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On-site identification of animal species in meat products

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On-site identification of animal species in meat products

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1 General Introduction

Meat production

Food production and trade in food are among the most important economic sectors in Germany, due to their economic added value and the high number of employees (1). Globally, improved trading conditions and the networking of different markets and economic systems are leading to the steady growth of the food industry. Globalization has meanwhile become an important building block in the food trade and in addition to the social advantages such as growth and prosperity also harbours significant risks: Saturated food markets in Europe create high pressure on innovation and prices (2). In combination with the high price differences for food raw materials from different countries of origin, the risk of food fraud increases (3). At the same time, the very complex networking of international trade and goods flows makes it difficult to control food. In this area of conflict, the most important is to improve the level of consumer protection in Europe through innovative control systems. This issue is clearly appointed in the regulation No 178/2002 of the European parliament and of the council, Article 8 “Protection of consumers’ interests”. By focusing on the meat production sector, animal species of which is contained in the meat product has to be declared on the label. Correct labelling is crucial for the consumers health and their religious as well as fulfilling personal preferences (4). However, the determination of the origin is based primarily on the information provided by the food manufacturer or the company that markets the products (5). An incorrect declaration can only be made more difficult or restricted by more stringent and regular controls. Despite these risks, there are always incidents, especially with meat products, in which expensive types of meat are mixed with less expensive meat species. In 2013, the so-called “horse meat scandal” dominated the press in Germany: beef was mixed with cheap horse meat over long trade routes with different intermediate suppliers and brought to the market. As a result, the food processing industry unwittingly processed horse meat and undeclared horse meat products were offered to consumers for purchase (6). In addition to these examples of false declarations, the non-declaration of small quantities of meat is becoming increasingly important. Since poultry is a good source of protein and is also cheaper than red meat, the risk of adulteration of beef with poultry increases (7). Furthermore,

turkey meat is more expensive than chicken meat, which is why the tendency to adulterate turkey products is also increasing (8).

Adulteration of exotic or inexpensive meat source can also cause the transmission of human pathogens to the consumers. Contaminated meat is not properly handled or prepared, which increase the risk of emerging infectious diseases (9). This can be seen in the current global pandemic outbreak of SARS-coronavirus-2 (10).

Therefore, an improvement of the surveillance system for food products and their raw material is urgently needed.

Diagnostic Methods

For the identification of contamination by foodborne pathogens or none-declared origin of the meat species, the gold standard analytical method is the polymerase chain reaction (PCR) (11). DNA amplification of a target sequence of up to 3 kbp is accomplished by the use of a heat-resistant enzymes and different temperature steps in PCR. The process starts with the denaturation of the DNA double strand at 95 °C, following the primer annealing at a primer specific temperature depending on the primers melting temperature between 55 to 65 °C. Elongation of the target sequence starting at the 3´ end of the primers is performed by the use of a thermostable DNA Polymerase at a temperature of 72 °C. These three steps are repeated in a thermocycler up to 45 times, which takes at least 90 minutes. For end point results, the amplification products can be separated by gel electrophoresis (e.g. conventional PCR) (12). The visualization of the DNA band on gel takes another 30 to 60 minutes. To speed up the data acquisition, real-time PCR using fluorescence label probe was introduced. During the amplification, the TaqMan probe with dual fluorophore is hybridized to the amplicon. The polymerase has an exonuclease activity leading the separation between the two dyes. This results in an emission of a fluorescence signal, which can be measured by fluorometer in real-time (13). The real-time PCR is a well-established method, however a highly equipped laboratory and trained personal is required. Therefore, samples have to be shipped to centralised laboratories. Using the PCR technology at point-of-need is unfortunately not

applicable. Performing tests directly in meat production facility will shorten the time needed to correct any deviations. On the top of that mobile diagnostics will allow second check point at the consumer end.

ISOTHERMAL DNA AMPLIFICATIONS TECHNOLOGIES

The selection of an isothermal amplification method could improve the identification of animal species or pathogens in meat products at point-of-need. Several isothermal amplification assays were developed. Based on different enzymes, all methods mentioned in table 1 can amplify DNA at single constant temperature. The amplification methods differ in the condition of the double strand DNA separation.

Table 1. Characteristics of different isothermal DNA Amplification methods

Name	Temperature (°C)	Speed (min)	Nr. of Primers	Probe
Loop-mediated Isothermal Amplification *(LAMP)*	60 or 65	30	4 - 6	+/-
Helicase-dependent amplification *(HDA)*	60	60	2	+
Rolling circle amplification *(RCA)*	31 - 37	90	1 - 2	-
Strand-invasion based amplification *(SIBA)*	42 - 44	20 - 30	2	-
Multiple displacement amplification *(MDA)*	30 then 65	150	6	-
Nucleic acid sequence-based amplification *(NASBA)*	65 then 41	90 - 120	2	+
Nicking Enzyme Amplification Reaction *(NEAR)*	55 - 59	2 - 5	2	+/-
Strand displacement amplification *(SDA)*	95 then 37	120	4	+
Recombinase Polymerase Amplification *(RPA)*	39 or 42	3-10	2	+

In case of the helicase-dependent amplification (HAD), the *Escherichia coli* UvrD helicase and T7 bacteriophage gp4 helicase to unwind double strand DNA were implemented. Single-strand-binding proteins stabilize the DNA single strand so the primer annealing and elongation by exonuclease-deficient DNA polymerase can take place (14-16). Unfortunately, the run time takes around 60 minutes (17). Nevertheless, real-time detection is possible by using fluorescent intercalating dye (18, 19).

The rolling circle amplification (RCA) employs circular DNA as template. Using T4 polynucleotide kinase and DNA T4 DNA circular primer-template-complex is formed (20-22). The elongation is accomplished by φ29 DNA polymerase (23). The biggest drawback is the run time exceeds that of the real-time PCR (24). The same is true for the multiple displacement amplification (25, 26), but random primer were used (27-29).

Strand displacement amplification (SDA) needs a DNA single strand as target template. A primer containing a *Hinc*II recognition sequences binds to template and initiates the DNA elongation. *Hinc*II nicks the amplification product at its recognition site and produces a new single strand for the oligonucleotide to primer (30, 31). This process takes at least 2 hours for an exponential DNA amplification (32). Using a hairpin-probe, detection in real-time can be conducted and whole genome amplification can be performed (33-35).

Strand-invasion based amplification (SIBA) is initiated by the insertion of a complex formed by a single-stranded invasion oligonucleotide and recombinase protein in the DNA double strand (36). The recombinase independent primer can now bind to the target specific sequence and extension by the polymerase can take place (37). The SIBA reaction produces results in 20 to 30 minutes, which can be monitored in real-time by applying a fluorescent intercalating dye (38).

The nucleic acid sequence-based amplification (NASBA) is specifically designed for the detection of RNA templates (39). The amplification is performed by the T7 RNA polymerase, RNase H, reverse transcriptase and two specific primer (40). Nevertheless, NASBA has the great advantage for the use of multiplex pathogen detection in real-time, which also can be included in a self-digitization (41, 42).

The nicking enzyme amplification reaction (NEAR) produces results within a few minutes. The advantage of the NEAR method is the PCR primers can be used (43,

44). Unfortunately, the technology is only used in Abbott ID now and not commercially available for the scientific community.

Loop-mediated isothermal amplification (LAMP) produces results in 30 minutes, uses DNA as target and amplifies amplicons up to 250 bp with four to six primers. The primers start with building a stem-loop DNA, which serves as starting point of the amplification. Using a strand-displacing DNA polymerase, a complementary amplicon of the target sequence is produced (45). Using fluorescent intercalating dye, real-time detection is possible. Several LAMP assays are established for the detection of animal DNA in meat. Within 30 to 90 minutes pork, horse, beef, sheep and ostrich DNA can be detected (46-51). Although the LAMP assays seem to be viable option for a point-of-need detection system for the meat product surveillance, the primer design for the four to six different oligonucleotide is not always convenient. Furthermore, the detection time is not always 30 minutes and can be also up to 90 minutes.

In the recombinase polymerase amplification (RPA), positive signal in high concentrated samples can be identified in 3 minutes. Low concentrated DNA samples produces results in 15 minutes. Moreover, only two primers with a length of 30 to 35 bp is needed to amplify the target sequence. For real-time detection, a fluorescence labelled probe (48 – 53 bp) is utilized (52). The RPA reaction starts with the binding of the recombinase T4 uvsX to the primers. This complex invades the double stranded DNA at the target sequence of the primers and form a D-loop. The complementary DNA strand stabilized by single-strand binding proteins (T4 gp32), thereafter, the strand displacing DNA polymerase *Bsu*I amplifies the target sequence (Figure 1) (53). Using a fluorescence labelled exo-probe, the amplification can be monitored in real-time. During the amplification, the exo-probe binds to the amplicon. The exo-probe contains a tetrahydrofuran abasic–site mimic (THF), which separates a fluorophore from a quencher. A double-strand–specific Escherichia coli endonuclease IV (Nfo) recognises this THF-residue and crop it. This results in a separation of fluorophore and quencher and a fluorescence signal can be measured

by a fluorescence detection device (54). The optimum temperature of RPA is between 39 to 42 °C (53).

All reagents can be freeze-dried and stored at room temperature. Therefore, no cold chain is required during the transport and use of the RPA reagents. The RPA test is easy to perform at low resource settings. This results in an ideal technique for point-of-need diagnostics, which is proven by scientific groups for the detection of different pathogens (54, 55).

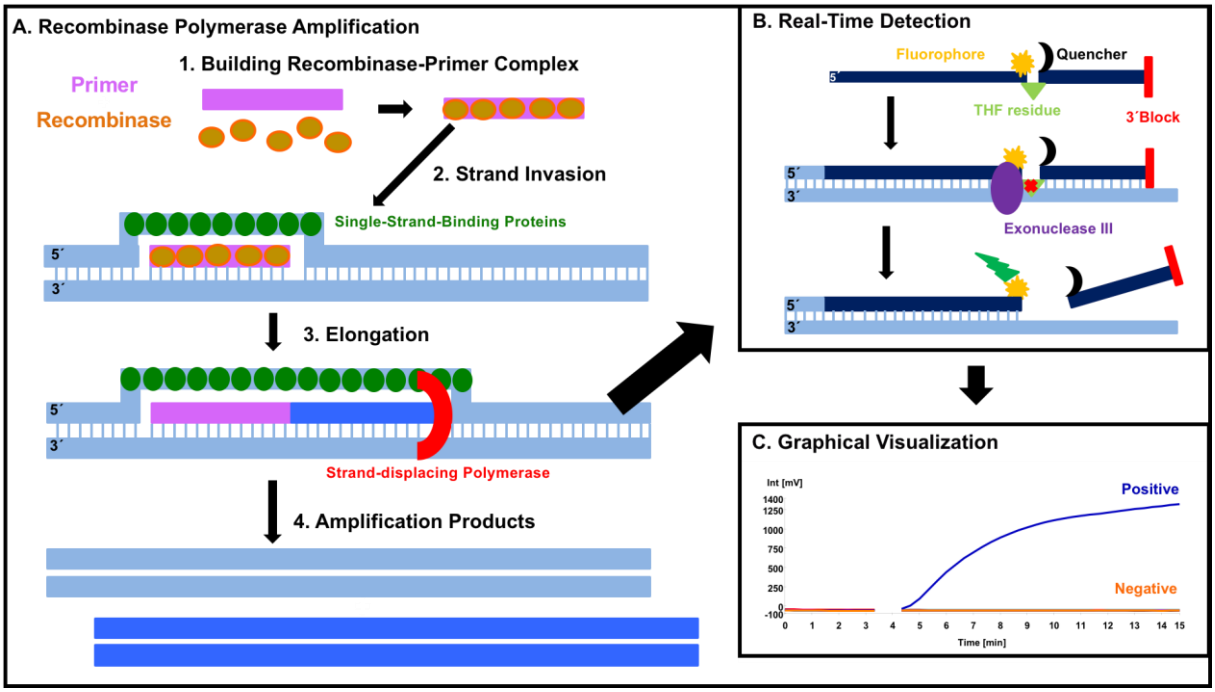

Figure 1. The RPA cycle (A) exo-probe (B) and amplification curves (C).

SEQUENCING

Using DNA amplification methods as diagnostic tools is advantageous when the species of interest is known. If the species is unknown, PCR or isothermal amplification assays reach their limits. At this point, DNA sequencing is the method of choice. The Sanger-Sequencing was first developed technology in this field (56), which evolved in Next Generation Sequencing (NSG). These technologies rely on sequencing by synthesis, in other words, the complementary DNA strand is produced

during the sequencing in order to identify the nucleotides of the target. However, they suffer from many challenges: point-of-need diagnostics is not possible; the devices cannot be transported; sample preparation is time-consuming; dependant on PCR before sequencing; a bioinformatic background is needed for analysis; and the costs are high. To overcame this difficulties, direct native DNA sequencing was achieved by pathing through a pore of nano-diameter in size *via* Oxford nanopore sequencing technology. Due to the size of the sequencing device MinION, this technology is applicable at point-of-need (57). Furthermore, library preparation can be performed without PCR steps and real-time basecalling is possible (58). Therefore, this technology is suited for a metagenomic sequencing for the identification of animal species in meat products on-site.

AIM OF THE WORK

The scope of this thesis is to develop point-of-need detection systems based on the RPA for the identification of animal species. The major part was to establish RPA assays for the detection of pork, horse, chicken, turkey, sheep and beef DNA in meat products. This should shorten “sample in” to “results out” during meat production, whereby the protection of consumer is guaranteed. Moreover, the developed species RPA assays can be implemented as internal positive control to assure the quality of the molecular diagnostics. An example was the deployment of the turkey RPA assay as an internal positive control in a duplex assay for the detection of avian influenza H5. Furthermore, the RPA was used to identify possible meat contamination with emerging pathogen as Monkey poxvirus (MPXV).

Since the RPA is limited to 6 targets as well as the need to screening for unpredictable meat adulteration or contamination, a rapid sequencing protocol deploying Oxford nanopore technologies was established.

These studies were performed to advance rapid point-of-need technologies especially in the field of surveillance of the meat production and consumption.

2 STUDIES PERFORMED

2.1 MANUSCRIPT I

Recombinase polymerase amplification assays for the identification of pork and horsemeat

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Analytical Methods

Recombinase polymerase amplification assays for the identification of pork and horsemeat

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ABSTRACT

Detection of animal species in meat product is crucial to prevent adulterated and unnecessary contamination during processing. Gold standard is the real-time PCR assays, which can be conducted at highly equipped laboratories. Toward the development of point-of-need test, two rapid molecular assays based on recombinase polymerase amplification (RPA) for the detection of pork and horse DNA were established. Target genes are the porcine mitochondrial ND2 and equine ATP 6–8 genes. The pork and horse_RPA assays detected 16 and one DNA molecules/μl in eleven to six minutes, respectively. The myoglobin in the meat did not influence the assays performances, while the presence of high background-DNA induced a one log decrease in the sensitivity. Both assays are highly specific and identify down to 0.1% of their target DNA in meat mixtures. Both RPA assays could be used on-site as a rapid and mobile detection system to determine contamination of meat products.

1. Introduction

Meat adulteration and fraud in its products are crucial issues in the food industry (Everstine, Spink, & Kennedy, 2013). Meat of higher quality is mixed with that of lower value without labelling (O'Mahony, 2013). In a huge meat adulteration scandal in 2013, several beef burger products contained pork and horsemeat (O'Mahony, 2013). Beside the consumer fraud, adulteration of meat products can entail health, social and religious consequences (Nakyinsige, Man, & Sazili, 2012).

Several detection methods like ELISA (Hsieh & Ofori, 2014), PCR (Ren, Deng, Huang, Chen, & Ge, 2017) and real-time PCR assays (Dooley, Paine, Garrett, & Brown, 2004) are available. Real-time PCR assays are used as gold standard method due to their high sensitivity and specificity (Mafra, Ferreira, & Oliveira, 2008). Nevertheless, this method is time-consuming, needs highly equipped laboratories and trained personal for the handling. On other hand, isothermal DNA amplification methods are promising technologies to implement molecular diagnostic at the point of need outside a diagnostic facility. One of these is the recombinase polymerase amplification (RPA) assay. This assay is based on enzymes, whereby the reaction is carried out between 39 and 42 °C and can produce results in a maximum of 15 min. The DNA amplification is initiated by the recombinase binding to the target specific primers (length from 30 to 35 base pairs). This complex invades the DNA double strand at the complementary site of the primers sequence. The resulting single strands are stabilized with single strand binding proteins, which tie up the opposite DNA strand. Thereby, any interference during the strand extension by strand displacing DNA polymerase is avoided (Piepenburg, Williams, Stemple, & Armes, 2006). For real-time detection, an *exo*-probe was applied to the RPA reaction. Upon binding to its complementary sequence, the *exo*-probe is sliced by an exonuclease leading to the release of a fluorescence signals, which can be measured by a portable device.

This study aimed to the establishment of two RPA assays for the real-time detection of pork and horse mitochondrial ND2 and ATP 6–8 genes, respectively, in order to identify the possible meat adulterations.

2. Material and methods

2.1. Molecular standard and genomic DNA

A molecular DNA standard containing both pork mitochondrial ND2 (GenBank accession number NC_000845, nt 4958–5318) and horse mitochondrial ATP 6–8 gene sequences (NC_001640, nt 7863–8124) was synthesized by GENEART AG (Regensburg, Germany). To determine the assay analytical sensitivity, a serial tenfold dilution (10^6–10^0 DNA molecules/μl) of the molecular standard was prepared.

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In addition, genomic DNA of pork and horse was purchased from Eurofins GeneScan Technologies GmbH (Freiburg, Germany) to determine the analytical sensitivity. The genomic DNA was diluted to 1000, 500, 100, 50, 10, 5 and 1 pg, which equal 3.7×10^{2} to 3.7×10^{-1} DNA molecules/reaction.

2.2. RPA oligonucleotides and condition

Pork and horse_RPA assays were designed to detect the mitochondrial ND2 and ATP 6–8 gene regions, respectively. For the pork_RPA assay, 20 combinations based on 5 forward and 4 reverse primers were tested to select the best oligos, which produce the highest RPA assay sensitivity and specificity, while 9 combinations were examined in the horse_RPA assay (Fig. S1). The *exo*-probe of the pork_RPA assay was designed in the same direction of the forward primer, while for the horse_RPA assay the reverse complementary sequence was used. This was done to avoid possible primer dimer between the hores_RPA forward primer and *exo*-probe, since the target sequence shows multiple repeats of adenine at the 5́-end and of thymine at the 3́-end (Fig. S1). All oligonucleotides were purchased from TIB MOLBIOL (Berlin, Germany). The TwistAmp Exo kits containing lyophilized RPA reagents (TwistDx Ltd, Cambridge, UK) were used as described previously (Hansen, Schafer, Fechner, Czerny, & Abd El Wahed, 2016; Kissenkotter et al., 2018). RPA assays were conducted in a solar powered suitcase lab (Fig. 1). TS1 reader (ESEquant Tubescanner, QIAGEN Lake Constance GmbH, Stockach, Germany) was used to collect and record the fluorescence signals. The threshold time was calculated by the tubescanner Studio Software (version 2.07.06).

2.3. Specificity and cross reactivity

To determine specificity and cross reactivity, the genomic DNA of cattle, chicken, donkey, turkey, duck, sheep, goat and rabbit in addition to the genomic pork and horse DNA were obtained from Eurofins GeneScan Technologies GmbH (Freiburg, Germany). In addition, vacuum-packed meat of eight animals (pork, cattle, chicken, turkey, duck, horse, lamb and rabbit) was purchased from a supermarket chain. The DNA was extracted as follows: 50 mg of each meat sample was mixed with 160 µl molecular biology grad H_2O in a precellys tube (Bertin Technologies, Montigny-le-Bretonneux, France) and then homogenised in the Precellys 24 (Bertin Technologies, Montigny-le-Bretonneux, France) at 6500 rpm for 30 s. Thereafter, QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany) were performed as described by the manufacturer.

