Original article

European Journal of Microbiology and Immunology 5 (2015) 1
DOI: 10.1556/EuJMI-D-14-00040

COMPARISON OF AN AUTOMATED NUCLEIC ACID EXTRACTION SYSTEM WITH THE COLUMN-BASED PROCEDURE

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Received: December 19, 2014; Accepted: December 23, 2014

Here, we assessed the extraction efficiency of a deployable bench-top nucleic acid extractor EZ1 in comparison to the column-based approach with complex sample matrices.

A total of 48 EDTA blood samples and 81 stool samples were extracted by EZ1 automated extraction and the column-based QIAamp DNA Mini Kit. Blood sample extractions were assessed by two real-time malaria PCRs, while stool samples were analyzed by six multiplex real-time PCR assays targeting bacterial, viral, and parasitic stool pathogens. Inhibition control PCR testing was performed as well.

In total, 147 concordant and 13 discordant pathogen-specific PCR results were obtained. The latter comprised 11 positive results after column-based extraction only and two positive results after EZ1 extraction only. EZ1 extraction showed a higher frequency of inhibition. This phenomenon was, however, inconsistent for the different PCR schemes. In case of concordant PCR results, relevant differences of cycle threshold numbers for the compared extraction schemes were not observed.

Switches from well-established column-based extraction to extraction with the automated EZ1 system do not lead to a relevantly reduced yield of target DNA when complex sample matrices are used. If sample inhibition is observed, column-based extraction from another sample aliquot may be considered.

Keywords: nucleic acid extraction, DNA, RNA, column, EZ1, automate, inhibition

Introduction

Nucleic acid extraction from biological samples is a prerequisite for diagnostic PCR in the microbiological routine laboratory. Automated extraction systems increasingly replace the traditional approach of column-based nucleic acid extraction [1–26], because they are easy-to-use and less consuming regarding hands-on time of technical assistants.

During military missions, hands-on time of laboratory technical assistants is limited. This impedes the broad implementation of molecular tools on deployment. To close this gap, we evaluated the automated EZ1 nucleic acid extraction system (Qiagen, Hilden, Germany) for its use in a field laboratory as a standard tool. Data from this study are based on first experiences of the German Armed Forces during a military deployment in tropical Koulikoro / Mali *(Fig. 1)*. Here, we assessed the performance of the EZ1 automate in comparison to well-established column-based extraction protocols from complex materials like stool and stored EDTA blood.

Materials and Methods

Samples

The here described harmonization analysis comprised a total of 129 complex sample materials from our diagnostic routine. Altogether, 48 EDTA blood samples from suspected malaria patients which had been frozen at −20 °C were analyzed. Repeated freeze–thawing of such samples can be applied to release the "malaria pigment" hemozoin for matrix-assisted laser-desorption–ionization time-of-flight mass spectrometry (MALDI–TOF–MS) analysis [27, 28] in the course of malaria diagnosis. Further, 43 stool samples from deployed soldiers with diarrhea which had been frozen at −20 °C to ensure storage and transport stability and 38 fresh stool samples from returnees from the tropics with history of diarrhea who presented at our out-patient department were assessed. All analyses were part of harmonization testing in the course of the implementation of automated EZ1-based nucleic acid extraction in our routine diagnostics.

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ISSN 2062-509X / $ 20.00 *© 2015 Akadémiai Kiadó, Budapest*

Fig. 1. Use of the deployable nucleic acid extraction system EZ-1 in a military field camp in Koulikoro/Mali

Diagnostic standard procedure for nucleic acid enrichment

Nucleic acid enrichment in our diagnostic department has been routinely performed by column-based enrichment kits (Qiagen, Hilden, Germany), i.e., the QIAamp DNA Blood Mini Kit (Qiagen) for molecular diagnosis of malaria from EDTA blood and the QIAamp DNA Stool Mini Kit (Qiagen) for nucleic acid enrichment from stool. These column-based kits were applied according to the manufacturer's instructions.

Parallel harmonization testing of the newly implemented automated EZ1 nucleic acid extraction system

From the same samples, nucleic acids were extracted and enriched using the automated EZ1 system (Qiagen). EZ1-based extraction from the frozen EDTA-blood samples with the EZ1 DNA Blood 200 µl Kit (Qiagen) was performed exactly as described by the manufacturer.

