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Rapid Genome Detection of Schmallenberg Virus and Bovine Viral Diarrhea Virus by Use of Isothermal Amplification Methods and High-Speed Real-Time Reverse Transcriptase PCR

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Over the past few years, there has been an increasing demand for rapid and simple diagnostic tools that can be applied outside centralized laboratories by using transportable devices. In veterinary medicine, such mobile test systems would circumvent barriers associated with the transportation of samples and significantly reduce the time to diagnose important infectious animal diseases. Among a wide range of available technologies, high-speed real-time reverse transcriptase quantitative PCR (RT-qPCR) and the two isothermal amplification techniques loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA) represent three promising candidates for integration into mobile pen-side tests. The aim of this study was to investigate the performance of these amplification strategies and to evaluate their suitability for field application. In order to enable a valid comparison, novel pathogen-specific assays have been developed for the detection of Schmallenberg virus and bovine viral diarrhea virus. The newly developed assays were evaluated in comparison with established standard RT-qPCR using samples from experimentally or field-infected animals. Even though all assays allowed detection of the target virus in less than 30 min, major differences were revealed concerning sensitivity, specificity, robustness, testing time, and complexity of assay design. These findings indicated that the success of an assay will depend on the integrated amplification technology. Therefore, the application-specific pros and cons of each method that were identified during this study provide very valuable insights for future development and optimization of pen-side tests.

Similar to human medicine, the demands for diagnostic tests that can be applied directly at the point of care are increasing in veterinary science also. These mobile "pen-side" tests would circumvent the delays in diagnosis associated with the transportation of the sample to a centralized laboratory and a resource-intensive processing. Furthermore, a rapid confirmation of clinical diagnosis directly on-site would enable timely intervention and implementation of control measures (e.g., during an outbreak of a transboundary animal disease). This has already been demonstrated for diagnosis of foot-and-mouth disease using rapid and simple lateral-flow devices (1, 2). However, over the past few years, a huge variety of innovative rapid technologies for amplification and detection of viral nucleic acid have been developed (3–5). These molecular approaches provide higher test sensitivity and specificity than the immunoassays mentioned before and are therefore attractive alternatives for integration into a new generation of mobile pen-side testing systems. Among the available technologies, high-speed reverse transcriptase quantitative PCR (RT-qPCR) and the two isothermal amplification techniques recombinase polymerase amplification (RPA) and loop-mediated isothermal amplification (LAMP) represent three promising candidates for applications in veterinary medicine. Real-time quantitative PCR (qPCR) is currently the most widely used method to detect genomes of viral pathogens, since it is highly sensitive and specific, allows quantitative analysis, and minimizes the risk of contamination. Nevertheless, commonly used RT-qPCR protocols require more than 1 h. Therefore, many efforts have been made to develop strategies that reduce reaction time to less than 20 min. However, the majority of these approaches required specialized PCR machines (6–8). Opposed to that, the application of high-speed RT-qPCR using conventional PCR machines represents a more feasible approach for common use as has recently been described (9).

The RPA method is based on the formation of a recombinase filament, a complex between oligonucleotide primers and a recombinase enzyme. Upon recognition of the target-specific sequence by the recombinase filament, strand exchange is initiated and primers are subsequently extended by a strand-displacing polymerase (10). Real-time detection can be performed by using TwistAmp exo probes. These probes contain an abasic nucleotide analogue (tetrahydrofuran [THF]), which is flanked by an internal fluorophore and a corresponding quencher group. Upon binding to the target sequence, the abasic site is recognized and cleaved by the DNA repair enzyme exonuclease III. This leads to separation of both the fluorophore and the quencher and subsequent generation of a fluorescent signal. RPA is a newly emerging technology, but present literature hints toward a promising tool for pen-side application (11–14).

In contrast, LAMP is the most widely researched and employed isothermal amplification method (15). It uses a strand-displacing DNA polymerase along with two internal primers (FIP and BIP)

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TABLE 1 Primers and probes used in this study

Assay	Target	Name	Sequence (5′→3′)[a]	Concn (pmol/reaction)	Reference
Standard and high-speed RT-qPCR	BVDV	Pesti-3F	CCTGAGTACAGGRTAGTCGTCA	10	23
		Pesti-4R	GGCCTCTGCAGCACCCTATCA	10	
		TQ-Pesti-Probe	FAM-TGCYAYGTGGACGAGGGCATGC-BHQ-1	1.875	25
	SBV	SBV-S-382F	TCAGATTGTCATGCCCCTTGC	10	30
		SBV-S-469R	TTCGGCCCCAGGTGCAAATC	10	
		SBV-S-408FAM	FAM-TTAAGGGATGCACCTGGGCCGATGGT-BHQ-1	1.875	
RPA	BVDV	BVDV1-F	CGAARAGAGGCTARCCATGCCCTTAGTAGG	7.5	This study
		BVDV1-R	TKTGGGCRTGCCCTCGTCCACGTGGCATCTCG	7.5	
		BVDV1-Probe	TGGAWGGCTKAAGCCCTGAGTACAGGG-**BT**-G-**F**-CGTCAGTGGTT CGAC-**PH**	2.5	
	SBV	SBV-F	TCCTCAAACTAGCTGAAGCTAGTGCTCAGATTG	10.5	This study
		SBV-R	AAAAGCATCAAGGAACATTTCGGCCCCAGGT	10.5	
		SBV-Probe[b]	ATCCAAGATACATTG-**BTF**-AACCATCGGCCCAGGTGCATCCCTTA ACCTC-**PH**	3	
LAMP	BVDV	BVDV1–F3	CATGCCCTTAGTAGGACTAGC	2.5	Modified after reference 31
		BVDV1–B3[b]	TTTTGTTTGTAWGTTTTGTATAAAAGTTCATT	2.5	
		BVDV1-FIP[c]	GGCRTGCCCTCGTCCACGTGTGGATGGCTKAAGCCCTGAG	25	
		BVDV1-BIP[c]	TGATAGGRTGCTGCAGAGGCCCACATGTGCCATGTACAGCAGAG	25	
		BVDV1-LF[b]	CGTCGAACCACTGACGACTAC	12.5	
	SBV	SBV-F3	CTTTTCGTGTAGTGTGTTGTGC	2.5	This study
		SBV-B3[b]	CTGCAAACATCAATGTAGTCAACA	2.5	
		SBV-FIP[c]	CTGAGGAGTAGAATGCAACACAGCTTGGGTTTGTAATGCCTTCTTCTG	20	
		SBV-BIP[c]	ACCACGGTGCATTGCATGCGACTAACTATRCGTTGACATCGTTCTT	20	
		SBV-LF[b]	AGTAAACAAGTGTGGATCGCTTTGC	10	
		SBV-LB	ATACCTTAGTATCTCTAAAGGAATGCGT	10	