2.4. Influence of red colour and background DNA on the performance of RPA assays

To determine if red colour of the meat juice could influence the performance of the RPA assays, 9 µl of beef juice was spiked with 1 µl of either pork or horse molecular DNA standard at concentrations of 10^{2} –10^{0} DNA molecules/µl.

To estimate the assays performance in presence of background DNA in the sample, DNA mixtures were prepared as follows: 4 µl of extracted DNA of either pork or horsemeat was spiked with 1 µl of the molecular DNA standard with concentrations 10^{2} to 10^{0} DNA molecules/µl (equivalent to 1.5–0.0015% DNA standard in one sample). For the validation of the pork_RPA assay, the samples with extracted horse DNA as background were used and for the horse_assay, the extracted pork DNA. The tests were performed in triplicates.

2.5. Fresh meat and salami mixtures

The ability of both RPA assays to detect target DNA in minced meat mixture and meat-products was determined using either 50 mg beef minced meat or salami spiked with either 10, 5, 1, 0.5 or 0.1% pork or horse meat. The meat and salami were purchased from a local supermarket chain. DNA extraction and RPA reaction were performed as described above (2.2 and 2.3).

2.6. Statistical analysis

A semi-logarithmic regression of the data set of the five RPA runs on 10^{6}–10^{0} DNA molecular standard and of the five runs on the genomic DNA 3.7×10^{2} to 3.7×10^{-1} DNA molecules/reaction was calculated with GraphPad PRISM version 6.07 software (GraphPad Software Inc., San Diego, California). Using STATISTICA software (StatSoft, Hamburg, Germany), from the section “higher models” the non-linear regression function “probit-regression” was chosen. For each used concentration, the logarithmic value was calculated and for each concentration the count of positive or negative hits were determined. A positive hit was assigned as 1, while a negative hit as 0. Using these variables, the probit- regression analyses was conducted and an exponential growth curve was produced to determine the limit of detection in 95% of the

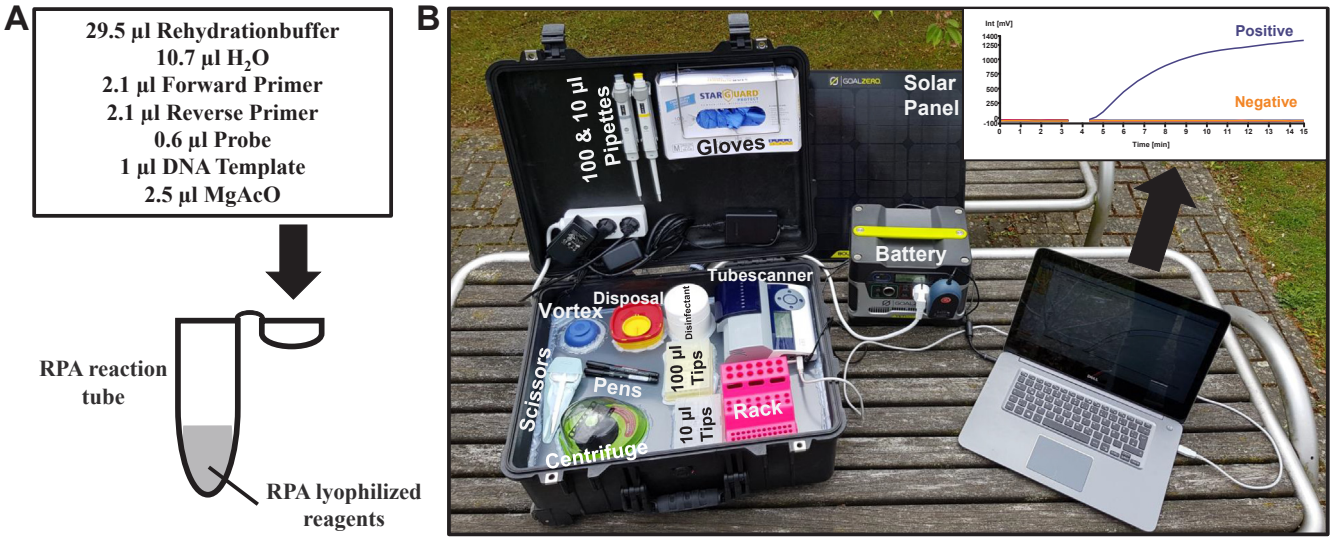

Fig. 1. Procedure of the detection of pork and horse DNA from meat products using a mobile suitcase lab. A: The RPA reaction, B: Mobile suitcase lab. All RPA needed reagents and equipment exist in a solar battery powered suitcase lab. The RPA results can be visualized in real-time due to the highly specific *exo*-probe, top right of B.

cases.

For the influence of red colour experiment, from each concentration, the means of the detection times of the analytical sensitivity of the samples with the meat juice and of the ones with background DNA were compared by performing a *t*-test by calculating an ANOVA ($p = 0.05$) analysing differences in variance.

3. Results & discussion

3.1. Selection of RPA primer and probe

For both RPA assays, mitochondrial genes were selected as target genes because of the high number of copies within a cell and unique sequences. Therefore, mitochondrial ND 2 and ATP 6–8 genes were preferred for species-specific primer and probe design for pork and horse_RPA assay, respectively (Fig. S1). The same region was targeted by a highly sensitive and specific real-time PCR assays (Kesmen, Gulluce, Sahin, & Yetim, 2009). In order to select sensitive RPA oligonucleotides, all possible primer combinations were screened using pork and horse molecular DNA standard at a concentration of 10^5 molecules/µl (Fig. S2). As a result, the primer combinations FP1 + RP4 (amplicon = 152 nt) and FP2 + RP3 (amplicon = 168 nt) revealed the best amplification curves for the pork and horse_RPA assays, respectively (Table 1). These combinations were then selected for further assay validation. All other primer combinations showed either a low or late fluoresces signal or build primer-dimer.

3.2. Analytical sensitivity

To determine the speed and limit of detection of the RPA assays, five RPA runs were performed on pork and horse molecular DNA standard with concentrations of 10^6 –10^0 DNA molecules/reaction. Both horse and pork_RPA assays required between eleven to 13.3 min to amplify and detect one or ten DNA molecules/reaction, respectively (Figs. 2 and 3A, S6A). Furthermore, by applying the probit regression analysis, the limit of detection in 95% of the cases for the pork_RPA assay was 22 DNA molecules/reaction (=59 pg), while the detection limit for the horse_RPA assay was two DNA molecule/reaction (=5.4 pg) (Fig. 3C). The well-established real-time PCR assays targeting the same genome region for detection of pork and horse DNA (Kesmen et al., 2009) detected down to 100 fg of DNA.

The same was done for the dilution range of 3.7×10^2–3.7×10^{-1} genomic DNA molecules/reaction. As 1 pg (=0.37 genomic DNA molecules/reaction) was detected in 5.3 to 7.5 min by the pork and horse RPA assays, respectively (Figs. 2, 3B). Applying a probit regression analysis to these data, the limit of detection in 95% of the cases was 1.23 pg (=0.45 genomic DNA molecules/reaction) and 1.28 pg (=0.47 genomic DNA molecules/reaction) for the pork and horse assay, respectively.

Two RPA assays for the detection of pork DNA were established (Cao et al., 2018; Szántó-Egész et al., 2016). Despite the use of the RPA technology in all studies, the DNA target and endpoint detection were completely different from our developed assays. In a study by Szántó-Egész et al., the Mangalica pork breed was identified by an amplification step using RPA and a detection step on lateral flow strip. Test time is around 35 min and a dilution step was necessary to transfer the amplified product to the lateral flow strip (Szántó-Egész et al., 2016). The assay has the advantage of not using a fluorescence reader, but the possibility of cross contamination due to the dilution step is high (Mondal et al., 2016). In another study, RPA assay for the identification of pig $_D$-loop DNA gene was achieved by RPA amplification step and a direct visualisation after adding SYBR Green I (Cao et al., 2018). The assay run time was more than 40 min as three procedures must be accomplished before reading the results (RPA amplification, amplicon purification and the SYBR Green addition). In contrast, our developed pork RPA assay produced results in a maximum of 15 min and there is no need to open the tube after adding the samples. Moreover, the data acquisition in real-time was conceivable *via* the fluorophore labelled *exo*-probe. Nevertheless, a fluorescence reader was crucial to conduct the experiment.

Other highly sensitive assays based on the Loop mediated isothermal amplification (LAMP) technology with analytical sensitivities ranging from 100 to 0.1 pg were established (Lee, Kim, Hong, & Kim, 2016; Ran et al., 2016; Zahradnik et al., 2015). The LAMP assays run time were around 60 min. Furthermore, a heating step of 95 °C before starting the LAMP reaction was required (Abdulmawjood et al., 2014; Liu, Shi, Teng, Wu, & Zhang, 2017; Ma, Dai, Fang, Wu, & Zhang, 2016; Yang et al., 2014). The amplification in LAMP assay require six primers which make the assay development is very complex, while the RPA assay utilized two primers for the amplification.

3.3. Cross-reactivity

Alignment of the pork and horse amplicons with the corresponding sequences of the other species showed the presence of mismatches within the primer and probe regions (Fig. S3). This was very important to avoid any non-specific amplification and detection, particularly, the amplification in the RPA is achieved in the presence of up to 5 to 11 mismatches in primer and probe regions (Abd El Wahed et al., 2013; Boyle et al., 2013; Kissenkotter et al., 2018; Patel et al., 2016). The lowest number of mismatches (n = 8) exists in the sequence of the donkey ATP 6–8 gene aligned with the horse amplicon. Nevertheless, the horse_RPA assay did not amplify the donkey genome (Table 2). All other species have more than 11 mismatches in the region of the pork and horse amplicons and therefore no cross-reactivity was recognized as shown in Table 2. While, the real-time PCR specific horse primers exhibit minimum cross-reaction to pork meat (Kesmen et al., 2009).

3.4. Influence of red colour and background DNA in samples on the performance of RPA assays

To validate the influence of the red colour of the meat juice on the RPA performances, beef juice was spiked with the corresponding pork or horse molecular standard DNA with a dilution range of 10^2 to 10^0 molecules/µl. In both assays, the detection time was not influenced significantly by the red colour of the meat juice. A performed ANOVA

Table 1
RPA primers and *exo*-probe combination yielding the highest analytical sensitivity in the pork and horse_RPA assays. QTF are sites of the quencher and fluorophore in the order quencher BHQ1-dt (Q), Tetrahydrofuran (T) and Fam-dT (F).

Name	Sequence (5′ to 3′)
PORK_RPA P1	CTAGTAATAATCAGCTCACACTGACTACTCA**QTF**GAATCGGATTCGAA
PORK_RPA FP1	CTACCCTTATCATAACAGTAATGTCCGGAACCAT
PORK_RPA RP4	TGTGGCTGCTTCTGTGGCTCGTG
HORSE_RPA P1	TGCTGGGAAATATGA**QGTF**CAGAATTACAATAGGGAGGCCTACTATTGT
HORSE_RPA FP2	CTGCCCCTTGAGAATCAAAATGAACGAAAATC
HORSE_RPA RP3	CTAGCCATTGTTGAATTGAGATTAGGCGATTGT

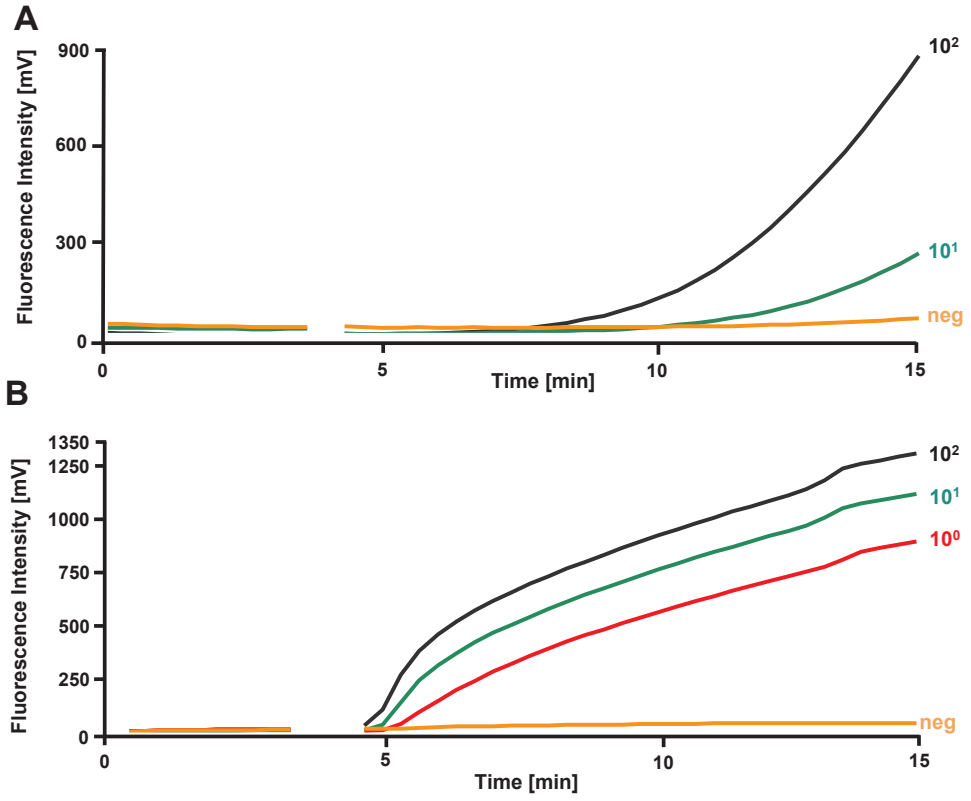

Fig. 2. The analytic sensitivity of pork (A) and horse_RPA assays (B). The primer combination FP1 + RP4 and FP2 + RP3 were used for pork and horse assays, respectively. The RPA assays limit of detection were determined with a dilution range of molecular DNA standard from 10^2 down to 10^0 molecules/µl. The pork_RPA assay detected down to 10 DNA molecules/reaction, while the horse assay identified one DNA molecule/reaction. After 4 min, the tubes were mixed and centrifuged, therefore, a gap appears in the graphs.

($p = 0.05$) revealed no difference between the means of the detection times of red coloured fluid to the means of clear one (Table 3). Indeed, the red colour causing myoglobin (Martens, Martens, & Stabursvik, 1982) did not influenced the RPA assays performances, likewise, haemoglobin, heparin and ethanol (Kersting, Rausch, Bier, & von Nickisch-Rosenegk, 2014). This is advantageous for the RPA as a simple lysis buffer can be used to extract the DNA without any further lengthy purification steps.

RPA reaction can be inhibited by the presence of high amount of DNA in the sample (Rohrman & Richards-Kortum, 2015). The pork_RPA assay performance in the presence of background DNA in the sample were tested by spiking 10^2 to 10^0 DNA molecules/µl or 1.5 to 0.0015% pork molecular standard DNA in extracts from horsemeat. The same was done to validate the horse_RPA assay. The background DNA significantly influenced the assays performances at the lowest concentration of 10^0 DNA molecules/µl. The effect was obvious in the pork_RPA assay (0/3 detected) more than the horse_RPA assay (1/3 detected). The assays run time was also altered particularly at the lower DNA concentration. A one-log decrease in the assays sensitivities was recorded, but this did not influence the overall lab performance of the assays, see below.

3.5. Fresh meat and salami mixtures

Fifty milligrams of minced beef meat or beef salami was mixed with 10, 5, 1, 0.5 or 0.1% of pork or horsemeat to validation the RPA assays. Both, pork and horse_RPA assays can detect a contamination with meat of their target species down to 0.1% in both minced meat (Fig. S4) and salami products (Fig. S5). According to the German Federal Office of Consumer Protection and Food Safety, 0.1% considered as the lower limit of detection of meat contamination (German Food and Feed Code §64 (LFGB)).

4. Conclusion

Determination of the animal sources in meat and meat products is crucial to prevent adulteration and fraud (Everstine et al., 2013). Therefore, several molecular techniques based on DNA detection were developed (Mafra et al., 2008). These assays are time consuming and only realisable in high-equipped laboratories. In this study, detection assays for pork and horse DNA based on RPA were developed and can be performed using portable devices (Abd El Wahed, Weidmann, & Hufert, 2015).

The developed RPA assays are highly sensitive and specific for the detection of pork and horse DNA in 15 min, which make it ideal for rapid detection of meat contaminations and adulteration. Moreover, the meat juice and background DNA do not hinder the performances of the RPA assays as down to 0.1% contamination with pork and horsemeat in beef and its products was detected. Thus, combination of the RPA assay with rapid homogenisation and DNA extraction protocols will be advantageous. Whereby possible adulterations of meat products would be detected faster and at point of need.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Jonas Kissenkötter, Susanne Böhlken-Fascher, Claus-Peter Czerny and Ahmed Abd El Wahed declare no conflict

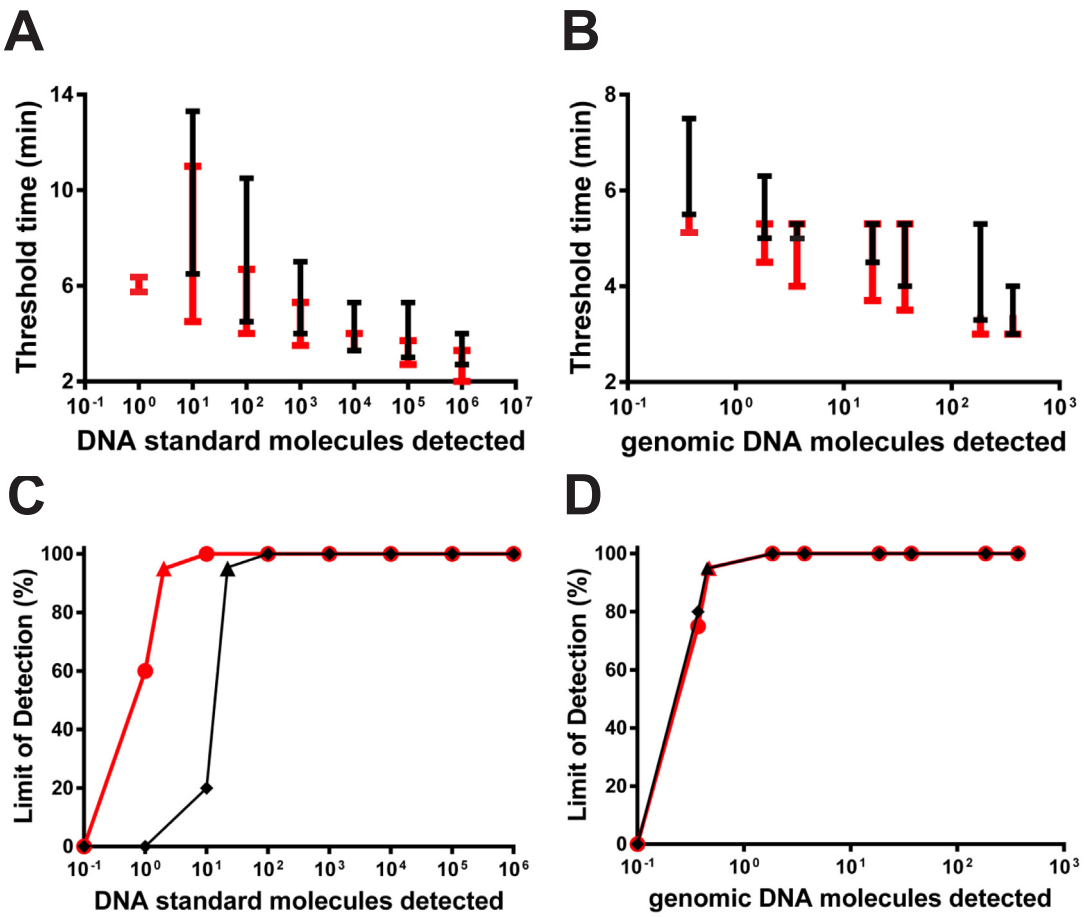

Fig. 3. The repeatability (A, B) and probit regression analysis (C, D) of the pork (black) and horse_RPA assays (red) determined with a DNA molecular standard (A, C) and genomic DNA (B, D). Using Prism Software, a semi-logarithmic regression of the threshold time data set of five runs of the pork and horse_RPA assays on a dilution range of the DNA molecular standard (10^6 –10^0 DNA standard molecules/reaction, A) and of genomic control DNA (3.7×10^2–10^{-1} genomic DNA molecules/reaction, B) were performed. The lowest concentration of the molecular standard were detected in 13.3 and 11 min, while that of the genomic control DNA were 7.5 and 5.3 min by the pork and horse RPA assays, respectively. In the pork_RPA assay, 10^6–10^2 DNA standard molecules were detected in 5/5, 10^1 in 4/5 and 10^0 in 0/5 RPA runs, while 3.7×10^2–1.85×10^1 genomic DNA were detected in 5/5 and 3.7×10^{-1} in 4/5. In the horse assay, 10^6–10^1 DNA standard molecules were detected in 5/5 and 10^0 in 4/5 RPA runs. As 3.7×10^2–1.85×10^1 produce 4/5 and 3.7×10^{-1} in 3/5 positive results using the horse assay. These data was applied for the probit regression analysis showed in C and D. Using STATISTICA software, the limit of detection at 95% probability were 22 DNA standard molecules/reaction and 0.45×10^0 genomic DNA molecules/reaction in the pork_RPA assay, while the horse assay detected 2 DNA molecule/reaction and 0.47×10^0 genomic DNA molecules/reaction.

