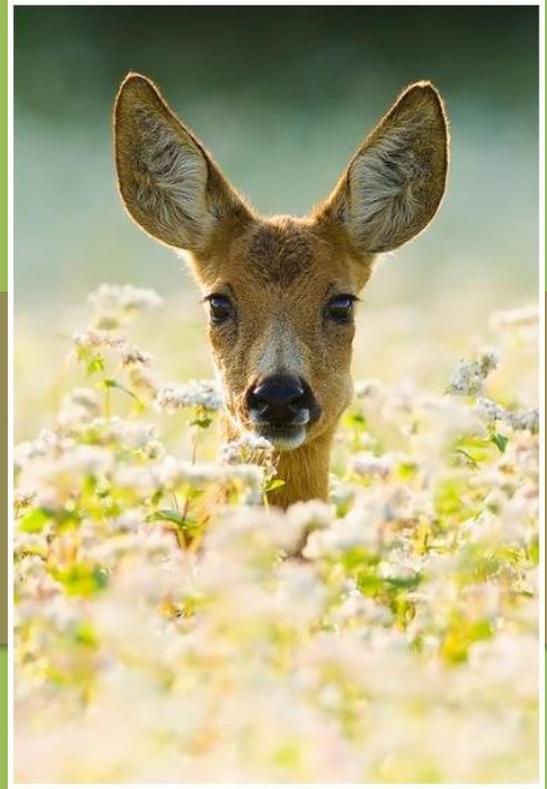


The quality and quantity of DNA in
blood samples taken from roe deer
(*Capreolus capreolus*) in the
Netherlands in 2009-2010



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“We wish to discuss a structure for the salt of deoxyribose nucleic acid (D.N.A.). This structure has novel features which are of considerable biologic interest.”

~ Rosalind Franklin

Abstract

Obtaining blood samples of roe deer (*Capreolus capreolus*) is a common way of use to perform the surveillance and determine the prevalence of diseases using polymerase chain reaction (PCR) technique. To the best of our knowledge nothing is known about the quality of samples used in PCR tests and it is suggested that DNA degradation affect test outcome. The purpose of this study was to determine if DNA purified from ethylenediaminetetraacetic acid (EDTA) whole blood samples obtained from Dutch roe deer in 2009 and 2010 is of sufficient quality and quantity. In this retrospective study we hypothesized that the methods for sample collection, transport and storage in 2009 and 2010 have no effect on DNA quality. Hereto, we investigated 482 EDTA whole blood samples collected by 261 hunters of different Dutch wildlife management units (WBE's). We linked the quality of host species DNA to the quantity of PCR products. We amplified the satellite sequence CCsatIII (consisting of 2.2 kb) and a part of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (consisting of 254 bp). Based on the ability to detect these PCR products, we drew conclusions with regards to DNA quality. Furthermore, a flowchart was developed to evaluate the different steps that could affect the quality and quantity of the samples in the pre-analytical process. The results reveal that in 37.6% of cases, the quantity of DNA was decreased; indicating that some of these samples may be of insufficient quality for further diagnostics. A high chance of false negative results in PCR reactions therefore have to be taken into account. Hence, methods for sample collection, transport and storage should be reconsidered before research on the prevalence and spatial distribution of diseases in the Dutch roe deer population is conducted.

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1. Introduction

1.1. Background

Roe deer (*Capreolus capreolus*) is the most common species of wild ruminant in the Netherlands. In recent decades, the roe deer population has increased significantly, and they are present in over 80% of Dutch wildlife management units (WBE's) (1, 2). It is estimated that there were between 40,000 and 50,000 roe deer in the Netherlands in 2000 and over 64,000 in 2013 (3). This growing roe deer population can interact with humans and other species, and there is a risk of disease transmission. Roe deer can be a source of infectious diseases for humans (zoonotic diseases), as well as livestock and vice versa (2). Therefore, knowledge about the infectious diseases occurring, or emerging, in roe deer in the Netherlands is important.

To gain this knowledge, the Dutch Wildlife Health Centre (DWHC) and the Central Veterinary Institute of Wageningen-UR (CVI) undertook a pilot study to assess the prevalence of Epizootic Hemorrhagic Disease Virus (EHDV) in roe deer in 2009. The study was performed in collaboration with the Koninklijke Nederlandse Jagersvereniging (KNJV) and Faunabeheereenheden (FBE) (1). The different WBE's sampled a given number of roe deer according to a predetermined sampling scheme – taking into account roe deer density and age-class related hunting periods – to obtain representative samples.

Blood samples were collected by 261 hunters between December 2009 and September 2010 from Dutch roe deer. The hunters participating in the study had received an instruction folder and sampling material through their WBE (Appendix I). The blood samples were collected in ethylenediaminetetraacetic acid (EDTA) monovettes and serum tubes, and sent by post to the DWHC where they were processed and stored (1, 4).

However, the quality and quantity of the DNA in the whole blood samples were unknown. Both DNA quality and quantity in the EDTA whole blood samples could have been negatively affected by the processes involved from culling the roe deer until samples reaching the laboratory for storage (5). It is known from human studies that factors during the pre-analytic process (sampling and handling of biological samples) can influence laboratory results (6).

1.2. Study design

The suggestion that DNA degradation may have occurred in the whole blood samples led to the research question being addressed in this paper. The purpose of this study is to determine whether the DNA purified from the EDTA whole blood samples is of sufficient quality and quantity. Specifically, it will be determined whether an adequate amount of host species DNA can be amplified from the EDTA whole blood samples, to give an indication of the DNA quality.