[a] RPA assay sequence abbreviations: **B**, thymidine nucleotide carrying BHQ-1 quencher; **T**, abasic tetrahydrofuran site; **F**, thymidine nucleotide carrying FAM fluorophore; **PH**, phosphate.
[b] Designed on the antisense strand.
[c] HPLC purified.

and two outer primers (F3 and B3), which recognize 6 different regions on the target gene (16). Two additional primers (Loop-F, Loop-B) that anneal at the loop structures of the LAMP amplicons enhance reaction speed and specificity (17). An animation that is helpful for understanding the amplification principle can be found online (http://loopamp.eiken.co.jp/e/lamp/index.html). An abundance of pathogen-specific assays have already been described as having performance equal to or better than that of the equivalent PCR. This also includes assays for the detection of transboundary animal diseases, such as, e.g., foot-and-mouth-disease virus (18), classical swine fever virus (19), and avian influenza (20).

The aim of the present study was to evaluate high-speed RT-qPCR, RPA, and LAMP and to define their application-specific pros and cons with regard to integration in molecular pen-side tests. In order to enable a fair and valid comparison, novel optimized pathogen-specific assays were developed for the detection of bovine viral diarrhea virus (BVDV) and Schmallenberg virus (SBV). BVDV is classified as a member of the genus *Pestivirus* within the family *Flaviviridae* (21). It possesses a single-stranded positive-sense RNA genome that encodes one single large polyprotein. The 5′ untranslated region (UTR) is used to assign species and genotype and harbors the majority of pestivirus-specific RT-qPCR assays (22–25). A diagnostic tool for rapid detection of persistent BVDV-infections in the field would be highly attractive, since identification and subsequent elimination of persistently infected cattle are essential for a successful BVD eradication strategy (26). SBV is an *Orthobunyavirus* from the family *Bunyaviridae* and belongs to the Simbu serogroup viruses (27). It has a segmented single-stranded RNA genome of negative polarity that comprises a small (S) segment, medium (M) segment, and large (L) segment. SBV was detected for the first time in Europe in autumn 2011, and over the last 2 years, it has spread rapidly over large parts of northwestern Europe. Adult animals develop no or mild clinical disease, whereas transplacental infection can lead to severe congenital malformations (28, 29).

The diagnostic accuracy of the newly developed SBV- and BVDV-specific tests was assessed in comparison to that of established standard RT-qPCRs. Special emphasis was placed on the suitability of the tests for rapid and reliable detection of viral infections in the field.

MATERIALS AND METHODS

Standard RT-qPCR. Previously established standard RT-qPCR assays were used to assess the diagnostic accuracy of the newly developed tests (23, 25, 30). The primers and probes are indicated in Table 1 along with the applied concentrations. Unless stated otherwise, all primers were synthesized by Metabion (Martinsried, Germany). Reactions were carried out in a 12.5-μl volume using the SuperScript III One-Step RT-PCR system with Platinum *Taq* (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RT-qPCR was performed using an Eco real-time PCR system (amplifa Labortechnik GmbH, Wasserburg, Germany) and the following thermal profile: reverse transcription for 15 min at 50°C and then polymerase activation for 2 min at 95°C, followed by 45 cycles of 95°C for 15 s, 56°C for 30 s, and 68°C for 30 s.

High-speed RT-qPCR. The primers and probes and their concentrations used in the different assays are indicated in Table 1. Reactions were

TABLE 2 Details of the BVDV- and SBV-specific assays used in this study

Assay	Target virus	Target gene region	Amplicon position (bp)	Amplicon length (bp)	GenBank reference sequence accession no.
Standard and high-speed RT-qPCR	BVDV	5′ UTR	192–365	173	AJ133738.1
	SBV	S segment	360–447	87	HE649914.1
RPA	BVDV	5′ TR	94–259	165	AJ133738.1
	SBV	S segment	335–465	130	HE649914.1
LAMP	BVDV	5′ UTR	107–433		AJ133738.1
	SBV	L segment	1601–1861		HE649912.1

conducted in a total reaction volume of 12.5 μl using the SuperScript III One-Step RT-PCR system with Platinum *Taq* (Invitrogen) according to the manufacturer's instructions with 1 μl of 5 mM magnesium sulfate added per reaction. In order to omit the reverse transcription step of the qPCR, 2.5 μl template RNA was added to the master mix at room temperature. Amplification was performed on an Eco real-time PCR system, using the Eco software version 4.0 (amplifa Labortechnik GmbH).

For the BVDV-specific assay, the following thermal profile was used: polymerase activation for 1 min at 95°C, followed by 45 cycles of denaturation at 98°C for 3 s and annealing and extension at 60°C for 1 s. For the SBV-specific assay, the denaturation time was shortened to 1 s, and the annealing temperature increased to 64°C.