Table 2
Specificity of the RPA assays. Genomic DNA (Eurofins GeneScan Technology GmbH, Freiburg, Germany) and extracted DNA from various animal species were tested to determine the specificity of the pork and horse_RPA assays. Both assays did not show any cross reactivity and only detected their target genome.

Organism	Pork_RPA assay		Horse_RPA assay	
	Genomic DNA	Extracted DNA	Genomic DNA	Extracted DNA
Sus scorfa domestica	+	+	–	–
Bos taurus	–	–	–	–
Gallus gallus	–	–	–	–
Meleagris galllopavo	–	–	–	–
Anas platyrhynchos	–	–	–	–
Equus caballus	–	–	+	+
Equus asinus	–	not tested[1]	–	not tested[1]
Ovis aries	–	–	–	–
Capra hircus	–	not tested[1]	–	not tested[1]
Oryctolagus cuniculus	–	–	–	–

[1] Meat of this animal sources could not be obtained with the pure quality, which was needed for the experiments.

Table 3
Influence of the red colour of the meat juice and background DNA on the performance of the pork and horse_RPA assays. Mean values of threshold time were compared by performing a *t*-test by following an ANOVA analysing differences in variance. Significant difference was recorded only for the horsemeat at a molecular concentration of one DNA molecule/reaction in the presence of high background DNA.

Concentration [molecules/µl]	Pork_RPA assay [minutes]			Horse_RPA assay [minutes]		
	Analytical sensitivity	Meat juice	Background DNA	Analytical sensitivity	Meat juice	Background DNA
10^2	8.125	8.67	9.47	5.4	5.3	5.3
10^1	10	11.5	11.57	5.47	5.43	5.67
10^0	[11]	[11.3]	negative	5.54	5.5	[14]*

[ ] DNA concentration was detected only one time out of all performed runs.
* significantly difference.

of interest. Matthew S. Forrest and Olaf Piepenburg were employees at TwistDx Ltd. RPA technology is subject to background IP protection.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.126759.

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2.2 MANUSCRIPT II

Recombinase polymerase amplification assay for rapid detection of Monkeypox virus

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ABSTRACT

In this study, a rapid method for the detection of Central and West Africa clades of Monkeypox virus (MPXV) using recombinase polymerase amplification (RPA) assay targeting the G2R gene was developed. MPXV, an *Orthopoxvirus*, is a zoonotic dsDNA virus, which is listed as a biothreat agent. RPA was operated at a single constant temperature of 42°C and produced results within 3 to 10 minutes. The MPXV-RPA-assay was highly sensitive with a limit of detection of 16 DNA molecules/μl. The clinical performance of the MPXV-RPA-assay was tested using 47 sera and whole blood samples from humans collected during the recent MPXV outbreak in Nigeria as well as 48 plasma samples from monkeys some of which were experimentally infected with MPXV. The specificity of the MPXV-RPA-assay was 100% (50/50), while the sensitivity was 95% (43/45). This new MPXV-RPA-assay is fast and can be easily utilised at low resource settings using a solar powered mobile suitcase laboratory.

1. Introduction

Monkeypox virus (MPXV) belongs to the genus *Orthopoxvirus* (OPXV, subfamily *Chordopoxvirinae*, family *Poxviridae*), which is an enveloped double stranded DNA virus (Parker et al., 2007). It is subdivided into two clades: the West African and the Congo Basin clades. The latter is more pathogenic (Likos et al., 2005) and the clinical signs of MPXV infections are similar to that of smallpox but in a milder form and with lower mortality (1 to 10%). The majority of deaths occurs at a young age due to the lack of immunization (Khodakevich et al., 1988). Rodents (Squirrels and Gambian rats) are the primary hosts (Falendysz et al., 2015, 2017), which can transmit the virus to monkeys and humans through direct contact with blood and bodily fluids (Nolen et al., 2015). The handling and consumption of infected monkeys and squirrels were documented as major infection sources in Africa (Cantlay et al., 2017). Furthermore, human-to-human transmission can occur through exposure to fomites and air droplets (Fleischauer et al., 2005). A specific vaccine for use in humans is not available, but cross protection in humans vaccinated against smallpox has been documented (Rimoin et al., 2010). This protection however, has been waning because when smallpox was declared eradicated in 1980, nationwide vaccination against smallpox has stopped (Breman and Henderson, 2002). The antiviral tecovirimat for treatment of accidental smallpox infections has been shown to reduce symptoms and to improve survival of MPXV infected macaques if applied up to 5 days post infection (Russo et al., 2018).

Human MPXV infections are endemic in West and Central Africa (McCollum and Damon, 2014). The first MPXV outbreak outside Africa was reported in 2003 in the USA after the shipment of animals from Ghana (Di Giulio and Eckburg, 2004). The latest outbreak was in Nigeria with 113 laboratory confirmed cases and seven deaths from September 2017 until August 2018 (Yinka-Ogunleye et al., 2018). Two recent zoonotic MPVX infections imported in the UK highlight ongoing MPXV activity in Nigeria (Vaughan et al., 2018).

Several diagnostic methods for the detection of MPXV are established with real-time PCR as the gold standard because of its high sensitivity and specificity (Li et al., 2006). To use this diagnostic tool, a highly equipped laboratory and specialized technicians are needed, which are not available in areas where MPXV infections

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occur. Therefore, an easy to handle simple molecular diagnostic method would improve the detection and surveillance of MPXV. Isothermal amplification methods have been proven to be an alternative to real-time PCR. Recombinase polymerase amplification (RPA) is one of these methods, in which an enzymatic based DNA amplification can be achieved at a temperature range of 37 to 42°C within 15 minutes (Piepenburg et al., 2006). The amplification is initiated by a primer-recombinase-complex. This complex invades the DNA double strand at the homologues sequences of the primer, where single-strand-binding proteins stabilize the reaction. Then, a strand-displacing polymerase DNA conducts the extension step. For real-time detection, a fluorophore/quencher-probe is used. Since RPA reagents are freeze-dried, the RPA kit can be stored at room temperature for several months. This allows the use of the RPA assay at point of need making them even more versatile through a mobile suitcase laboratory (Abd El Wahed et al., 2015a).

In this study, we have developed a rapid detection method specific for both clades of MPXV using a recombinase polymerase amplification (RPA) assay targeting the tumor necrosis factor (TNF) binding protein gene, which is present in duplicate as ORF G2L and G2R in the inverted terminal repeats of the MPXV genome.

2. Materials and Methods

2.1. Molecular MPXV DNA Standard and RPA Oligonucleotide

For assay validation, a molecular DNA standard based on 300 bp of the TNF binding protein gene (ORF: G2R, Accession number: DQ011153, nucleotides: 195915 - 196964), was synthesized by GeneArt (Regensburg, Germany). Three forward primers (FP), three reverse primers (RP) and one exo-probe were designed (Fig. S1). All oligos were synthesized by TIB MOLBIOL GmbH (Berlin, Germany).

2.2. RPA Assay Conditions

The TwistAmp exo kit (TwistDx Ltd, Cambridge, UK) was used. Per reaction, 29.5 μl rehydration buffer, 10.7 μl H_2O, 2.1 μl of each primer (10 μM) and 0.6 μl of 10 μM exo-probe were added into the lid of the reaction tube containing the freeze-dried pellet. After adding 2.5 μl of 280 mM magnesium acetate and 1 μl template, the reaction mixture was centrifuged, mixed, centrifuged and placed immediately into the tube scanner ESEQuant (QIAGEN Lake Constance GmbH, Stockach, Germany). The reaction was incubated at 42°C for 15 minutes. To increase the sensitivity, a mixing and centrifugation step was performed after 230 seconds of starting the measurement. A positive result was measured by the FAM channel of the ESEQuant tube scanner and analysed with the Tubescanner studio software (version 2.07.06, QIAGEN Lake Constance GmbH, Stockach, Germany).

2.3. MPXV RPA Assay Analytical Sensitivity

In total, nine primer combinations were tested with the MPXV DNA standard with concentration of 10^5 DNA molecules/μl. The best combination, which produced the earliest and highest fluorescence signal, was selected for further assay validation. The ability of the selected primer combination to amplify 10^4 to 1 DNA molecules/μl of the MPXV standard DNA was checked in order to test the analytical sensitivity and to determine the limit of detection.

2.4. MPXV RPA assay cross reactivity

The specificity of the MPXV-RPA-assay was tested with DNA of viruses of the two MPXV clades, six other poxviruses and other pathogens of clinical importance, see Table 1.

2.5. Clinical samples

The MPXV-RPA-assay performance was validated with plasma samples of infected (n=25) and uninfected (n=23) monkeys. The animals were looked after by experienced personnel from the German Primate Center and kept according to the German Animal Welfare Act, which is in compliance with the European Union Guidelines on the use of non-human primates for biological research and the Weatherall report. Sampling from MPXV-infected monkeys was approved by the Lower Saxony State Office of Consumer Production and Food Safety with the project license 33.9.42502-04/019/07, that from uninfected animals with the project license 33.9.42502-04-15/1769. In addition, 20 positive (4 whole blood, 16 serum) and 27 negative (8 whole blood, 19 serum) human samples from the recent MPXV outbreak in Nigeria (November 2017) were tested with the RPA-MPXV-assay. The samples were collected for diagnostics purposes and handled anonymously. The DNA from these samples was isolated using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer instructions.

2.6. Real-time PCR

For comparison, the molecular DNA standards as well as clinical samples were tested with a reference MPXV real-time PCR assay

Table 1
Reactivity of the MPXV_RPA assay to the genome of poxviruses and other pathogens. MPXV_RPA assay detected both clades of MPXV, but not other poxviruses and pathogens.

Pathogen	Clade/ Source	Concentration [ng/μl]	RPA	Real-time PCR
Monkeypox	Central Africa		+	+
Monkeypox	West Africa		+	+
Vaccinia	Elstree	7.6	-	-
Cowpox	2	3.6	-	-
Camelpox	-	18	-	-
Sheeppox	Russia	4.6	-	-
Goatpox	India	3.1	-	-
Orf	Burghessler	3	-	-
Calpox virus	-	6.1	-	-
Herpes-simplex-Virus 1	Quality Control for Molecular Diagnostics (QCMD)	1.7	-	-
Herpes-simplex-Virus 2		3.6	-	-
Varicella-zoster Virus		3.1	-	-
Staphylococcus aureus	DSMZ ID: 1104	4.2	-	-
Clostridium perfringes	DSMZ ID: 756	40.2	-	-
Enterococcus faecialis	DSMZ ID: 20478	35.2	-	-
Plasmodium falciparum	University of Ibadan, Nigeria	2.8	-	-
Rickettsia rickettsia	BNITM Hamburg, Germany	4.7	-	-
Rickettsia africae		4.3	-	-

Table 2
RPA primers and exo-probe combination, yielding the earliest and highest signal in the MPXV RPA assay. QTF are sites of the quencher and fluorophore in the following order BHQ1-dt (Q), Tetrahydrofuran (T) and Fam-dT (F).

Name	Sequence (5´ to 3´)
MPXV RPA P1	ACAGAAGCCGTAATCTATGTTGTCTATCG**QTF**CCTCCGGGAACTTA
MPXV RPA FP3	AATAAACGGAAGAGATATAGCACCACATGCAC
MPXV RPA RP3	GTGAGATGTAAAGGTATCCGAACCACACG

targeting the same gene region of the developed RPA assay (Li et al., 2010). The G2R-G real-time PCR assay detects both MPXV clades and the real-time PCR reaction was performed as described previously (Kissenkotter et al., 2018) using the LightCycler DNA-Master HybProbe kit and the LightCycler 480 (Roche Mannheim, Germany).

2.7. Statistical Analysis

The limit of detection of the MPXV-RPA-assay was calculated by performing a probit regression analysis on the data set of eight RPA assay using STATISTICA software (StatSoft, Hamburg, Germany) in order to determine the number of DNA molecules/µl, which were detected in 95% of the cases. Furthermore, the detection time was calculated by performing a semi-logarithmic regression on the same data set with GraphPad PRISM 7 software (GraphPad Software Inc., San Diego, California).

3. Result

3.1. Selection of RPA Primers and Probe

In order to select sensitive RPA oligonucleotides, all possible primer combinations were tested using a MPXV DNA molecular standard at a concentration of 10^5 DNA molecules/µl. As a result, the primer combination FP3 + RP3 (Table 2) produced the best amplification curves (Fig. S1) and was selected for further assay validation.

3.2. Analytical Sensitivity and Specificity

To determine the analytical sensitivity, the performance of the best primer combination FP3 and RP3 was evaluated with a tenfold dilution range of the MPXV DNA standard (10^4 to 1 DNA molecules/µl, Fig. 1) in eight replicates. The MPXV-RPA-assay detected the molecular MPXV DNA standard with the concentration from 10^4 to 10^2 molecules/µl in all eight RPA runs and the concentration of 10^1 molecules/µl in four runs, while no amplification was observed in the tube containing one molecule/µl. With this data set, a probit regression analysis was performed and revealed a detection limit of 16 DNA molecules/µl in 95% of the cases (Fig. 2). Seven minutes is the maximum time needed to amplify as low as 10 DNA molecules by the MPXV RPA assay (Fig. 3). FP3 and RP3 primers were able to amplify the two clades of MPXV but did

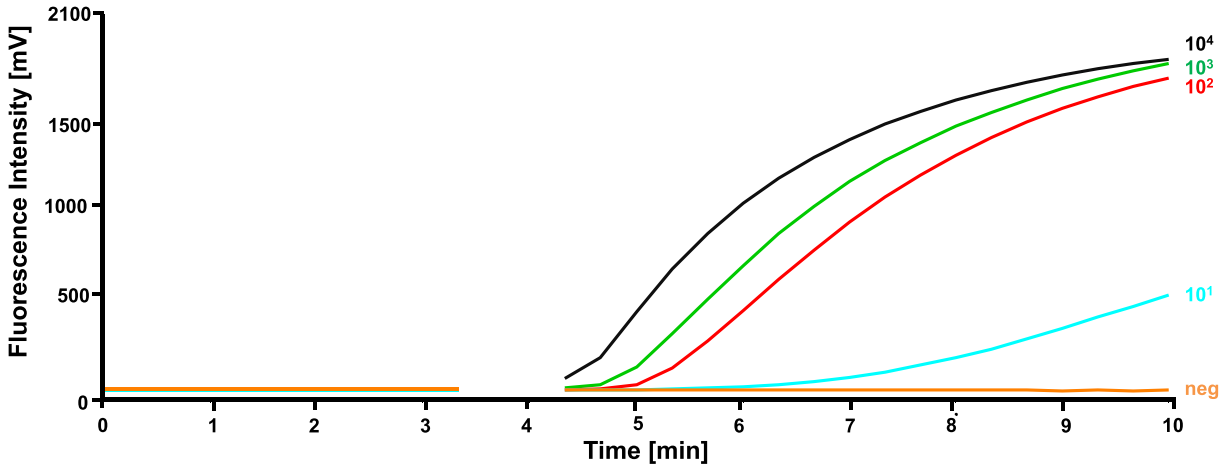

Fig. 1. Analytical sensitivity of the MPXV-RPA-assay tested with a tenfold dilution of the molecular DNA standard (10^4 – 10^0 DNA molecules/µl). The primer combination FP3 + RP3 detected the concentration 10^4 – 10^1 DNA molecules/µl. After 230 seconds a mixing step was performed.

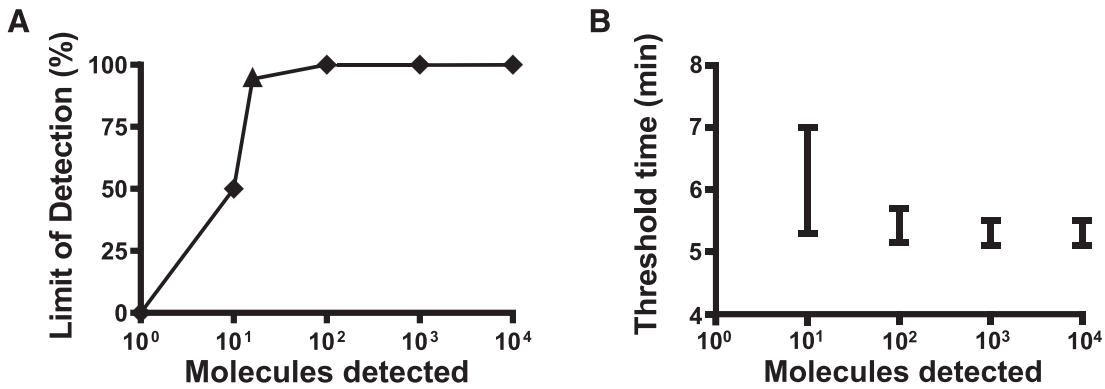

Fig. 2. Probit regression analysis of the dataset of the eight repetitions of the analytical sensitivity test of the MPXV-RPA-assay for the determination of the detection limit (A) and semi-logarithmic regression of the detection time (B). Performing the probit regression analysis on the dataset revealed a detection limit of 16 DNA molecules/µl in 95% of the cases (A). Using Prism Software, a semi-logarithmic regression of the data from the eight runs on a dilution range of the molecular DNA standard (10^4-10^0 DNA molecules/reaction) were performed. The lowest concentration of 10^1 DNA molecules/µl was detected after a maximum of seven minutes (B).