The results will give information on the usefulness of these blood samples in determining the prevalence of disease in the host population. The null hypothesis predicts that the methods used for sample collection (from roe deer), transport and storage in 2009 and 2010 have no effects on the host species DNA quality. If this study shows that the DNA is of poor quality and quantity, the null hypothesis will be rejected. When this happens it will indicate that some of these samples may be of insufficient

quality for further diagnostics with a high chance of obtaining false negative results with polymerase chain reaction (PCR). The methods for blood collection, transport and storage will have to be reconsidered before further research is conducted on the prevalence and spatial distribution of diseases in the Dutch roe deer population.

The present study is set up in two parts. The first part involves laboratory work to determine the yield and subsequently the quality of DNA in the samples. PCR technique will be used to analyse the DNA in the samples. This method consists of a cyclic process whereby a specific DNA fragment is amplified. However, when DNA is degraded into very small pieces by nucleases, the specific DNA fragment cannot be amplified (7). Therefore the PCR test can be used to evaluate the quality of DNA in blood based on the PCR fragment length. The second part involves a schematic overview of the different steps which possibly affect the quality and quantity of the samples in the pre-analytical process. Statistical analysis will be performed on the factors for which data are available to determine their effects on test outcome.

2. Materials and methods

2.1. EDTA whole blood samples from Dutch roe deer

Whole blood samples from 482 Dutch roe deer were available (Table 1) from the EHDV pilot study. All roe deer were hunted professionally in the period from December 2009 to September 2010 in the Netherlands. The individual animals were randomly selected; therefore the study group consisted of animals from both sexes and various age-groups. These animals formed part of a sample survey, which was based on a list of roe deer counted in 2006 per WBE. It was assumed that the sample survey was representative of the roe deer population in the Netherlands (4).

Hunters shot the animals and collected the blood samples. They collected blood from either the heart or vena cava, axillary blood vessels, inguinal blood vessels, thoracic cavity or elsewhere from the corpse (Table 2). Mixed samples were taken from some of the roe deer from multiple sites, although most of the EDTA whole blood samples were taken from the chest – roe deer were shot in the heart or lungs – and the collection of blood from the chest was therefore easier (4). The whole blood samples were collected in EDTA monovettes and were sent to DWHC. Most of the collected samples were haemolytic upon arrival at the laboratory.

Left over blood were stored for retrospective studies to detect any other pathogens present in the blood. The EDTA whole blood samples were stored at -80°C.

Table 1. Make up of the randomly selected population of roe deer according to sex and age-group.

Sex and Age	Animals (N)
Female adult	137
Female yearling	59
Female calve	118
Female of unknown age	6
Male adult	72
Male yearling	49
Male calves	36
Male of unknown age	1
Unknown seks yearling	1
Unknown sex calve	1
Unknown in sex and age	2
Total	482

Table 2. Blood collection sites.

Location	Blood samples (N)
Heart + vena cava	58
Axillary blood vessels	9
Thoracic cavity	305
Inguinal blood vessels	16
Elsewhere from the corpse	23
Mixed samples	62
Unknown	9
Total	482

2.2. DNA purification, measuring DNA concentrations and PCR

The blood samples were thawed at 4°C. Qiagen DNA Tissue Kit (Qiagen, 2006, Hilden; Germany) was used for DNA purification according to the protocol (Appendix II). After the DNA was purified from the blood samples, it was stored at -20°C. First the quantity of DNA was determined before the quality was ascertained. The DNA samples were thawed at 4°C and the DNA concentrations were measured with Qubit® 2.0 Fluorometer (Appendix IV).

Specific primers were needed to amplify the chosen DNA sequences (7). Therefore, the primers for roe deer satellite CCsatIII were used resulting in PCR products with a length of 2.2 kb (8). In the present study it was assumed that the DNA quality was good when PCR products of this size were obtained. PCR was performed on the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in samples that were negative for CCsatIII products. These GAPDH primers formed PCR products that were smaller with a length of 254 bp. This was a measure of the extent of DNA degradation in the blood samples.

A positive control for the PCR had previously been created with muscle tissue from a Dutch roe deer (Appendix III) which was stored at -20°C. The satellite sequence was amplified by using the primers Sat3reeF2, 5'-CCCTCGCTCTCC AATGAA-3'; and Sat3reeR, 5'-ACTCCATTTTCTGAATGAGCT-3' (Appendix V) (8). Primers CapreoGapDHF, 5'-TCATGACCACTTCGGCATCGTGG-3'; and CapreoGapDHR, 5'-GGCGGC AGGTCAGATCCACAAC-3' were used for the amplification of the GAPDH sequence (Appendix VI). The obtained PCR products were analyzed with gel electrophoresis on a 1% agarose gel which contained 0.5 µg/ml ethidiumbromide run at 100 Volt (Appendix VII). PCR products were visualized using the Gel Doc 1000 (Bio-Rad) system.

2.3. Flowchart and literature study

A flowchart was developed to visualize the whole blood sample procedure from the collection until lab analysis, taking into account the factors potentially affecting sample quality and quantity (Figure 1). Influenceable factors included the location of sampling, timeframe from shooting until sampling, EDTA tube mixing, transport, storage and lab analysis. A literature study was conducted to determine which of these factors could alter the yield or the quality of DNA in the samples.

2.4. Data analysis

The data were analyzed by performing the one-way ANOVA test and the Student's *t*-test in Excel. The calculations for the Chi-square test were performed by using the program 'Interactive Chi-Square Tests'.