Recombinase polymerase amplification. Sequences of primers and probes used for RPA, as well as details of the assay design are shown in Tables 1 and 2, respectively. Both TwistAmp exo RPA probes were synthesized by TIB Molbiol (Berlin, Germany) with an inverse arrangement of fluorophore (6-carboxyfluorescein [FAM]) and quencher (black hole quencher 1 [BHQ-1]). RPA reactions were performed in a 25-μl volume using the enzyme pellets of the TwistAmp exo kit (TwistDx, Cambridge, United Kingdom), 1.7× rehydration buffer, 1.5 μl of 280 mM magnesium acetate (TwistDx), 2 mM dithiothreitol (DTT) (Invitrogen); and 5 U Transcriptor reverse transcriptase (Roche Diagnostics, Mannheim, Germany). Mixtures of primers and probes according to the concentrations indicated in Table 1 were prepared and added to an empty 0.2-ml reaction tube. A master mixture containing the rehydration buffer, DTT, water, and Transcriptor RT was prepared separately and added to the dried enzyme pellets. Twenty microliters of the resuspended pellet was then added to the primer-probe mixtures. Finally, magnesium acetate was pipetted into the tube lid, and 1 μl of template RNA was added to the reaction mixture. The lids were closed, and the magnesium acetate was centrifuged into the tubes. The tubes were then immediately placed into an ESEQuant tube scanner (Qiagen, Hilden, Germany). Fluorescence measurements using the FAM channel were performed for 20 min at 42°C. Optimal reaction conditions were defined after testing different incubation temperatures (39 to 42°C), as well as different concentrations of template (0.5 to 2 μl), magnesium acetate (1 to 2.5 μl), and DTT (2 to 4 mM). For interpretation of the collected fluorescence signals, a signal slope analysis combined with a 2nd derivative analysis was performed (Tube Scanner Studio software; Qiagen).

Loop-mediated isothermal amplification. The BVDV LAMP assay described in Tables 1 and 2 was designed based on a previously published primer set (31). The original primers were modified using a sequence alignment of the 5′ UTR of BVDV-1 strains available in GenBank. In addition, a Loop-F primer was included in the set. Placement of a Loop-B primer was not possible due to low sequence conservation in the respective genomic region of the 5′ UTR.

The L segment was chosen as target for the SBV LAMP. Sequences available in GenBank were aligned using ClustalW in order to find conserved regions. The final primer set (Tables 1 and 2) was constructed by using the LAMP primer design software Primer Explorer V4 (http://primerexplorer.jp/elamp4.0.0/index.html). For both assays, FIP and BIP primers were purified by high-performance liquid chromatography (HPLC).

The RT-LAMP reactions were carried out in a 12.5-μl reaction volume containing the primer concentrations indicated in Table 1; a 1× concentration of ThermoPol buffer (New England BioLabs [NEB], Ipswich, MA), 8 mM magnesium sulfate (Invitrogen), 0.8 M betaine (Sigma-Aldrich, St. Louis, MO), 1.4 mM each deoxynucleoside triphosphate (dNTP) (Qiagen, Hilden, Germany), 0.25 μl ResoLight dye (Roche Diagnostics), 3 U of Bst DNA polymerase (large fragment; NEB), and 3 U of cloned avian myeloblastosis virus (AMV) reverse transcriptase (Invitrogen). Finally, 2.5 μl of template RNA was added to the reaction mixture. Optimization was performed by testing different concentrations of magnesium sulfate (4 to 10 mM) and betaine (0.6 to 1 M) as well as different reaction temperatures ranging from 60 to 65°C. For the final assays, amplification was performed on the Eco real-time PCR system (amplifa Labortechnik GmBH), using 60 cycles of 1 min at 63°C followed by a standard melting curve analysis. Real-time data were analyzed in conjunction with melt curve data to exclude nonspecific fluorescence interference (Eco software version 4.0; amplifa Labortechnik GmbH).

Viruses, reference RNA, and clinical samples. Simbu serogroup viruses (Sabo, Sango, Shamonda, Shuni, Aino, Simbu, Peaton, Douglas, and Sathuperi viruses) were kindly provided by Peter Kirkland (Elizabeth Macarthur Agricultural Institute, Australia) and Robert Tesh (University of Texas Medical Branch). Full-length viral RNAs from BVDV strains 1a, 1b, 1d, 1e, 1f, 1 h, 1x, 2a, and 2c, as well as from classical swine fever virus (CSFV), border disease virus (BDV), and atypical pestiviruses, were taken from the EPIZONE pestivirus reference RNA panel (32). SBV reference RNA was produced from cell-culture-grown SBV. The RNA copy number of the starting dilution was determined using an external SBV standard.

Previously *in vitro*-transcribed and quantified RNA from a BVDV-DI9 plasmid construct (33, 34) was used to determine the analytical sensitivity of the BVDV assays. BVDV-positive blood and serum samples were supplied by various veterinary diagnostic laboratories from different parts of Germany as well as by the BVDV National Reference Laboratory at the Institute of Diagnostic Virology of the Friedrich-Loeffler-Institut (FLI). SBV-positive blood and serum samples were obtained during animal trials conducted at the FLI. All experimental protocols were reviewed by a state ethics commission and have been approved by the competent authority (State Office for Agriculture, Food Safety and Fisheries of Mecklenburg-Vorpommern, Rostock, Germany; reference no. LALLF M-V TSD/7221.3-1.1-004/12). Additional blood and tissue samples were taken from the collection of SBV-positive field samples at the Institute of Diagnostic Virology of the FLI.

SBV and BVDV reference RNAs were tested in three independent runs to determine the analytical sensitivity of the assays. Clinical samples were tested in duplicate by standard and high-speed RT-qPCR, and the mean value of the replicates was calculated. For RPA and LAMP assays, only samples yielding negative results in the first run were tested a second time.

