Original article

European Journal of Microbiology and Immunology 6 (2016) 3, pp. 244–252
DOI: 10.1556/1886.2016.00028

COMPARISON OF FIVE COMMERCIAL NUCLEIC ACID EXTRACTION KITS FOR THE PCR-BASED DETECTION OF *BURKHOLDERIA PSEUDOMALLEI* DNA IN FORMALIN-FIXED, PARAFFIN-EMBEDDED TISSUES

Sonja Obersteller[1], Heinrich Neubauer[2], Ralf Matthias Hagen[3,+], Hagen Frickmann[4,5,+,*]

[1]Chemical Faculty, University of Hamburg, Hamburg, Germany
[2]Friedrich Loeffler Institute, Federal Research Institute for Animal Health, Jena, Germany
[3]NATO Center of Excellence for Military Medicine (MilMedCOE), Deployment Health Surveillance Capability (DHSC), Munich, Germany
[4]Department of Tropical Medicine at the Bernhard Nocht Institute, German Armed Forces Hospital of Hamburg, Hamburg, Germany
[5]Institut for Medical Microbiology, Virology and Hygiene, University Medicine Rostock, Rostock, Germany

Received: August 1, 2016; Accepted: August 26, 2016

The extraction and further processing of nucleic acids (NA) from formalin-fixed paraffin-embedded (FFPE) tissues for microbiological diagnostic polymerase chain reaction (PCR) approaches is challenging. Here, we assessed the effects of five different commercially available nucleic acid extraction kits on the results of real-time PCR.

FFPE samples from organs of *Burkholderia pseudomallei*-infected Swiss mice were subjected to processing with five different extraction kits from QIAGEN (FFPE DNA Tissue Kit, EZ1 DNA Tissue Kit, DNA Mini Kit, DNA Blood Mini Kit, and FlexiGene DNA Kit) in combination with three different real-time PCRs targeting *B. pseudomallei*-specific sequences of varying length after 16 years of storage.

The EZ1 DNA Tissue Kit and the DNA Mini Kit scored best regarding the numbers of successful PCR reactions. In case of positive PCR, differences regarding the cycle-threshold (Ct) values were marginal.

The impact of the applied extraction kits on the reliability of PCR from FFPE material seems to be low. Interfering factors like the quality of the dewaxing procedure or the sample age appear more important than the selection of specialized FFPE kits.

Keywords: formalin-fixed, paraffin-embedded, tissue, PCR, nucleic acid extraction, *Burkholderia pseudomallei*

Introduction

Formalin-fixed paraffin-embedded (FFPE) tissues are appropriate sample materials for histopathological or forensic diagnostic approaches in the medical diagnostic laboratory [1, 2]. Such materials are basically inappropriate for cultural approaches and poorly suited for molecular diagnostics like polymerase chain reaction (PCR). The main difficulties concerning the extraction of DNA or RNA from FFPE tissues and the subsequent amplification using PCR are the deamination of cytosine to uracil caused by formalin fixation [2, 3] as well as single strand breaks [2, 3] that can result in failure of the PCR reaction [3]. The latter problem can in part be overcome by the use of PCRs targeting particularly short sequence fragments [3].

If the possibility of infectious etiology has not been considered initially, it is a frequent problem that only FFPE samples have been taken by the physician in charge. If additional acquisition of sample materials is technically demanding, painful or even risky for the patient, a reliable molecular diagnosis of the infectious agent from FFPE samples is desirable in spite of the abovementioned tech-

* Corresponding author: Hagen Frickmann; Department of Tropical Medicine at the Bernhard Nocht Institute, German Armed Forces Hospital of Hamburg, Bernhard Nocht street 74, 20359 Hamburg, Germany; Phone: 0049-40-6947-28700; Fax: 0049-40-6947-28709; E-mail: Frickmann@bni-hamburg.de

[+] These authors contributed equally to this work.

nical limitations. Further, the option of long-term storage of patient tissue samples in paraffin wax also provides an important source of material for forensic diagnostic analyses by polymerase chain reaction (PCR) months or even years after the sample has been taken [2, 3].

For the extraction of DNA or RNA from FFPE tissues and other demanding sample materials, numerous protocols have been developed and specialized PCR approaches have been designed to amplify the extracted nucleic acids [2].

This study assessed the effects of different modes of DNA extraction after dewaxing of bioptic FFPE tissue samples on the results of diagnostic real-time PCR. Five different extraction approaches were compared using tissue samples of mice, which had been experimentally infected with *Burkholderia pseudomallei* [4].

B. pseudomallei is a saprophytic, Gram-negative bacterium, and the causative agent of melioidosis (also referred to as Whitmore's disease) [4–6]. Its natural reservoir is soil or surface waters in the tropics and subtropics, and it can bear extreme pH values as well as lack of nutrition, making it very adaptable to a broad range of habitats [5, 6]. Melioidosis mainly occurs in Southeast Asia and northern Australia, but there were also a few cases in the United States, in which patients did not have any travel history to endemic areas [6]. If undetected and, therefore, not treated properly or soon enough, melioidosis reaches mortality rates up to 55% [7]. This is why fast and reliable diagnosis is needed to efficiently treat the patient. The use of FFPE tissue and real-time PCR for diagnostic purposes can allow the analysis from older patient samples as a safe alternative, if the diagnostic gold standard, i.e., cultivation of this biosafety level (BSL) 3 pathogen under suitable laboratory conditions, has been missed or is impossible due to infrastructural limitations like the unavailability of a BSL 3 laboratory.