For stool samples, pretreatment with ASL-buffer (Qiagen) and InhibitEx tablets (Qiagen) to reduce inhibiting effects of the matrix was performed as described by the manufacturer for the QIAamp DNA Stool Mini Kit (Qiagen). In detail, ASL-buffer was preheated at 70 °C to dissolve precipitates. Afterwards, either 200–300 mg formed stool or 200 µl of unformed stool were vortex-mixed with 1.4 ml ASL-buffer for 1 min. Then, the samples were again heated at 70 °C for 5 min, followed by vortex-mixing for 15 s and centrifugation at 20.000*g* for 1 min. A total of 1.2 ml supernatant was transferred to another laboratory cup, and the pellet was discarded. The InhibitEx tablet was added and vortex-mixing was performed for 1 min until it was completely dissolved. Afterwards, the samples were incubated at room temperature for 1 additional minute before they were centrifuged at 20.000*g* for 6 min. The supernatant was transferred into another laboratory cup, and the pellet was discarded. Again, the samples were centrifuged at 20.000*g* for 3 min, before 200 µl of the supernatants was used for nucleic acid extraction with the EZ1 Virus Mini Kit v2.0 (Qiagen) as described by the manufacturer.

Applied PCR systems

For the analysis of DNA extraction quality, only well-characterized and evaluated real-time PCR systems were used.

From the nucleic acid extractions from the blood samples, two real-time PCRs for the detection of plasmodia were applied. A real-time PCR targeting *Plasmodium* (*P.*) *falciparum*, *P. malariae*, *P. ovale*, and *P. vivax* in a SybrGreen-melting curve analysis-based approach was performed as described [29]. Further, the *Plasmodium* spp.-specific real-time PCR RealStar Malaria PCR Kit 1.0 (altona DIAGNOSTICS, Hamburg, Germany) was applied according to the manufacturer's instructions.

From the nucleic acid extractions of the assessed stool samples, PCRs for bacterial, protozoan and viral enteric pathogens were performed. The PCRs for bacterial pathogens comprised an in-house real-time multiplex PCR targeting the invasive enteropathogenic bacteria *Salmonella* spp., *Shigella* spp./enteroinvasive *Escherichia coli* (EIEC), *Campylobacter jejuni*, and *Yersinia* spp. as previously detailed [30, 31]. Diarrhea-associated *Escherichia coli* were assessed using the commercial RidaGene (R-Biopharm, Darmstadt, Germany) real-time PCR kits "EAEC," "EHEC-EPEC," and "ETEC-EIEC" to identify marker genes of enteroaggregative *E. coli* (EAEC), enterohaemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enterotoxic *E. coli* (ETEC), and *Shigella* spp./EIEC.

DNA of the enteropathogenic protozoa *Entamoeba histolytica*, *Giardia duodenalis*, *Cyclospora cayetanensis,* and *Cryptosporidium* spp. was amplified by a recently described [32–34] in-house real-time multiplex PCR from the German National Reference Centre for Tropical Diseases Bernhard Nocht Institute Hamburg with a minor modification. In detail, the described primer–probe sets for *Entamoeba histolytica*, *Giardia duodenalis*, and *Cryptosporidium* spp. were complemented by a primer-probe-set for *Cyclospora cayetanensis* from another publication [33] as the only change of the described protocol [32, 34].

In addition, viral enteric pathogens norovirus genogroups I (G1) and II (G2), astrovirus, rotavirus, adenovirus, and sapovirus were detected using the commercial Fast-track Diagnostics (Sliema, Malta) PCR kit "Viral gastroenteritis."

A previously detailed phocid herpesvirus PCR [34–36] was applied for inhibition control purposes. The respective primer–probe composition was implemented in both applied in-house multiplex PCRs for enteric pathogens. The commercially available PCR kits had own inhibition control protocols. The in-house malaria real-time multiplex protocol was run without additional inhibition control.

Analysis

In case of concordant positive PCR results after column-based and EZ1 extraction, achieved cycle threshold- (Ct-) values were assessed. Median and mean values as well as standard deviation (SD) were calculated. In addition, cases of identified PCR inhibition were assessed.