RNA extraction. RNA was extracted from 140 μl of sera or infected cell culture supernatant, 75 μl of whole blood, or 140 μl of homogenized tissue by using the QIAamp viral RNA minikit (Qiagen) or the RNeasy

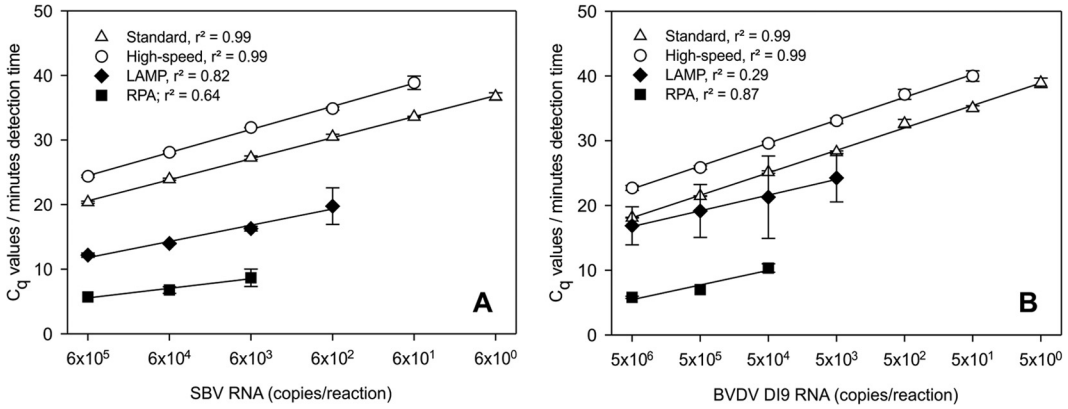

FIG 1 Analytical sensitivity and standard curves for (A) SBV-specific assays and (B) BVDV-specific assays. Serial 10-fold dilutions of reference RNA samples were tested in three independent runs. Linear regression analysis was performed using C_q values (white symbols) for standard and high-speed RT-qPCR and the time (minutes) to detection (black symbols) for LAMP and RPA.

mini kit (Qiagen) according to the manufacturer's instructions. All samples were eluted in 100 μl.

Statistical analysis. Linear regression analyses, the Kruskal-Wallis test, and Dunn's test were performed using the SigmaPlot software v11 (Systat Software GmbH, Erkrath, Germany). PCR efficiency (E) was calculated using the following equation: $E = 10^{(-1/\text{slope})} - 1$.

RESULTS

Assay design and optimization. (i) High-speed RT-qPCR. For BVDV, a number of previously described primers have been evaluated (22–24). Primers Pesti-3F and Pesti-4R (23) in conjunction with the TQ-Pesti-probe (25) yielded the best results using the high-speed profile. The SBV-specific assay could be established by using the previously published RT-qPCR (30). Both of the protocols were optimized by variations of the denaturation time and of the annealing and extension temperature. Using the Eco cycler, the final run times were 26 min for the BVDV-specific assay and 22 min for the SBV-specific assay, respectively.

(ii) LAMP. A BVDV-RT-LAMP primer set located in the same genomic region on the 5′ UTR as the selected RT-qPCR assay has been described before (31). However, amplification of BVDV RNA could only be achieved after manual modification of the published primer set (Tables 1 and 2). For the SBV LAMP, initial primer sets designed for the S segment repeatedly produced nonspecific amplification products. For this reason, additional assays were also designed for target regions in the M and L segments. Among those, only one primer set located in the L segment allowed specific amplification of SBV RNA and was therefore chosen for the final assay. The specificity and rapidity of the assay could be further optimized by variation of the outer primer B3. The concentration of the individual primers and their ratio to each other were found to have a crucial influence on the specificity of the LAMP assays. Optimal performance was achieved using primer ratios of 10:1:5 (inner to outer to loop) for the BVDV-specific assay and 8:1:4 for the SBV-specific assay.

(iii) RPA. For each RPA, several forward and reverse primers were designed and evaluated in combination with the respective TwistAmp exo probe. During initial experiments, the original 50-μl volume of the RPA reaction was successfully reduced to 25 μl. The optimal concentration of primers and probes was found to be assay specific (Table 1). After evaluation of several RT enzymes, the Transcriptor RT was selected since it outperformed the remaining candidates with regard to amplification speed (data not shown).

Analysis of assay parameters. Serial 10-fold dilutions of *in vitro*-transcribed BVDV-1-specific RNA were used to determine the analytical sensitivity of the newly developed BVDV assays. Five RNA copies per reaction could be amplified using the standard RT-qPCR, whereas the detection limits were 50 copies for the high-speed assay, 5×10^3 copies for LAMP, and only 5×10^4 copies for RPA (Fig. 1B). Accordingly, the analytical sensitivity of the SBV-specific assays was defined using serial 10-fold dilutions of SBV reference RNA. The standard RT-qPCR protocol (30) was able to amplify the dilution series down to 6 genome copies per reaction. In comparison, the sensitivities of the high-speed RT-qPCR, LAMP, and RPA were 1-, 2-, and 3-$\log_{10}$ steps lower, respectively (Fig. 1A).

Quantitative parameters of all assays were further assessed by linear regression analysis. Calculations were performed using the quantification cycle (C_q) values for the standard and high-speed RT-qPCR assays and the detection time (in minutes) for the respective LAMP and RPA assays. Consequently, the standard curves presented in Fig. 1A and B do not allow a direct comparison of the reaction times. An overview of statistical analyses is given in Table S1 in the supplemental material. In summary, r^2 values reached >0.9 for standard and high-speed RT-qPCR, whereas for LAMP and RPA, the r^2 values were <0.9 (see Table S1).