3. Results

3.1. DNA purification and measuring DNA concentrations

When pipetting the EDTA whole blood samples, there were 101 (21.0%) of the 482 EDTA whole blood samples which contained blood clots or blood clots with contamination. Twenty-three (4.8%) of these were excluded in this study because these contained too many blood clots or blood clots with contamination.

The DNA of remaining 459 (95%) blood samples was successfully purified according to the protocol and the concentrations were measured with Qubit® 2.0 Fluorometer. The samples which contained blood clots with contamination had significantly (Student's *t*-test; $p = 0.04$) higher levels of DNA yields (average DNA concentration of 5.19 ng/ μ l) than the remaining blood samples (average DNA concentration of 3.89 ng/ μ l). The DNA concentration of the positive control was 60.8 ng/ μ l.

3.2. PCR

3.2.1. Development and optimization of PCR

The PCR mixture contained the satellite sequence primers Sat3reeF, 5'-CCCTCGCTCTCCAATGAAGC-3' and Sat3reeR, 5'-CGAGGACTCCATTTTCTCCATTTTCTGAATG-3' and the protocol according to the article of Buntjer et al. (1998) was used (8). This program consisted of 2 minutes at 95°C; 40 cycles of 30 seconds at 95°C, 30 seconds at 53°C, 2 minutes at 72°C and 7 minutes at 72°C (8). PCR products were not obtained initially and we checked primers using Basic Local Alignment Search Tool (BLAST) (9). The forward primer was corrected by skipping two nucleotides on the 3'end (Sat3reeF2, 5'-CCCTCGCTCTCCAATGAA-3') and adapting the annealing temperature from 53°C to 45.8°C. PCR products of the right size were generated from then on.

To optimize the PCR test on satellite sequence CCsatIII, 0.1 μ l (6.08 ng/ μ l) dilutions of the positive control were used. We equalized the DNA concentrations of all the samples with the DNA concentration of a 0.1 unit dilution of the positive control (6.08 ng/ μ l). Thus, different volumes of various samples were used to achieve this and the samples were divided into volumes of 1 μ l, 2 μ l, 3 μ l, 6 μ l and 10 μ l (Table 3). To clarify this: the DNA concentrations of the samples were equalized to a certain amount of microliters, which had to be used to get the same concentration as the positive control. This meant when a sample contained 0.608 ng/ μ l, 10 μ l of the sample was used to get 6.08 ng/ μ l.

Table 3. DNA per sample to obtain a concentration of 6.08 ng/ μ l.

Volume of the DNA sample	Blood samples (N)
1 μ l	91
2 μ l	133
3 μ l	111
6 μ l	87
10 μ l	36
Total	458

3.2.2. Evaluation of PCR products

After the PCR test, the PCR products were analyzed with gel electrophoresis and the intensity of the bands on the gel were assessed as good (+++), moderate (++) , poor (+) and negative (-) (Table 4). The samples which tested negative in the CCsatIII PCR were used in the PCR reaction with GAPDH primers. These samples were assessed in the same way as the CCsatIII PCR products (Table 5). One sample was excluded from analysis because it was erroneously not assayed with the CCsatIII PCR test but only with the GAPDH PCR reaction. This together with the previously described 23 inappropriate samples, excluded 24 (4.98%) of the 482 EDTA samples in the present study.

Table 4. Quantification of PCR product CCsatIII.

Quantity	Blood samples (N)	Percentages (%)
Good (+++)	113	24.7
Moderate (++)	50	10.9
Poor (+)	24	5.24
Negative (-)	271	59.2
Total	458	458

Table 5. Quantification of PCR product GAPDH.

Quantity	Blood samples (N)	Percentages (%)
Good (+++)	18	6.64
Moderate (++)	24	8.86
Poor (+)	57	21.0
Negative (-)	172	63.5
Total	271	100

3.2.3. Classification of sample quality according to PCR product quantity

The quantity of CCsatIII and GAPDH PCR products were classified according to DNA quality (Table 6). The 187 EDTA samples which were positive for CCsatIII sequence (classes good to poor) were classified as of good DNA quality. The 99 of 271 EDTA whole blood samples which were negative for CCsatIII sequence but positive for GAPDH sequence were classified as of moderate DNA quality. The 172 EDTA samples which were negative for both CCsatIII sequence and GAPDH sequence were classified as of poor or insufficient quality.

Table 6. Qualification of PCR products.

Quality	Blood samples (N)	Percentages (%)
Good	187	40.9
Moderate	99	21.6
Insufficient	172	37.6
Total	458	100

3.3. The influence of sampling, transport and storage

3.3.1. Development of the flowchart

For the development of the flowchart, the pre-analytical processes which the EDTA whole blood samples followed were determined by logical reasoning and a literature study. The factors, which possibly influenced the DNA yield and quality, were illustrated in a chain of events which consisted of six different steps (Figure 1).

Roe deer were killed before hunters collected the blood samples (1). The collection of blood samples took place directly after shooting or at a later time. This timeframe was the first step in the chain of events which affected the quantity, as the coagulation process had already begun. The coagulation made the collection of blood samples difficult and clots were formed. It was assumed that there was less DNA available in clotted samples, as a part of the total DNA amount was captured in the blood clots (9-12).

The second step was the location of blood sampling. Blood from the dead animal was obtained either from the heart or vena cava, axillary blood vessels, inguinal blood vessels, thoracic cavity or elsewhere from the corpse (1, 4). The location of blood collection could influence the DNA quality, as organ enzymes might degrade the DNA. These enzymes could have been released systemically due to organ damage by the penetrating bullet (4, 5). In this case, contamination is also a possibility.