Material and methods

Sample materials

Sixteen-year-old residual materials from a previously published study [4] were used. Shortly summarized, mice at 8-9 weeks of age were intraperitoneally infected with 200 colony-forming units (CFU) of a *B. pseudomallei* suspension. Starting 2 days after infection, the surviving mice 1, 2, 3, 5, 7, 10, and 15 were euthanized and their lungs, kidneys, livers, brains, and hearts were removed, fixed with 4% buffered formalin and paraffin-embedded. In parallel, bacterial load in the tissues had been culturally assessed. For all sample materials included in this study, the detected pathogen densities (in colony forming units (CFU) per gram of the respective organ) are depicted in *Table 1*.

Sample preparation

The paraffin-embedded organ samples were cut of the paraffin blocks with a scalpel and then transferred into 2.0 ml tubes (Eppendorf, Hamburg, Germany). To dewax the samples, 2.0 ml xylene was added to every sample and the tissues were then incubated for 45 min at 37 °C under constant shaking at 400 rounds per minute (rpm) in a ThermoMixer (Eppendorf, Hamburg, Germany). The samples were then centrifuged for 10 min at 13,200 rpm and the supernatant was discarded. This procedure was repeated until the paraffin was completely removed. Due to the varying paraffin content in the samples, the number of xylene steps required to remove the paraffin differed between organs and samples *(Table 2)*.

After treatment with xylene was completed, 1200 µl of ethanol (70%) was added to all of the samples. They

Table 1. Bacterial load in organs from Swiss mice intraperitoneally infected with 200 CFU of *B. pseudomallei* 6068 VIR [8]: CFU (colony forming units) counts were measured by colony counting on agar. Organs were sampled at days 2, 3, 4, 5, and 7 after infection

Organs	CFU	Mice No./day after Infection						
		No. 1/d + 2	No. 2/d + 2	No. 3/d + 3	No. 5/d + 4	No. 15/d + 4	No. 7/d + 5	No. 10/d + 7
Liver	CFU^B	$6.32 \cdot 10^3$	$4.13 \cdot 10^4$	$8.17 \cdot 10^3$	–	–	–	10
Spleen	CFU^B	$2.09 \cdot 10^6$	$2.21 \cdot 10^7$	69	$1.46 \cdot 10^8$	–	$1.57 \cdot 10^9$	$2.64 \cdot 10^8$
Lungs	CFU^B	$9.81 \cdot 10^2$	$4.03 \cdot 10^3$	0	$3.49 \cdot 10^3$	–	$3.03 \cdot 10^5$	0
Kidneys	CFU^B	0	$2.26 \cdot 10^2$	0	$1.88 \cdot 10^4$	–	$3.21 \cdot 10^5$	0
Brain	CFU^B	0	14	0	0	$5.04 \cdot 10^2$	$1.19 \cdot 10^2$	0
Heart	CFU^B	0	0	0	0	$7.47 \cdot 10^3$	$2.72 \cdot 10^5$	0
Bone marrow	CFU^B	0	$8.00 \cdot 10^2$	0	$1.37 \cdot 10^4$	$1.37 \cdot 10^4$	$1.41 \cdot 10^5$	$1.50 \cdot 10^6$
Peritoneal exudate	CFU^B	$3.60 \cdot 10^3$	$3.80 \cdot 10^3$	0	80	80	$3.43 \cdot 10^7$	$1.43 \cdot 10^7$

Numbers are individual data expressed in CFU. Values were obtained by colony counting on agar (CFU^B), of the respective organ samples from one mouse
No. = number, – = sample not available

Table 2. Varying xylene-treatment schemes as required for the dewaxing of the different samples

Samples	Treatment
He15, Br15, Li1, He1, Lu1, He5, Br5, Ki5, Lu5, He2, Lu2	xylene-treatment 2× for 45 min, 1× overnight
Ki1, Br1, Br2, Ki2	xylene-treatment 1× for 45 min, 1× overnight
He7, Ki7, Lu7, Br7, Li3, Ki3, Lu3, He3, Br3, Ki10, He10, Li2	xylene-treatment 1× for 45 min, 1× overnight, 1× for 60 min
Br10, Li10, Lu10, Li5	xylene-treatment 1× overnight, 1× for 60 min
Sp samples complete	xylene-treatment 2× for 45 min each

He = heart, Br = brain, Li = liver, Lu = lung, Ki = kidney, Sp = spleen

were vortexed for 5 min and then centrifuged for 10 min at 13,200 rpm at room temperature. This step was repeated twice. Subsequently, the samples were stored in ethanol (70%) at 4 °C overnight.

For the next step, ethanol was removed by centrifugation of the samples for 10 min at 13,200 rpm. Afterwards, the supernatant was discarded. To remove the remaining ethanol residuals, the samples were air-dried in a heat block for 15 min at 37 °C.

After dewaxing, the bioptic samples from the organs were frozen using liquid nitrogen and then grinded using a TissueLyser LT device (QIAGEN, Venlo, Netherlands). Each sample was treated for 5 to 10 min depending on its tissue properties. The liver and kidney samples were treated for 10 min, and the brain, heart, lung, and spleen for 5 min.