Using the detection time for comparison of the individual assays, high-speed RT-qPCR, LAMP, and RPA evidently required less time to detect equal amounts of target RNA than the respective standard RT-qPCR (Fig. 2A and B). Statistical analysis (Kruskal-Wallis test followed by Dunn's test) confirmed that these differences with regard to reaction speed were significant for both the BVDV- and SBV-specific assays ($P < 0.05$).

Diagnostic sensitivity and specificity. (i) SBV. The applicability of the SBV assays was tested using samples from SBV-infected animals (Table 3). Analysis revealed similar levels of performance of LAMP and high-speed RT-qPCR, with slightly reduced sensitivity of LAMP for samples with low viral loads. Using RPA, 8 false-negative results were obtained. This included samples with C_q values of >27 as well as 2 tissue samples. No amplification of nontarget RNA could be observed using previously characterized SBV-negative samples (data not shown). The cross-reactivity of

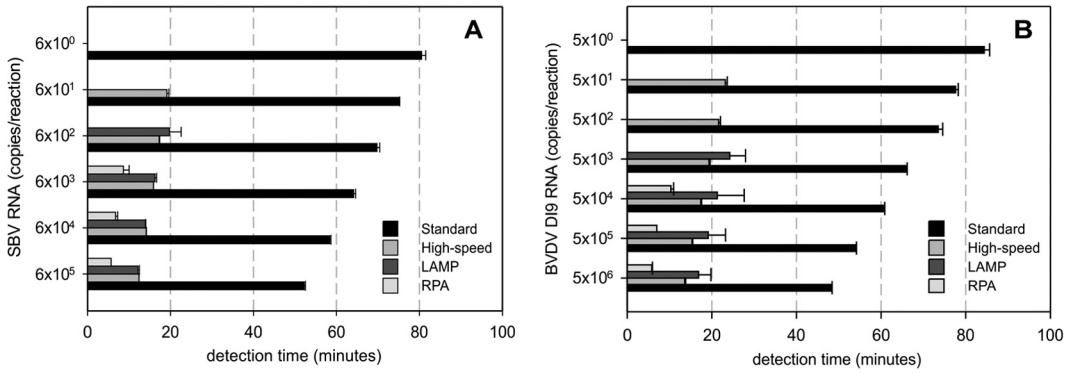

FIG 2 Assay times of the different SBV-specific (A) and BVDV-specific (B) detection systems. The time (minutes) until detection of a positive signal is plotted against the concentration of target RNA in the sample. For standard and high-speed RT-qPCR assays, the time to detection was calculated from the obtained C_q values.

the assays was evaluated using 9 viruses of the Simbu serogroup. Standard and high-speed RT-qPCR, as well as RPA, cross-detected several of these viruses. In contrast, the LAMP assay proved to be highly specific for SBV (Table 3).

(ii) BVDV. The suitability of the BVDV assays to detect viremic cattle in the field was investigated using serum and blood samples collected in different parts of Germany. All virus-positive samples were readily detected by high-speed RT-qPCR and LAMP. In contrast, RPA yielded 5 false-negative results (Table 4). Each assay identified positively all of the investigated BVDV-1 reference strains (Table 4). Using standard and high-speed RT-qPCR assays, all of the additional pestiviruses included in the EPIZONE reference panel could be amplified, whereas the RPA detected only BVDV-2 strains and the atypical pestivirus Giraffe (Table 4). The BVDV LAMP assay was specific for BVDV-1, with the exception of one BVDV-2a strain (Table 4). The latter could be distinguished from BVDV-1 strains by melting curve analysis (data not shown).

Finally, no amplification of nontargeted RNA was observed during testing of BVDV-negative samples with all systems (data not shown).

DISCUSSION

Over the past few years, a variety of rapid nucleic acid amplification technologies have been developed and used for integration into molecular pen-side tests. This includes PCR-based approaches and isothermal amplification techniques. However, each of these methods has application-specific pros and cons that make a valid comparison challenging. In order to identify the most suitable strategy for the future development of pen-side test systems, we aimed to directly compare the applicabilities of high-speed RT-qPCR, RPA, and LAMP. For this purpose, we developed novel pathogen-specific assays for the detection of BVDV and SBV and evaluated these assays in comparison with standard RT-qPCRs.

The high-speed RT-qPCR assays both showed a higher analytical sensitivity than the respective LAMP or RPA assays, with only a 1-$\log_{10}$-step reduction compared to the reference standard RT-qPCR (Fig. 1A and B). They further displayed a larger linear dynamic range and a higher reproducibility than the isothermal amplification techniques (see Table S1 in the supplemental material). The fast assay development was an additional very valuable advantage of the high-speed RT-qPCRs. As shown before, primers and probes of established RT-qPCR assays can be applied and optimized for the high-speed protocol (9). However, we also found that the size of the amplification product critically influenced the assay performance. In the case of BVDV, the primer pair Pesti-3F and Pesti-4R, which produced the shortest amplicon among all the primer candidates (173 bp), yielded the best results in the high-speed profile. Use of the SBV-specific assay, which amplifies a product of only 87 bp, allowed reduction of the denaturation time during two-step cycling to 1 s and an increase in the annealing and extension temperature to 64°C, which further decreased the total running time of the protocol. Consequently, the highest reaction speed can be achieved by using RT-qPCR assays with amplicons of less than 100 bp. Considering future applications in the field, the high-speed RT-qPCR protocols were established using the portable Eco real-time system (amplifa Labortechnik GmbH). In addition to a small size, the Eco cycler enables a sample ramping rate of 5.5°C/s, which is significantly faster than ramp rates of conventional Peltier block-based cyclers. Therefore, the reaction speed of the high-speed protocol depends on the available thermocycler, as was previously shown (9). This finding, together with the costly and nonstabilized PCR reagents, might restrict application of high-speed RT-qPCR in resource-limited settings.

