The development and implementation of genotype specific qRT-PCR's for the diagnosis of Avian Orthoreovirus (ARV)

November ´13 – January ´14

Utrecht University
Reinhoudt, D.H.J.
3155951

Supervisors
Utrecht University, Faculty of Veterinary Medicine, Department of Farm Animal Health, Utrecht, the Netherlands
– Dr. Francisca Velkers
University of Veterinary Medicine, Department for Farm Animals and Veterinary Public Health, University Clinic for Poultry and Fish Medicine, Vienna, Austria
– Prof. Dr. Michael Hess, Dr. Salome Troxler & Dr. Ivana Bilic

Contents

Contents 1
Foreword 2
Abstract 3
Introduction 4
    Genetics 4
        S1-gene: σC-protein 4
        S4- gene: σNS- protein 4
    Vaccination 4
    Diagnostics 5
    Polymerase chain reaction (PCR) and electrophoresis 5
    Research objective 6
Materials and methods 7
    Samples 7
    RT-PCR 7
    Optimization of PCR conditions 7
    qRT-PCR 9
Results 11
    RT-PCR 11
    qRT-PCR 41 RNA-samples 12
    Degenerated qRT-PCR 13
    Sequence alignment of RNA-samples 13
Discussion 14
References 16
Attachments 18
    Table 3: qRT-PCR results for 41 RNA-samples 18
    Figure 6: Sequence alignment of RNA-samples 19

Foreword

Students that attend the faculty of Veterinary Medicine at the University of Utrecht, the Netherlands are required to fulfill a three month research internship sometime during the last two years of their education program. This research internship is to be preceded by a research proposal and must be completed with a report and presentation.
The University of Veterinary Medicine in Vienna, Austria (VetMedUni) was kind enough to offer me a research internship from the beginning of November 2013 until the end of January 2014 at their facilities. During my stay, the main focus was on lab work and routine diagnostic testing for Avian Orthoreovirus. After a demonstration of the lab work by one of my supervisors, I was mostly allowed to work independently. Results were discussed with my supervisors, after which we decided on what the next step would be. I also assisted in field work by sampling laying hens and by performing necropsies on selected animals. During my internship details of the work in the lab were written in a lab notebook, which has remained property of VetMedUni and can be found there.
I would like to thank my supervisors at VetMedUni, Prof. Dr. Michael Hess, Dr. Salome Troxler and Dr. Ivana Bilic for the opportunity to work at their laboratory / clinic. I would also like to thank Dr. Francisca Velkers for her help and guidance throughout the internship. I have learned a lot and enjoyed my time in Vienna very much.

Abstract

Avian Orthoreovirus (ARV) is a double-stranded RNA-virus and is the causative agent of viral tenosynovitis. The virus causes economic losses in broiler flocks due to high feed conversion, lameness, culling and low carcass weights. Clinical disease is seen among broilers despite vaccination of breeder flocks. To improve immunity in young chicks, circulating field ARV's need to be determined to be incorporated in new vaccines. However, nucleotide variation is high in the viral genome and thus many ARV-subtypes exist, which makes development of a sensitive qRT-PCR for the detection of ARV difficult. The main objective of this study was to develop a qRT-PCR suitable for routine diagnostic detection of ARV in field samples.

In this study forty-one confirmed reovirus RNA-samples, collected from affected flocks, were subjected to a qRT-PCR with genotype specific primers and probes. Prior to assessment of the RNA-samples, testing conditions for qRT-PCR were optimized using a standard sample. qRT-PCR resulted in Ct-values for thirty-five RNA-samples. Six samples were without a Ct-value. Degenerated qRT-PCR performed on negative samples did not result in an increase in the amount of Ct-values. Results were confirmed by electrophoresis.

New sequence information was obtained from the RNA-samples included in this study. qRT-PCR worked on the majority of cluster 1 RNA-samples, but results for the samples of cluster 2, 4 and 5 varied. Further optimizing of qRT-PCR conditions is necessary and/or new primers and probes will have to be developed in order to improve diagnostic results.

Introduction

Avian Orthoreovirus (ARV) is a double-stranded non-enveloped RNA- virus and is a member of the family of Reoviridae (1, 3, 4, 8, 13, 14). Avian reoviruses are associated with several avian diseases including malabsorption syndrome, respiratory disease, myocarditis and hepatitis. However, it is mostly known as the causative agent of tenosynovitis and viral arthritis (4-6, 8, 11, 12, 14, 16).
Avian reoviruses can affect all ages of poultry, but susceptibility is highest in young chicks (15) and infection is mostly seen in broiler chicks two to five weeks of age (1, 4). Pathogenicity between different reovirus strains varies and many infections are not clinically recognizable but can be isolated from the gut and respiratory tract (1, 3, 5, 20).
Transmission of the virus can occur via eggs or fecal-oral route (1). Most birds are likely to become infected via the latter route, although infection via the respiratory tract or via broken skin of the feet may also occur (21). The virus initially replicates in the intestine and the bursa of Fabricius, enters the bloodstream and spreads to other parts of the body (21, 22). Outside of the body ARV may survive up to ten weeks in drinking water (14).
Affected animals show lameness and prefer not to move. Unilateral and bilateral swelling of tendons in the hock region is common and can be followed by tendon rupture. Growth is stunted, feed conversion is high and, although mortality is low, economic losses in broiler flocks can be high as a consequence of culling of affected birds, low carcass weights and 'downgrading' of carcasses at slaughter (1,4, 12-14).