After this treatment, volumes of 600 µl bi-distilled water were added to each dry sample for resuspension. Each sample was then split in six aliquots of 100 µl and transferred into new 1.5 ml cups (Eppendorf, Hamburg, Germany). The samples were then stored in the freezer at −20 °C until further processing.

Further sample preparation was performed using five different kits from QIAGEN: FFPE DNA Tissue Kit (QIAGEN), EZ1 DNA Tissue Kit (QIAGEN), DNA Mini Kit (QIAGEN), DNA Blood Mini Kit (QIAGEN), and FlexiGene DNA Kit (QIAGEN). With the exception of the FlexiGene DNA Kit, a spin column had to be used for nucleic acid purification and proteinase K (QIAGEN) for the lysis of the tissue with all other kits. For the FlexiGene Kit, no columns were needed because the purification is solely done by centrifugation and the "QIAGEN protease" was used for the lysis of the tissue. The DNA extraction was performed according to the manufacturers' instructions for all kits with only minor modifications.

These minor modifications comprised:

FFPE DNA Tissue Kit:

- Starting the protocol with step 10 due to the already performed dewaxing of the samples

EZ1 DNA Tissue Kit:

- Elution of the DNA in 50 µl instead of 200 µl to ensure identical initial DNA concentrations in all compared protocols
- Incubation of the samples of the mice M3, M10, and M15, and the Sp-samples of all mice with proteinase K for 1 h 30 min because tissue lysis was not complete after one hour

DNA Mini Kit**:**

- Elution of the DNA in 50 µl instead of 200 µl to ensure identical initial DNA concentrations in all compared protocols
- Incubation of the samples of the mice M3, M10, and M15, and the Sp-samples of all mice with proteinase K for 1 h 15 min to ensure complete tissue lysis

FlexiGene DNA Kit:

- One-hour incubation of samples of the mice M3, M10, M15, and Sp-samples of all mice with FG2/Protease instead of 10 min to ensure complete tissue lysis

The extracted DNA was diluted with a final volume of 50 µl bi-distilled water and stored in the freezer at −20 °C for further experiments.

Nucleic acid amplification tests

PhHV Control

To verify that the extraction kits did not cause PCR inhibition or degradation of the DNA, 10 µl phocid Herpes virus (PhHV)-1-DNA had been added to each sample as an extraction and internal control.

The samples were screened for the PhHV-1 DNA using a previously described real-time PCR protocol [9] with minor modifications. The PCR mixture contained 10 µl HotStarTaq-Mix 2× (Qiagen, Hilden, Germany), 30 pmol of the primers, 5 pmol of the Cy5-labeled probe *(Table 3)*, 3 mM $MgCl_2$, and 2 µl of each sample. The final reaction volume was 20 µl. The PCR was performed using the following temperature profile: an initial denaturation step for 15 min at 95 °C was followed by 40 cycles of denaturation for 15 s at 94 °C, annealing for 20 s at 56 °C, and elongation for 30 s at 72 °C.

B. pseudomallei-*specific 16S rRNA gene PCR*

The PCR targeting a 566-base pair fragment of the 16S rRNA gene of *B. pseudomallei* was performed as described [10] with minor modifications. The PCR mixture contained 12.5 µl HotStarTaq Mastermix 2× (Qiagen), 3.0 mM $MgCl_2$, 0.4 pmol of each primer, and 0.4 pmol of the FAM-labeled probe *(Table 3)* as well as 2.5 µl of the extracted sample DNA. The final reaction volume was 25 µl. The PCR was performed using the following temperature profile: an initial denaturation step for 2 min at

Table 3. Polymerase chain reaction primers and probes used for the amplification of *Burkholderia*-specific DNA in the samples. Lyophilized primers and probes were diluted to a concentration of 10 pmol prior for use

Primers	Sequence
PhHV-267s	5′ GGG CGA ATC ACA GAT TGA ATC 3′
PhHV-337as	5′ GCG GTT CCA AAC GTA CCA A 3′
PhHv-305tq	5′ Cy5.5 TTT TTA TGT GTC CGC CAC CAT CTG GAT C 3′ BBQ650
Burk 16S	5′ TTC TGG CTA ATA CCC GGA GT 3′
Burk 16R	5′ GCC CAA CTC TCA TCG GGC 3′
Burk 16TM	5′ FAM TAA CTA CGT GCC AGC AGC CGC GGT 3′ BHQ1
fup-1	5′ GTG GAG CTT CTT CGG CAG CAT 3′
fup-2	5′ ATG ACG ACG ATT CTT TTG AA 3′
rpsU-L2 T	5′-FAM-AGG-CGC-TTG-TGC-AGG-CGC-BHQ1-3′
BpTTS1 fw	5′ CGA ATT GTC GTT GGA CTT TCT TC 3′
BpTTS1 rev	5′ GCG AGC GTA CTA ACG GGA ATC 3′
BpTTS1 T	5′ FAM CAT CCA GCG ACG CAT CGG GC BHQ1 3′

95 °C was followed by 45 cycles of denaturation for 15 s at 94 °C and by annealing and amplification for 30 s at 58 °C.