Opposed to that, the RPA technology has several important advantages with regard to field use. (i) RPA reagents are available in a lyophilized format, with only the separately added RT enzyme requiring refrigeration. (ii) The very-small-footprint ESEQuant tube scanner can be easily transported and installed on site. (iii) The low reaction temperature of 42°C is an advantage with regard to miniaturization and integration into battery-driven devices (3). This has already been proven by the development of a microfluidic lab-on-a-foil system and a digital RPA SlipChip (35, 36). However, in the present study, the newly developed SBV and BVDV RPA tests showed a low analytical sensitivity (Fig. 1A and B). As indicated in Tables 3 and 4, virus-positive samples with RT-qPCR C_q values of >27 were not reliably detected using RPA. In case of BVDV, the RPA detected only 27 out of 32 field samples collected from persistently infected cattle. This was surprising, since the 5 false-negative samples yielded C_q values of <26 in the corresponding reference RT-qPCR (Table 4). The failure of the test can therefore not be explained by low viral loads in the samples. More likely, the selected primers and probe do not optimally recognize all of the currently circulating BVDVs. Thus, the assay is not suit-

TABLE 3 Evaluation of SBV-specific high-speed RT-qPCR, LAMP, and RPA assays in comparison to standard RT-qPCR using samples from SBV-infected animals and supernatants of cells infected with different Simbu serogroup viruses

Classification	Sample	Detection time, min (corresponding C_q value)			
		RT-qPCR[a]			
		Standard	High speed	LAMP	RPA
SBV exptl infection	R07/4-S	52.3 (20.4)	11.9 (23.4)	12.1	5.7
	R07/4-B	52.4 (20.4)	11.7 (23.0)	11.3	5.7
	R07/3-B	54.8 (21.9)	12.5 (24.6)	12.0	6.0
	R08/3-S	73.6 (32.7)	17.4 (35.2)	24.1	6.3
	668/4-S	60.0 (24.8)	14.0 (27.8)	14.0	6.7
	790/4-S	60.5 (25.2)	14.1 (28.0)	14.6	7.7
	R08/4-B	60.5 (25.2)	14.1 (28.1)	13.3	7.0
	R08/3-B	60.1 (24.9)	14.3 (28.5)	13.3	8.0
	790/4-B	61.1 (25.5)	14.2 (28.2)	13.6	15.3
	R12/5-S	62.4 (26.2)	14.7 (29.4)	14.8	7.0
	R3/8-S	63.2 (26.7)	15.0 (30.1)	14.2	8.0
	R5/4-B	64.8 (27.6)	15.7 (31.4)	16.2	Negative[b]
	R12-4-S	65.7 (28.1)	15.6 (31.2)	15.4	13.0
	R10/5-S	64.5 (27.5)	15.5 (31.1)	14.8	13.7
	R14/4-B	65.6 (28.1)	16.0 (32.1)	17.7	7.3
	R10/6-S	66.3 (28.5)	16.1 (32.3)	18.3	10.7
	790/5-S	66.3 (28.5)	16.9 (34.1)	15.9	9.0
	R14/5-S	68.3 (29.6)	18.3 (37.2)	19.2	Negative
	R11/5-S	68.9 (30.0)	18.4 (37.4)	ND[c]	Negative
	687/5-B	70.1 (30.7)	19.3 (39.2)	22.7	ND
	790/3-B	76.5 (34.4)	20.3 (41.4)	21.5	ND
SBV-positive field samples	BH199/12-5	71.1 (31.3)	17.5 (35.3)	18.0	ND
	BH199/12-6	79.5 (36.1)	21.2 (43.4)	Negative	ND
	BH305/12-2	60.8 (25.3)	14.4 (28.7)	14.2	ND
	BH305/12-3	48.7 (18.3)	11.4 (22.3)	11.3	5.5
	BH305/12-5	62.4 (26.3)	15.1 (30.2)	16.2	Negative
	BH305/12-6	53.1 (20.9)	12.5 (24.5)	11.8	5.7
	BH316/12-1	56.7 (23.0)	13.6 (26.9)	13.8	8.7
	BH316/12-4	66.5 (28.6)	16.3 (32.7)	22.5	Negative
	BH316/12-6	64.1 (27.2)	15.6 (31.3)	15.2	Negative
	BH316/12-7	61.7 (25.9)	14.8 (29.6)	15.1	5.3
	BH316/12-8	55.7 (22.4)	13.1 (26.0)	8.9	6.3
	BH316/12-9	64.0 (27.1)	15.4 (30.8)	13.4	ND
	BH316/12-10	59.8 (24.7)	14.2 (28.2)	13.6	ND
	BH316/12-12	57.9 (23.6)	13.9 (27.7)	13.4	7
	BH652/12-1	52.5 (20.5)	12.3 (24.2)	11.9	5.7
	BH648/12-1.1	51.2 (19.8)	12.2 (24.0)	11.5	5.3
	BH641/12-1	66.1 (28.4)	15.9 (31.9)	15.8	9
	BH641/12-2	65.2 (27.9)	16.2 (32.5)	18.0	8.3
	BH453/12-6	59.8 (24.7)	15.8 (31.7)	17.7	Negative
	BH453/12-10	56.7 (23.0)	15.7 (31.5)	16.3	Negative
	BH318/12-2	52.4 (20.4)	13.5 (26.8)	13.3	5.7
	BH315/12-9	72.6 (32.1)	18.6 (37.8)	Negative	ND
Simbu serogroup viruses	Sabo	79.1 (35.9)	19.4 (39.4)	Negative	Negative
	Sango	Negative	Negative	Negative	Negative
	Shamonda	51.0 (19.6)	Negative	Negative	5.3
	Shuni	Negative	Negative	Negative	Negative
	Aino	Negative	Negative	Negative	Negative
	Simbu	78.4 (35.59)	Negative	Negative	Negative
	Peaton	80.0 (36.4)	19.7 (40.2)	Negative	Negative
	Douglas	53.9 (21.4)	19.2 (38.9)	Negative	5.7
	Sathuperi	Negative	Negative	Negative	5.3

[a] Shown are mean values from 2 replicates.
[b] Negative results were those negative in 2 consecutive runs.
[c] ND, not determined.