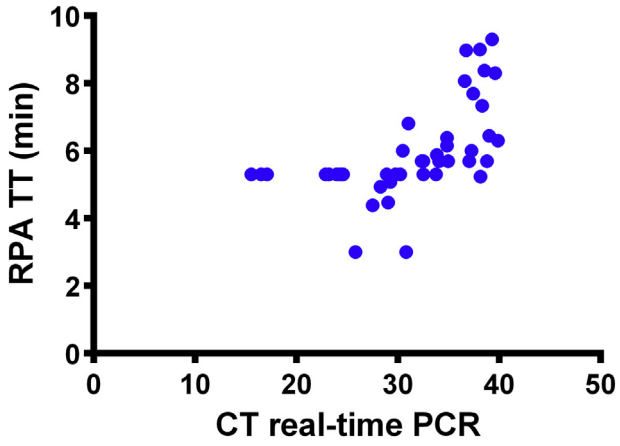

Fig. 3. Screening of 45 blood, plasma or serum samples from MXPV infected macaques and humans by real-time PCR and RPA assays. Linear regression analysis of real-time RT-PCR cycle threshold values (Ct) and RPA threshold time in minutes (TT) were determined. No correlation was found between TT and Ct values since the RPA is much faster than the real-time PCR. Diagnostic sensitivity of real-time PCR assay was 100%, while that of MPXV-RPA-assay was 95 %(43/45).

not detect high concentration DNA of related poxviruses or other pathogens (Table 1).

3.3. Clinical Samples

All collected samples were screened in parallel with both real-time PCR and the RPA assays. Employing the real-time PCR assay, all 45 samples tested positive, while by the RPA assay 43/45 were identified as positive. Fifty samples (23 monkey plasma and 27 human serum and whole blood samples) were negative in both methods. With this data, the clinical specificity and sensitivity of the MPXV-RPA-assay could be calculated as 100 and 95%, respectively.

4. Discussion

Infection with MPXV occurs in West Africa and the Congo Basin (McCollum and Damon, 2014). The most affected regions suffer from limited resources, infrastructure and diagnostic capacities, beside insufficient accessibility to remote and conflict areas. Thus, identification of MPXV infected cases is difficult (Thomassen et al., 2013). Therefore, a simple point of need diagnostic test is crucial in order to limit the spread of MPXV and control the outbreaks.

Applying the MPXV-RPA-assay both the West Africa and the Congo Basin clade were detected within seven minutes with a detection limit of 16 DNA molecules/µl. The RPA oligonucleotides target the TNF receptor gene as no mismatch between both MPXV clades was identified and thus cover the currently known diversity of MPXV, while between 13-31 mismatches were identified when this sequence was compared to those of other poxviruses (Fig. S3). The number of mismatches between the targeted MPXV gene sequence and the sequences of closely related poxviruses was the key to a specific RPA assay. Two samples were negative in the RPA assay but weakly positive in real-time PCR (CT: 38.8 and 39.97). Eight samples with CT values around 38-39 and eight samples with CT values 35-37 were scored positive in the RPA. All these samples had low DNA levels and lack of positive scoring of two samples in the RPA lay within the probability of missing weak positives as shown by the probit anaylsis.

Real-time PCR assays for MPXV detection need at least 90 minutes and highly sophisticated thermal cycler (Li et al., 2010). Although freeze-dried PCR reagents are slowly becoming available (Babonneau et al., 2015), they are as yet not in widespread use, whereas the RPA kits per se are freeze-dried and stable under different environmental conditions including temperatures above 30°C (Abd El Wahed et al., 2013). This is a huge advantage in areas where highly equipped laboratories are not available. When comparing the performance of the MPXV-RPA-assay with the real-time PCR assay on clinical samples with linear regression analysis, no correlation was found between TT and Ct values since the RPA is much faster than the real-time PCR (Fig. 3). One reason for this observation for several RPA assays (Abd El Wahed et al., 2013, 2015b; Patel et al., 2016) is that the RPA reaction is optimized for maximal enzymatic activity at one temperature leading to very dynamic non linear amplification (Piepenburg et al., 2006), whereas the real-time PCR reaction depends on different temperature steps for denaturation, annealing and amplification yielding a close to exponential amplification (Deepak et al., 2007).

Another isothermal amplification assay based on loop-mediated isothermal amplification for the detection of MPXV is available (Iizuka et al., 2009). This assay has a clinical sensitivity of 72%. However, our MPXV-RPA-assay proved to be more sensitive (95 % sensitivity). The LAMP MPXV assay requires 6 primers to amplify the MPXV DNA in around 60 minutes, while RPA uses two primers and one probe producing a result within 15 minutes.

The MPXV-RPA-assay appears an appropriate assay for the point of need detection of active MPXV cases as RPA is fast, highly sensitive and specific as well as utilizing cold-chain independent reagents.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.diagmicrobio.2019.03.015.

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2.3 MANUSCRIPT III

Evaluating two approaches for using positive control in standardizing the avian influenza H5 reverse transcription recombinase polymerase amplification assay

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ABSTRACT

Highly pathogenic avian influenza H5N1 virus causes heavy losses in poultry farms worldwide. Molecular diagnostic techniques like RT-PCR and real-time RT-PCR are considered the gold standard for identification of H5 influenza viruses in clinical samples. These techniques are hampered by the need of well-equipped laboratories, large space requirement, and relatively long time-to-result. Recombinase polymerase amplification (RPA) assay represents an excellent alternative to PCR since it is more simple, rapid, economic, and portable. Reverse transcription RPA (RT-RPA) assay was recently developed for sensitive and specific detection of H5N1 virus in 6–10 min. To ensure the accuracy of the developed assay, two approaches for using a positive control were evaluated in this study. These approaches included: 1) all-in-one (internal positive control; IPC), 2) two-tubes-per-one-sample (external positive control; EPC). Sigma virus (SIGV) RNA and turkey mitochondrial DNA were tested as positive controls in both approaches. For all-in-one approach, both targets (H5 and IPC) were strongly inhibited. In contrast, very good amplification signals were obtained for the two types of EPC with no effect on the analytical sensitivity and specificity of H5 RT-RPA assay in two-tubes-per-one-sample approach. The performance of EPC-based H5 RT-RPA was further validated using 13 tracheal swabs. The results were compared to real-time RT-PCR and proved superior specificity in detecting H5N1 but not H5N8 viruses. Inclusion of EPC did not affect the aptitude of both assays in terms of sensitivity, specificity and reproducibility. In conclusion, the two-tubes-per-one-sample approach was more reliable to control the false negative results in H5 RT-RPA assay.

1. Introduction

Highly Pathogenic Avian Influenza (HPAI) is an acute contagious disease that affects domestic poultry causing severe economic losses. Infrequently, the disease can be directly transmitted from infected birds to humans and results in severe public health threats [1]. The HPAI caused by H5N1 avian influenza virus was originally developed in China in 1996, and consequently spread towards Europe and Africa crossing central and western Asia. The strains descended from this outbreak continued to spread in more than sixty countries, with emergence of several distinct phylogenetic clades [2,3].

HPAI viruses belong to genus *alphainfluenzavirus* in the family *Orthomyxoviridae*. Eight segments of negative sense single stranded RNA are complexed with the nucleoprotein and enclosed with the viral polymerase subunits in a lipid envelope. Two major glycoproteins that determine the viral antigenicity are projecting from the surface of the lipid envelope; hemagglutinin (HA) and neuraminidase (NA) [4].

Molecular techniques are the gold standard for routine identification of H5 influenza viruses in endemic countries [5]. Methods like reverse transcription PCR (RT-PCR) and quantitative RT-PCR (qRT-PCR) are demanding at the levels of infrastructure, containment and expertise. Time-to-result is relatively long [6]. Recombinase polymerase amplification (RPA) is a recently developed isothermal amplification technique that employed phage recombinase and co-factor to

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eliminate the need for denaturation and annealing steps in traditional PCR [7]. This technique combined robustness with minimum space requirement and the ability to be used at point-of-need [8]. RPA and RT-RPA were utilized for rapid detection of many viruses of veterinary importance [6,9–13]. RT-RPA was recently described for diagnosis of H5N1 virus in clinical samples in just 6–10 min. The developed assay has proven high specificity, sensitivity, reproducibility, and robustness comparable to qRT-PCR. It was able to detect as low as one copy of *in vitro* transcribed RNA Standard [11].

RPA reaction validation is a critical requirement to ensure the quality and accuracy of the test. False negative results may arise from inhibitory substances, poor performance of the enzymes, and/or incorrect RPA mixture. The use of positive controls is a common strategy to avoid false negative results in PCR (and certainly in RPA). With the aim to improve the performance of the recently developed H5 RT-RPA in field applications, positive controls were tested and compared in different settings. Sigma virus (SIGV) RNA and turkey mitochondrial DNA were evaluated using two approaches: All-in-one approach (internal positive control; IPC), and two-tube-per-sample approach (external positive control; EPC).

2. Materials and methods

2.1. Quantitative molecular standards, RPA primers and exo-probes

Influenza A H5 molecular RNA standard was synthesized by amplification of a 970 bp fragment of HA2 gene of the Egyptian H5N1 reference strain A/chicken/Egypt/1273CA/2012 using RT-PCR. The amplified fragment was ligated into pCRII vector by TA Cloning and the RNA standard was *in vitro* transcribed and quantified [11]. The quantitative RNA standard of SIGV was generated according to the method developed by Weidmann et al. [14]. Turkey DNA standard was synthesized by GeneArt (Thermofisher, Regensburg, Germany) as a short string of 300 bp of conserved *Meleagris gallopavo* mitochondrial DNA sequence. The primers and exo-probes used for detection of Influenza A H5N1 and SIGV were prepared according to Yehia et al. [11] and Euler et al. [8], respectively. Few modifications were introduced to the exo-probe used for detection of SIGV to adapt TAMRA and/or ROX channel detection (TIB Molbiol GmbH, Berlin, Germany). All primers and exo-probes (Table 1) were synthesized to fit with the requirements of Twist Amp™ exo RT kit (Twist Dx, Cambridge, UK).

2.2. RPA reaction setup and result interpretation

RPA was performed in 50 μl reaction volumes using TwistAmp™ Exo-RT kit for H5N1 influenza virus and SIGV, and TwistAmp™ Exo kit for turkey DNA (TwistDx, Cambridge, UK). The reaction mixture contained 420 nM of each RPA primer and 120 nM of the exo-probe, 14 mM of magnesium acetate, and TwistAmp rehydration buffer. One microliter of the DNA/RNA template was added to the mixture after dispensing into the reaction tubes containing dried enzyme pellets, and the reaction volume was completed by nuclease-free water. The tubes were briefly centrifuged and placed directly in ESEQuant Tube Scanner™ (Qiagen, Lake Constance, Germany) at 42 °C for 15 min. Fluorescence measurement was performed each 20 s in the FAM channel for H5N1, TAMRA or ROX channel for SIGV, and ROX channel for Turkey DNA. Specific amplification was verified by the tubescanner studio by increase in the fluorescence intensity in 1st derivative analysis and over time.

2.3. Optimization of IPC-based H5 RT-RPA assay (all-in-one approach)

The effect of including different types of non-competitive IPC on the analytical sensitivity of the previously developed H5 RT-RPA assay was evaluated in a single-tube setting. A 10-fold dilution series of the *in vitro* transcribed H5 RNA standard was prepared – in triplicates – from 10^6 to 10^0 copies/μl. The sensitivity limit was determined as the highest dilution that showed exponential amplification above the threshold of the negative control within 6–10 min. Similar dilution sets of the quantitative standards of SIGV and turkey DNA were tested in parallel for determination of the suitable concentrations for use as IPCs; highest dilutions that generated amplification curves after 6–7 min. Duplex reactions that include both H5 and IPC primers and exo-probes were prepared in groups. Each group received constant concentration of the IPC standard and a dilution series of H5 RNA standard. All groups were tested in triplicates including separate no-template-control tubes.

2.4. Optimization of EPC-based H5 RT-RPA assay (two-tube-per-sample approach)

To avoid potential assay inhibition, two separate monoplex reactions; one for H5 and the second for the positive control, were tested in parallel. The 10-fold dilution series of H5 RNA standard and the previously determined constant dilution of each positive control were assayed in single RPA runs. No-template control was included in all assays. The performance of each EPC in this approach was analyzed in triplicates within and among assays.

2.5. Validation of assay performance on clinical samples

Thirteen tracheal swabs were collected from chicken showing indicative clinical signs for infection with HPAI virus from different Egyptian governorates between 2016 and 2018. Five samples were collected from specific-pathogen-free chicken and served as negative controls. Viral RNA was isolated using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. All samples were concurrently tested using the gold standard real-time RT-PCR [11] and the EPC-based H5 RT-RPA assay. The performance of both assays in detection of H5N1 virus and positive control was compared and analyzed in terms of sensitivity, specificity, and

Table 1
Sequence of RPA primers and exo-probes.

Name	Sequence (5′- 3′)
H5 RPA-FP	TAACGGTTGTTTCGAGTTCTATCACAGATG
H5 RPA-RP	ACTTATTTCCTCTCTTTTTAATCTTGCTTC
H5 Exo-probe	GTATGGAAAGTGTAAGAAACGGAACGTA (BHQ1-dT) THF(FAM-dT)TACCCGCARTATTC-PH
SIGV RPA-FP	TGACCATCCTAACTCTGTGACATTCCAAGT
SIGV RPA-RP	GTTGACAGTGAGCTCTTGAATCTCTGGGTT
SIGV RPA-P-TAMRA	ACTGATTTCCCTCCGTGTCCTCCCGGTACCAC-(BHQ2-dT) THF(TAMRA-dT)-CCAAACTGCCGTTGTG-PH
SIGV RPA-P-ROX	ACTGATTTCCCTCCGTGTCCTCCCGGTACCAC-(BHQ2-dT) THF(ROX-dT)-CCAAACTGCCGTTGTG-PH
Turkey RPA-FP	CTAATAACAACAACCATATTCTTATCATTAACCC
Turkey RPA-RP	GCCGGCTAGAGATAGGAGTGCAAGTATTATAG
Turkey RPA-P-ROX	ATCATTAACCCAGATCAAAGTCCTGAAAC-(BHQ2-DT)THF (ROX-DT)CAACAATACTCATC-PH

FP: forward primer; RP: reverse primer; P: probe, BHQ-dT: thymidine nucleotide carrying Blackhole quencher, THF: tetrahydrofuran, FAM-dT: thymidine nucleotide carrying Fluorescein, PH: phosphate group.

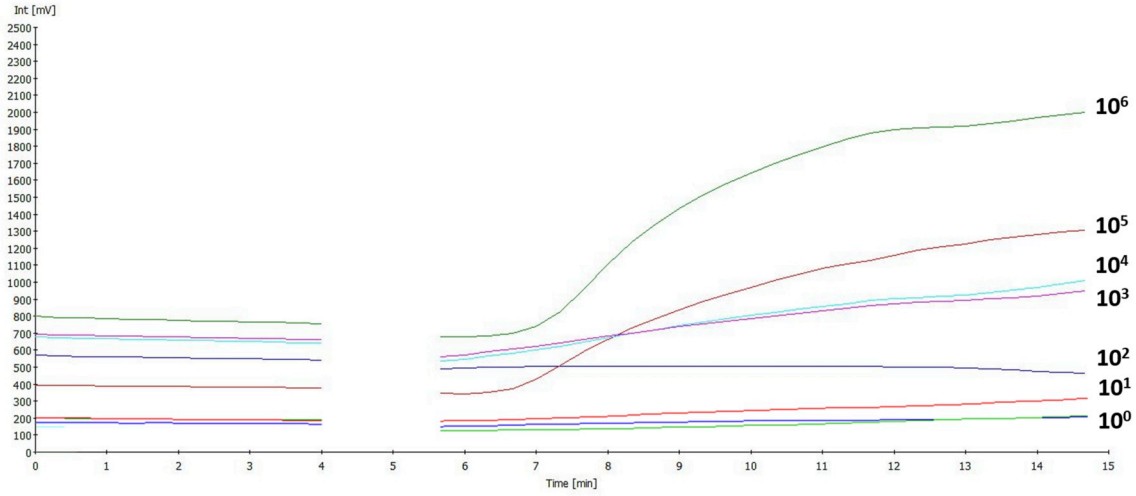

Fig. 1. Analytical sensitivity of H5 RT-RPA assay. A graph generated by ESEQuant Tube Scanner™ software showing fluorescence development overtime of the *in vitro* transcribed H5 RNA molecular standard (dilution range of 10^6 to 10^0 copies/reaction). The concentration of the RNA standard is indicated at the right side of the corresponding curve.

reproducibility.

3. Results and discussion

3.1. Analytical sensitivity of single-target (monoplex) RPA assays

The different RPA assays targeting Influenza A H5N1 virus, SIGV, and turkey DNA were independently evaluated for their performance before combined detection of multiple targets was examined. A 10-fold dilution series (10^6 to 10^0 copies/μl) of each standard was prepared in triplicates and was tested using the relevant primers and exoprobe. The analytical sensitivity of H5 RT-RPA assay ranged from 10^2-10^3 molecules per reaction (Fig. 1), whereas the detection limit in RPA assays used for detection of SIGV (either using ROX- or TAMRA-labelled exoprobes) and turkey DNA was 10^3 molecules per reaction (Table 2).

In an earlier report, the H5 RT-RPA assay was developed and detected down to a single-copy of the *in vitro* transcribed RNA standard [11]. Likewise, the detection limit of SIGV RNA standard was previously reported to range from 10^1-10^2 copies per reaction [8]. This indicates that the analytical sensitivity of the current assays are 1–3 logs lower than those described before. Since there was no specific difference between current and previous assays in terms of design, instrumentation, and personnel, it may be expected that the lower sensitivity of the current assays is regarded to: i) the use of different reagent batches; ii) the deterioration of primer and exoprobe aliquots by repeated freezing and thawing, iii) the gradual decrease in instrument aptitude over time. This observation is not surprising since even in a well established quatitative real time PCR systems, fine changes in the experimental procedures or reagent sources can drop the analytical sensitivity up to 6 folds [15]. However, validation of all reagents and instrumentation are deemed essential in reproducibility and repeatability of quatitative molecular biology experimentations including RPA [16,17].

3.2. Performance of IPC-based H5 RT-RPA (duplex) assays

Exogenous IPC is often added to the amplification reactions to ensure quality control of the results and to avoid false negatives [18]. Non-competitive IPC; that is not homologous to the target(s), is mostly preferable for several reasons including: ease of design and synthesis, low capacity of inhibition of one or both reactions due to competition, and ability of using the same IPC in different assays simultaneously [19]. Two non-competitive IPCs were evaluated in the current study; 1) SIGV; an insect rhabdovirus, which is theoretically impossible to be present as a contaminant in chicken samples at any instance [20], 2) turkey mitochondrial DNA, which is characteristic for turkey cells and is completely different from similar sequences found in chicken tissues. Mitochondrial DNA is routinely used for tracing the history and evolution of domestic and wild turkeys [21]. Both types of IPC were optimized in RPA assays and their sensitivity limits were determined as

Table 2
Analytical sensitivity of RT-RPA assays used for optimization of All-in-one approach.

Concentration (Copies/μl)	Monoplex Reactions				Duplex Reactions		
	H5 STD FAM	SIGV STD TAMRA	SIGV STD ROX	Turkey DNA STD ROX	H5 STD FAM SIGV STD TAMRA[a]	H5 STD FAM SIGV STD ROX[a]	H5 STD FAM Turkey DNA STD ROX[a]
10^6	+	+	+	+	–	–	–
10^5	+	+	+	+	–	–	–
10^4	+	+	+	+	–	–	–
10^3	+	+[c]	+	+	–	–	–
10^2	±[b]	–	–	–	–	–	–
10^1	–	–	–	–	–	–	–
10^0	–	–	–	–	–	–	–

STD: Standard.
[a] A single concentration (10^4) of the CIPC was used in the reaction.
[b] Weak and inconsistent signal.
[c] Weak but consistent signal.

shown above. The concentration of 10^4 copies per reaction was chosen for all IPCs in optimization of duplex assays as it represents the least amount of DNA/RNA standard that yielded consistent amplification signals.

The performance of H5 RT-RPA assay in the presence of non-competitive IPC was evaluated but unfortunately no amplification signals were developed for both targets; H5 and IPC (Table 2). No variation in the results was observed by changing the type of IPC, the detection channel, and the concentration of the two targets.