rpsU *PCR*

The *rpsU* PCR targeting a 179-base pair fragment of the ribosomal protein subunit 21 gene *(rpsU)* of *Burkholderia* spp. and phylogenetically closely related genera [5, 11–13] was converted into a real-time PCR as follows. The PCR mixture was composed of 12.5 µl of HotStarTaq Mastermix 2× (Qiagen), 3.0 mM $MgCl_2$, 0.4 pmol of each primer, and 0.4 pmol of the FAM-labeled probe *(Table 3)* as well as 2.5 µl of the extracted sample DNA. The inner rpsU-L2-primer was used as the probe. The final reaction volume was 25 µl. The following temperature profile was used: an initial denaturation step for 10 min at 95 °C was followed by 35 cycles of denaturation for 60 s at 94 °C, annealing for 60 s at 59 °C, and elongation for 60 s at 72 °C.

BpTTS1 *PCR*

The *BpTTS1* PCR targeting a 65-base pair fragment of the gene of the type three secretion system (TTS) of *B. pseudomallei* was performed as described [14] with minor modifications. The PCR mixture contained 12.5 µl of HotStarTaq Mastermix 2×, 3.0 mM $MgCl_2$, 0.4 pmol of each primer, and 0.4 pmol of the FAM-labeled probe *(Table 3)* as well as 2.5 µl of the extracted sample DNA. The final reaction volume was 25 µl. The following temperature profile was used: an initial denaturation step for 10 min at 95 °C was followed by 40 cycles of denaturation for 20 s at 94 °C, annealing for 20 s at 59 °C, and elongation for 20 s at 72 °C.

Statistics

Descriptive statistical approaches were applied. First of all, the used PCR schemes were compared to identify the PCR approach leading to the most reliable results by means of the number of failed reactions. Only PCR approaches leading to positive results were afterwards used for the comparison of the nucleic acid extraction schemes.

The assessment of the nucleic acid extraction schemes was based on a qualitative comparison of the numbers of positive results per scheme. Afterwards, a quantitative assessment based on the measured Ct values of real-time PCR was performed using Wilcoxon-matched pairs testing with the help of the Software GraphPad Instat (GraphPad Software Inc., La Jolla, CA, USA), version 3.10.

Ethics statement

The experiments with the mice had been approved by the C.R.S.S.A. Emile Pardë Animal Care Committee (No. 3/99) [4].

Results

Comparison of applied PCR schemes

The samples were screened for the DNA of *B. pseudomallei* using three different real-time PCR approaches. Hereby, the most suitable real-time PCR approach for the testing of the efficiency of the different extraction kits for FFPE tissues was identified.

PhHV real-time PCR

None of the extraction kits relevantly inhibited or degraded the extracted PhHV-DNA. The averaged Ct value of the PhHV-PCR about all samples and extraction schemes was 20.37 (±3.14). In detail, the Ct values of the PhHV-PCR were 20.86 (±1.60) from the samples treated with the DNA

Mini Kit, 22.16 (±4.88) from the samples treated with the DNA Blood Mini Kit, 18.82 (±3.63) from the samples treated with the FFPE DNA Tissue Kit, 20.71 (±1.95) from the samples treated with the FlexiGene DNA Kit, and 18.90 (±0.97) from the EZ1-DNA Tissue Kit. Accordingly, the mean values after all extraction schemes were within the standard deviation of the averaged Ct values about all samples and extraction schemes.

16S rRNA gene real-time PCR

No sample tested positive for *B. pseudomallei*-specific DNA.

rpsU *gene real-time PCR*

With few exceptions *(Table 4),* the most of the samples were tested negative. *Burkholderia*-specific DNA was only found in the spleen samples of the mice M2, M5, M7, and M10, in one kidney sample of mouse M2 and in one brain sample of mouse M10.

BpTTS1 *real-time PCR*

The results of the PCR revealed a higher number of samples positive for *B. pseudomallei* DNA than in the abovementioned approaches *(Table 4)*. The majority of the positive samples were spleen samples; except for mouse M3, every mouse spleen was detected positive for *B. pseudomallei*. The organ samples of mouse M7, the DNA of which had been extracted with the QIAamp DNA Mini Kit and the QIAamp EZ1 DNA Kit, were all positive for *B. pseudomallei*. Also, the mice M2, M10, and M5 showed positive results in liver (M2), kidney (M2, M5), brain (M5, M15), and lung (M10). Positive PCRs were further observed in brain and heart samples of mouse M15.

Comparison of applied nucleic extraction schemes

To identify the best method for the purification of nucleic acids from FFPE tissue, the FFPE DNA Tissue Kit, the EZ1 DNA Tissue Kit, the DNA Mini Kit, the DNA Blood Mini Kit, and the FlexiGene DNA Kit were compared.

Qualitative assessment

In *Table 4*, the results of the three PCR approaches in combination with the five extraction kits are shown.