Genetics

The dsRNA genome of the virus is enclosed in a double layered protein capsid and consists of 10 segments which can be divided in three large (L1, L2, L3), three medium (M1, M2, M3) and four small segments (S1, S2, S3, S4) (3, 5-8, 10, 12, 13, 15, 16). The genome encodes for ten structural (12, 16) and four non-structural proteins (12, 13, 16).

S1-gene: σC-protein

The S1 segment of the genome is tricistronic and consists of three sequential overlapping reading frames (ORFs). The third and longest ORF encodes for the *sigma capsid protein* (σC) (3-5, 7, 15), a structural protein (3, 4, 7, 16). The σC-protein plays a role in cell attachment and environmental stability of the virus (3, 6, 7, 14-16). Additionally, it induces the production of antibodies by the host during infection (3, 7, 14-16) as well as apoptosis of the host cell (12, 14, 16).
The σC-protein consists of 362 amino acids has the most variable nucleotide sequence in the ARV genome (15, 16). By using PCR methods and analyzing nucleotide sequences of the σC-ORF, viral strains can be aligned and grouped into one of five clusters, according to cluster-specific sequences (14).

S4- gene: σNS- protein

The S4 segment encodes for the non-structural sigma non-structural protein (σNS) (10, 13, 16) which consists of 367 amino acids (10, 13). The gene contains a single ORF with a length of 1185 base pairs (10). Compared to the σC-ORF, the σNS-protein encoded on the S4 gene is better conserved in the viral genome but is still prone to variations in nucleotide sequence.

Vaccination

Prevention of ARV is possible by vaccinating breeder flocks to ensure adequate maternal immunity in young chicks (4, 14, 15). Unfortunately, numerous outbreaks of ARV have been described despite vaccination of breeder stock (7, 8, 14, 15). A recent article by Troxler et al.

(4) reports numerous cases of arthritis and tenosynovitis in offspring of vaccinated broiler breeders due to ARV's in different regions in France.

Available vaccines are commonly based on strains S1133, 2408, 1733, UMI 203, 81-5 and 2177 of which most belong to the same serotype and cluster (14, 16). As mentioned above, variation in the viral genome causes diversity among reovirus strains and subtypes tend to differ from these vaccine strains (4, 14, 15)
The diversity between the vaccine strains and field isolates may explain why vaccination is often not efficacious (15).

Diagnostics

ARV can be detected and diagnosed in different ways. Clinical symptoms and post mortem lesions can contribute to diagnosis. Virus isolation from the intestine is not very useful as non-pathogenic strains are easily found here (1). Samples from affected tendons can be used for histology and cell culture (4, 8). Serological ELISA testing, fluorescent antibody assays and agar-gel precipitation may be used. However, cell culture takes time and the serological testing mentioned above has a lower sensitivity compared to *real time-reverse transcriptase PCR* and is of little diagnostic value in vaccinated animals (8, 11).
The development of a sensitive qRT-PCR for the detection of ARV is difficult because of the genetic diversity between ARV strains (16). Suitable locations for primer and probe design and construction are scarce.
In order to ensure good immunity in young chicks, it is important to determine the circulating field ARV's so that these viruses can be incorporated in commercial vaccines. The use of molecular diagnostics is of great use here (1, 3, 4, 7, 11, 14).

Polymerase chain reaction (PCR) and electrophoresis

ARV can be detected in samples by using the '*Polymerase Chain Reaction (PCR)*' technique. Before continuing on to Chapter 'Materials and Methods', the basics of the conventional PCR and the different PCR techniques used in this research internship will be explained here.
The conventional PCR is used to amplify DNA and qualitatively detect the presence of DNA or RNA. During the first cycling step (denaturation step) template DNA is melted into separate strands. The next step (annealing step) consists of forward and reverse primers binding to the 3' ends of both template DNA strands after which DNA-polymerase is bound. In the third step (elongation step) new DNA strands are synthesized by DNA-polymerase using dNTPs. These new DNA strands are complementary to the template DNA. The three steps form a cycle which is repeated a number of times after which the template DNA has been amplified exponentially (17, 18).

As mentioned above ARV has a dsRNA genome and amplification by PCR requires an extra step. Before cycling can begin, the template RNA must first be converted to DNA using reverse transcriptase. The resulting DNA can then be used for amplification. The PCR technique in which RNA is converted into DNA and then amplified exponentially is called '*reverse transcriptase PCR (RT-PCR)*' (17, 18)

After a PCR has been completed, *electrophoresis* can confirm the presence of amplified DNA (the PCR product). This is done by loading the PCR onto an agarose gel containing ethidium bromide. An electric field applied to the gel causes the DNA fragments to separate according to their size. By using UV-light the gel is then illuminated. The presence of a PCR product can be seen as a fluorescent band in the gel. Alongside the PCR, a DNA size marker can be loaded onto the gel. A PCR product with a certain (known) length can then be compared to this DNA-ladder (17, 18).

The quantitative detection of DNA or RNA is possible by using a different PCR method. This method, known as *real-time PCR* or *quantitative PCR (qPCR)* amplifies DNA and measures the amount of PCR product using fluorescent probes. Although a number of different probes exist, only the *Taqman-probe* used during the research internship will be described here.