Matched pairs of recorded Ct values were compared using Wilcoxon matched-pairs signed ranks testing with the software GraphPad InStat, version 3.06 (GraphPad Software Inc., La Jolla, CA, USA). Significance was accepted in case of a two-tailed P-value ≤ 0.05. The nonparametric Spearman correlation coefficient was calculated (GraphPad Software Inc., La Jolla, CA, USA) to confirm effective pairing.

Nonparametric testing was chosen because the test values did not pass normality testing (KS-test, GraphPad InStat) in all instances.

Results

Concordant and discordant PCR results after EZ1-based and column-based nucleic acid extraction

From the 48 assessed frozen EDTA plasma samples, in-house malaria PCR after EZ1-extraction and after column based extraction led to the identification of 37 malaria cases in perfect concordance. In detail, melting curve analysis allowed for the identification of *Plasmodium falciparum* in 32 instances, of *P. vivax* in 4 instances, and of *P. ovale* in 1 instance. The RealStar Malaria PCR Kit 1.0 (altona DIAGNOSTICS) identified even 38 cases of *Plasmodium* spp. but did not allow for any discrimination on species level. Again, there were concordant results after EZ1-extraction and column-based extraction.

In one out of the 38 assessed fresh stool samples from returnees from the tropics, *Cryptosporidium* spp. DNA was identified. Again, the results after EZ1 extraction and column-based extraction were concordant. No other pathogen was detected.

PCR from 43 frozen stool samples from patients with diarrhea in the tropics led to a more differentiated results pattern. In total, DNA of *Cryptosporidium* spp. was identified in three instances, of *Giardia duodenalis* in one instance, of *Salmonella* spp. in one instance, of *Shigella* spp./EIEC in three instances for all three applied PCR protocols, of labile toxin producing ETEC in 15 instances, of stabile toxin producing ETEC in 11 instances, of EPEC in 16 instances, of EAEC in 13 instances, of norovirus G1 in two instances, of norovirus G2 in five instances, of astrovirus in one instance, and of sapovirus in one instance, respectively. From those positive results, one labile toxin producing ETEC (Ct 29 after EZ1 extraction) and one stabile toxin producing ETEC (Ct 24 after EZ1 extraction) were missed after column-based extraction. In contrast, the following pathogens were missed after EZ1-extraction although they were detected after column-based extraction: one *Cryptosporidium* spp. (Ct 27 after column-based extraction), one *Salmonella* spp. (Ct 27 after column-based extraction), one labile toxin producing ETEC (Ct 20 after column-based extraction), three stabile toxin producing ETEC strains (Ct 14, 18, 20 after column-based extraction), three EPEC strains (Ct 21, 22, 25 after column-based extraction), one norovirus G1 (Ct 34 after column-based extraction), and one sapovirus (Ct 32 after column-based extraction). All other PCR results showed concordance after both kinds of nucleic acid enrichment, resulting in 147 concordant results and 13 discordant results.

Sample inhibition after EZ1-based and column-based nucleic acid extraction

The internal inhibition control PCRs within the applied PCR assays showed a differentiated reaction pattern *(Table 1)*. Relevantly increased inhibition after EZ1 extrac-

Table 1. Inhibition as observed after EZ1-based nucleic acid extraction and after column-based nucleic acid extraction for various applied PCR test kits. The in-house malaria PCR did not include an internal inhibition-control approach

Applied PCR kit	Number of analyzed samples (*n*)	Inhibition after EZ1 extraction only (*n*)	Inhibition after column-based extraction only (*n*)	Inhibition after both extraction schemes (*n*)
Malaria PCR Kit 1.0 (altona DIAGNOSTICS)	48	4	0	0
In-house real-time multiplex PCR for enteroinvasive bacteria according to [30, 31]	81	7	0	1
ETEC-EIEC kit (RidaGene)	81	0	0	1
EHEC-EPEC kit (RidaGene)	81	0	0	1
EAEC kit (RidaGene)	81	1	0	1
In-house real-time multiplex PCR for enteropathogenic protozoa according to [32–34]	81	10	0	2
Viral gastroenteritis kit (Fast-track Diagnostics)	81	0	0	0

tion in comparison to column-based extraction was identified for the RealStar Malaria PCR Kit 1.0 and the two applied in-house real-time PCR approaches. The latter used the same inhibition control PCR primers in conjunction with the target-specific oligonucleotides. The commercial *E. coli* and virus multiplex PCRs did not show relevantly increased inhibition after EZ1 extraction in comparison to column-based extraction. Furthermore, those PCR reactions were not strongly affected by inhibition at all. Of note, there is a marked discrepancy between the low rate of inhibition of the commercial *E. coli* PCRs and the high number of failed *E. coli* PCR reactions as detailed above.