While the 16S PCR did not show positive results for any of the used kits and for any of the mouse tissues, the *rpsU* PCR allowed for the detection of *B. pseudomallei* DNA in the spleen samples of the mice M5 and M10 after nucleic acid extraction with all kits with the exception of the FlexiGene DNA Kit, which did not allow for the detection of positive *rpsU* results in any samples. Using the FFPE Kit, the fully automated EZ1 Kit, and the Mini Kit DNA, *Burkholderia* DNA was isolated from the spleen of mouse M2 as well. The highest number of positive samples in combination with the *rpsU* PCR approach, i.e., four out of seven spleen samples, was found in the lot where the DNA had been extracted using the QIAamp DNA Mini Kit, followed by the FFPE DNA Tissue Kit, the EZ1 DNA Tissue Kit, and the DNA Blood Mini Kit, with three out of seven samples each.

Best results for all analyzed nucleic acid extraction kits

Table 4. Results of the five different nucleic acid purification kits in combination with the three subsequent real-time PCR approaches

		16S PCR						*rpsU* PCR						*BpTTS1* PCR					
		Ki	Li	Lu	Br	He	Sp	Ki	Li	Lu	Br	He	Sp	Ki	Li	Lu	Br	He	Sp
QIAamp DNA FFPE Tissue Kit	M1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	M2	–	–	–	–	–	–	+	–	–	–	–	+	–	+	–	–	–	+
	M3	–	–	–	–	–	–	–	–	–	–	–	–	/	/	/	/	/	–
	M5	–	/	–	–	–	–	–	/	–	–	–	+	–	/	–	–	–	+
	M7	–	/	–	–	–	–	–	/	–	–	–	–	–	/	+	–	+	+
	M10	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–	–	+
	M15	/	/	/	–	–	/	/	/	/	–	–	/	/	/	/	+	+	/
QIAamp EZ1 DNA Tissue Kit	M1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
	M2	–	–	–	–	–	–	–	–	–	–	–	+	+	+	–	–	–	+
	M3	–	–	–	–	–	–	–	–	–	–	–	–	/	/	/	/	/	–
	M5	–	/	–	–	–	–	–	/	–	–	–	+	–	/	–	+	–	+
	M7	–	/	–	–	–	–	–	/	–	–	–	–	+	/	+	+	+	+
	M10	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	+
	M15	/	/	/	–	–	/	/	/	/	–	–	/	/	/	/	–	–	/

Table 4. *(cont'd)*

		16S PCR						*rpsU* PCR						*BpTTS1* PCR					
		Ki	Li	Lu	Br	He	Sp	Ki	Li	Lu	Br	He	Sp	Ki	Li	Lu	Br	He	Sp
QIAamp DNA Mini Kit	M1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
	M2	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–	–	+
	M3	–	–	–	–	–	–	–	–	–	–	–	–	/	/	/	/	/	–
	M5	–	/	–	–	–	–	–	/	–	–	–	+	–	/	–	+	–	+
	M7	–	/	–	–	–	–	–	/	–	–	–	+	+	/	+	+	+	+
	M10	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–	–	+
	M15	/	/	/	–	–	/	/	/	/	–	–	/	/	/	/	–	+	/
QIAamp DNA Blood Mini Kit	M1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
	M2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
	M3	–	–	–	–	–	–	–	–	–	–	–	–	/	/	/	/	/	–
	M5	–	/	–	–	–	–	–	/	–	–	–	+	–	/	–	–	–	+
	M7	–	/	–	–	–	–	–	/	–	–	–	+	+	/	+	–	–	+
	M10	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	+
	M15	/	/	/	–	–	/	/	/	/	–	–	/	/	/	/	–	–	/
QIAamp FlexiGene DNA Kit	M1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
	M2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
	M3	–	–	–	–	–	–	–	–	–	–	–	–	/	/	/	/	/	–
	M5	–	/	–	–	–	–	–	/	–	–	–	–	+	/	–	+	–	+
	M7	–	/	–	–	–	–	–	/	–	–	–	–	+	/	+	–	–	+
	M10	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
	M15	/	/	/	–	–	/	/	/	/	–	–	/	/	/	/	–	–	/

+ = positive results, – = negative results, / = no samples

Table 5. Ct values of the samples depending on the different extraction methods and the different real-time PCR approaches

		QIAamp DNA FFPE Tissue Kit	QIAamp EZ1 DNA Tissue Kit	QIAamp DNA Mini Kit	QIAamp DNA Blood Mini Kit	QIAamp Flexi-Gene DNA Kit
rpsU PCR	SP 2	35.43	35.56	36.39	–	–
	SP 5	35.94	35.25	35.72	36.89	–
	SP 7	–	–	37.11	35.82	–
	SP 10	34.87	32.77	33.76	33.48	–
BpTTS1 PCR	SP1	–	33.99	34.79	36.7	35.78
	SP 2	28.41	28.84	30.43	32.66	31.44
	SP 5	33.78	30.07	28.92	29.94	27.72
	SP 7	27.34	26.67	27.22	28.9	26.92
	SP 10	34.61	28.68	29.02	28.98	26.31
	Ki2	–	37.83		–	36.39
	KI7	–	30.88	31.47	33.34	33.27
	Li2	37.08	34.7	–	–	–
	Lu7	32.92	33.61	32.81	36.75	35.53
	Br5	–	35.79	36.65	–	36.17
	Br7	–	36.06	36.39	–	–
	He7	32.57	32.35	35.16	–	–
	Hel5	36.43	–	34.85	–	–

were observed with the *BpTTS1* PCR *(Table 4)*. For the samples extracted with the EZ1 DNA Tissue Kit and the DNA Mini Kit, 13 positive results were detected each. The remaining three kits led to the detection of *B. pseudomallei*-specific DNA in ten (FlexiGene Kit), nine (DNA FFPE Tissue Kit), and eight (DNA Blood Mini Kit) samples. The most positive PCR results were obtained from spleen samples (four to five out of seven samples, respectively).