The Taqman-probe is a sequence-specific fluorescent probe which contains a fluorophore at the '5 end of the probe and a quencher at the 3' terminus. When in close proximity to each other, the quencher inhibits the production of a fluorescent signal by absorbing the energy emitted by the fluorophore. During PCR the Taqman-probe binds to specific sequences in the DNA strands and emits no signal. When DNA-polymerase reaches the probe during elongation, the probe is hydrolyzed releasing the fluorophore and the quencher. This results in a greater distance between fluorophore and quencher allowing a fluorescent signal to develop (17-19).

As with conventional PCR, the quantitative detection of RNA requires the conversion of RNA into DNA by DNA-polymerase before real-time PCR can begin. This PCR is called *real-time reverse transcriptase PCR (rt-RT-PCR)* or *quantitative reverse transcriptase PCR (qRT-PCR).*

Research objective

The main objective of this research project was to develop a genotype specific qRT-PCR suitable for the routine diagnostic testing of field samples for Avian Orthoreovirus. To achieve this, qRT-PCR conditions were optimized and primers and probes specific for the S4 gene were tested on confirmed reovirus RNA-samples.

Materials and methods

Samples

Forty-one confirmed reovirus RNA-samples were extracted from 200µL cell culture supernatants using the QIAamp® cador® Pathogen Mini Kit (QIAGEN) according to protocol. These cell cultures were derived from tissue samples that were collected from poultry flocks in Austria during 2011 (6 samples), 2012 (21 samples) and 2013 (14 samples). RNA-samples belonged to either cluster 1 (32 samples), 2 (3 samples), 4 (5 samples) or 5 (1 sample) according to cluster-specific nucleotide sequences of the σC-gene on segment S1 of the viral genome. Cluster 3 RNA-samples were not included in this research.

RT-PCR

Reverse- transcriptase PCR (RT-PCR) was performed on eighteen RNA-samples to convert viral RNA into complementary DNA followed by DNA amplification using the primers S4-F13 and S4-R1133. The primers S4-F13 and S4-R1133 encode for part of the σNS-protein located on segment S4 of the viral genome. 5µL of RNA-template were added to a mastermix containing 5µL OneStepBuffer, 1µL S4-F13 (10pM/µL), 1µL S4-R1133 (10pM/µL), 1µL dNTP, 0.5µL RNase out, 1µL Enzyme Mix and 10.5µL water resulting in a total volume of 25µL per reaction. RT-PCR was performed in a Bio-Rad thermal cycler together with a positive RNA control sample and a negative RNA-extraction control sample.

RT-PCR (5'- 3')

Forward primer:	S4-F13	(GTGCGTGTTGGAGTTTCCCG)
Reverse primer:	S4-R1133	(TACGCCATCCTAGCTGGA)

Thermal conditions consisted of 50°C for 30 minutes and 94°C for 15 minutes, These steps were followed by 35 cycles of 94°C for 30 seconds, 53°C for 1 minute and 72°C for 1 minute. The last step was made up of 72°C for 10 minutes after which the PCR could be kept in the thermal cycler at 4°C endlessly.

Subsequently the PCR product was loaded on to 1.5% gel (1.5g agarose per 100ml 1xTAE buffer) and separated by gel electrophoresis. Fluorescent bands with a DNA length of 1120 base pairs were cut out after which the DNA was extracted using the QIAquick Gel Extraction Kit (QIAGEN) according to manufacturer's instructions. The extracted DNA was then sent to LGC Genomics in Germany for sequencing.

Optimization of PCR conditions

(Quantitative) reverse transcriptase PCR (qRT-PCR) was performed on sample 11-12523 under different circumstances to determine optimal testing conditions prior to evaluation of all RNA-samples included in this research. Results of the PCR were analyzed by examining the Ct-value and electrophoresis results produced under each set of conditions.

qRT-PCR: Primer concentration

qRT-PCR was performed on sample 11-12523 using different primer concentrations. Forward primer S4-rt-F1 and reverse primer S4-rt-R1 were each added to a Brilliant III Ultra-Fast SYBR® Green QRT-PCR mastermix (Agilent Technologies) in 16 different concentration combinations. Primer concentrations were either 150nM, 300nM, 400nm or 500nM. The mastermix had a total volume of 20µL including 2µL RNA-template and thermal settings were according to manufacturer's instructions.

qRT-PCR (5´-3´)

Forward primer:	S4-rt-F1	(ATCAAAGAGCAAGGTTTCGC)
Reverse primer:	S4-rt-R1	(ACCATTTCGGATTTCGAGAC)

Primers and probe for qRT-PCR were designed according to the article by Guo et al. (2011).

<u>qRT-PCR: Serial dilutions</u>
Sample 11-12523 was subjected to a qRT-PCR in which six ten-fold serial dilutions of RNA-sample were combined with various primer concentrations. 2µL of RNA-template were added to a Brilliant III Ultra-Fast SYBR® Green qRT-PCR mastermix together with either 300nM, 400nM or 500nM of each primer. Dilutions were tested in duplicates and PCR included a negative no-template control.