RPA has proven superior performance in detecting different pathogens (bacteria, fungi, protozoa, viruses, and others) that infect a wide array of vertebrate, invertebrate and plant hosts [22]. However, optimizing multiplex RPA assays that are capable to detect multiple targets simultaneously or that include IPC was mostly ineffective. A possible explanation is that RPA conditions cannot tolerate the presence of a cocktail of nucleic acids and are strongly inhibited when background DNA exists in particular concentrations [23].

However, the ability to develop multiplex assays was only achieved when RPA was combined with other technologies that enabled: 1) immobilization of the primers on a solid surface (microfluidic chips) to combine the performance of RPA with the multiparameter analysis of microarray systems [24], 2) visual detection of the amplicons using lateral flow readouts [23,25,26]. However, the use of lateral flow reduced the analytical sensitivity of individual target molecules in the multiplex reaction [25], 3) simultaneous extraction, amplification, and fluorescent detection in a centrifugal chip [27], 4) product detection achieved using surface-enhanced Raman Scattering labelled nanotags; RPA-SERS [28]. Even though, in most cases, the targets detected by the multiplex RPA assays do not simultaneously exist in the clinical samples, and consequently the assay is performed as a monoplex reaction [25–27,29,30]. The use of multiplex RPA to simultaneously detect RNA targets is not available so far. Therefore, in the current protocol, we have evaluated a duplex assay that included a previously optimized RNA target (H5N1 influenza virus) and an IPC of either RNA (SIGV) or DNA (turkey mitochondrial DNA) nature. The failure of the reaction to generate positive signals under all conditions provides additional evidence that the available RPA conditions are demanding for use in multiplexing. It may require further improvements in the reaction-setup, instrumentation, and supporting technologies; yet maintaining the potential of RPA in simplicity, speed, and ability to use in point-of-need facilities.

3.3. Aptitude of the two-tube-per-sample as an alternative approach

The effect of co-amplification of H5 and EPC in two-dependent monoplex RPA reactions was analyzed for use as an alternative to the inefficient IPC-based RT-RPA assays. The different EPC tested (SIGV-ROX, SIGV-TAMRA, and turkey mitochondrial DNA-ROX) produced clear amplification signals after 6–7 min, while maintaining the analytical sensitivity of H5 RT-RPA assay. No significant intra-assay or inter-assay variation was determined between the replicates.

False negative results in amplification reactions is mostly attributed to three main factors: 1) failure of one or more of the reaction components, 2) existence of inhibitors, 3) instrument failure [31]. Although IPCs are routinely used to efficiently control the three elements in several amplification-based assays, their insufficiency in RPA may require shift to the use of EPCs. The use of EPC controls for successful instrument performance and integrity of the reaction mixture, but it does not exclude the presence of inhibitors in the reaction. Together with the ease of design and optimization of the reaction, the use of EPC is satisfactory right now and provides a good alternative to the IPC, with no need for combination of the RPA assay with sophisticated and complicated platforms/procedures.

3.4. Analysis of clinical samples

The performance of EPC-based H5 RT-RPA assay in field applications was validated using 13 tracheal swabs suspected to be infected with HPAIV. All samples were tested positive with H5 real-time RT-PCR with a mean CT value of 20.3 ± 5.46. Three samples were identified as H5N1 (CT value: 25.3 ± 8.5) and 10 samples as H5N8 (CT value: 20.1 ± 4.8) using type-specific real-time PCR. As the H5 RT-RPA assay was designed to amplify specific sequences of clade 2.2.1 H5 sequences (i.e. H5N1 viruses) but not clade 2.3.4.4b (i.e. H5N8 viruses) [11], only the three samples positive for H5N1 have developed amplification curves between 6 and 8 min with H5 RT-RPA assay. All H5N8 positive samples as well as the five negative controls either generated negative or invalid results. These results further confirm the specificity of the developed H5 RT-RPA assay on clinical samples. The inclusion of either type of EPC in the RT-RPA and real-time RT-PCR assays did not affect the specificity, sensitivity, and reproducibility of both assays.

CRediT authorship contribution statement

Basem M. Ahmed: Data curation, Investigation, Writing - original draft. **Haitham A. Amer:** Validation, Visualization, Writing - original draft. **Jonas Kissenkoetter:** Investigation, Software. **Ahmed Abd El Wahed:** Conceptualization, Supervision, Writing - review & editing. **Mahmoud M. Bayoumi:** Resources, Investigation. **Susane. Böhlken-Fascher:** Investigation. **Mahmoud A. Elgamal:** Investigation. **Nahed Yehia:** Resources, Investigation. **Ausama A. Yousif:** Validation, Supervision. **Mohamed A. Shalaby:** Conceptualization, Funding acquisition, Resources.

Declaration of competing interest

The author declares that he has no conflict of interest.

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2.4 MANUSCRIPT IV

Flesh ID: nanopore sequencing combined with offline BLAST search for the identification of meat source

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Abstract

Detection of animal species in meat product is crucial to prevent adulterated and unnecessary contamination during processing, in addition to avoid allergy and religious consequences. Gold standard is the real-time PCR assays, which has a limited target capability. In this study, we have established a rapid sequencing protocol to identify animal species within hours. Sequencing was achieved by nanopore sequencing and data analysis *via* offline BLAST search. The whole procedure was conducted in a mobile suitcase lab. As per national and international regulations, the developed assay detected adulteration of pork meat with 0.1 % of Horse, Chicken, Turkey, Cattle, Sheep, Duck, Rabbit, Goat and Donkey. The developed test could be used on-site as a rapid and mobile detection system to determine contamination of meat products.

Introduction

Undeclared or incorrectly declared species in food can lead to considerable health risks or attack religious taboos (1). In addition to the religious exclusion of some animal species from consumption, certain animal species pose a high health risk for consumers (2). Adulteration of meat products with exotic meat increases the risk of introducing parasites. On top of that, many incidents of expensive types of meat were mixed with less expensive types (3). In the horse meat scandal in 2013, the food processing industry unknowingly processed horse meat and offered it incorrectly declared to customers for sale (4). The fundamental problem remains that the inspection of meat that is supplied or processed is posing increasing challenges to the food industry and to official food control (5). So far, the meat origin can only be clarified by very specific analytical methods such as immunological assays or DNA-based amplification technologies (6, 7). The gold-standard in the authentication process of meat and meat products is the real-time PCR (8). Recently, many isothermal amplification assays were established (9, 10). However, these molecular tests are limited to a maximum of eight targets and are unable to identify species outside the narrow target range. As a consequence, delay in diagnosis, false negative and increase costs can occur. Un-targeted or targeted metagenomic sequencing can be a promising solution to overcome these limitations (11). A number of assays relying in high throughput second generation or Next Generation Sequencing (NGS) technologies were developed to detect and genetically characterize animal species (12, 13). However, challenges remain with dependence on PCR based amplification, cumbersome end-point result analysis, logistic demand, cost, applicability in field site and restriction to laboratory settings. Fourth generation sequencing such as nanopore technology confers a promising alternative to offer feasible, field deployable and rapid sequencing option with a real-time data acquisition (14, 15). Therefore, we are aiming to evaluate the performance of this metagenomic sequencing based on nanopore technology in detecting animal species in meat as well as developing user friendly offline-BLAST search for data analysis.

Material and Methods

Meat samples

Vacuum-packed meat of pig, cattle, sheep, horse, chicken, turkey, duck and rabbit was purchased from a local supermarket. The DNA of goat and donkey with a concentration of 10^3 molecules/µl (equals proportion of 0.1 %) were provided by Eurofins GeneScan Technologies GmbH (Freiburg, Germany). Fifty milligram of pork meat was mixed with 0.1 % of each of the other nine meat or DNA. Total nucleic acid from the meat mixture was extracted by applying a lysis buffer (200 mM NaOH) for one hour at room temperature, following a neutralization step with Tris-HCL (0.04M pH 7.5).

Sequencing Library preparation

For library preparation, the rapid sequencing kit (SQB-RBK004) and Flongle from Oxford Nanopore technologies (Cambridge, United Kingdom) were used. A total of 200ng DNA of the meat mixture was incubated with rapid adapters incubated for one minute at 30 °C for one minute and then inactivated at 80 °C for each one minute. The sequencing buffer and loading beads were prepared as instructed by the manufacturer. It is important to mention that the loading style of the mix to the Flongle must be conducted by attaching the filter tips of 200 µl automatic pipette to the sample port, then rotate the volume adjustment knob in clockwise manner. Pushing the fluid using the plunger can destroy the nanopore membrane (16).

Sequencing

Sequencing was conducted on the MinION device including both Flongle adaptor and cell. Data acquisition and basecalling was carried out in real-time by the MinKNOW software. The equipment and software were purchased or downloaded from Oxford Nanopore technologies (Cambridge, United Kingdom). Sequencing were performed for 18 hours using -180 Voltage and either high-accuracy or fast bascalling.

Data analysis

For data analysis, all generated data files in Fastq-format were transferred to the Software Geneious 10.2.3. Here, the sequences of all available chromosomes of Pig, Horse, Chicken, Turkey, Cattle, Sheep, Duck, Rabbit, Goat and Donkey were downloaded from the NCBI database (Table S1). The accuracy of the selected database for the offline BLAST-search was tested by using reference sequences of six additional animal species (dog, NC_002008.4; impala, NC_020675; lion, CM_018460.1; bison, NC_12346; camel, NC_009849.1; japanese quail, NC_003408.1). The speed of species identification was measured by analysing sequence data generated after 0.5, 1, 3 and 9 hours of the sequence run.

Results

Data acquisition

The MinKnow software saved Fastq sequence files directly on the computer hard desk. In total, 34,811 reads were collected after 48 hours of the high-accuracy sequencing run. One very important issue was that the What's in my pot of the Epi2Me software (Oxford Nanopore technologies, Cambridge, United Kingdom) did not identify any of the meat species. Therefore, the establishment of an offline-BLAST search using Geneious software was the next step. All reads with a length lower than 900 basepairs were deleted and the remaining reads were used for further analysis (Fig. 1).

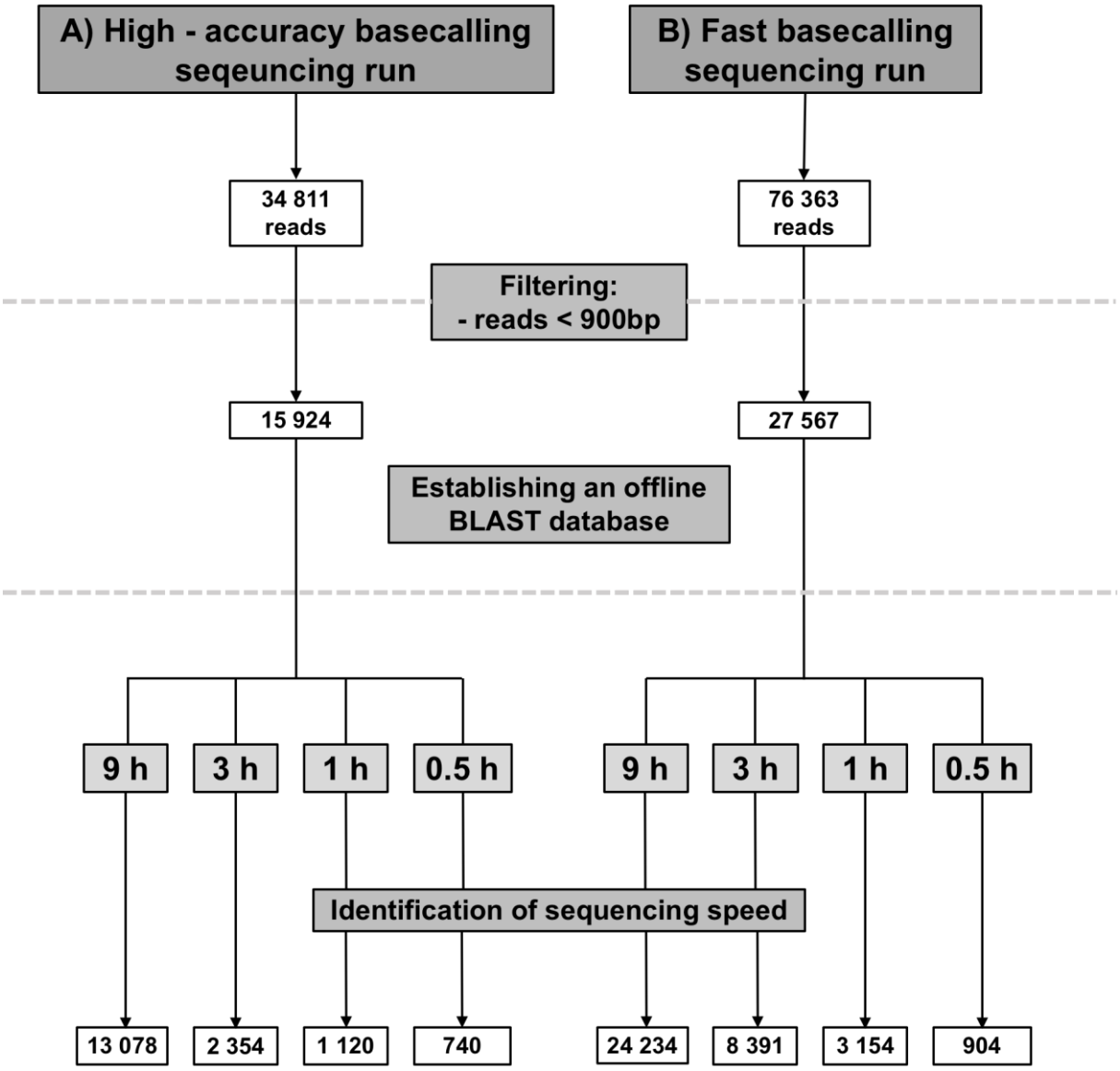

Figure 1. Number of reads of the fast (A) and high-accuracy (B) basecalling run in total, after filtering and after different times.

Offline BLAST-search

The filtered reads of the high-accuracy run were blasted against all selected sequences (Table S1). For the BLAST-search, the fast and high similarity Megablast program were chosen, with only a Query-centric alignment and a maximum of one hit per read. The e-value was set to 1e-100. All expected animal species could be identified (Figure 2, S1). As anticipated, the highest number of hits were assigned to the pig reference sequence, while the poultry species produced lower number of hits than the mammal species.

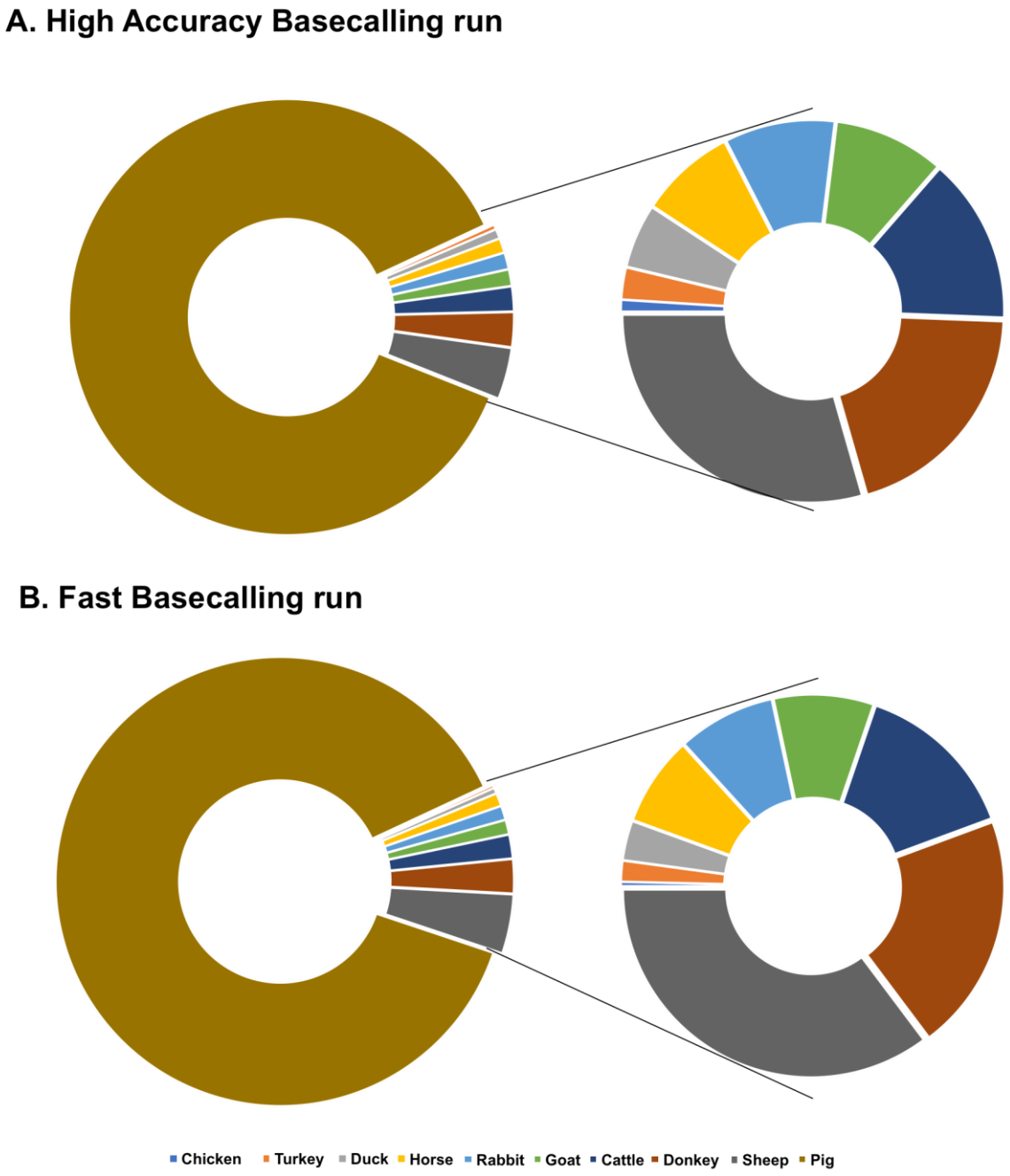

Figure 2. Results of the offline BLAST-search by appling the Flesh-ID database to the sequencing data of the high-accuracy (A) and fast (B) basecallling run. In both sequence runs all 10 animal species could be identified using the Flesh-ID database.

Identification of sequencing speed

For identification of the sequencing run duration, the reads produced within the first 30 minutes, one, three and nine hours of the high-accuracy were analysed. Surprisingly, the reads produced in the first hour against chromosome one of pig, sheep, goat, horse and duck, chromosome 2 of chicken and cattle, chromosome 3 of turkey and chromosome 7 of rabbit were sufficient to identify all ten animal species of the meat mixture. The pairwise identity between the hits and the corresponding reference sequences ranged from 80.33 to 85.96 % (Table S2).
To validate the results, the sequence run was repeated using the fast basecalling model. In total 76, 363 reads were obtained. All species was identified within one hour of sequencing except chicken was detected first after 9 hours (Table S2).

Database specificity

To assess the accuracy of the database to correctly identify the possible meat adulteration, the whole reference sequence of the mitochondrial genome of five unrelated animal species (dog, impala, camel, bison and the japanese quail) and one shotgun sequence of the lion genome were chosen as a negative database. Performing an offline BLAST-search with this database on the sequence reads of the high-accuracy and fast basecalling runs, no hits were assigned to these sequences, which indicate high specificity of the offline BLAST-search.