The third step involved was gently mixing the collected blood samples in the EDTA monovettes. These blood samples had to be mixed directly after collection to prevent coagulation. Clots arose when samples were not mixed, which might influence the DNA yield in the samples (10-13).

Subsequently the fourth step was the transport of EDTA whole blood samples. The EDTA whole blood samples were sent to the DWHC laboratory for analysis. Samples could have been shaken or dropped during transport. Haemolysis of blood cells results in the leakage of intracellular components which can have an effect on the DNA quality (6). The ambient temperatures and transport time from shooting until arrival at the laboratory might influence the DNA quality. These factors could activate enzymes such as endo- and exonucleases resulting in DNA degradation. Samples decompose at high

ambient temperatures and when transport time increases (5). To the best of our knowledge samples were kept at 4°C or at a maximum temperature of 23°C. However, ambient temperatures below zero had to be avoided as this induces cell lysis in the samples, since both freezing and thawing (freeze-thaw cycle) have a negative effect on the blood samples and must be avoided as much as possible (13, 14).

After the arrival at the DWHC laboratory, the fifth step was performed. The EDTA monovettes were gently mixed before samples were transferred into smaller tubes for storage at -80°C. It was assumed that when the monovettes were not mixed first, the blood cells would not be distributed equally in the samples which would result in unevenly distributed DNA. Further, during the freezing process DNA degradation can occur which could influence the DNA quality (13).

In order to be able to obtain the total amount of DNA in the last step of the chain of events, the EDTA tubes had to be mixed before lab analysis. Pipetting errors could occur during the lab process. After the samples were used for DNA extraction they were stored in the freezer at -20°C. Again, the freeze-thaw cycle for further lab analysis could influence the quality of the extracted DNA (13, 14).

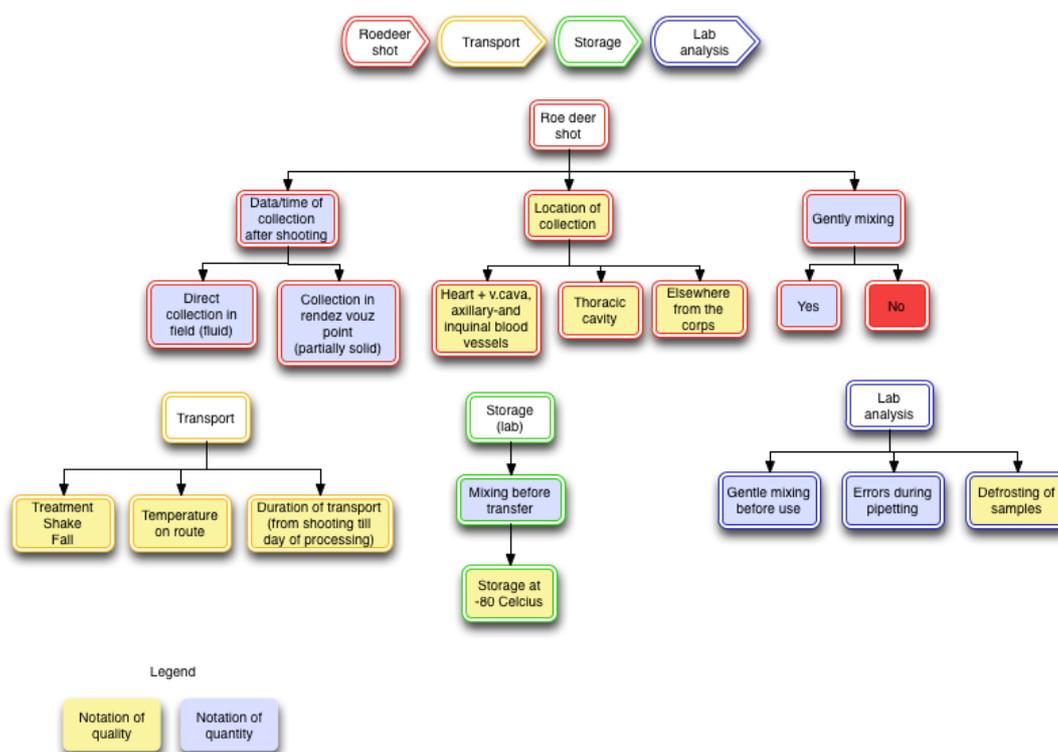


Figure 1: Flowchart of the procedure of the whole blood samples taken from roe deer at post mortem up until laboratory analysis. The different factors - location of collection, timeframe of collection, the mixing of EDTA tubes, transport, storage and lab analysis - were colored according to their effect on the quality (yellow) and quantity (blue) of DNA.

3.3.2. Analysis of sampling effects

During the collection of samples there were three factors, which influenced the yield and quality of the DNA: the time between shooting the roe deer and the collection of the samples, the sampling site and the way the samples were mixed in the EDTA monovettes.

There were no data available about the timeframe between the shooting of the roe deer and collecting the samples. The 458 samples were taken from either the heart or vena cava, axillary blood vessels, inguinal blood vessels, thoracic cavity or elsewhere from the corpse (Table 7). Although, the location of sampling could influence the DNA quality, there was no significant difference ($\text{Chi}^2 = 2.085$; $\text{df} = 1$; $p = 0.148$) between the quality of DNA in samples from the different sites. The quantity of PCR product was significantly decreased for both PCR tests for flood samples with contamination in comparison to the remaining blood samples ($\text{Chi}^2 = 16.69$; $\text{df} = 1$; $p < 0.01$). This supports the idea that contamination of samples will negatively affect their quality. After blood was collected into EDTA monovettes, it is unknown if the coagulation of the blood was prevented by gently mixing the monovettes (which has an effect on the quantity of the DNA).