<u>qRT-PCR: Primer and probe concentration</u>
Various concentrations of S4-rt-primers and S4-rt-probe were tested on dilutions of sample 11-12523. Primers were each added to a One-Step RT-PCR Kit (QIAGEN) in a concentration of either 400nM or 600nM per reaction. Furthermore probe was added in either 200nM or 300nM per reaction. In order to enhance DNA-polymerase activity, 0.75µL of magnesium chloride was added to the mastermix. Thermal settings were according to manufacturer's instructions.

qRT-PCR (5´-3´)

Probe	S4-rt-P1	([6FAM]CGGTCTGGTCCAACTCGCCC[BHQ1])

<u>qRT-PCR: RNA- samples</u>
qRT-PCR was performed on twelve RNA-samples using the One-Step RT-PCR Kit. Primers S4-rt-F1 and S4-rt-R1 were each added in a concentration of 600nM as well as 200nM probe S4-rt-P1 and 0.75µL magnesium chloride. Thermal settings consisted of 50°C for 30 minutes and 94°C for 15 minutes followed by 45 cycles of 94°C for 5 seconds and 60°C for 20 seconds. Fluorescence was measured at the end of each cycle. No-template samples were included in the PCR as negative controls.

<u>qRT-PCR: Degenerated primers and probe</u>
Following qRT-PCR on twelve RNA-samples using the conventional S4-rt-primers and probe, the degenerated primers, S4-rt-F1deg and S4-rt-R1deg, were tested under the same conditions on sample 11-12523 and 12-6009. Degenerated primers are mixtures of primers in which a number of base positions within the primer contain different nucleotides. Sample 12-6009 produced no Ct value in the previous qRT-PCR and was therefore included in the degenerated PCR. Sample 11-12523 served as a positive control whereas a no-template sample was added as a negative control.

Degenerated qRT-PCR (5´-3´)

Forward primer:	S4-rt-F1deg	(ATCAARGARCARGGYTTYGC)
Reverse primer:	S4-rt-R1deg	(ACCATTHCKGATYTCRAGAC)
Probe:	S4-rt-P1deg	([6FAM]CGDTCYGGTCCDACTCGDCC[BHQ1])

<u>qRT-PCR: Annealing temperature degenerated primers and probe</u>
Samples 11-12523 and 12-6009 where subjected to qRT-PCR with 600nM degenerated primers and 200nM degenerated probe per reaction. Reference dye was added to the One-Step RT-PCR Kit mastermix to normalize variations in fluorescence unassociated with the PCR.

Thermal conditions for the annealing step were adjusted to the primer melting temperature (Tm-value) of the degenerated primers. This value was estimated using the formula $Tm = 2°C \times (A+T) + 4°C (G+C)$. Degenerated nucleotides were included in the formula and multiplied with 3°C. Adjusted thermal conditions were as follows: 50°C for 30 minutes and 94°C for 15 minutes followed by 45 cycles of 95°C for 5 seconds, 50°C for 20 seconds and 72°C for 10 seconds.

Gradient RT-PCR: degenerated primers

Sample 11-12523 was incorporated in a RT-PCR using the One-Step RT-PCR Kit to determine the optimal annealing temperature for the degenerated primers S4-rt-F1deg and S4-rt-R1deg. The annealing temperature ranged from 45°C to 60°C and was simultaneously tested with different primer concentrations of 400nM, 600nM or 800nM. Gradient RT-PCR was run in a Bio-Rad thermal cycler with a temperature gradient function.

qRT-PCR: adjusted annealing temperature degenerated primers and probe

The degenerated primers and probe were tested on sample 11-12523 and 12-6009 using the One-Step RT-PCR Kit. Annealing temperature was set at 54°C according to thermal gradient results. Primers were added to the mastermix at a concentration of 600nM or 800nM per reaction.
Thermal setup consisted of 50°C for 30 minutes and 94°C for 15 minutes followed by 40 cycles of 95°C for 15 seconds, 54°C for 20 seconds and 72°C for 10 seconds.

qRT-PCR: Comparison of real-time PCR machines

The One-Step RT-PCR Kit was compared using sample 11-12523 in the Stratagene Mx3000P® QPCR System (Genomics) and the Rotor-Gene Q real-time PCR (QIAGEN) machine. Thermal conditions were identical in both machines and comprised 50°C for 30 minutes and 94°C for 15 minutes followed by 45 cycles of 94°C for 5 seconds and 60°C for 20 seconds.

qRT-PCR

qRT-PCR on all RNA-samples

One-step qRT-PCR was performed on all RNA-samples using the One-Step RT-PCR Kit in a Rotor-Gene Q real-time PCR machine. Forward S4-rt-F1 and reverse S4-rt-R1 primers were each added to the mastermix in a 600nM concentration alongside with 200nM of probe S4-rt-P1 and 2µL of RNA-sample per reaction. In addition, the mastermix contained 5µL OneStepBuffer, 1µL dNTP mix, 0.5µL RNase inhibitor, 1µL Enzyme Mix and 7.25µL of water per reaction.
Thermal setup consisted of 30 minutes at 50°C and 15 minutes at 94°C followed by 45 cycles of 5 seconds at 94°C and 20 seconds at 60°C.
Negative control samples produced during RNA extraction were included in the qRT-PCR as negative controls. The presence of a PCR product was checked by electrophoresis.

Degenerated qRT-PCR all RNA-samples

Five samples without a Ct-value ensuing conventional qRT-PCR were subjected to qRT-PCR with degenerated primers and probe. Both degenerated primers were added to the mastermix at a concentration of 600nM, together with 200nM probe and 2µL of RNA-sample per reaction. The degenerated qRT-PCR was carried out with the One-Step RT-PCR Kit on a Rotor-Gene Q real-time PCR machine.