Discussion

For the identification of animal species in meat products, nanopore sequencing was combined with a novel offline BLAST-search. The DNA was extracted in one hour using alkaline lysis buffer. Library preparation was conducted in 10 minutes and the sequencing run in 18 hours. The offline BLAST-search in Geneious was achieved in less than 20 Minutes.
Oxford nanopore developed two basecalling options, the high-accuracy (Flip-flop basecalling) and the fast model. While the high-accuracy basecalling produces a higher raw read quality with a basecalling speed of 4.4k bases/sec, the fast model has a speed of 36k bases/sec, which results in a lower raw read accuracy (17). In our

experiment, the double number of the reads was collected by the fast basecalling, but both methods produced similar sequence accuracy (Fig 1., Table S2). Nevertheless, the data of both basecalling method lead to the identification of all animal species in the meat mixture. The only difference was the speed as all species were identified after one hour in the high-accuracy sequencing run, while nine hours was needed for the fast basecalling (Table S2).

Oxford nanopore offers a range of online data analysing tools. Sequencing data can be uploaded to the cloud-based *Epi2Me* platform for real-time analysis workflows (18). Unfortunately, only virus, bacteria, fungi and archaea sequences can be recognized by *Epi2Me*. Therefore, for the identification of animal species in meat mixtures, the offline BLAST-search Flesh-ID database was developed and data analysis was conducted in Geneious software. In our database, reference sequences of varioust chromosomes of the animal species were included (Table S1). In other studies, specific genes were selected for the identification process. Most commonly mitochondrial genes like the COI (19), the cyt b (20), the 16S (21) and the 12S gene (22). The authors performed an amplification step using PCR before sequencing, which resulting in a more complex and time-consuming library preparation. Using nanopore sequencing combined with offline-BLAST search, whole genome sequencing is possible as no amplification step is needed during library preparation. Another advantage is, that the use of portable sequencing device, the MinION, that can easily be implemented at point of need (23).

Compared to PCR or isothermal amplification assays, the sequencing method is not limited to specific species as any desired animal species can be easily included in the Flesh-ID database. Amplification dependent assays are designed to specific targets and for each new species of interest, a new assay has to be developed. Moreover, performing several amplification assays for several animal species of interest is time-consuming.

Conclusion

In this study, rapid alkaline lysis was combined with nanopore sequencing technology and offline BLAST for the identification of species in meat mixtures in around 4 hours. The whole procedure was conducted in a mobile suitcase lab, which facilitates the use at point of need. However, a highly trained person must operate the developed assay and the prices is still high. Furthermore, the stability of reagents must be improved to allow long storage at room temperature. In the long run, sequencing will be the standard of molecular diagnostics but data analysis and handling still a great obstacle.

Conflict of interest

The authors declare that they have no competing interests.

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Authors' Contributions

Conceived and designed the experiments: JK and AAW; Performed the experiments: JK and SBF; Analysed the data: JK and AAW; Wrote the paper: all the authors. First author: JK.

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3 General Discussion

In this study, rapid point-of-need detection systems were developed. Focussing on the aim to develop on-site identification of animal species in meat and meat products, two different systems were established. The first is based on the isothermal DNA amplification method, RPA, while the second is based on oxford nanopore technologies. Both approaches can be implemented in a mobile suitcase and therefore conducted at point-of-need. Furthermore, a RPA assay for the identification of monkeypox virus infection was established and two approaches for the using of a positive control in the avian influenza H5 reverse transcription RPA assay were evaluated.

The RPA assay was selected as method of choice because of many advantages: results are available in 15 minutes and all reagents can be freeze-dried (59). However, the primer design is the bottleneck as several oligonucleotides were tested to select the most sensitive pair to achieve DNA amplification. All designed RPA primers and exo-probes were listed in table S1. The length of the primers appears from 23 to 34 bp. Although pork RP4 did not has the recommend length, this primer produced in combination with the forward primer the highest sensitivity for the pork assay. During the RPA assays development, a total amount of 215 primer combination were tested. Therefore, primer with a length from 20 to 40 bp were also tested. Comparing the two turkey assays, a change of the forward primer and the probe resulted in an increase of the sensitivity from 100 to 30 molecules/reaction. Therefore, in this study the following remarks on primer design were noticed: 1) RPA primer should have a length between 20 to 34 bp and a GC content of 30 to 70 %, 2) Primer-primer interactions, secondary structures and hairpin loops should be avoided, 3) The oligonucleotide with more C contents assure better strand invasion.

In this study, six RPA assays were established for the detection of pork, horse, chicken, turkey, sheep and beef DNA in meat products. To decrease the cost per run and shorten turn out time, multiplexing is crucial. Establishment of duplex or triplex RPA assays is highly complicated as the oligonucleotides are competing on the

recombinase enzyme, consequently, the sensitivity of the assays is decreased (60). In case of the approach of combining influenza H5 RPA assay with the turkey RPA assay, the single plex assays have a sensitivity of 100 molecules/reaction, while no signal is measured in the duplex assays. Furthermore, an RNA mosquito virus, Sigma virus, inhibited the influenza H5 RPA assay (61). Despite both targets are RNA based where a reverse transcriptase is needed, the addition of extra reverse transcription inhibited the DNA amplification step in the RPA reaction (60). The scenario is different in DNA based identification as the duplex RPA assay based on animal species target produced similar sensitivities to the single plex RPA assay table 2. All three duplex assays detected 0.1 % contamination in meat mixtures, which considered as the lower limit of detection of meat contamination (German Food and Feed Code §64 (LFGB)). Nevertheless, not all DNA assays can be merged together as the combination of the assays as listed in table 2 were not possible. Interestingly, the successful duplex RPA assay require the adjustment of the final concentration of the oligonucleotide.

Table 2. Duplex RPA assays for the detection of six animal species in meat products**.**

Duplex Assay	Species	Fluorophore	Probe ratio	Primer ratio	Detection Limit [DNA molecules/µl]	Contamination in meat mixtures
1	**Turkey**	FAM	1	0.5	100	**0.1 %**
	Sheep	ROX	1	1	100	**0.1 %**
2	**Horse**	FAM	1	0.5	100	**0.1 %**
	Pork	ROX	2	0.75	100	**0.1 %**
3	**Chicken**	FAM	1	0.75	100	**0.1 %**
	Beef	ROX	1	1	100	**0.1 %**

Compared to PCR and other isothermal amplification assays, the RPA assays produces the same level of sensitivity (46-51, 62). All molecular assays are specific for and produce excellent results with clinical or diagnostic samples. But the RPA has a great advantage, which is the speed. Furthermore, PCR assay need pure and high-quality DNA samples, which require the performance of long DNA extraction protocols, while the RPA reaction is more robust as non-purified DNA yielded from simple alkaline lysis was sufficient (manuscript in preparation with Animal ID ZIM project).

There are two published RPA assays for the detection of animal species available (63, 64). One assay is for the detection of a high value pig species, where a lateral flow strip was used for the detection. Therefore, an additional dilution step was necessary and the test time is around 35 minutes (64). The other study deployed SYBR green I, fluorescent intercalating dye, after the amplification reaction for the identification of duck, chicken, cow, sheep and pig. The procedure conducted in more than 40 minutes (63). Using a fluorescence labelled exo-probe in this study has improve the assays speed and specificity, but the exo-probe did not have a great role in assay sensitivities as it composed of one short and one longer end. At the 5´-end the probe, 30 to 35 bp exist and at 3´-end, 15 bp. The two ends are link *via* two thymine nucleotides, these are labelled with the fluorophore and the quencher. In between those to T´s, one to 4 basepairs should occur (TwistDx, (53)).

The developed six RPA assays for the detection of pork, horse, chicken, turkey, sheep and beef are well suited for the implementation at point of need using a suitcase lab (Manuscript I, Figure 1B.). This mobile laboratory with the measurements of 62+49+30 cm is powered by a solar battery (59). Combining the RPA technology with simple alkaline lysis as descript in manuscript IV, identification of animal species in meat products can be performed within 75 minutes from collecting the sample to receiving the result.

This novel and rapid animal DNA detection system could improve the surveillance of meat products on-site, whether it is used during the meat production as in-house

quality control measurement, during external audits or in the supermarket for the surveillance of the end product.

The oxford nanopore sequencing technology can be performed at point-of-need, where the source of adulteration, contamination or infection is unknown (65). With the developed offline BLAST-search for the identification of sixteen animal species, the metagenomic sequencing exceeded the number of targets per run in molecular assay. Moreover, the sequencing has no limit as more targets can be included during the data analysis. The established sequencing protocol in this study produced results within around 4 hours. To use the mobile suitcase lab for the nanopore sequencing, MinION, high-performance laptop, cooling rack for the sequencing reagents, DNA purification magnetic beads, magnetic rack, and a 1000 µl pipette and filer tips are needed (Figure 2.).

Figure 2. Mobile suitcase lab for the use of oxford nanopore sequencing technology at point-of-need (58).

The huge advantage of the nanopore sequning technique is the size of the sequencing device and the speed. The MinION has the length of a pen and can easily be transported, whereas other Next Generation Sequencing devices cannot be transported and are very expensive. Unfortunately, the nanopore sequencing reagents cannot be freeze-dried and are not stable at room temperature. In addition, the test must be performed by a highly trained person.

CONCLUSION

The following aspects can be concluded in this thesis:

1. A rapid on-site detection system for the identification of animal species in meat and its products was developed.
2. Based on the RPA, six assays for the detection of pork, horse, chicken, turkey, sheep and beef DNA were established.
3. Using the RPA technology, a test for the detection of monkeypox virus can identify infections in ten minutes.
4. The whole procedure can be operated by a mobile suitcase laboratory.
5. Three duplex RPA assays for the detection of animal species were validated and showed the necessary detection limit of 0.1 % contamination in minced-meat and salami mixtures.
6. Duplex RPA assays for the combination of RNA and DNA detection in one tube lack of sensitivity and do not produce satisfying results. Further study is needed to elucidate the complexity of the RNA targeted RPA assays.
7. Combined with an alkaline lysis, the RPA reaction can produce results in 75 minutes at point-of-need.
8. For the identification of exotic or unexpected animal species in meat products a sequencing protocol and an offline-BLAST search were developed.

OUTLOOK

A microfluidic device of automated DNA extraction and RPA test is necessary to simplify the use of the developed assays at point of need. Despite the great opportunity for the nanopore sequencing in molecular diagnostics, development of cooling-independent sequencing kits will be a plus to assure thermostability. Moreover, uncomplicated data analysis workflow is urgently needed.

4 SUMMARY

The control of meat and its products is essential for sales and consumer protection. Due to personal preferences, but also religious and health reasons, the correct labelling is crucial. For meat species inspection, samples have to be sent to a well-equipped laboratory, where a qualified technician extract the DNA from the meat and conduct real-time PCR. The extraction alone takes at least five hours. The real-time polymerase chain reaction (PCR) is used as the gold standard method for the detection of animal species in meat products. The mechanism of real-time PCR is based on three different temperature steps for DNA denaturation (95 °C), primer annealing (60 °C) and elongation (72 °C) of the DNA string. In addition, the use of a fluorescence-labelled probe enables real-time detection of positive signals. In real-time PCR, highly sophisticated, big and expensive devices are required. Furthermore, the run time is around 90 minutes. For the above mentioned reasons, the aim of this doctoral thesis was to determine a rapid detection method for the identification of animal species in meat and meat products in order to simplify on site screening during production or in the sales outlets to enable immediate execution.
An isothermal DNA amplification, recombinase polymerase amplification (RPA), was chosen as the detection method. The RPA amplifies its target gene at a constant temperature between 39 and 42 ° C using enzymes and recombinant protein. Similar to the real-time PCR, the successful amplification is visualized by a fluorescence-labelled probe in a maximum of 15 minutes.

For the development of the RPA assays for the identification of animal species in meat products, primers and probes were targeting the mitochondrial genes of pork, horse, chicken, turkey, cattle and sheep. The sensitivity of each assay was evaluated by performing eight independent runs using serial concentration of molecular DNA standard of each species (10^2 to 10^0 DNA molecules/reaction) and the datasets were subjected to probit-regression analysis. The selected primers were able to amplify their target species with a sensitivity between one and 30 DNA molecules/reaction in a maximum of 11 minutes. No cross-reactions were observed, in other words, each primer combination detects only its target animal species. For field validation of the developed assays, meat and salami mixed samples spiked with various concentration (10, 5, 1, 0.5 and 0.1%) of foreign meat were produced. Each RPA

assay was successfully able to detect meat concentration down to of 0.1% in tested samples. The 0.1 % is the lower recommended value by the German Food and Feed Code §64 (LFGB). Moreover, two different fluorescent dyes (FAM and ROX) were applied to detect meat contamination in duplex, whereby one sample can be tested for up to two species in one reaction. No loss of sensitivity in the duplex assays was noticed. This step was important to reduce the assay running costs while maintaining the same productivity.

Another important issue in meat industry is the freedom from food borne pathogens. Infectious agents can be ingested through the consumption of contaminated meat and lead to food poisoning in humans. In Africa, however, eating "bush meat" can lead to more contagious and deadly infection like monkey pox (MPXV). In order to diagnose such an infection as early as possible and to start the control measures, the use of a rapid test is beneficial. To make this possible, another RPA assay that detects the monkeypox tumor necrosis factor (TNF) binding protein gene was developed. Both monkey pox clades can be determined with a sensitivity of 16 DNA molecules/µl in 10 minutes. With the selected primer pairs, there is no cross-reaction with the closely related tested viruses or monkey genome. The clinical performance of the MPXV-RPA-assay was tested, revealing a specificity of 100% (50/50), while the sensitivity was 95% (43/45). This assay will pave the way for the identification of food borne infectious agents at low resource settings.

Upon presenting my data to the scientific community and end user in international meetings, many individuals have raised the importance of screening more than six animal species. Both RPA and real-time PCR is restricted to the number of the developed assays as well as the fluorescence channels in the detection devices. On other hand, next generation sequencing represents a method with no target limit. For the identification of an unknown adulteration animal source an Oxford nanopore sequencing protocol was tested. The method was combined with offline BLAST search to allow sequencing and data analysis in less than one hour. The developed procedure was successfully detected the contamination of the mock pork sample with 0.1% beef, sheep, goat, horse, donkey, chicken, turkey, duck and rabbit meat. The specificity of the technology was challenged with sequences of exotic animal species as dog, camel, lion, impala, bison and japanese quail. The nanopore sequencing

combined with the Offline BLAST search has proven adequate sensitivity and specificity for species identification and represent the future of molecular diagnostics.

In summary, in the PhD thesis, not only a rapid on-site detection system for the identification of six animal species in meat products based on the recombinase polymerase amplification were developed, but also a rapid sequencing protocol for the use of the Oxford Nanopore technologies. Both detection methods can be combined with an easy to perform DNA extraction method and all methods can be carried out in a mobile suitcase lab, whereby screening of meat and its products can be performed at point of need. The current work will pave the way for the implementation of such technologies for the benefit of the community and consumers.

5 ZUSAMMENFASSUNG

Die Kontrolle von Fleisch und seinen Produkten ist für den Vertrieb und den Verbraucherschutz von essentieller Bedeutung. Aus persönlichen Vorlieben, aber auch religiösen und gesundheitlichen Gründen ist die korrekte Kennzeichnung ausschlaggeben. Zur Überprüfung der Tierarten in Fleischprodukten müssen entnommene Proben zunächst in ein gut ausgestattetes Labor gesendet werden, dort extrahiert qualifiziertes Personal die DNA aus den Fleischproben und führt eine real-time Polymerase-Kettenreaktion (PCR) durch. Die Extraktion alleine dauert mindestens fünf Stunden. Die real-time PCR wird als Goldstandardmethode zum Nachweis von Tierarten in Fleischprodukten verwendet. Deren Mechanismus basiert auf drei unterschiedlichen Temperaturschritten für die DNA-Denaturierung (95 °C), das Primer-Annealing (60 °C) und die Elongation (72 °C) des DNA-Stranges. Darüber hinaus ermöglicht die Verwendung einer fluoreszenzmarkierten Sonde die Erfassung positiver Signale in Echtzeit. Für die Durchführung werden jedoch hochentwickelte, große und teure Geräte benötigt. Außerdem beträgt die Laufzeit der real-time PCR weitere 90 Minuten. Aus diesen oben genannten Gründen war es das Ziel dieser Doktorarbeit, ein Schnellnachweisverfahren zur Identifizierung von Tierarten in Fleisch und Fleischprodukten zu ermitteln, um die Vor-Ort-Kontrolle während der Produktion oder in den Verkaufsstellen zu vereinfachen und eine sofortige Durchführung zu ermöglichen.

Als Nachweismethode wurde eine isotherme DNA-Amplifikation, die Rekombinase-Polymerase-Amplifikation (RPA), gewählt. Die RPA amplifiziert das Zielgen bei einer konstanten Temperatur zwischen 39 und 42 ° C unter Verwendung von Enzymen und einem rekombinanten Protein. Ähnlich wie bei der real-time PCR wird die erfolgreiche Amplifikation durch eine fluoreszenzmarkierte Sonde sichtbar gemacht, jedoch innerhalb von 15 Minuten.

Für die Entwicklung der RPA-Assays zur Identifizierung von Tierarten in Fleischprodukten wurden Primer und Sonden für mitochondriale Gene von Schwein, Pferd, Huhn, Pute, Rind und Schaf designt. Die Sensitivität der Assays wurde bestimmt, indem acht unabhängige Läufe unter Verwendung einer seriellen absteigenden Verdünnungsreihe eines molekularen DNA-Standards jeder Spezies (10^2 bis 10^0 DNA-Moleküle/Reaktion) durchgeführt wurden. Die erhaltenen

Datensätze wurden mit einer Probit-Regressions-Analyse validiert. So konnte für die sechs Assays eine Sensitivität zwischen einem und 30 DNA-Molekülen/Reaktion bestimmt werden. Die maximale Laufzeit betrug außerdem maximal 11 Minuten und es wurde keine Kreuzreaktion festgestellt. Zur Feldvalidierung der entwickelten Assays wurden Fleisch- und Salamimischproben hergestellt, die mit verschiedenen Konzentrationen (10, 5, 1, 0,5 und 0,1 %) an Fremdfleisch versetzt waren. Jeder RPA-Assay war erfolgreich in der Lage, Fremdfleischkonzentrationen von bis zu 0,1 % in den getesteten Proben nachzuweisen. Dies entspricht der empfohlenen Nachweisgrenze nach §64 des deutschen Lebensmittel- und Futtermittelgesetzbuches (LFGB). Darüber hinaus wurden zwei verschiedene Fluoreszenzfarbstoffe (FAM und ROX) zum Nachweis von Fleischkontaminationen im Duplexverfahren eingesetzt, wobei eine Probe in einer Reaktion auf bis zu zwei Arten getestet werden kann. Bei den Duplex-Assays kommt es zu keinem Sensitivitätsverlust im Vergleich zu den singleplex-Assays. Dieser Schritt war wichtig, um die laufenden Kosten des Assays bei gleicher Produktivität zu senken.

Ein weiteres wichtiges Thema in der Fleischindustrie ist die Freiheit von durch Lebensmittel übertragenen Krankheitserregern. Infektionserreger können durch den Verzehr von kontaminiertem Fleisch aufgenommen werden und beim Menschen zu Lebensmittelvergiftungen führen. In Afrika kann der Verzehr von sogenanntem "Buschfleisch" jedoch zu ansteckenden und tödlichen Infektionen wie zum Beispiel mit Affenpocken (MPXV) führen. Um eine solche Infektion so früh wie möglich zu diagnostizieren und die Bekämpfungsmaßnahmen einzuleiten, ist der Einsatz eines Schnelltests von Vorteil. Um dies zu ermöglichen, wurde ein weiterer RPA-Assay entwickelt, der das TNF-Bindungsprotein-Gen des Affenpocken Genoms nachweist. Beide Affenpocken-Kladen können mit einer Sensitivität von 16 DNA-Molekülen/Reaktion in 10 Minuten bestimmt werden. Bei den ausgewählten Primerpaaren findet keine Kreuzreaktion mit den eng verwandten getesteten Viren oder dem Affengenom statt. Die klinische Leistung des MPXV-RPA-Assays wurde getestet und ergab eine Spezifität von 100% (50/50), während die Sensitivität 95% (43/45) betrug. Dieser Assay ebnet den Weg für die Identifizierung von durch Lebensmittel übertragenen Infektionserregern an Orten mit wenig verfügbaren Ressourcen.