Table 7: The collection sites of the samples which were analyzed in the present study.

Sampling site	Blood samples (N)
Heart or vena cava	54
Axillary blood vessels	8
Thoracic cavity	295
Inguinal blood vessels	15
Elsewhere from the corpse	22
Mixed samples	59
Unknown	5
Total	458

3.3.3. Analysis of transport factors effects

The transport of the samples to the DWHC laboratory could influence the quality of the DNA. The treatment of these samples during transport was unknown. The average ambient temperature under zero occurring during transport was analyzed with available data from the Royal Netherlands Meteorological Institute (KNMI) (14). No significant difference (one-way ANOVA test; $p = 0.433$) was detected for the average ambient temperature during transport (average of the daily mean ambient temperatures for the days of transport) among the sample groups of good-, poor- and insufficient DNA quality. The influence of an ambient temperature below zero on the quality of DNA in all samples was not significantly different ($\text{Chi}^2 = 0.354$; $\text{df} = 2$; $p = 0.838$).

The journey time of the samples varied between 1 and 11 days with an average duration of 3 days (1). There was no significant difference (one-way ANOVA test; $p = 0.398$) in the time of journey and among the groups of good-, poor- and insufficient DNA quality of the samples.

3.3.4. Storage and lab analysis

When the samples arrived at DWHC, they were transferred from the EDTA monovettes into smaller tubes. It was unknown if the samples were mixed thoroughly before transfer which could affect the DNA yield from the EDTA whole blood samples. After transferring, the samples were stored at -80°C.

The samples were thawed from -80°C to 4°C in the refrigerator and came to room temperature during the laboratory procedures which could affect the DNA quality. DNA was purified successfully from the samples, but errors during pipetting could not be excluded. After laboratory work the DNA samples were stored at -20°C in the freezer.

4. Discussion and conclusion

In the present study PCR was the method of choice to determine the quality of DNA in blood samples. With this technique the level of DNA degradation could be evaluated by the detection of two host species DNA sequences of different lengths (5). Based on the results it was clear that PCR method was of added value to analyze DNA in the 458 EDTA whole blood samples. In order to determine the quality of DNA, the primer sets for the satellite CCsatIII sequence and the housekeeping gene GAPDH sequence were used. The satellite CCsatIII element is specific for roe deer DNA and frequently occurs as a tandem repeat unit in approximately 5% to 10% of the roe deer genome (8). The housekeeping gene GAPDH sequence is often used in comparisons of gene expression data studies because of its consistency (16). These primer sets were used because of the different sizes of the amplified PCR products that indicate the progression of DNA breakdown in the samples.

PCR with the satellite primer CCsatIII, was initially performed according to the protocol in the article of Buntjer et al. (1998) with an adjustment of the forward primer (8, 9). We optimized the PCR test by equalizing the DNA concentration of the various samples with the DNA concentration of the positive control. We assumed that if the monovettes were not mixed before transfer the blood cells would be unevenly distributed which would result in unevenly distributed DNA. However, the differences in the DNA concentration of the various samples were probably due to the lack of consistency in filling the EDTA monovettes. It was reported that several hunters had problems to fill up the EDTA monovettes which resulted in some under filled EDTA monovettes (1, 4).

We reviewed the DNA quality in the EDTA whole blood samples by determining the amount of PCR products. The satellite CCsatIII sequence could not be amplified in 59.2% of the samples and 37.6% of these were also negative for the PCR reaction with the housekeeping gene GAPDH. Several good DNA quality samples are used as a control in the GAPDH PCR.

In conclusion, the two host species DNA sequences were of too poor quality to be detected by PCR test in more than a third of the samples which indicated DNA degradation was occurred in the EDTA whole blood samples obtained from Dutch roe deer from 2009 till 2010. DNA degradation can occur in post-mortem samples e.g. by activation of endo- and exonucleases, or growth of microorganisms. Furthermore, hydrolysis and oxidation can also degrade and modify DNA structures but this will take place after a longer period of time (5).

By using the developed flowchart we investigated the possible factors responsible for DNA degradation in the EDTA whole blood samples.

Our results revealed that EDTA whole blood samples which contained blood clots with contamination were of insufficient DNA quality but had significant higher DNA concentrations than the remaining samples. We observed that these samples were decomposed which was probably due to contamination with microorganisms. This might be the cause of the degradation while DNA extracted from bacteria might explain the high DNA concentrations.

It should be pointed out that high levels of DNA degradation occurred in 59.2% of the samples. These samples were acquired by several hunters from different WBE's (4). According to the EHDV pilot study, there was a high response rate of hunters who collected the samples and most of these samples were haemolytic (1, 4). Some of these samples were inappropriate and were excluded before lab analysis began. Hunters who

received an instruction folder describing the collection method, but without any practical knowledge could be a reason for insufficient DNA quality in 37.6% of the samples. Studies reported education and management guidelines are required for blood sampling collection, since proper knowledge of these topics can prevent haemolysis, clots and contamination in blood samples (6, 17, 18).

Haemolytic and clotted samples are usually rejected by laboratories (6, 17). Few studies reported that clotted blood samples can be used for genetic studies (10, 13, 19). However, the purified DNA yields are greater in anticoagulated blood, than from clotted blood samples (13). A reason for this is probably the multiple wash steps to remove haemoglobin from the clotted samples (19). One study reported that they could amplify DNA fragments with sizes ranging from 215 bp up to 2064 bp from clotted samples (12). We can therefore assume our two sequences could be amplified from clotted samples, because in our study we used the satellite CCsatIII PCR with a product size of 2.2 kb and GAPDH PCR which results in a fragment of 254 bp.