The thermal settings comprised 50°C for 30 minutes and 94°C for 15 minutes followed by 45 cycles of 94°C for 5 seconds and 60°C for 20 seconds. Subsequent to the qRT-PCR, results were confirmed by electrophoresis.
Sample 11-12523 was included as a positive control in the assay together with a (no-template) negative control.

Results

RT-PCR

Due to pre-existing sequence knowledge of certain strains and lack of time, not all samples were subjected to RT-PCR or sent for sequencing. Nineteen out of 41 RNA-samples were evaluated using RT-PCR. Seventeen samples produced a fluorescent band with a DNA-length of 1120 base pairs. Sample 12-1179 did not show up on electrophoresis. The band produced by sample 12-1182 is questionable due to its irregular edges (see Figure 1).

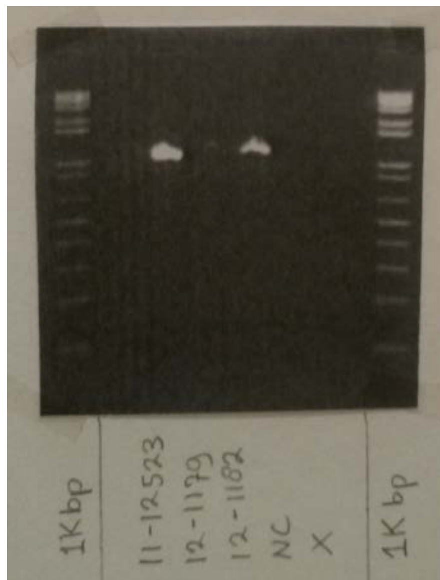


Figure 1: Electrophoresis on samples 11-12523 (slot 1), 12- 1179 (slot 2), 12-1182 (slot 3) and negative control (slot 4) ensuing RT-PCR.

Optimizing PCR conditions

Prior to qRT-PCR on 41 RNA-samples, testing conditions for qRT-PCR were optimized using sample 11-12523. The lowest Ct-values were produced using the undiluted RNA-sample together with primer concentrations of 600nM each and a probe concentration of 200nM. The Ct-value differed by only 0.09 between the duplicates with an average Ct-value of 25,52.

	Primer 400nM		Primer 600nM	
Probe 200nM	25.87	26.2	**25.47**	**25.56**
Probe 300nM	25.88	25.91	25.63	25.05

Table 1: Ct-values for sample 11-12523 in qRT-PCR using primer concentrations of 400nM or 600nM and probe concentrations of 200nM or 300nM.

The exact amount of RNA in each sample was unknown. Therefore, the standard curves produced by qRT-PCR on dilutions of sample 11-12523 were relative.

<u>Degenerated primers and degenerated probe</u>

The degenerated primers and degenerated probe were tested using samples 11-12523 and 12-6009. Both samples produced a negative result during qRT-PCR. Subsequently, the annealing temperature was adjusted to match the degenerated primers' estimated Tm-values (see below). The outcome of this qRT-PCR was also negative.

forward Tm = 2°C x (5+3) + 4°C x (4+3) +3°C x (5) = 59°C
reverse Tm = 2°C x (5+4) + 4°C x (2+5) +3°C x (5) = 58°C

A thermal gradient RT-PCR was applied to test multiple annealing temperatures together with different primer concentrations. Sample 11-12523 was visible on electrophoresis with fluorescent bands strongest between 45,0°C – 56,0°C and at primer concentrations of 600nM and 800nM. A clear fluorescent band was visible for an RT-PCR run with an annealing temperature of 54°C. This temperature is 5°C under the calculated primer Tm-values and was incorporated as the annealing temperature in a subsequent degenerated qRT-PCR.

Degenerated qRT-PCR was repeated on sample 11-12523 and 12-6009 using an annealing temperature of 54°C and degenerated primer concentrations of 600nM and 800nM. Sample 11-12523 produced the lowest Ct-values when using primer concentrations of 800nM each. No positive outcome was obtained for sample 12-6009.

The Ct-values for a qRT-PCR run on sample 11-12523 were compared in the Stratagene Mx3000P® QPCR System and the Rotor-Gene Q real-time PCR machine. Ct-values were lower for qRT-PCR run in the Rotor-Gene machine (see Table 2). Additionally, a Ct-value was obtained for a 10-4 dilution of sample 11-12523 in the Rotor-Gene. The same sample dilution produced no Ct-value in the Stratagene machine.

Ct values	Rotor-Gene	Stratagene
Dilution 10-1	**23.92**	28.57
Dilution 10-2	**27.16**	31.35
Dilution 10-3	**29.95**	34.59
Dilution 10-4	**32.93**	No Ct value

Table 2: Ct-values for sample 11-12523 run in a Stratagene and a Rotor-Gene machine.

qRT-PCR 41 RNA-samples

qRT-PCR was performed on forty-one RNA-samples using the optimized conditions described above. Samples which produced a Ct-value were considered positive, whereas samples without a Ct-value were considered negative. Results of qRT-PCR were confirmed by the presence of a fluorescent band with a length of 118 base pairs on electrophoresis.
A Ct-value was obtained for thirty-five RNA-samples. Six RNA-samples were negative (see Table 3 in the attachment). Out of 32 cluster-1 samples, 30 tested positive. Samples 12-1179 and 12-1184 were negative. Cluster-2 samples were positive in two cases, one was negative. Five cluster-4 samples were tested of which three produced a Ct- value. Only one cluster-5 sample was included in the qRT-PCR. This sample did not produce a Ct-value. Electrophoresis confirmed the presence or absence of the Ct-values. Samples without a Ct-value were also without a fluorescent band (see Figures 2, 3 and 4).