Durch Präsentieren dieser Daten vor der wissenschaftlichen Gemeinschaft und Endnutzern auf internationalen Kongressen, wurde auf den Nachweis von weiteren Tierarten hingewiesen. Sowohl die RPA als auch die real-time PCR ist auf die Anzahl der entwickelten Assays sowie die Fluoreszenzkanäle in den Nachweisgeräten beschränkt. Hier gegenüber stehen Methoden der Next Sequencing Generation, bei denen keine solche Beschränkungen vorkommen. Zur Identifizierung einer unbekannten Kontaminationsquelle wurde ein Oxford-Nanoporen-Sequenzierungsprotokoll getestet. Diese Methode wurde mit einer Offline-BLAST-Suche kombiniert, um Sequenzierung und Datenanalyse in weniger als einer Stunde zu ermöglichen. Mit dem entwickelten Verfahren konnte die Kontamination von 0,1% Rind-, Schaf-, Ziegen-, Pferde-, Esel-, Hühner-, Puten-, Enten- und Kaninchenfleisch in einer im Labor hergestellten Schweinefleischprobe erfolgreich nachgewiesen werden. Die Spezifität der Technologie wurde mit einer Reihe exotischer Tierarten wie Hund, Kamel, Löwe, Impala, Bison und japanischer Wachtel in Frage gestellt. Die Nanoporen-Sequenzierung in Kombination mit der Offline-BLAST-Suche hat sich als ausreichend sensitiv und spezifisch für die Identifizierung von Arten erwiesen und ist die Zukunft der molekularen Diagnostik.

Zusammenfassend wurde in dieser Dissertation nicht nur ein schnelles vor-Ort Nachweissystem zur Identifizierung von sechs Tierarten in Fleischprodukten basierend auf der Rekombinase-Polymerase-Amplifikation entwickelt, sondern auch ein schnelles Sequenzierungsprotokoll für den Einsatz der Oxford Nanopore-Technologien. Beide Nachweismethoden können mit einer einfach durchzuführenden DNA-Extraktionsmethode kombiniert und alle Methoden in einem mobilen Kofferlabor durchgeführt werden. So kann die Kontrolle von Fleisch und seinen Produkten vor-Ort durchgeführt werden. Die aktuelle Arbeit wird den Weg für die Implementierung solcher Technologien zum Nutzen der Gemeinschaft und der Verbraucher ebnen.

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7 APPENDIX

AUTHORS CONTRIBUTION

Manuscript I

Recombinase polymerase amplification assays for the identification of pork and horsemeat

Conceptualization: **JK**, AAW, MSF, OP, CPC; Investigation: **JK**, SBF; Methodology: **JK**, MSF, OP, AAW; Project administration: **JK**, AAW, CPC; Supervision: AAW, CPC; Validation: **JK**, SBF, AAW; Visualization: **JK**, SBF; Software: **JK**; Writing - Original Draft Preparation: **JK**, AAW; Writing - Review and Editing: all authors; First Author: **JK.**

Manuscript II

Recombinase polymerase amplification assay for rapid detection of Monkeypox virus

Conceptualization: AAW, CPC, FTH, OGA, MW, AAS, OF; Investigation: SDD, **JK**, MF, CSH, OF, SBF; Methodology: **JK**, FTH, AAW, MW; Project administration: AAW, CPC, FTH, OGA, MW, AAS, OF; Supervision: AAW, CPC, FTH, OGA, MW, AAS, OF; Validation: SDD, **JK**, MF, OF; Visualization: SDD, **JK**, MF; Software: SDD, **JK**, MF; Writing - Original Draft Preparation: SDD, **JK**, MF, AAW; Writing - Review and Editing: all authors; equal First Author: SDD, **JK**, MF.

Manuscript III

Evaluating two approaches for using positive control in standardizing the avian influenza H5 reverse transcription recombinase polymerase amplification assay

Basem M. Ahmed: Data curation, Investigation, Writing – original draft. Haitham A. Amer: Validation, Visualization, Writing – original draft. **Jonas Kissenkoetter**: Investigation, Software. Ahmed Abd El Wahed: Conceptualization, Supervision, Writing - review & editing. Mahmoud M. Bayoumi: Resources, Investigation. Susane. Böhlken-Fascher: Investigation.

Mahmoud A. Elgamal: Investigation. Nahed Yehia: Resources, Investigation. Ausama A. Yousif: Validation, Supervision. Mohamed A. Shalaby: Conceptualization, Funding acquisition, Resources.

Manuscript IV

Flesh ID: nanopore sequencing combined with offline BLAST search for the identification of meat source

Conceived and designed the experiments: **JK** and AAW; Performed the experiments: **JK** and SBF; Analyzed the data: **JK** and AAW; Writing - Original Draft Preparation: **JK** and AAW; Review and Editing: all the authors. First author: **JK**

ABBREVATIONS

°C	Degrees centrigrade
µl	Microliter
µM	Micromolar
bp	basepair
DNA	Desoxyribonucleic acid
FP	Forward Primer
HDA	Helicase-dependent amplification
k	Kilo
LAMP	Loop-mediated Isothermal Amplification
mV	Millivoltage
MPXV	Monkeypox Virus
MDA	Multiple displacement amplification
NEAR	Nicking Enzyme Amplification Reaction
nt	Nucleotid
NASBA	Nucleic acid sequence-based amplification
P	Probe
RPA	Recombinase Polymerase Amplififcation
RNA	Ribonucleic acid
RCA	Rolling circle amplification
sec	seconds
SDA	Strand displacement amplification
SIBA	Strand-invasion based amplification

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SUPLEMENTARY DATA

MANUSCRIPT I

Recombinase polymerase amplification assays for the identification of pork and horsemeat

Jonas KISSENKÖTTER[1], Susanne BÖHLKEN-FASCHER[1], Matthew S. FORREST[2], Olaf PIEOENBURG[2], Claus-Peter CZERNY[1], Ahmed ABD EL WAHED[1, §]

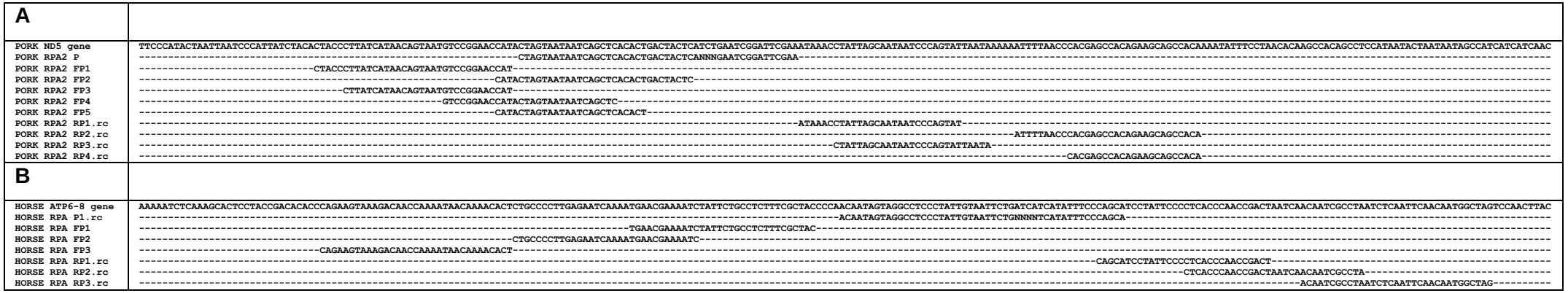

Figure S1. Pork and horse_RPA assays amplicon as well as primer and probe sequences. (A) PORK assay (nucleotides 4958-5318; Genbank accession number: NC_000845): five forward and four reverse primer were tested. (B) HORSE assay (nucleotides 7863-8124; Genbank accession number: NC_001640: three forward and three reverse primer were tested. RC: reverse complementary sequence.

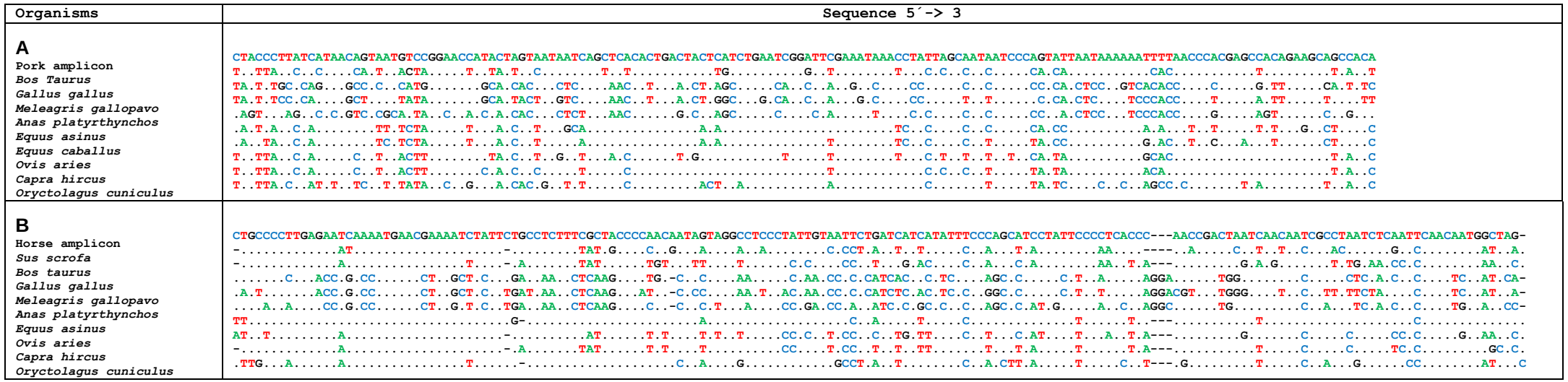

Figure S3. Alignment of the pork and horse_RPA amplicon to the tested corresponding sequence of the other tested animal species. (A) pork_RPA assay: nt 5106-5258, Genbank accession number: NC_000845 (B) horse_RPA assay: nt 7946-8114, Genbank accession number: NC_001640

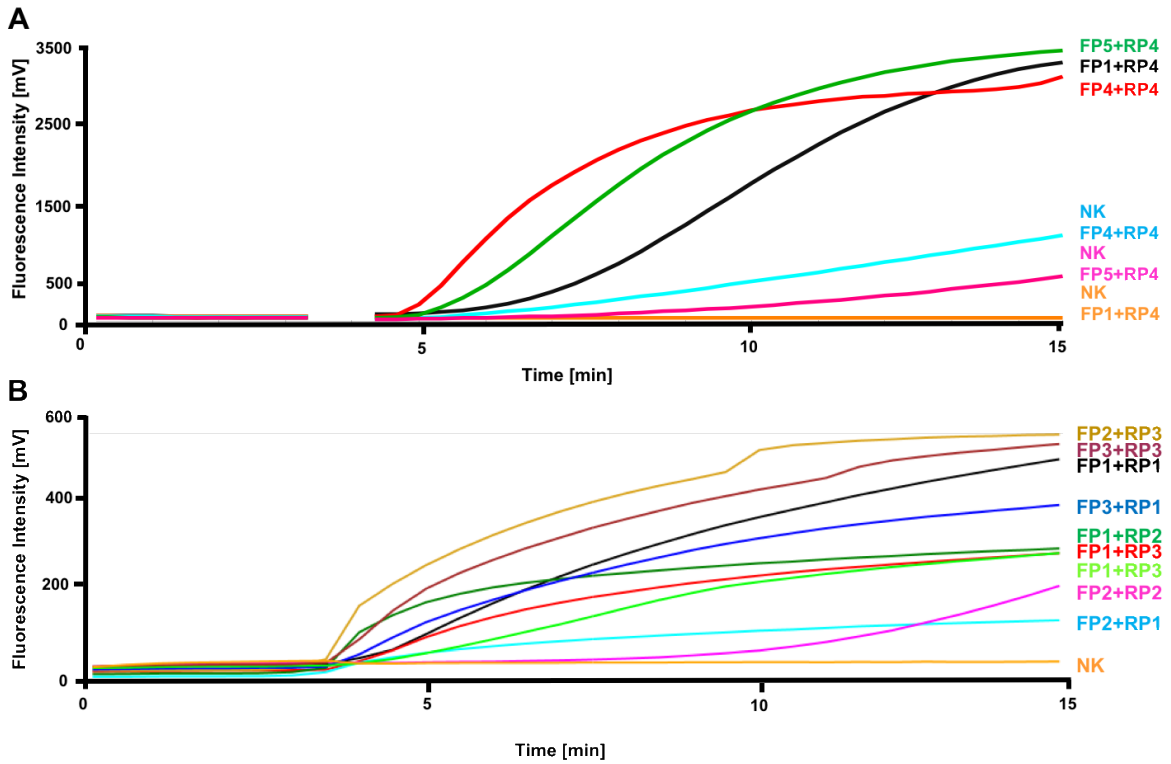

Figure S2. Amplification curves of screening possible primer combinations targeting the two RPA assays (pork (A) and horse_RPA assay(B)). 20 and nine primer combination were tested for the pork and horse_RPA assay, respectively. The combinations with the highest sensitivity were chosen for further screening (pork assay: FP1+RP4; horse assay: FP2+RP3).

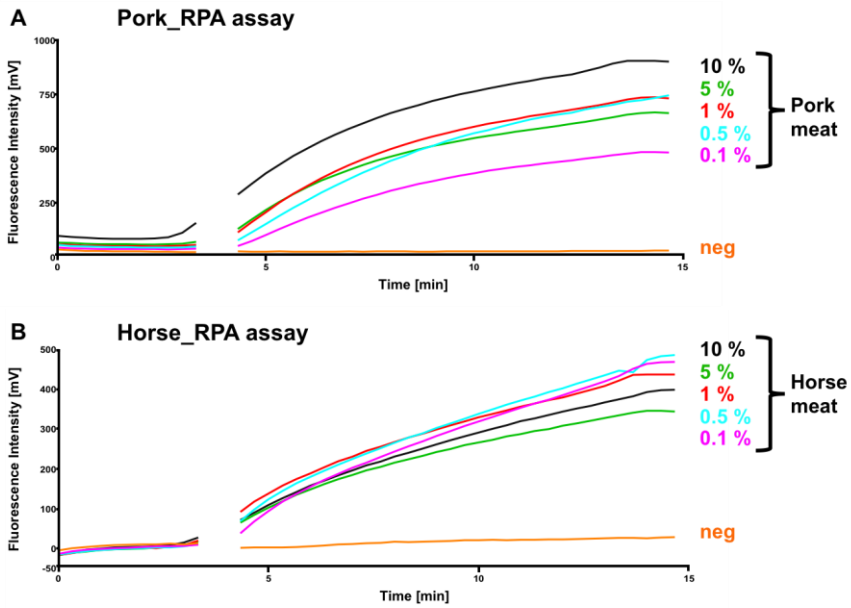

Figure S4. Detection of pork or horsemeat in beef minced meat mixtures. Both, the pork (A) and the horse_RPA assay (B) detect down to 0.1 % of their traget meat in beef minced meat mixtures. DNA extraction were performed by using QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany). After 4 minutes, the tubes were mixed and centrifugated, therefore, a gap appears in the graphs.

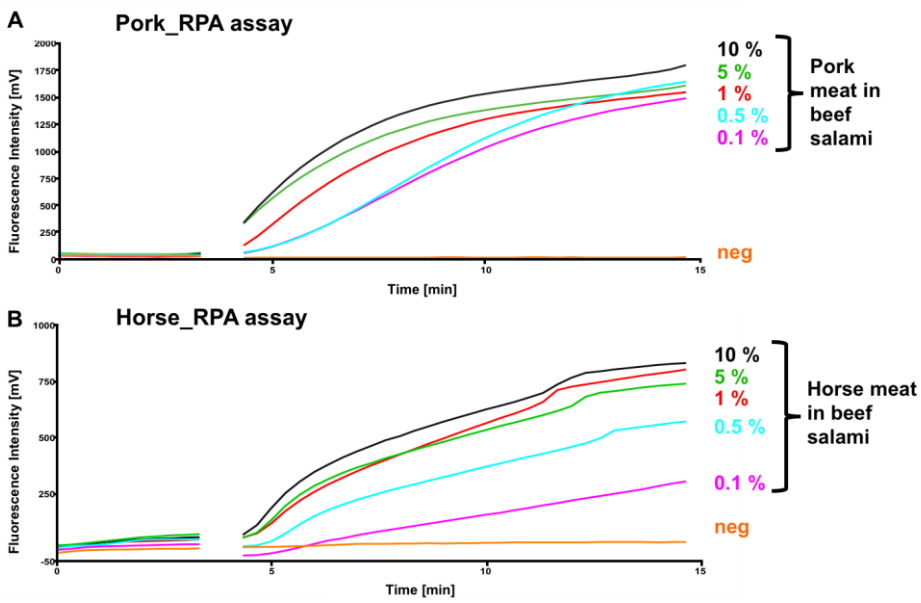

Figure S4. Determination of the detections limit of the pork (A) and horse_RPA assays (B) of their target species in salami products. The assays detect down to 0.1 % of pork or horsemeat in beef salami mixtures. DNA extraction were performed by using QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany). After 4 minutes, the tubes were mixed and centrifugated, therefore, a gap appears in the graphs.

MANUSCRIPT II

Recombinase polymerase amplification assay for rapid detection of Monkeypox virus

Saskia Dede Davi[1, *], Jonas Kissenkötter[2, *], Martin Faye[3, *], Susanne Böhlken-Fascher[2], Christiane Stahl-Hennig[4], Oumar Faye[3], Ousmane Faye[3], Amadou A. Sall[3], Manfred Weidmann[5], Olusegun George Ademowo[6], Frank T. Hufert[1], Claus-Peter

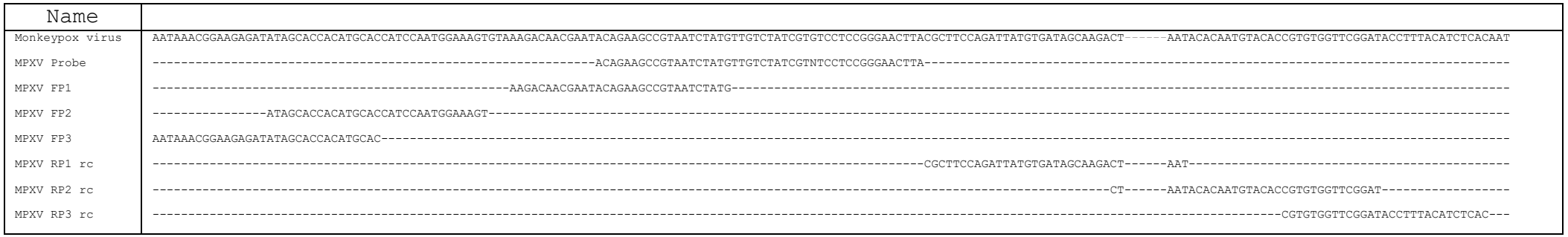

FIGURE S1. MPXV RPA ASSAYS AMPLICON AS WELL AS PRIMER AND PROBE SEQUENCES. MPXV_ASSAY (NUCLEOTIDES 194120-194304; GENBANK ACCESSION NUMBER: NC_003310: THREE FORWARD AND THREE REVERSE PRIMERS WERE TESTED. RC: REVERSE COMPLEMENTARY SEQUENCE.