As described earlier, haemolytical samples will mostly be rejected in laboratories, because of the intracellular components which can interfere with lab analysis (6, 17). It is known that haemolysis can cause DNA strand breaks in white blood cells (18, 20). Haemolysis can occur either when blood is not taken properly or blood is diluted for example by rain due to hypo-osmolality (21). Guder et al. (2013) reported that this phenomenon is increased in winter and can be prevented by using closed systems (15). In our study we used closed vacutainers, but it is reported some hunters opened the monovettes which made the blood collection easier. However, this phenomenon cannot be excluded if the wound which was created by the bullet came into direct contact with rain. A part of the hunting season was during the winter and according to the KNMI there were rainy days during the hunting periods (1, 4, 15). Underfilled EDTA monovettes could also result in haemolysis due the blood volume being disproportionate to the EDTA volume in the tube (6, 17). In contrast, Lahiri and Schnabel (1993) reported that EDTA can inhibit DNA degradation (14). The hunters indicated that it was easier to collect blood from the thoracic cavity, where the roe deer was shot. It was reported that haemolytic blood samples were obtained from the chest (1, 4). Trauma as a result of the bullet would contribute to haemolysis.

Sample collection from the chest could also increase the chance of contamination with enzyme containing organ fluids, which could break down the DNA (4, 5). An open wound was created during shooting, which led to contamination with environmental microorganisms. Hereby a bigger timeframe between shooting and blood sample collection also plays a large part, whereby a higher DNA degradation in decomposing tissue occurs (5).

Steinberg et al. (1997) reviewed a few studies to evaluate the influence of ambient temperatures on the whole blood samples. Cushwa et al. (1993) cited by Steinberg et al. (1997) suggested that blood had to be processed quickly to prevent DNA degradation. To purify good quality DNA the same investigators ascertained the journey time had to be less than three days with a maximum ambient temperature of 23°C. Nonetheless, it was recommended that the ambient temperatures during transport of whole blood samples had to be 4°C (13). Our results show there was no significant difference in DNA quality and quantity when ambient temperatures were under zero or above zero during the hunting periods, shipping and storage. The suggestion was that the ambient temperatures during the hunting periods were not extreme enough to induce lysis of the red blood cells. The average ambient temperature in the laboratory was 23°C.

According to some studies EDTA whole blood could be stored at -20°C for several months before DNA purification. On the contrary, Gustincich et al. (1991) cited by Steinberg et al. (1997) reported a decrease of 30% to 40% in DNA yields when blood samples (without pretreatment) were stored either at 4°C for longer than four days or at -20°C. Despite this the purified DNA can be stored for years at -20°C before PCR test are performed (13). The EDTA whole blood samples obtained from the EHDV pilot study did not undergo pretreatment where inhibitors were removed for molecular techniques but were stored immediately at -80°C in the laboratory for over two years. These samples underwent the freeze-thaw cycle before lab analysis. Cheng et al. (2001) explained DNA degradation can occur due to white blood cells being more sensitive to freezing processes because of red blood cell lysis during freezing-thaw cycle (22). Lahiri and Schnabel (1993) showed that DNA degradation took place when samples underwent more than four freeze-thaw cycles (14).

In summary, we conducted research on the DNA quality and quantity of the EDTA whole blood samples obtained from Dutch roe deer collected in the years 2009 and 2010. PCR technique was used and a flowchart was developed which gave a schematic overview of the different steps which affected the quality and quantity of the samples in the pre-analytical process. It was hypothesized that the methods for sample collection, transport and storage from roe deer in the years 2009 and 2010 have no consequences on the DNA quality. However, in more than a third of the cases the samples were of poor quality and the null hypothesis is rejected. This signifies that some of the samples are possibly insufficient for further diagnostics and it has to be taken into account that there will be a high chance of false negative results in PCR tests. Hence, the methods for collecting the samples, transport and storage from roe deer have to be reconsidered before we conduct research on the prevalence and spatial distribution of diseases in the Dutch roe deer population.

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Appendices

Appendix I: Collection of whole blood samples

PRAKTISCHE UITVOERING BEMONSTEREN

BLOED NEMEN

1. **Bloed nemen.** bv. uit de lies of uit de oksel, na het doorsnijden van de huid en de vaten. Het kan ook uit het hart en de grote vaten, en eventueel uit de borstholte (over het algemeen geldt dat bloed direct uit het vat minder verontreinigd is en meer betrouwbare testuitslagen geeft).



2. **Eerst de twee kleine rode (EDTA) monovettes vullen.** U kunt met de monovettes direct bloed op zuigen. U neemt het dopje van de monovette af (linker foto) en zuigt het bloed op tot de buis vol is. net als met een spuit (rechter foto).



3. U doet het dopje er weer goed op, breekt de steel af, **en HEEL BELANGRIJK zwenkt (niet schudden) het een keer of 10 heen en weer.** Door te zwenken mengt het de stof die er voor zorgt dat het bloed niet stolt zich goed met het bloed.



4. **Daarna de grote monovette met bruine dop (serum) vullen (mits er voldoende bloed is).** U doet het dopje er weer goed op, en breekt de steel af. **Deze niet schudden of zwenken.** Indien mogelijk de buis goed vullen, want dan schudt het bloed minder tijdens transport (bij schudden is de kans dat bloed cellen kapot gaan groter en kan ook invloed hebben op de betrouwbaarheid van tests).



5. **Nu de "besmette" handschoenen uit doen:** ze binnenste buiten trekken, en zolang even op een schone plek leggen.