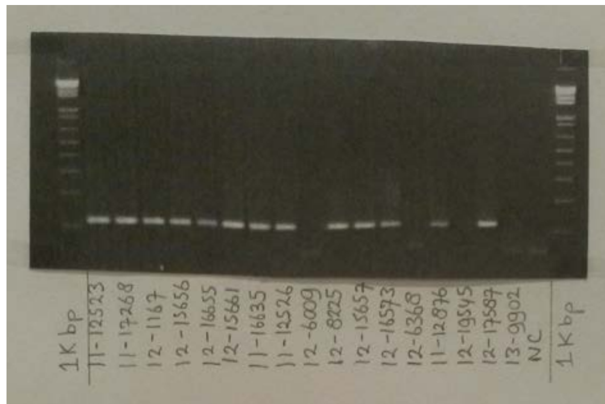
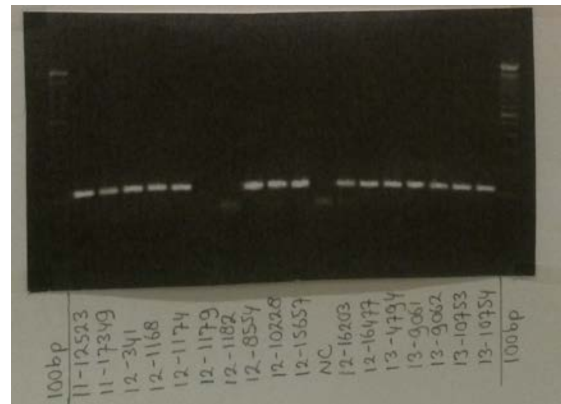


Figure 2: qRT-PCR on 18 samples including standard sample 11-12523 and a negative control.



Fiqure 3: qRT-PCR on 18 samples including standard sample 11-12523 and a negative control.

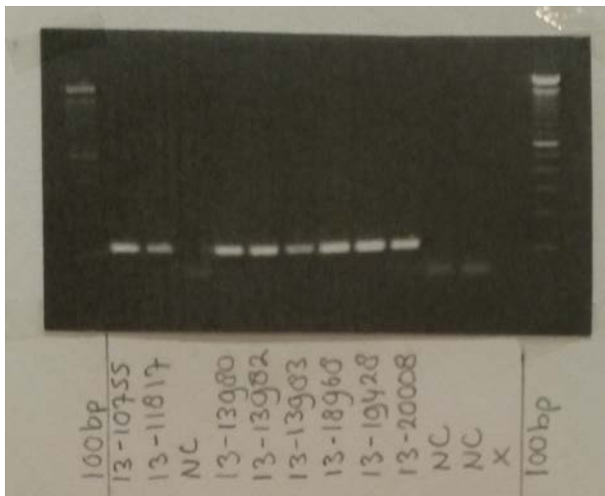


Figure 3: qRT-PCR on 11 samples including 3 negative controls.

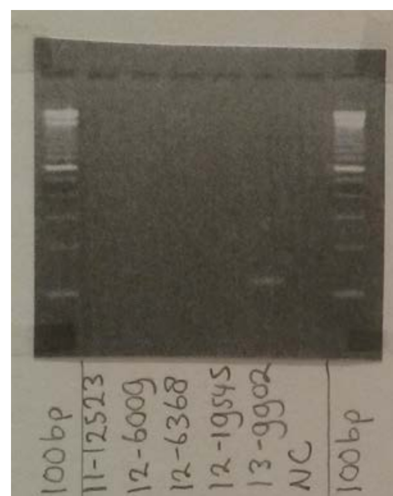


Figure 2: Degenerated qRT-PCR on 6 samples including standard sample 11-12523 and a

Degenerated qRT-PCR

Four samples without a Ct-value for qRT-PCR were subjected to qRT-PCR with degenerated primers and a degenerated probe. None of the samples produced a Ct-value for the degenerated qRT-PCR (see Table 3 in the Attachment). These results were confirmed by the absence of fluorescent bands on electrophoresis (see Figure 5). The standard sample 11-12523 was also negative.
Samples 12-1179 and 12-1184 were not included in the degenerated qRT-PCR due to the absence of fluorescent bands on RT-PCR electrophoresis.

Sequence alignment of RNA-samples

Seventeen RNA-samples which were sent for sequencing were added to the ARV alignment database and compared to the ARV consensus strain. In Figure 6 (see Attachment), nucleotides that are identical to the consensus strain are represented by dots. Letters represent a nucleotide which differs from the consensus strain.
Four samples are marked by a red dot in the left column. These samples did not produce a Ct-value during (degenerated) qRT-PCR. The amount of nucleotide differences between these strains and the consensus strain is higher than in other strains, especially for the position of the probe S4-rt-P1.

Discussion

The primers S4-rt-F1, S4-rt-R1 and probe S4-rt-P1 work on the majority of cluster 1 RNA-samples included in this research project. However, results vary for the samples grouped under cluster 2, 4 and 5. The negative results can be due to a number of causes.