Quantitative assessment

For tissues with positive PCR results, quantitative comparison of the achieved Ct values was performed. All measured Ct values for the *rpsU* PCR and the *BpTTS1* PCR are depicted in *Table 5*.

Wilcoxon-matched pairs testing for the comparison of the extraction schemes led to the results as depicted in *Table 6* for the *rpsU* PCR and in *Table 7* for the *BpTTS1* PCR.

As shown in *Tables 6* and *7*, PCR results after the five extraction methods did not show relevant differences between most of the kits. In combination with the *rpsU* real-time PCR, there were no significant differences at all, the *P* values ranged from 0.25 to 0.99. For the *BpTTS1* real-time PCR, there was a significant difference between the EZ1 DNA Tissue Kit and the DNA Blood Mini Kit, the DNA Mini Kit and the DNA Blood Mini Kit, and the FlexiGene DNA Kit and the DNA Blood Mini Kit (*P* value = 0.0313 each), but for none of the other kits. When Bonferroni correction for multiple testing [15] was applied, significance got lost.

Discussion

As confirmed by this study, extraction of nucleic acids from FFPE tissue and subsequent downstream detection and identification procedures like PCR are still troublesome, since the fixation with formalin causes cross-linking and single-strand breaks in DNA and RNA [2, 3]. The degradation of the nucleic acids increases over the period of time during which the tissues are embedded in paraffin [16]. Further, the way how the samples were treated before and during the embedding procedure also affects the results of molecular diagnostic approaches [17]. Of note, the assessed samples used for this study were formalin-fixed and paraffin-embedded in 1999 under biosafety level 3 (BSL-3) laboratory conditions which made the laboratory work more challenging and processing errors, therefore, more likely. However, there are up-to-date reports about successful nucleic acid purification from FFPE material even from the 1970s [1].

Table 6. Wilcoxon-matched pairs testing for the comparison of the extraction schemes based on the Ct values achieved in the *rpsU* PCR

Extraction method 1	Average Ct value ± standard deviation	Extraction method 2	Average Ct value ± standard deviation	Significance	Significance level (*P*)
FFPE DNA Tissue Kit	35.413 ± (0.535)	EZ1 DNA Tissue Kit	34.5271 ± (1.529)	Not significant	0.5000
FFPE DNA Tissue Kit	35.413 ± (0.535)	DNA Mini Kit	35.2901 ± (1.367)	Not significant	0.7500
FFPE DNA Tissue Kit	35.405 ± (0.757)	DNA Blood Mini Kit	35.1851 ± (2.411)	Not significant	>0.9999
EZ1 DNA Tissue Kit	34.527 ± (1.529)	DNA Mini Kit	35.2901 ± (1.367)	Not significant	0.2500
EZ1 DNA Tissue Kit	34.010 ± (1.754)	DNA Blood Mini Kit	35.1851 ± (2.411)	Not significant	0.5000
DNA Mini Kit	34.7401 ± (1.386)	DNA Blood Mini Kit	35.1851 ± (2.411)	Not significant	>0.9999

Table 7. Wilcoxon-matched pairs testing for the comparison of the extraction schemes based on the Ct values achieved in the *BpTTS1* PCR

Extraction method 1	Average Ct value ± standard deviation	Extraction method 2	Average Ct value ± standard deviation	Significance	Significance level (*P*)
FFPE DNA Tissue Kit	32.387 ± (3.428)	EZ1 DNA Tissue Kit	30.703 ± (2.926)	Not significant	0.2188
FFPE DNA Tissue Kit	32.294 ± (3.286)	DNA Mini Kit	31.201 ± (3.109)	Not significant	0.4688
FFPE DNA Tissue Kit	31.412 ± (3.305)	DNA Blood Mini Kit	31.446 ± (3.333)	Not significant	>0.9999
FFPE DNA Tissue Kit	31.412 ± (3.305)	FlexiGene DNA Kit	29.584 ± (3.876)	Not significant	0.6250
EZ1 DNA Tissue Kit	31.694 ± (3.179)	DNA Mini Kit	32.286 ± (3.374)	Not significant	0.1602
EZ1 DNA Tissue Kit	32.456 ± (3.447)	DNA Blood Mini Kit	32.467 ± (3.375)	Significant	0.0313
EZ1 DNA Tissue Kit	31.818 ± (3.691)	FlexiGene DNA Kit	32.178 ± (4.209)	Not significant	0.4961
DNA Mini Kit	30.666 ± (2.580)	DNA Blood Mini Kit	32.467 ± (3.375)	Significant	0.0313
DNA Mini Kit	31.414 ± (3.191)	FlexiGene DNA Kit	31.651 ± (4.171)	Not significant	0.7422
DNA Blood Mini Kit	32.467 ± (3.375)	FlexiGene DNA Kit	31.006 ± (4.050)	Significant	0.0313

Although high titers of *B. pseudomallei* had been detected in the assessed Swiss mice by culture before [4], only a minor proportion of samples was detected positive for *Burkholderia* DNA by three different PCR assays.

