 minutes at 30 rps. Afterwards lysis was performed.

Sodium hypochlorite (NaClO)

Sodium hypochloride (chlorine content 12 % (Carl Roth GmbH & Co. KG, Karlsruhe, Art.-No. 9062.3) at various strengths was used for the cleaning of steel and tungsten carbide beads (incubation time: 20 minutes) (Tab. 1).

For decontamination, 1 ml NaClO was added to each bead; each reaction vessel was kept at room temperature for 20 minutes and was shaken every 2 minutes.

NaClO was removed with a Pasteur pipette, afterwards the beads were washed with 1 ml double distilled water, placed into a new reaction vessel and a grinding step with TLB for 5 minutes at 30 rps was simulated.

Scanning electron microscopy

The impact on the surface of tungsten carbide beads treated with PAA and NaClO was documented with a scanning electron microscope. Simultaneously, an untreated tungsten carbide bead was used for comparison.

Results

Steel beads

Physical methods and decontamination with DNAaway® and Exonuclease III at various concentrations were ineffective. PAA removed DNA only when used at a concentration of 0.25 %. NaClO was successful if diluted 1:1 (active chlorine 5.4 %) or 1:0.9 buffered (pH 8.5; active chlorine 4.7 %) (Tab. 2).

Positive and negative controls of each PCR corresponded to the expected results.

Positive and negative controls showed expected results for each experiment.

Tungsten carbide beads

For tungsten carbide beads, only those decontamination methods were used that were shown to be previously

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TABLE 2: *Steel beads: results of decontamination technique (five repetitions).*

Decontamination technique	PCR: positive result (number of beads)	PCR: negative result (number of beads)
Washing (3 times)	4	1
Autoclaving	3	2
UV irradiation, 20 minutes (polystyrene cuvettes)	5	0
UV irradiation, 20 minutes (UV cuvettes)	2	3
DNAaway®	5	0
Exonuclease III (100 Units)	5	0
Exonuclease III (200 Units)	5	0
0.1 % PAA, pH 5	1	4
0.1 % PAA, pH 7	4	5
0.25 % PAA, pH 5	0	5
0.25 % PAA, pH 7	0	5
NaClO dilution 1:10 (active chlorine 1.1 %)	4	1
NaClO dilution 1:5 (active chlorine 2.2 %)	2	3
NaClO dilution 1:2.5 (active chlorine 3.0 %)	3	2
NaClO dilution 1:1 (active chlorine 5.4 %)	0	5
NaClO dilution 1:1 buffered, pH 7 (active chlorine 3.7 %–2.2 %)	2	3
NaClO dilution 1:0.9 buffered, pH 8.5 (active chlorine 4.7 %)	0	5

effective with steel beads. Here, even at high concentration, PAA did not decontaminate or incompletely decontaminated the tungsten carbide surface.

Only decontamination with NaClO dilution 1:1 (active chlorine 5.4 %) and NaClO dilution 1:0.9 buffered (pH 8.5; active chlorine 4.7 %) removed DNA from bead surfaces (Tab. 3).

Bead surface

The decontamination substances had an impact on the surface of the beads (Fig. 1–3). The surface of an untreated tungsten carbide bead was smooth and without any depressions (Fig. 1).

TABLE 3: *Tungsten carbide beads: results of decontamination technique (five repetitions).*

Decontamination technique	PCR: positive result (number of beads)	PCR: negative result (number of beads)
0.25 % PAA, pH 5	5	0 (PCR solution turns greenish)
0.25 % PAA, pH 7	5	0
0.5 % PAA, pH 7	3	2
0.75 % PAA, pH 7	1	4
NaClO dilution 1:1 (active chlorine 5.4 %)	0	5 (bead surfaces blackening)
NaClO dilution 1:0.9 buffered, pH 8.5 (active chlorine 4.7 %)	0	5 (bead surfaces blackening)

In comparison, beads showed corrosion after decontamination with PAA and NaClO.

Concentration of PAA at 0.25 %

After a 1-hour incubation with PAA at 0.25 % (pH 5), the colour of the solution became green, although the surface of tungsten carbide bead appeared visually unchanged.