1. Insufficient RNA in the sample.
2. Genetic diversity between virus strains.
3. Design of primers and probe are not optimal.

The amount of RNA in a sample can be too low for adequate amplification to take place. Insufficient amplification causes insufficient fluorescence resulting in an outcome of 'no Ct' in qRT-PCR and the absence of a fluorescent band in electrophoresis. Two cluster 1 RNA-samples did not produce a Ct-value during qRT-PCR. This was confirmed by electrophoresis. Electrophoresis performed on RT-PCR products also either lacked a fluorescent band for these samples (12-1179) or produced a weak result (12-1184). The primers S4-F13 and S4-R1133 used for the RT-PCR were suitable for all reovirus strains and should have been able to amplify the RNA in samples 12-1179 and 12-1184. The absence of a (clear) fluorescent band in both RT-PCR and qRT-PCR gels therefore suggests a lack of template RNA in the samples. In order to determine that both samples are truly negative, new RNA-samples must be extracted from identical cell culture supernatants and subjected to RT-PCR and qRT-PCR.

Negative results can also be caused by genetic diversity between the virus strains. (Degenerated) primers and probes used in qRT-PCR are gene specific and hybridization between primers, probe and template-DNA is dependent on complementarity. Nucleotide mismatches between primer, probe and template-DNA will prevent primers and probe from binding. This is especially important for mismatches at the 3' end of the primer. Alignment of the RNA-samples used in this research shows the level of genetic diversity in the S4 segment of the viral genome (see Figure 6). The nucleotide sequences framed by the yellow, orange and green boxes represent the locations for the qRT-PCR (degenerated) primers and probe. In most RNA-samples the three locations contain a number of mismatches.
The strains marked with a red dot produced no Ct during qRT-PCR. Closer examination of the nucleotide sequences of these samples shows an increased amount of mismatches compared to other samples. The amount of differences between these strains and the (degenerated) primers and probe could be the cause of no DNA amplification and thus no of Ct-value.

During gradient PCR sample 11-12523 was used for testing. This sample was positive for qRT-PCR but negative for degenerated qRT-PCR. However, the sample produced fluorescent bands in electrophoresis following gradient PCR with degenerated primers. This PCR did not contain the degenerated probe. The positive result for gradient PCR without use of the degenerated probe might suggest that the probe is the cause of negative results for the degenerated qRT-PCR.

In order to obtain more positive test results, it might be necessary to design new (degenerated) primers and probes for the S4-gene. This can prove to be a difficult task due to the amount of nucleotide differences between virus strains for this genome segment. Another solution would be to develop primers and probes for a different gene with better conserved nucleotide sequences. Another option would be to optimize and develop the current qRT-PCR for the detection of cluster 1 RNA-strains only.

Before qRT-PCR can be incorporated in routine diagnostic testing, further research, as suggested above, will have to be done before field samples can be tested for ARV using genotype specific qRT-PCR.

The main goal of this research project was to develop a genotype specific qRT-PCR suitable for the routine diagnostic testing of field samples for Avian Orthoreovirus in order to determine circulating field ARV's. With the results of this study, new sequence information has been obtained from the RNA-samples included in this research. It was shown that qRT-PCR works for the majority of cluster 1 samples and qRT-PCR works for some samples from clusters 2, 4 and 5. Further optimizing of qRT-PCR conditions will have to take place and/or new primers and probes will have to be developed to obtain better results.

References

1. Overview of viral arthritis in poultry - Merck Veterinary Manual
Accessed in January 2014
http://www.merckmanuals.com/vet/poultry/viral_arthritis/overview_of_viral_arthritis_in_poultry.html?qt=viral%20arthritis%20poultry&alt=sh

2. Sigma-C capsid protein – Uniprot:
Accessed in January 2014
http://www.uniprot.org/uniprot/Q992I2

3. DAY, J.M. (2009) The diversity of the orthoreoviruses: Molecular taxonomy and phylogenetic divides. Infection, Genetics and Evolution 9 (4): 390-400

4. TROXLER, S,. RIGOMIER, P., BILIC, I., LIEBHART, D., PROKOFIEVA, 1., ROBINEAU B., HESS, M. (2013) Identification of a new reovirus causing substantial losses in broiler production in France, despite routing vaccination of breeders. Veterinary Record 172 (21) 556 originally published online May 1 2013. doi: 10.1136/vr. 101262

5. KANT, A., BALK, F., BORN, L., ROOZELAAR van, D., HEIJMANS, J., GIELKENS, A., HUURNE ter, A. (2003) Classification of Dutch and German avian reoviruses by sequencing the sigmaC protein. Veterinary Research 34 (2): 203-212

6. LIU, H.J., LEE, L.H., HSU, H.W., KUO, L.C., LIAO, M.H. (2003) Molecular evolution of avian reovirus: evidence for genetic diversity and reassortment of the S-class genome segments and multiple co-circulating linages. Virology 314 (1): 336-349

7. GOLDENBERG, D., PASMANIK-CHOR, M., PIRAK, M., KASS, N,. LUBLIN, A., YEHESKEL, A., HELLER, D., PITCOVSKI J. (2010) Genetic and antigenic characterization of sigma C protein from avian reovirus. Avian Pathology 39 (3): 189-199

8. GUO, K., DORMITORIO, T.V., OU, S., GIAMBRONE, J.J. (2011) Development of TaqMan real-time RT-PCR for detection of avian reoviruses. Journal of Virological Methods 177 (1): 75-79

9. LEEUWEN van, W.B., VINK, C. (first edition 2009) Molecular Diagnostics techniques and applications.

10. CHIU C. J., LEE L. H. (1997) Cloning and nucleotide sequencing of the S4 genome segment of avian reovirus S1133. Archives of Virology 142 (12): 2515 -2520

11. RECK C., MENIN A., CANEVER M. F., MILETTI L.C. (2013) Rapid detection of mycoplasma synoviae and avian reovirus in clinical samples of Poultry using multiplex PCR. Avian Diseases, 57 (2): 220-224.