TABLE 4 Evaluation of BVDV-specific high-speed RT-qPCR, LAMP, and RPAs in comparison to standard RT-qPCR using BVDV-positive field samples and pestivirus reference RNA

Classification	Sample	Detection time, min (corresponding C_q value)			
		RT-qPCR[a]			
		Standard	High speed	LAMP	RPA
BVDV-positive field samples	699/12-8	55.0 (22.0)	15.2 (25.7)	32.1	10
	699/12-9	58.5 (24.0)	15.8 (26.7)	24.8	9.3
	699/12-10	54.7 (21.8)	16.1 (27.2)	27.5	7.7
	699/12-11	60.0 (24.8)	16.3 (27.6)	27.6	9
	699/12-12	54.8 (21.8)	14.8 (24.8)	22.8	7
	696/12-1	62.3 (26.2)	16.6 (28.1)	37.0	Negative[b]
	696/12-2	60.2 (24.9)	16.1 (27.1)	25.5	9.3
	696/12-3	57.0 (23.1)	15.4 (26.0)	28.3	Negative
	696/12-4	55.4 (22.2)	15.3 (25.8)	25.9	8.3
	720/12-1	58.0 (23.7)	16.3 (27.7)	27.7	10
	720/12-2	54.0 (21.4)	15.1 (25.5)	23.5	8.7
	720/12-3	57.8 (23.6)	17.3 (29.4)	25.8	12
	699/12-1	51.4 (19.9)	14.8 (24.9)	21.1	8
	699/12-4	55.5 (22.3)	15.7 (26.4)	19.6	9.7
	699/12-5	52.7 (20.6)	15.0 (25.2)	27.7	11
	699/12-15	52.7 (20.7)	14.6 (24.5)	18.2	8.3
	696/12-14	59.6 (24.6)	16.9 (28.7)	23.8	7
	696/12-15	55.9 (22.5)	15.8 (26.7)	23.5	6.3
	720/12-9	53.1 (20.9)	14.2 (23.8)	18.5	8.3
	720/12-10	59.6 (24.6)	16.9 (28.6)	26.0	11
	720/12-12	53.1 (20.9)	14.6 (24.5)	22.6	8.3
	720/12-13	57.0 (23.1)	15.9 (26.9)	25.4	9
	720/12-16	57.8 (23.6)	15.7 (26.5)	20.1	8.3
	720/12-17	61.6 (25.8)	16.8 (28.5)	22.2	Negative
	720/12-18	56.7 (23.0)	15.3 (25.8)	30.3	7.7
	720/12-20	57.3 (23.3)	15.8 (26.7)	29.9	9.3
	720/12-21	51.4 (19.9)	14.5 (24.3)	17.7	8.7
	720/12-23	60.3 (25.0)	16.8 (28.4)	27.8	Negative
	720/12-24	59.8 (24.8)	16.8 (28.5)	29.5	Negative
	720/12-25	53.9 (21.3)	15.0 (25.2)	26.7	8.7
	720/12-19	53.1 (20.8)	15.1 (25.4)	19.6	8.3
Reference RNA					
BVDV-1	BVDV-1a	51.4 (19.9)	14.1 (23.7)	22.8	7.3
	BVDV-1b	51.1 (19.7)	14.5 (24.3)	26.9	6
	BVDV-1d	57.5 (23.4)	15.9 (26.9)	21.9	6.7
	BVDV-1e	53.3 (21.0)	15.3 (25.7)	24.4	7.7
	BVDV-1f	58.9 (24.2)	18.8 (32.0)	31.8	9.7
	BVDV-1h	60.2 (25.0)	17.9 (30.4)	27.3	7.7
	BVDV-1x	60.8 (25.3)	17.6 (29.9)	49.8	9
BVDV-2	BVDV-2a US	61.8 (25.9)	19.1 (32.5)	Negative	Negative
	BVDV-2a G	59.3 (24.4)	18.1 (30.8)	38.87	7.3
	BVDV 2c	62.0 (26.0)	19.0 (32.4)	Negative	9.7
	BVDV 2c NRW	56.5 (22.8)	16.6 (28.1)	Negative	8.3
Pestivirus	CSFV Alfort 187	53.0 (20.8)	16.4 (27.8)	Negative	Negative
	CSFV Pader	57.6 (23.5)	18.0 (30.6)	Negative	Negative
	CSFV Koslov	56.8 (23.0)	17.1 (29.1)	Negative	Negative
	CSFV Uelzen	55.1 (22.0)	17.2 (29.1)	Negative	Negative
	BDV Gifhorn	53.2 (20.9)	15.1 (25.4)	Negative	Negative
	BDV Moredun	54.0 (21.4)	16.8 (28.5)	Negative	Negative
	Hobi	60.9 (25.4)	20.5 (35.1)	Negative	Negative
	Giraffe	55.6 (22.3)	16.2 (27.5)	Negative	9

[a] Shown are mean values from 2 replicates.
[b] Negative results are those negative in 2 consecutive runs.

able as a screening test for the detection of a broad range of different BVDV strains.