Ct value comparison of matched pairs of positive PCR reactions after EZ1-based and column-based nucleic acid extraction

Matched Ct values after EZ1-based and column-based nucleic acid extraction were compared *(Table 2)*. Wilcoxon matched-pairs testing suggested significant ($P < 0.0001$) lower Ct values after column-based DNA preparation for the in-house malaria multiplex PCR only. The quantitative effect was negligible with a mean Ct value of 17 after column-based extraction and of 18 after the EZ1-based procedure, accounting for less than one decadic logarithmic step regarding malaria DNA copy numbers. Of note, the effect could not be demonstrated for the RealStar Malaria PCR Kit 1.0 (altona DIAGNOSTICS). Further, no significant Ct value differences between both extraction methods were detectable for matched pairs of any positive PCR results from stool samples. Tendencies were observed in both directions, suggesting equal baseline DNA amounts in case of positive PCR reactions. Effective pairing was confirmed for all instances.

Discussion

This work describes a comparison of nucleic acid extraction and enrichment from native stool samples, frozen stool samples, and frozen EDTA blood samples as examples for complex sample matrices by well-established column-based nucleic acid extraction and automated extraction using a bench-top EZ1 system. The latter uses magnetic particles [2]. First successful applications of the EZ1 system, which requires considerable less hands-on time of technical laboratory assistants than tradition column-based extraction systems [23], were published in the fields of microbiology [24] and forensic medicine [25, 26] as early as in 2005. The EZ1 system was shown to be useful for the isolation of target DNA in low quantities, e.g., of fetal DNA in maternal plasma [21], or of DNA in partially degraded biological specimens [22]. Successful EZ1-based DNA extraction from formalin-fixed, paraffin-embedded tissue samples [9], or otherwise long-term preserved tissue specimens [8] has been demonstrated. Even DNA extraction from previously stained smears with the EZ1 system works reliably [6]. The EZ1 system extracts not only DNA but also RNA depending on the chosen program [1].

EZ1-based nucleic acid extraction is in use and has been validated for various PCR approaches in the microbiological laboratory. Those approaches comprise virus diagnostics for cytomegalovirus [13, 16, 20], hepatitis virus B and C [18], human immunodeficiency virus [12, 18], PCRs for bacterial [15] and viral [4, 15] respiratory pathogens, stool pathogens [14] or commensalic bacteria from stool [7], and biothreat agents [5], as well as PCR diagnostics for parasitic diseases like toxoplasmosis [17] and for fungal pathogens like *Aspergillus fumigatus* [3].

EZ1 extraction is affected by various preanalytic factors like sample type and chosen preprocessing protocol

Table 2. Comparison of Ct values of samples which were associated with positive PCR reactions after EZ1-based nucleic acid extraction and after column-based nucleic acid extraction.