In total, *Burkholderia*-specific DNA was only detected in maximum eight and 13 out of 42 samples by the *rpsU* PCR and the *BpTTS1* PCR, respectively. The age of the samples was presumably an important factor. Formalin-associated effects may explain the big difference between the three PCR approaches. In contrast to the 16S PCR which amplifies a very long fragment of 566 bp (base pairs), the *rpsU* PCR and the *BpTTS1* PCR result in rather short amplicons with 179 bp and 65 bp, respectively. PCRs with shorter amplicons are known to be more suitable for the amplification of DNA from FFPE tissue [2, 16, 17]. Thus, the phenomenon of progressed DNA degradation is confirmed by this combined application of long-range and short-range PCR approaches, as the degenerated DNA is not only mutated but also fragmented by single-strand breaks [2, 3] as well, jointly resulting in the high number of observed failed PCR approaches. The 16S PCR was not even able to detect any positive sample, whereas the adapted *rpsU* PCR did at least show positive results for most of the spleen samples of the mice. The *BpTTS1* PCR detected *Burkholderia* DNA in eight to 13 samples, depending on the kit that was used to purify the DNA. Again, most positive samples were spleen tissues. This might be explained by the fact that *B. pseudomallei* is known to infect the spleen with particular high titers. A comparison with the cultural results supported this hypothesis. During the initial cultural analysis, the spleen appeared to be the organ being affected by the highest bacterial loads [4]. Besides the spleen, *B. pseudomallei* DNA was found in brain, lungs, and kidney samples as well. These organs are known as typical sites of infection as well in melioidosis patients [4]. Apart from the tissue-specific tropism of *B. pseudomallei*, tissue-specific PCR inhibitors might also explain the differentiated reaction pattern. This is, however, unlikely, because the inhibition control PCR did not indicate relevant differences between tissues from different organs as indicated by low standard deviations for the PhHV-PCR.

Addressing the primary focus of the study, the comparison of the nucleic acid extraction kits suggested best results of the fully automated EZ1 DNA Tissue Kit and the DNA Mini Kit in the qualitative assessment and poorer results of the DNA Blood Mini Kit. Regarding the quantitative assessments, Wilcoxon-matched pairs testing of the measured Ct values of the *BpTTS1* real-time PCR indicated significant differences between the DNA Blood Mini Kit and the other kits with the exception of the FFPE DNA Tissue Kit. In fact, the Ct values after the extraction with the DNA Blood Mini Kit were slightly higher than after extractions with the FlexiGene DNA Kit, the EZ1 DNA Tissue Kit, and the DNA Mini Kit. However, the effects get lost when Bonferroni adjustment for multiple testing is applied [15].

Considering the modes of DNA extraction by the different kits, this result might in part be explained by the fact that all of them except for the FlexiGene Kit use spin columns and the same buffers for nucleic acid purification. Only the FlexiGene Kit uses solely centrifugation for the purification. Nevertheless, the DNA blood Mini Kit scored worst in both the qualitative and the quantitative assessments.

In spite of all mentioned limiting factors of PCR from FFPE tissue, the low number of positive results remains surprising considering the high number of cultured bacteria from the samples [4]. One possible reason and an undeniable limitation of the study is the fact that only one mode of dewaxing and tissue disruption could be applied due to the low quantities of available sample material. Dewaxing included whole bioptic tissue samples instead of tissue slices from the microtome. Especially small organs like the brain and the spleen as well as segmented organs like the liver are difficult to remove from paraffin blocks with a scalpel. High quantities of remaining paraffin required the repetition of the xylene incubation steps for up to four times to sufficiently accomplish the dewaxing procedure. Remaining paraffin traces might, nevertheless, have partly inhibited the nucleic acid extraction process; however, the results of the extraction control PCR showed nonsuspicious results. A comparison with an alternative dewaxing method would have been desirable but could not be performed due to the limited amounts of sample material available.

Further, the success of tissue disruption after the dewaxing step depended on the kind of tissue. Although the liver and the kidney samples were treated with liquid nitrogen and later disrupted with beads in a cell disruptor for 10 min, the effect of the procedure was in part dissatisfying. A possible reason for the incomplete homogenization could be the fact that storage in ethanol overnight dehydrated the tissue samples and made them more solid. The sample-depending changes of the standard extraction protocols that became necessary during the sample preparation make the interpretation of the comparison difficult. This phenomenon also confirms the fact that standardization of molecular diagnostic approaches for FFPE tissues is challenging and that negative PCR results from FFPE samples have to be interpreted with care.

Conclusion

The extraction of nucleic acids from FFPE tissue and the processing of DNA with subsequent downstream analysis approaches like real-time PCR remain difficult due to formalin-associated degradation of DNA. If possible, approaches targeting short amplicons should be used since the DNA becomes fragmented when getting in contact with formalin during the fixation process [2, 3]. Analyzing the qualitative PCR outcomes, best extraction results were achieved using the EZ1 DNA Tissue Kit and the DNA Mini Kit together with the *BpTTS1* real-time PCR. Regarding

the quantitative assessment of the Ct values, best results were achieved with the EZ1 DNA Tissue Kit, the DNA Mini Kit, and the FlexiGene DNA Kit. The good results of the FlexiGene DNA Kit indicate that using spin columns to extract the nucleic acids from FFPE tissue is not indispensable. Interestingly, the FFPE DNA Tissue Kit did not show outstandingly good results, although this kit was specifically developed for the extraction of nucleic acids from FFPE tissue. The quantitative difference in comparison of Ct values with the other approaches was marginal. This finding challenges the concept that special kits for FFPE tissue are indispensable to extract DNA from such tissues. In contrast, different extraction methods enabled the purification of DNA from FFPE tissues in a similar way.