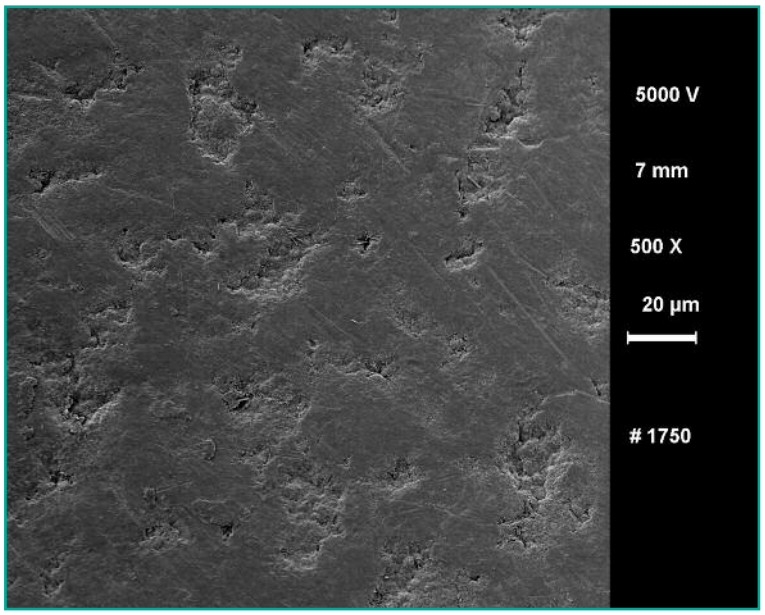

FIGURE 1: *Tungsten carbide bead, untreated (Surface of tungsten carbide, scanning electron microscopy, (500 x) sputtering 45 sec).*

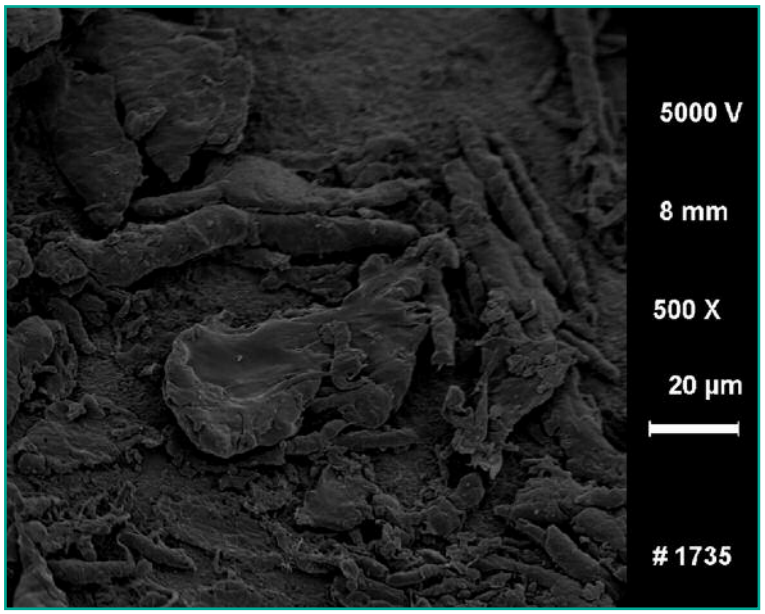

FIGURE 2: *Tungsten carbide bead, after decontamination with PAA 0.75 % (Surface of tungsten carbide, scanning electron microscopy, (500 x) sputtering 45 sec).*

After the addition of DNA extraction reagents, the plastic reaction vessel was affected after 5 minutes of grinding and plastic particles appeared in the solution.

Concentration of PAA at 0.75 %

After treatment with PAA at 0.75 %, affects on the surface of the vessel were visible by scanning electron microscopy (Fig. 2).

NaClO dilution 1:1

After 5 minutes of incubation with NaClO at a dilution of 1:1 (active chlorine 5.4 %), the surface of the bead appeared black. After 20 minutes, the solution was also black.

NaClO dilution 1:0.9

After treatment with NaClO dilution 1:0.9 buffered (pH 8.5; active chlorine 4.7 %), the surface of the beads appeared black after a few seconds. During the last shake, suddenly the caps from 2 of 5 reaction vessels snapped open. In a scanning electron microscopy, the surfaces of the beads were porous (Fig. 3).

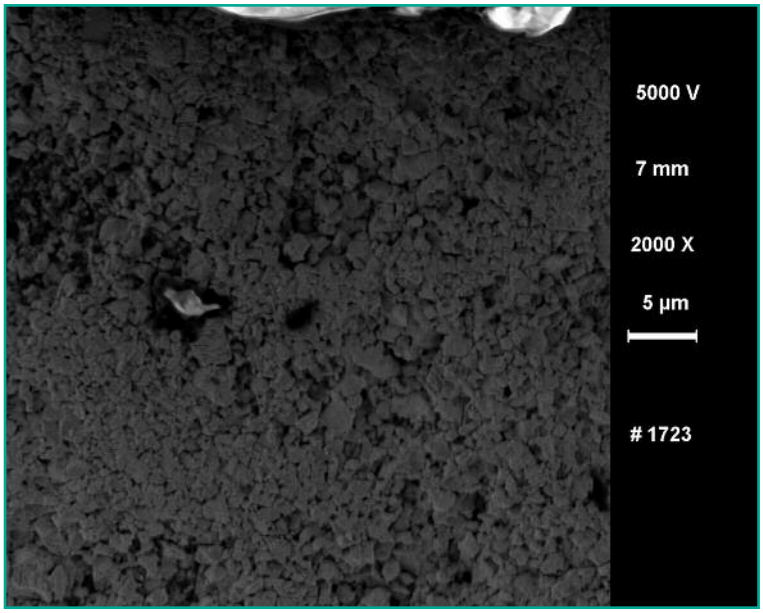

FIGURE 3: *Tungsten carbide bead, after decontamination with NaClO (Dilution 1:0.9, 24 ml NaClO + 20 ml PBS + 2 ml 2M H_2SO_4) (Surface of tungsten carbide, scanning electron microscopy, (2000 x) native).*

Discussion

The beads

Because of the permanent risk of DNA transfer during PCR, the use of one-way materials is considered to be safer then the use of reusable materials (Kwok and Higushi 1989, Borst 2004). In this study, several decontamination techniques have been compared in order to assess their role in a possible transfer route. No decontamination technique for preparation of tungsten carbide beads prior to reuse was offered, several decontamination techniques were tested.