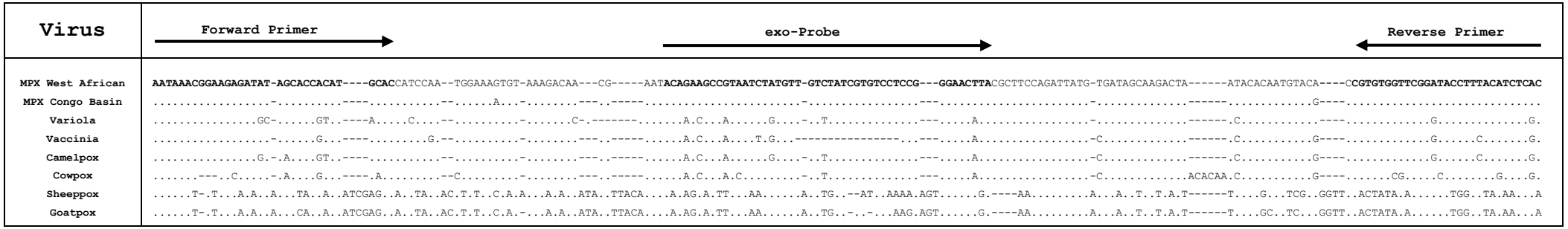

Figure S3. Alignment of the MPXV-RPA-assays amplicon with the Congo Basin clade and other *Chordopoxvirinae* of interest. Using Geneious (Version: 11.1.2, Biomatters Limited, New Zealand) the target sequence of the G2R gene of the monkeypox West African virus (Genebank accession number: DQ011153, nucleotides: 195962 – 1969143) was compared with monkeypox Congo Basin virus (accession number: NC_003310, nt: 194120 – 194301), variola virus (accession number: NC_001611, nt: 182618 - 182749), vaccinia virus (accession number: NC_006998, nt: 189299 – 189472), camelpox virus (accession number: NC_003391, nt: 201497 – 201678) ,cowpox virus (accession number: NC_003663, nt: 219885 – 220071), sheeppox virus (accession number: NC_004002, nt: 112967 – 113171) and goatpox virus (accession number: NC_004003, nt: 112695 – 112892). Between 1 to 31 mismatches could be identified in the primers and probe sequences.

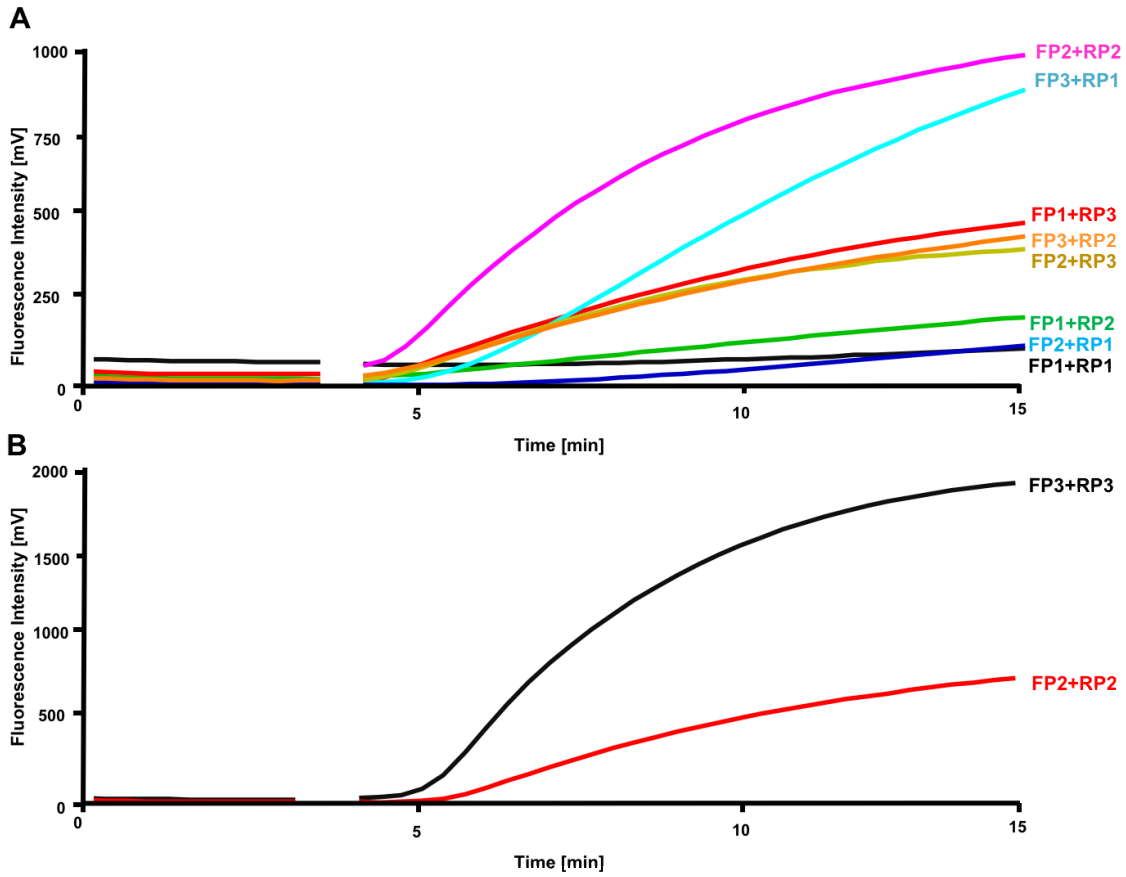

Figure S2. Testing of all possible primer combination of the MPXV-RPA-assay. All nine primer combination were tested with the molecular DNA standard with a concentration of 105 DNA molecules/µl. A mixing step was conducted after 230 sec. The combination FP3 + RP3 showed the earliest and highest fluorescence signal and was therefore chosen for further assay validation.

MANUSRIPT IV

Flesh ID: nanopore sequencing combined with offline BLAST search for the identification of meat source

Jonas Kissenkötter[1], Susanne Böhlken-Fascher[1], Ahmed Abd El Wahed[1&2]

Table S1. Number of sequencing hits to each chromosome of eight animal species. All available chromosome sequences of the eight species included in 0.1% in the meat mixture were screened against the dataset of the high-accuracy basecalling run. The chromosome sequence with the highest number of hits was highlighted in green.

Chromosome	Species															
	Chicken		Turkey		Goat		Duck		Rabbit		Horse		Cattle		Sheep	
	AC	Hits	AC	Hits	AC	Hits	AC	Hits	AC	Hits	AC	Hits	AC	Hits	AC	Hits
1	NC_006088.5	11	NC_015011.2	12	NC_030808.1	174	NC_040046.1	24	NC_013669.1	47	NC_009144.3	64	NC_037328.1	172	NC_040252.1	219
2	NC_006089.5	15	NC_015012.2	10	NC_030809.1	170	NC_040047.1	15	NC_013670.1	48	NC_009145.3	26	NC_037329.1	175	NC_040253.1	216
3	NC_006090.5	10	NC_015013.2	16	NC_030810.1	160	NC_040048.1	17	NC_013671.1	44	NC_009146.3	34	NC_037330.1	147	NC_040254.1	195
4	NC_006091.5	3	NC_015014.2	4	NC_030811.1	153	NC_040049.1	11	NC_013672.1	28	NC_009147.3	26	NC_037331.1	148	NC_040255.1	151
5	NC_006092.5	7	NC_015015.2	6	NC_030812.1	158	NC_040050.1	11	NC_013673.1	25	NC_009148.3	24	NC_037332.1	152	NC_040256.1	154
6	NC_006093.5	4	NC_015016.2	6	NC_030813.1	141	NC_040051.1	4	NC_013674.1	23	NC_009149.3	25	NC_037333.1	143	NC_040257.1	149
7	NC_006094.5	4	NC_015017.2	5	NC_030814.1	164	NC_040052.1	6	NC_013675.1	51	NC_009150.3	25	NC_037334.1	153	NC_040258.1	144
8	NC_006095.5	6	NC_015018.2	4	NC_030815.1	154	NC_040053.1	11	NC_013676.1	30	NC_009151.3	29	NC_037335.1	166	NC_040259.1	153
9	NC_006096.5	2	NC_015019.2	1	NC_030816.1	145	NC_040054.1	7	NC_013677.1	36	NC_009152.3	28	NC_037336.1	153	NC_040260.1	152
10	NC_006097.5	3	NC_015020.2	5	NC_030817.1	148	NC_040055.1	7	NC_013678.1	24	NC_009153.3	27	NC_037337.1	157	NC_040261.1	137
11	NC_006098.5	3	NC_015021.2	3	NC_030818.1	145	NC_040056.1	6	NC_013679.1	35	NC_009154.3	14	NC_037338.1	158	NC_040262.1	121
12	NC_006099.5	3	NC_015022.2	3	NC_030819.1	138	NC_040057.1	6	NC_013680.1	30	NC_009155.3	13	NC_037339.1	135	NC_040263.1	131
13	NC_006100.5	1	NC_015023.2	3	NC_030820.1	134	NC_040058.1	5	NC_013681.1	34	NC_009156.3	12	NC_037340.1	145	NC_040264.1	135
14	NC_006101.5	1	NC_015024.2	2	NC_030821.1	149	NC_040059.1	8	NC_013682.1	43	NC_009157.3	40	NC_037341.1	140	NC_040265.1	132
15	NC_006102.5	0	NC_015025.2	1	NC_030822.1	133	NC_040060.1	9	NC_013683.1	29	NC_009158.3	30	NC_037342.1	145	NC_040266.1	146
16	NC_006103.5	2	NC_015026.2	2	NC_030822.1	134	NC_040061.1	5	NC_013684.1	32	NC_009159.3	24	NC_037343.1	148	NC_040267.1	140
17	NC_006104.5	3	NC_015027.2	0	NC_030824.1	124	NC_040062.1	4	NC_013685.1	41	NC_009160.3	27	NC_037344.1	134	NC_040268.1	135
18	NC_006105.5	2	NC_015028.2	0	NC_030825.1	130	NC_040063.1	4	NC_013686.1	29	NC_009161.3	42	NC_037345.1	128	NC_040269.1	142
19	NC_006106.5	2	NC_015029.2	5	NC_030826.1	123	NC_040064.1	3	NC_013687.1	26	NC_009162.3	20	NC_037346.1	129	NC_040270.1	136
20	NC_006107.5	1	NC_015030.2	1	NC_030827.1	136	NC_040065.1	3	NC_013688.1	21	NC_009163.3	16	NC_037347.1	140	NC_040271.1	122
21	NC_006108.5	0	NC_015031.2	2	NC_030828.1	142	NC_040066.1	4	NC_013689.1	18	NC_009164.3	21	NC_037348.1	138	NC_040272.1	122
22	NC_006109.5	0	NC_015032.2	0	NC_030829.1	130	NC_040067.1	1	Not available	-	NC_009165.3	14	NC_037349.1	129	NC_040273.1	132
23	NC_006110.5	1	NC_015033.2	0	NC_030830.1	121	NC_040068.1	2	Not available	-	NC_009166.3	27	NC_037350.1	120	NC_040274.1	142
24	NC_006111.5	0	NC_015034.2	2	NC_030831.1	132	NC_040069.1	1	Not available	-	NC_009167.3	16	NC_037351.1	142	NC_040275.1	107
25	NC_006112.4	0	NC_015035.2	0	NC_030832.1	109	NC_040070.1	3	Not available	-	NC_009168.3	11	NC_037352.1	110	NC_040276.1	124
26	NC_006113.5	1	NC_015036.2	2	NC_030833.1	129	NC_040071.1	3	Not available	-	NC_009169.3	15	NC_037353.1	132	NC_040277.1	114
27	NC_006114.5	1	NC_015037.2	0	NC_030834.1	115	NC_040072.1	0	Not available	-	NC_009170.3	18	NC_037354.1	117	Not available	-
28	NC_006115.5	0	NC_015038.2	1	NC_030835.1	124	NC_040073.1	0	Not available	-	NC_009171.3	15	NC_037355.1	125	Not available	-
29	Not available	-	NC_015039.2	1	NC_030836.1	124	NC_040074.1	0	Not available	-	NC_009172.3	12	NC_037356.1	121	Not available	-
30	NC_028739.2	0	NC_015040.2	1	Not available	-	Not available	-	Not available	-	NC_009173.3	9	Not available	-	Not available	-

31	NC_028740.2	1	Not available	-	Not available	-	Not available	-	Not available	-	NC_009174.3	13	Not available	-	Not available	-
32	NC_006119.4	0	Not available	-	Not available	-	Not available	-	Not available	-	Not available	-	Not available	-	Not available	-
33	NC_008465.4	1	Not available	-	Not available	-	Not available	-	Not available	-	Not available	-	Not available	-	Not available	-
X	Not available	-	Not available	-	Not available	-	Not available	-	NC_013690.1	33	NC_009175.3	39	NC_037357.1	169	NC_040278.1	170
W	NC_006126.5	3	NC_015042.2	1	Not available	-	Not available	-	Not available	-	Not available	-	Not available	-	Not available	-
Z	NC_006127.5	10	NC_015041.2	9	Not available	-	NC_040075.1	11	Not available	-	Not available	-	Not available	-	Not available	-
mt		0		0		0		0		0		0		0		0

Table S2. Results of the identification of the sequencing runs speed. The sequencing data presented after 0.5, 1, 3, 9 and 18 hours of the high-accuracy and the fast basecalling run were analyzed to identify the time after which all species are correctly detected. The number of hits for each species and the pairwise identity to the reference sequences are shown.

	High Accuracy Basecalling										Fast Basecalling									
	0.5 h		1 h		3 h		9 h		18 h		0.5 h		1 h		3 h		9 h		18 h	
	Hits	Pairwise Identity [%]	Hits	Pairwise Identity [%]	Hits	Pairwise Identity [%]	Hits	Pairwise Identity [%]	Hits	Pairwise Identity [%]	Hits	Pairwise Identity [%]	Hits	Pairwise Identity [%]	Hits	Pairwise Identity [%]	Hits	Pairwise Identity [%]	Hits	Pairwise Identity [%]
Chicken	0	-	1	84	1	84	3	81.37	4	80.9	0	-	0	-	0	-	2	81.25	2	81.25
Turkey	2	87.5	2	87.5	4	88.28	11	85.96	11	85.96	0	-	1	88.6	2	84.65	10	84	10	84
Duck	2	83.65	2	83.65	2	83.65	17	83.05	22	83.44	1	80.8	3	82.87	5	83.68	16	84.31	19	83.96
Horse	1	79.9	2	79.4	2	79.4	26	80.97	33	80.82	3	83.87	7	82.23	16	82.9	37	82.5	43	82.26
Rabbit	2	87.45	3	88.3	5	88.06	31	84.82	38	85.14	2	85.75	5	86.2	19	85.08	43	84.91	47	84.7
Goat	1	78.4	2	81.4	4	84.46	32	82.26	38	82.3	3	84.33	7	83.7	19	82.95	47	82.93	48	82.94
Cattle	2	87.8	2	87.8	10	82.12	48	82.88	57	83.2	4	80.85	12	81.95	26	82.21	74	81.76	79	81.65
Donkey	3	82.73	5	81.76	13	81.42	62	79.95	80	80.01	1	77.8	16	80.35	54	79.63	110	80.3	114	80.33
Sheep	4	81.05	7	81.96	23	82.94	100	82.94	118	82.93	10	82.96	27	84.35	69	84.1	185	82.95	197	82.93
Pig	124	84.93	189	84.94	405	85.28	2190	84.69	2691	84.8	151	82.28	485	85.35	1337	85.19	3651	84.59	4067	84.47

Suplementary Data: Table S2

Table S1. List of all developed RPA assays with primer and probe sequences, their GC content (%) and length (bp), as well as the sensitivity of each assay. QTF are sites of the quencher and fluorophore in the order quencher (Q), Tetrahydrofuran (T) and fluorophore (F).

Species	Primer/Probe	Sequence (5`- 3´)	GC Content (%)	Length (bp)	Sensitivity (molecules/reaction)
Pork	RPA_FP1	CTACCCTTATCATAACAGTAATGTCCGGAACCAT	41.18	34	22
	RPA_RP4	TGTGGCTGCTTCTGTGGCTCGTG	60.87	32	
	RPA_P1	CTAGTAATAATCAGCTCACACTGACTACTCA**QTF**GAATCGGATTCGAA	40.00	48	
Horse	RPA_FP2	CTGCCCCTTGAGAATCAAAATGAACGAAAATC	40.63	32	2
	RPA_RP3	CTAGCCATTGTTGAATTGAGATTAGGCGATTGT	39.39	33	
	RPA_P1	TGCTGGGAAATATGA**Q**G**TF**CAGAATTACAATAGGGAGGCCTACTATTGT	42.22	48	
Chicken	RPA_FP3	ATCCTAGCCTTCTCATCCATCTCCCATTTA	43.33	30	2
	RPA_RP3	GGTTTTAGTTCATGAGATGAGTAGTGTTGACAGT	38.24	34	
	RPA_P1	ATTATCTCCTATAACCCACAACTCACTAT**QTF**CACCTTCATCCTCT	36.53	46	
Turkey (SpeciesID)	RPA_FP2	CACCTTTGCATTGTATTCACTAATAACAACAAC	33.33	33	30
	RPA_RP2	GGCCGGCTAGAGATAGGAGTGCAAGTATTATAG	48.48	32	
	RPA_P2	ATCATTAACCCAGATCAAAGTCCTGAAAC**QTF**CAACAATACTCATC	37.21	46	
Beef	RPA_FP1	TAATACCTATTATCCTACTAGTCTTCGCAGCC	40.63	32	15
	RPA_RP9	ATTGGAGTAAGTTGAGGTTTTGTACATAATCAGTA	31.43	35	
	RPA_P2	TCGCAGCCTGTGAAGCAGCCCTAGGTCTATC**QTF**ACTAGTAATAGTATC	47.83	49	
Sheep	RPA_FP1	CACAATAATATTCATCCACACAGGACA	37.04	27	30
	RPA_RP2	GATCATGTAACGAATAGTGCTACTGGAACG	43.33	30	
	RPA_P4	GTTTGAGGGTTTGGATGGTTAGTCAGTGTCA**QTF**GAGATAATTATTTCT	36.96	49	
MPXV	RPA FP3	AATAAACGGAAGAGATATAGCACCACATGCAC	40.63	32	16
	RPA RP3	GTGAGATGTAAAGGTATCCGAACCACACG	48.26	29	
	RPA P1	ACAGAAGCCGTAATCTATGTTGTCTATCG**QTF**CCTCCGGGAACTTA	46.51	46	
H5	RPA-FP	TAACGGTTGTTTCGAGTTCTATCACAGATG	40.00	30	100
	RPA-RP	ACTTATTTCCTCTCTTTTTAATCTTGCTTC	30.00	30	
	Exo-P	GTATGGAAAGTGTAAGAAACGGAACGTA**QTF**TACCCGCARTATTC	40.48	45	
SIGV	RPA-FP	TGACCATCCTAACTCTGTGACATTCCAAGT	43.33	30	100
	RPA-RP	GTTGACAGTGAGCTCTTGAATCTCTGGGTT	46.67	30	
	Exo-P	ACTGATTTCCCTCCGTGTCCTCCCGGTACCAC**QTF**CCAAACTGCCGTTGTG	58.33	51	
Turkey (IPC)	RPA-FP	CTAATAACAACAACCATATTCTTATCATTAACCC	29.41	34	100
	RPA-RP	GCCGGCTAGAGATAGGAGTGCAAGTATTATAG	46.86	32	
	Exo-P	ATCATTAACCCAGATCAAAGTCCTGAAAC**QTF**CAACAATACTCATC	37.21	45	

DECLARATION

1. I, hereby, declare that this Ph.D. dissertation has not been presented to any other examination body either in its present or a similar form.

   Furthermore, I also affirm that I have not applied for a Ph.D. at any other higher school of education.

   Göttingen, 25.05.2020

   .....

   Jonas Kissenkötter

2. I, hereby, solemnly declare that this dissertation was undertaken independently and without any unauthorized aid.

   Göttingen, 25.05.2020

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   Jonas Kissenkötter