Acknowledgements

Simone Priesnitz is gratefully acknowledged for excellent technical assistance.

Declaration of interest

The authors declare that there are no conflicts of interest.

Funding sources

There has been no source of funding.

References

1. Ludyga N, Grünwald B, Azimzadeh O, Englert S, Höfler H, Tapio Soile, Aubele M: Nucleic acids from long-term preserved FFPE tissues are suitable for downstream analyses. Virchows Arch 460, 131–140 (2012)
2. Dietrich D, Uhl B, Sailer V, Holmes E, Jung M, Meller S, Kristiansen G: Improved PCR performance using template DNA from formalin-fixed and paraffin-embedded tissues by overcoming PCR inhibition. PLoS One 8, 1–10 (2013)
3. Huijsmans CJJ, Damen J, Linden JC, Van Der Savelkoul PHM, Hermans MHA: Comparative analysis of four methods to extract DNA from paraffin-embedded tissues: effect on downstream molecular applications. BMC Res Notes 3, 239 (2010)
4. Gauthier YP, Hagen RM, Brochier GS, Neubauer H, Splettstoesser WD, Finke E-J, Vidal DR: Study on the pathophysiology of experimental *Burkholderia pseudomallei* infection in mice. FEMS Immunol Med Microbiol 30, 53–63 (2001)
5. Hagen RM, Gauthier YP, Sprague LD, Vidal DR, Zysk G, Finke E-J, Neubauer H: Strategies for PCR based detection of *Burkholderia pseudomallei* DNA in paraffin wax embedded tissues. Mol Pathol 55, 398–400 (2002)
6. Benoit TJ, Blaney DD, Gee JE, Elrod MG, Hoffmaster AR, Doker TJ, Bower WA, Walke HT; Centers for Disease Control and Prevention (CDC): melioidosis cases and selected reports of occupational exposures to *Burkholderia pseudomallei* – United States, 2008–2013. MMWR Surveill Summ 64, 1–9 (2015)
7. Wuthiekanun V, Peacock SJ: Management of melioidosis. Expert Rev Anti Infect Ther 4, 445–455 (2006)
8. Chambon L: Analyse serologique de l'antigene somatique de *malleomyces pseudomallei*. Ann Inst Pasteur 98,405–415 (1960)
9. Niesters HGM: Quantitation of viral load using real-time amplification techniques. Methods 25, 419–429 (2001)
10. Tomaso H, Scholz HC, Al Dahouk S, Pitt TL, Treu TM, Neubauer H: Development of 5' nuclease real-time PCR assays for the rapid identification of the *Burkholderia mallei*/*Burkholderia pseudomallei* complex. Diagn Mol Pathol 13, 247–253 (2004)
11. Frickmann H, Chantratita N, Gauthier YP, Neubauer H, Hagen RM: Discrimination of *Burkholderia mallei*/*pseudomallei* from *Burkholderia thailandensis* by sequence comparison of a fragment of the ribosomal protein S21 (*rpsU*) gene. Eur J Microbiol Immunol (Bp) 2, 148–156 (2012)
12. Frickmann H, Neubauer H, Loderstaedt U, Derschum H, Hagen RM: *rpsU*-based discrimination within the genus *Burkholderia*. Eur J Microbiol Immunol (Bp) 4, 106–116 (2014)
13. Ostermann MF, Neubauer H, Frickmann H, Hagen RM: Correlation of *rpsU* gene sequence clusters and biochemical properties, GC–MS spectra and resistance profiles of clinical *Burkholderia* spp. isolates. Eur J Microbiol Immunol (Bp) 6, 25–39 (2016)
14. Zhang B, Wear D J, Kim H S, Weina P, Stojadinovic A, Izadjoo M: Development of hydrolysis probe-based real-time PCR for identification of virulent gene targets of *Burkholderia pseudomallei* and *B. mallei* – a retrospective study on archival cases of service members with melioidosis and glanders. Mil Med 177, 216–221 (2012)
15. Perneger TV: What's wrong with Bonferroni adjustments. BMJ 316, 1236–1238 (1998)
16. Frickmann H, Tenner-Racz K, Eggert P, Schwarz NG, Poppert S, Tannich E, Hagen RM: Influence of parasite density and sample storage time on the reliability of *Entamoeba histolytica*-specific PCR from formalin-fixed and paraffin-embedded tissues. Diagn Mol Pathol 22, 236–244 (2013)
17. Frickmann H, Loderstaedt U, Racz P, Tenner-Racz K, Eggert P, Haeupler A, Bialek R, Hagen RM: Detection of tropical fungi in formalin-fixed, paraffin-embedded tissue: still an indication for microscopy in times of sequence-based diagnosis? Biomed Res Int 2015, 93872 (2015)