6. **Het verpakkingsmateriaal**

(nr 2 t/m 5): nr 2 is schuimachtig materiaal, dat veel vocht op kan vangen, nr 3 een plastic zak die afgesloten kan worden, nr 4 is een kleine envelop, en nr 5 is een gewatteerde envelop. Dit is voor veilig transport: als de buisjes lekken tijdens transport, dan wordt het bloed door de verpakking tegengehouden en geabsorbeerd



7. **Een paar schone handschoenen aandoen** (die vindt u bij het verpakkingsmateriaal).



8. ***Schoon inpakken – stap 1:*** een van de twee schone handschoenen als “***besmet***” bestempelen, ***alleen met deze hand de monovettes aanraken***. Dit omdat de buitenkant van de monovette mogelijk in aanraking geweest is met het dier of besmeurd met bloed is. Hoewel het in de meeste gevallen een gezond dier was en het vlees voor consumptie geschikt is, moet volgens de postwet de buitenkant van de monovette voor opsturen met de post behandeld worden als zijnde “besmet”. Het is ook belangrijk om niet het verwijt te krijgen dat door het onderzoek bepaalde ziektes werden verspreid in Nederland. Met de “besmette” hand pakt u de monovettes op, met de “schone” houdt u verpakkingsmateriaal nr 2 open en schuift u een monovette in ieder van de drie vakjes.



9. ***Schoon inpakken – stap 2:*** U trekt vervolgens de “besmette” handschoen binnenstebuiten uit. U stopt met de “schone” hand het opgerolde verpakkingsmateriaal nr 2 in de plastic zak (verpakkingsmateriaal nr 3).



Als het voor u praktisch voor het weggooien is, kunt u nu de drie eerder binnenstebuiten getrokken handschoenen erbij in doen, en als laatste de nu ook uitgetrokken “schone” handschoen (foto). **Vervolgens verzegelt u de plastic zak (sluiting goed dichtdrukken), nu kan er niets meer uitlekken.**



10. **Schoon inpakken – stap 3:** Daarna het geheel eerst in de papieren envelop (verpakkingsmateriaal nr 4) stoppen.



11. **Schoon inpakken – stap 4: gegevens erbij:** In principe neemt u dus bloed van de eerste afschot in het geiten/kalveren seizoen, vanaf het moment dat u de monovettes en het verpakkingsmateriaal in huis heeft. U bepaalt dus niet van te voren of het een geit, vrouwelijk kalf of mannelijk kalf is, en ook niet of het

gezond of ziek is. Wat het uiteindelijk geworden is, vult u in op het **formulier** dat u met het bloed opstuurt. Op dit formulier vragen wij u om in te vullen contact gegevens, ree gegevens, datum en plaats van afschot, genomen monsters, en plaats van afname bloed. U kunt er ook eventuele opmerkingen bij schrijven.

NB: In **Noord Brabant** kan in plaats van het ingevulde formulier, een **print-out van de Faunaregistratie** in de envelop bij de monsters gedaan worden. Dan wel graag ook aangeven vanuit welke plaats in de ree het bloed genomen is (zie de punten onderaan het DWHC formulier).



12. **Schoon inpakken – stap 5: Op de post:** Envelop nr 4 gaat nu samen het ingevulde formulier of de print-out in de gefrankeerde envelop (verpakkingsmateriaal nr 5; foto rechts boven), en kan gepost worden.



Nog vragen?

U kunt dan met het DWHC bellen (030-2537925) of mailen (dwhc@uu.nl)

Appendix II: DNA purification from whole blood samples

Protocol: Purification of Total DNA from Animal Blood or Cells (Spin-Column Protocol)

1. Nonnucleated: Pipet 20 μ l proteinase K into a 2 ml microcentrifuge tube (not provided). Add 50 μ l anticoagulated blood. Adjust the volume to 220 μ l with 150 μ l PBS.
2. Add 200 μ l Buffer AL (without added ethanol). Mix thoroughly by vortexing, and incubate at 56°C for 10 min.
3. Add 200 μ l ethanol (99%) to the sample, and mix thoroughly by vortexing.
4. Pipet the mixture from step 3 into the DNeasy Mini spin column placed in a 2 ml collection tube (provided). Centrifuge at $\geq 6000 \times g$ (8000 rpm). Discard flow-through and collection tube.*
5. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 μ l Buffer AW1, and centrifuge for 1 min at $\geq 6000 \times g$ (8000 rpm). Discard flow-through and collection tube.*
6. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 μ l Buffer AW2, and centrifuge for 3 min at 20,000 $\times g$ (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube.
7. Place the DNeasy Mini spin column in a clean 2 ml microcentrifuge tube (not provided), and pipet 200 μ l Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at $\geq 6000 \times g$ (8000 rpm) to elute.

*Flow-through contains Buffer AL or Buffer AW1 and is therefore not compatible with bleach.