Steel beads

Steel beads were used to test decontamination techniques described in the literature.

Decontamination of steel bead surfaces was complete with chemical methods PAA 0.25 % and NaClO (dilution 1:1 (concentration of active chlorine 5.4 %) and dilution 1:0.9 buffered (pH 8.5; concentration of active chlorine 4.7 %)).

None of the other decontamination techniques removed DNA completely making them impractical for molecular analysis.

Tungsten carbide beads

Tungsten carbide beads were used because of the possibility for reuse them. Only PAA and NaClO in concentrations effective on steel bead surface for decontamination were performed.

Decontamination techniques

Washing

It was suggested that multiple washing steps may remove DNA from bead surface. However, positive PCR results indicate that DNA was transferred to the new reaction vessel.

Autoclaving

Autoclaving reagents prior to use in PCR laboratory is frequently used to avoid DNA contamination and false positive results (Dwyer and Saskena 1992) but results vary in different studies (Dwyer and Saksena 1992, Gefrides et al. 2010).

Autoclaving DNA on bead surface was ineffective, too. Gefrides et al. (2010) found that autoclaving saliva samples at 121 °C was effective for DNA elimination after 60 minutes (75 % of the profile was lost) and after 120 minutes 100 % of the profile was gone. Here autoclaving was performed at 121 °C for 15 minutes only. We conclude, that usual autoclaving procedures (as provided in microbiological laboratories) are not sufficient for PCR because DNA fragments remained on the bead surfaces.

UV-irradiation

Goldenberger and Altwegg (1995) described effective UV irradiation in transparent disposal microtubes of amplificated DNA. So we used polysterene cuvettes, too. UV irradiation with these cuvettes was ineffective, therefore UV-cuvettes were used. Results indicated a potential efficiency. Possibly, UV rays did not reach all parts of the beads because of the threedimensional situation and the rays did not reach parts being in the “shadow”.

Exonuclease III

Exonuclease III degrades double stranded DNA at 3’-hydroxyl termini (Weiß 1976). Single-stranded DNA will not be affected (Richardson et al. 1964) and could be a substrate for amplification (Hoheisel 1993) and detected by PCR. In addition DNA could not be degraded from 3’ terminus if it is protruded, because 5’ mononucleotides could not be removed (Sambrook et al. 1989). Richardson et al. (1964) found a stop of enzyme activity when 35–45 % of the DNA were degraded. Remaining DNA fragments can then be amplificated and positive PCR results will appear. Our results confirm the lack of efficacy indicated in these papers.

DNAaway®

DNAaway® is a commercial DNA decontamination preparation kit. Champlot et al. (2010) mentioned that efficacy of DNAaway® has not yet been evaluated systematically for short DNA fragments (200–100 bp). Their results indicate removal of only two thirds of surface-attached DNA.

It could be supposed that DNAaway® is not effective enough for complete removal of DNA as used here.

PAA and NaClO

PAA and NaClO at concentrations tested here affected the surface of tungsten carbide beads, possibly reacting with

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tungsten carbide, which is a transition metal: Compounds with carbon produce metal carbides in which atomic carbon is in a non-bound state which facilitates reaction with other elements. In tungsten carbide, non-metal and metal are stacked in an alternate lattice structure, forming new sublattices, which determine the hardness grade of tungsten carbide (Meyer 2007a, b).

Because of its functional group, PAA is a powerful oxidant (Falbe and Regnitz 1995) and carbon atoms of tungsten carbide might have been oxidised.

Our observations on tungsten carbide beads (blackening, porous surface) after decontamination with NaClO might be attributable to the oxidation of carbon. In addition, the oxidation of carbon and dissolution out of the crystalline lattice might trigger the porous surface structure of tungsten carbide beads (Lekutat 2011, personal communication).

Conclusion

For PCR, disposable beads should be used, as has already been suggested for reagents and expandable items such as gloves and pipettes in PCR laboratories (Kwok and Higuchi 1989).

Concerning reusable substances, decontamination techniques used here on tungsten carbide beads affected their surfaces. This makes the reuse of these beads inappropriate.

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Korrespondenzadresse:
Dr. med. vet. Nina Langkabel
Panel VPH
Institute of Meat Hygiene and Technology
Veterinary Medicine, FU Berlin
Brümmerstraße 10
14195 Berlin
langkabel.nina@vetmed.fu-berlin.de