PCR-target	Applied kit	Number of positive reactions after both extraction schemes (*n*)	EZ1 nucleic acid extraction			Column-based nucleic acid extraction			Paired Ct value differences of EZ-1 extraction and column-based nucleic acid extraction			Significance level by Wilcoxon matched-pairs testing (*P*)
			Median Ct value	Mean Ct value	Standard deviation (SD)	Median Ct value	Mean Ct value	Standard deviation (SD)	Median difference	Mean difference	Standard deviation (SD)	
PCRs from frozen EDTA blood												
Plasmodium falciparum, *P. vivax*, *P. malariae*, *P. ovale*	In-house according to [29]	37	18	18.4	3.5	17	17.0	3.6	1	1.4	1.0	<0.0001 (Significant)
Plasmodium spp. (*P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, *P. knowlesi*)	Malaria PCR Kit 1.0 (altona DIAGNOSTICS)	38	17	16.7	4.4	16	16.6	4.1	0	0.1	1.1	0.42 (Not significant)
PCRs from frozen and fresh stool samples												
Shigella spp. / EIEC	In-house according to [30, 31]	5	17	17.4	3.0	17	17.8	3.3	0	-0.4	0.5	0.50 (Not significant)
Shigella spp. / EIEC	EHEC-EPEC kit (RidaGene)	5	13	18.8	1.1	14	14.0	1.2	0	-0.2	0.8	0.75 (Not significant)
Shigella spp. / EIEC	ETEC-EIEC kit (RidaGene)	5	13	13	1.2	13	13	1.2	0	0	0.7	>0.99 (Not significant)
ETEC (labile toxin gene positive)	ETEC-EIEC kit (RidaGene)	13	17	17.2	3.2	18	17.5	3.1	0	-0.2	0.7	0.38 (Not significant)
ETEC (stabile toxin gene positiv)	ETEC-EIEC kit (RidaGene)	7	13	14.7	3.0	13	14.4	2.4	0	0.3	1.5	0.75 (Not significant)
EPEC	EHEC-EPEC kit (RidaGene)	13	20	19.8	2.7	21	20.5	2.7	-1	-0.6	1.3	0.13 (Not significant)
EAEC	EAEC kit (RidaGene)	13	20	19.8	2.7	19	18.9	2.8	0	0.9	2.2	0.25 (Not significant)
Cryptosporidium spp.	In-house according to [30–34]	3	24	27.3	6.7	23	26.3	6.7	1	1	0	n.a. (Too low sample count)

n.a. = not applicable

2. Table. *(cont.)*

PCR-target	Applied kit	Number of positive reactions after both extraction schemes (*n*)	EZ1 nucleic acid extraction			Column-based nucleic acid extraction			Paired Ct value differences of EZ-1 extraction and column-based nucleic acid extraction			Significance level by Wilcoxon matched-pairs testing (*P*)
			Median Ct value	Mean Ct value	Standard deviation (SD)	Median Ct value	Mean Ct value	Standard deviation (SD)	Median difference	Mean difference	Standard deviation (SD)	
Giardia duodenalis	In-house according to [30–34]	1	23	23	n.a.	24	24	n.a.	–1	–1	n.a.	n.a. (Too low sample count)
Norovirus G1	Viral gastroenteritis kit (Fast-track Diagnostics)	1	20	20	n.a.	20	20	n.a.	0	0	n.a.	n.a. (Too low sample count)
Norovirus G2	Viral gastroenteritis kit (Fast-track Diagnostics)	5	16	18	6.0	14	16.6	5.1	2	1.4	1.9	0.50 (Not significant)
Astrovirus	Viral gastroenteritis kit (Fast-track Diagnostics)	1	30	30	n.a.	30	30	n.a.	0	0	n.a.	n.a. (Too low sample count)

n.a. = not applicable

[11]. E.g., nucleic acid extraction from cotton swabs yields higher DNA percentages in comparison to nylon swabs [10]. Altogether, the extraction quality of the EZ1 system is comparable with other automated extraction systems [11].

In our comparison of EZ1 extraction and column based extraction with complex sample matrices like frozen blood and stool, EZ1 extraction was associated with increased sample inhibition, which is a point of concern. Of note, this phenomenon was not reproducible for all accessed PCR systems but only for PCR protocols with self-designed inhibition control, i.e., the in-house multiplex PCRs, or with a very sensitive inhibition control approach like in case of the RealStar Malaria PCR Kit 1.0 (altona DIAGNOSTICS).

Another point of concern is a marked discrepancy of PCR results after the two assessed extraction systems which cannot be explained with sample inhibition alone. Next to 147 concordant PCR results, altogether two positive PCR reactions were observed after EZ1 extraction alone and 11 positive PCR reactions after column-based nucleic acid extraction alone. The apparently failed reactions were particularly frequent for PCRs targeting diarrhea-associated *E. coli*. In contrast, those PCRs were affected by only few detectable cases of sample inhibition. Incidental contamination events during sample preparation are not completely excluded. However, the events were randomly distributed, the respective negative control PCRs remained negative and the laboratory work was executed by a highly experienced technical laboratory assistant. Accordingly, sample contamination in such a high frequency with a wide distribution is not completely excluded but highly unlikely. The relatively low Ct values of the samples with discordant results do not suggest close proximity to the detection limit as a potential explanation of the phenomenon.