Appendix III: DNA purification from tissue

Protocol: DNA purification from tissue using the Magnalyser and DNeasy Blood & Tissue Kit (Qiagen)

Purification of DNA

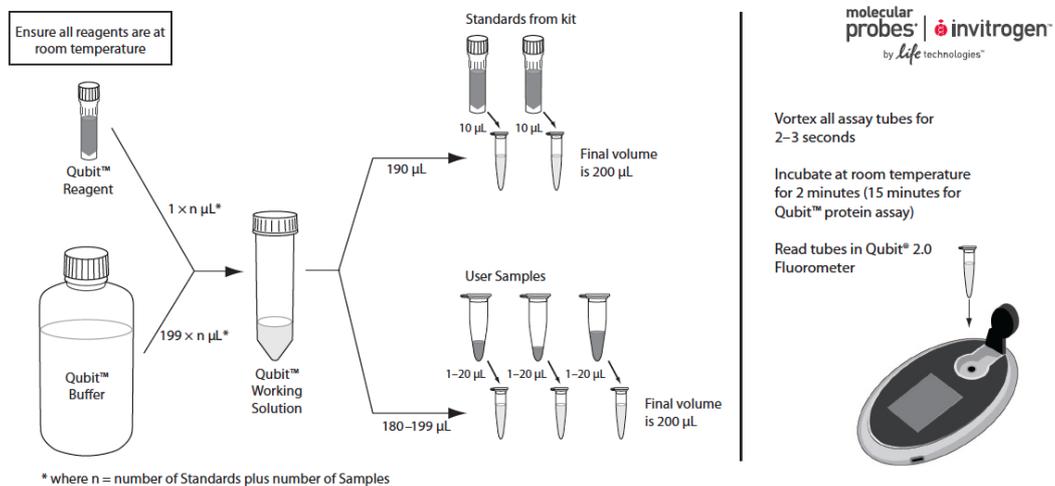
1. Cut up to 37,5 mg of tissue with a clean scalpel into small pieces. Put the pieces in a Magnalyser Green Beads tube.
2. Add 270 μ l Buffer ATL.
3. Add 30 μ l of a proteinase K solution.
4. Homogenize the tissue with the Magnalyser. In case of prolonged time: cool the sample in between on ice.
5. Incubate at 56 °C (If possible under shaking conditions) until the tissue is completely lysed. (if necessary: o.n.)
6. Pipet 200 μ l into a fresh eppendorf tube.
7. Add 200 μ l Buffer AL and mix thoroughly.
8. Add 200 μ l ethanol 96-99% and mix thoroughly.
9. Pipet the mixture into the DNeasy Mini Spin column.
10. Centrifuge 1 min at 6000 x *g*.
11. Discard flow-through.
12. Add 500 μ l Buffer AW1.
13. Centrifuge 1 min at 6000 x *g*.
14. Discard flow-through.
15. Add 500 μ l Buffer AW2.
16. Centrifuge 3 min at 6000 x *g*.
17. Discard flow-through.
18. Centrifuge 1 min at max speed.
19. Discard flow-through.

Elution

1. Add 100 μ l Buffer AE.
2. Incubate 1 min at room temp.
3. Centrifuge 1 min \geq 6000 x *g*.
4. Store flow-through at 4 °C until further use.

Appendix IV: Measuring DNA concentration with Qubit® 2.0 Fluormeter

1. Prepare Qubit® Working Solution for 1 sample (Figure 2):
 - Qubit® Buffer 199.0 µl
 - Qubit® Reagent 1.0 µl
2. Preparing User samples in Qubit® assay 500 µl tubes (Figure 2):
 - Qubit® Working Solution 198.0 µl
 - DNA sample 2.0 µl



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Figure 2: Qubit quick refcard (23).

Appendix V: PCR on roe deer specific Satellite CCsatIII

1. Prepare mixture for PCR using Taq polymerase (Fermentas life sciences) containing:

- Sample	1.0 μ l
- Forward primer 100ng/ μ l	1.0 μ l
- Reverse primer 100ng/ μ l	1.0 μ l
- dNTPs 10mM	1.0 μ l
- Taq polymerase %U/ μ l	0.2 μ l
- DreamTaq, Green Buffer + MgCl ₂	10X 5.0 μ l
- Aquadest	40.8 μ l
Total volume	50.0 μ l

2. Mix the samples gently and spin down.

3. PCR protocol

1 cycle:	Initial denaturation	95°C, 2 minutes
40 cycles:	Denaturation	95°C, 30 seconds
	Annealing	45.8°C, 30 seconds
	Elongation	72 °C, 2 minutes
1 cycle:	Final extension	72°C, 7 minutes

4. After PCR the sample can be used for gel electrophoresis.

Appendix VI: PCR on GAPDH roe deer

1. Prepare mixture for PCR using Taq polymerase (Fermentas) containing:

- Sample	1.0 μ l
- Forward primer 100ng/ μ l	1.0 μ l
- Reverse primer 100ng/ μ l	1.0 μ l
- dNTPs 10mM	1.0 μ l
- Taq polymerase %U/ μ l	0.2 μ l
- Buffer + KCl 10X	5.0 μ l
- Aquadest	40.8 μ l
Total volume	50.0 μ l

2. Mix the samples gently and spin down.

3. PCR protocol

1 cycle:	Initial denaturation	95°C, 2 minutes
40 cycles:	Denaturation	95°C, 30 seconds
	Annealing	54°C, 30 seconds
	Elongation	72 °C, 20 seconds
2 cycle:	Final extension	72°C, 7 minutes

4. After PCR the sample can be used for gel electrophoresis.

Appendix VII: Gel electrophoresis

- Tris-borate-EDTA (TBE) buffer 50.0 mL
- Agarose 1% 0.5 g

The agarose gel is brought to boil in a microwave. Let the gel cool down for 5 minutes. 2.5 µl of ethidium bromide is added to the 50.0 mL agarose gel. Gently mix this till the ethidium bromide is homogeneously divided. Then the gel can be cast in the gel tray with gel casting gates. After 40 minutes remove the gel casting gates and load 5.0 µl of each sample into the wells. In the gel electrophoresis chamber 1 µl of loading dye is added and the power supply is put on 100 V. The gel has to be run as far as possible.