The SBV RPA assay correctly identified samples from experimentally infected animals that were sampled at the peak of viremia. However, field samples yielding C_q values of >25 were not reliably detected (Table 3). Therefore, the RPA technology in its current format is not suitable for field detection of transiently SBV-infected animals with only low to moderate viral loads. Furthermore, the test did not detect SBV RNA in two tissue samples with C_q values of <25 in standard RT-qPCR. This indicates that the reaction might be inhibited by the complex genomic background present in those samples. However, since tissue samples are not applicable for pen-side testing, this finding is of minor importance. More significant drawbacks of the RPA are the requirement of relatively long primers (30 nucleotides [nt]) in combination with a probe with a length of at least 50 nt. The probe further requires internal modifications that are restricted to T residues with fewer than 6 intervening nucleotides (*TwistAmp Combined Manual*, TwistDx, Cambridge, United Kingdom). This makes the assay design challenging, especially in the case of highly variable viruses. Furthermore, all candidate primers and probes have to be evaluated empirically, which renders assay development not only time-consuming and labor intensive but also quite expensive. Nevertheless, with regard to reaction speed, the RPA was superior to LAMP and high-speed RT-qPCR, since it yielded positive results in less than 10 min. For this reason, the technique represents a promising tool for rapid local decision-making during a confirmed outbreak of a highly contagious disease, such as, e.g., foot-and-mouth disease.

In contrast to the experiences with RPA, testing of various clinical samples using the SBV and BVDV LAMP assays showed good agreement with RT-qPCR (Tables 3 and 4). This implies the suitability of these assays for use in the field, even though the analytical sensitivity was lower than for standard RT-qPCR (Fig. 1A and B). The reaction speed of the SBV-specific LAMP was comparable to that of the tested high-speed RT-qPCRs; i.e., a positive result was obtained in less than 20 min. The BVDV-LAMP required longer reaction times, which can be explained by the lack of a Loop-B primer. Hence, a significant enhancement of reaction speed can only be expected by using a combination of two Loop primers (17). In comparison to RPA and to high-speed RT-qPCR, the LAMP assays displayed a very high specificity. This finding can be explained by the principle of the LAMP reaction using a set of 6 primers that recognize 8 distinct regions on the target sequence. Amplification occurs only when all 8 regions within the target gene are correctly recognized by the primers (16, 17). Consequently, the BVDV LAMP assay specifically amplified BVDV-1 strains, whereas the RT-qPCR and RPAs cross-detected several other pestivirus strains, as indicated in Table 4. In a similar manner, the SBV LAMP assay proved to be specific for SBV and did not detect any of the related Simbu serogroup viruses (Table 3). Thus, the LAMP assays represent attractive tools for confirmatory diagnosis and rapid differentiation of target viruses. However, it has to be considered that due to its high specificity, the LAMP assay might not be suitable for reliable detection of highly variable viruses and for initial screening investigations, which require a maximum of test sensitivity. Real-time monitoring using an intercalating dye was chosen as the detection strategy for LAMP, in order to prevent contamination and to enable a direct comparison to RT-qPCR and RPA. This approach has previously been described and successfully applied (18, 37, 38). However, the LAMP reaction can also be performed using a simple heat block or a water bath. Furthermore, visual monitoring by the naked eye of the reaction is possible through color change by addition of a fluorescent dye (39). Thus, LAMP is not dependent on sophisticated equipment, which makes the technique especially attractive for application in resource-limited settings and for integration into pen-side tests. An additional feature of the LAMP method is its previously described tolerance to various biological substances that inhibit PCR (40). Successful amplification has been described with little or no sample preparation (41–43). This indicates that the extraction step can be omitted in LAMP, which saves time, labor, and costs. However, we found the complex primer design to be the major drawback of LAMP. Even though primer design software is available online, the whole process is time-consuming, and the success of the LAMP reaction relies on the selected primer set. Thus, careful primer design and evaluation of several primer sets for different target regions are required. We further had the experience that the primers created by the software do not guarantee optimal performance. During development of the SBV LAMP, several primer sets were designed for different target regions on the S segment and M segment. However, with each of these sets, nonspecific amplification products were detected, probably due to primer dimer formation. Among additional primers designed for the SBV L segment, only 1 out of 4 sets specifically amplified SBV RNA.

TABLE 5 Level of suitability of high-speed RT-qPCR, LAMP, and RPA with regard to important properties of a pen-side test

Test parameter	Level of suitability of[a]: High-speed RT-qPCR	LAMP	RPA
High sensitivity	+++	++	+
High specificity	++	+++	++
Reaction speed of <20 min	++	++	+++
High robustness	+++	++	++
Simple, portable equipment	+	+++[b]	+++
Stabilized reagents	+	+	++
Cost-effective	+	+++	++
Rapid assay design	+++	+	+

[a] +++, very high; ++, high; +, medium to low.
[b] Based on the possibility to perform LAMP with a simple heat block or water bath.

Contrary problems occurred using a previously published BVDV-specific LAMP (31): amplification of target RNA was not possible using the described assay. A successful amplification of BVDV RNA was achieved only after several manual modifications of the primers. These experiences illustrate the importance of careful primer design. However, they also demonstrate that assay development can be complicated and labor intensive. As discussed before, similar experiences were made using the RPA technology. Thus, both isothermal techniques are not suitable for a rapid establishment of novel pathogen-specific assays. In our view, the complexity of the test design might even represent one of the major obstacles for routine application of LAMP and RPA.

Conclusions. In our study, newly developed high-speed RT-qPCR, RPA, and LAMP assays enabled rapid detection of BVDV and SBV in less than 30 min. However, the tests revealed major differences with regard to sensitivity and specificity, robustness, assay time, complexity of test design, and field applicability (Table 5). Based on these findings, we conclude that none of the investi-

gated amplification techniques represents a generic platform that can be used across a variety of diagnostic questions and for a broad range of pathogens. Since the success of a pen-side test relies on the integrated amplification strategy, the application-specific properties of the available technologies have to be assessed carefully prior to assay development. Thus, the findings of the present study deliver a valuable contribution to the future development of rapid and reliable molecular pen-side test systems.

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