The fact that no extraction control, e.g., by adding the nucleic acid for the inhibition control PCR to the samples prior to nucleic acid extraction, was implemented, is a limitation of the analysis. Such a procedure would have allowed for the detection of occasional failure of nucleic acid extraction, potentially explaining the observed discordant results. Without extraction control PCR results, discordant target-specific PCR results might be either due to failed PCR reactions in the one extraction process or due to sample contamination in the other one.

For all but one tested real-time PCR systems, there was no significant difference between the assessed extraction schemes regarding the measured Ct values of concordantly positive PCR results, suggesting comparable quantities of target nucleic acids in the samples. For the in-house malaria PCR alone but not for the commercial malaria PCR, Ct values after EZ1-extraction were significantly increased for unknown reasons. However, the quantitative dimension of this phenomenon was negligible with a mean difference of only one Ct-step, suggesting a mean reduction of target-DNA amounts by less than one decadic logarithmic step. Accordingly, there were no relevant differences regarding the yield of target nucleic acids for column-based extraction and EZ1 extraction.

Some previous evaluation results regarding the extraction efficiency of the EZ1 system are contradictory. In a recently published comparison of different extraction protocols, the EZ1 system scored best for the extraction of cytomegalovirus DNA from plasma [13]. In another recent comparison of three nucleic acid extraction schemes from cotton and nylon swabs, an assessed EZ1 protocol scored worst in comparison to other approaches with, however, altogether still acceptable performance [10]. In comparison with easyMag (bioMérieux, Nürtingen, Germany) extraction of nucleic acids of respiratory pathogens, the EZ1 system showed a slightly higher detection limit but a considerably lower operational complexity [19]. Of note, the easyMag extractor is a high-throughput system which is not easily deployable for use in a field laboratory.

The difference between 37 positive malaria PCR results by in-house PCR and 38 positive PCR results by commercial malaria PCR does not necessarily indicate a disturbed PCR reaction of the in-house system. The respective sample was positive by MALDI–TOF–MS analysis for hemozoin [27, 28] but negative in microscopy, suggesting a low parasite density (data not shown). If a patient is infected by South-East Asian *Plasmodium knowlesi*, the in-house PCR which has been designed to cover African plasmodia only [29] will consequently show a negative result. In contrast, all species of human pathogenicity are covered by the commercial *Plasmodium* spp. PCR which does not discriminate on species level.

In summary, our analysis suggests comparative extraction efficiency of column-based and EZ1-based nucleic acid extraction from difficult sample matrices like frozen EDTA blood and stool samples. The higher frequency of confirmed sample inhibition after EZ1 extraction remains a point of concern. The high frequency of 8.1% (13 out of 160) discordant positive PCR results following the two compared extraction procedures could not be resolved by the applied approaches.

Conclusion

In case of concordant positive PCR results, EZ1 extraction leads to comparable extraction results like well-established column-based extraction from complex sample materials such as frozen EDTA plasma and stool. EZ1 extraction considerably reduces the required hands-on time for technical laboratory assistants.

Due to the observed moderately increased number of inhibited samples after EZ1 extraction, the preparation of two aliquots seems advisable if EZ1 extraction is intended as the extraction procedure of first choice. Thereby, repeated freeze–thawing for aliquot-acquisition from stool samples should be avoided to preserve the quality of target nucleic acids. In individual cases of PCR inhibition after EZ1 extraction, column-based extraction can be added from the other aliquot of the respective samples.

Due to excellent concordance of positive results of the malaria PCRs in spite of a slightly increased inhibition frequency, we adapted EZ1 extraction as the method of first choice for frozen blood samples in our laboratory. In contrast, considerable numbers of discordant results for PCRs from stool samples which are not explained by inhibition alone convinced us to keep on with traditional column-based extraction from stool materials. If EZ1 extraction from stool materials is intended, we strongly recommend for adding test nucleic acid for inhibition control PCR already prior to nucleic acid extraction to ensure extraction control.

The analysis further demonstrates the need for validation of each individual PCR protocol in conjunction with the applied nucleic acid extraction scheme. In case of changes of the extraction method without new evaluation testing, negative PCR results do not necessarily indicate the absence of target pathogen DNA.

Acknowledgements

Simone Priesnitz is gratefully acknowledged for excellent technical assistance.

Declaration of interest

The authors declare that there are no conflicts of interest.

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