12. TENG L., XIE Z., XIE L., LIU J., PANG Y., DENG X., XIE Z., FAN Q., LUO S., FENG J., KHAN M. I. (2013). Sequencing and phylogenetic analysis of an avian reovirus genome. Virus Genes (2014) 48 (2): 381-386

13. TOURIS-OTERO F., MARTINEZ-COSTAS J., VAKHARIA V. N., BENAVENTE J. (2005) Characterization of the nucleic acid-binding activity of the avian reovirus non-structural protein σNS. Journal of General Virology 86 (4): 1159-1169

14. LUBLIN A., GOLDENBERG D., ROSENBLUTH E., HELLER D. E., PITCOVSKI J. (2011) Wide-range protection against avian reovirus conferred by vaccination with representatives of four defined genotypes. Vaccine 29 (47): 8683-8688

15. GOLDENBERG D., PASMANIK-CHOR M., PIRAK M., KASS N., LUBLIN A., YEHESKEL A., HELLER D., PITCOVSKI J. (2010) Genetic and antigenic characterization of sigma C protein from avian reovirus. Avian Pathology 39 (3): 189-199

16. GUAN K. M., HSUEH C. L., LIANG K. Y., WEN J.T., JULIUS C. L. C., MING L.H., TIEN C. J., HUNG L. J. (2006) Development of a quantitative Light Cycler real-time RT-PCR for detection of avian reovirus. Journal of Virological Methods 133 (1): 6-13

17. MACKAY I. M., ARDEN K. E., NITSCHE A. (2002) Real-time PCR in virology. Nucleic Acids Research 30 (6): 1292-1305

18. MACKAY I. (2004) Real-time PCR in the microbiology laboratory. Clinical Microbiology and Infection 10 (3): 190-212

19. EPSY M. J., UHL J. R., SLOAN L. M., BUCKWALTER S. P., JONES M. F., VETTER E. A., YAO J. D. C., WENGENACK N. L., ROSENBLATT J. E., COCERILL F. R., SMITH T. F. (2006) Real-Time PCR in Clinical Microbiology: Applications for Routine Laboratory Testing. Clinical Microbiology Reviews 19(1): 165-256

20. SWAYNE D.E. Diseases of Poultry. Thirteenth Edition. 2013 Wiley & Sons Imc. Chapter 11: Reovirus infections. Richard C. Jones. Pages 351- 373

21. JONES R.C. Avian reovirus infections (2000). Revue Scientifique et Technique de l'office international des epizooties 19 (2), 614-625

22. JONES R.C., ISLAM M.R., KELLY D.F. (1989) Early pathogenesis of experimental reovirus infection in chickens. Avian pathology 18 (2): 239-253

Attachments

Table 3: qRT-PCR results for 41 RNA-samples

Sample	Cluster	RT-qPCR	deg RT-qPCR	Sample	Cluster	RT-qPCR	deg RT-qPCR
11 12523	1	positive	negative	12 1179	1	negative	negative
11 17268	1	positive	n.a.	12 1182	1	negative	negative
12 1167	1	positive	n.a.	12 6009	2	negative	negative
12 15656	1	positive	n.a.	12 6368	4	negative	negative
12 16655	1	positive	n.a.	12 19545	4	negative	negative
12 15661	1	positive	n.a.	13 9902	5	negative	negative
11 16635	1	positive	n.a.				
11 12526	1	positive	n.a.				
11 17349	1	positive	n.a.				
12 341	1	positive	n.a.				
12 1168	1	positive	n.a.				
12 1174	1	positive	n.a.				
12 8554	1	positive	n.a.				
12 10228	1	positive	n.a.				
12 15657	1	positive	n.a.				
12 16203	1	positive	n.a.				
12 16477	1	positive	n.a.				
13 4794	1	positive	n.a.				
13 9061	1	positive	n.a.				
13 9062	1	positive	n.a.				
13 10753	1	positive	n.a.				
13 10754	1	positive	n.a.				
13 10755	1	positive	n.a.				
13 11817	1	positive	n.a.				
13 13980	1	positive	n.a.				
13 13982	1	positive	n.a.				
13 13983	1	positive	n.a.				
13 18968	1	positive	n.a.				
13 19428	1	positive	n.a.				
13 20008	1	positive	n.a.				
12 8225	2	positive	n.a.				
12 15657	2	positive	n.a.				
12 16573	4	positive	n.a.				
11 12876	4	positive	n.a.				
12 17587	4	positive	n.a.				

Table 3: Results for qRT-PCR and degenerated qRT-PCR for all samples included in this research. Samples with a Ct-value were considered positive whereas samples with no Ct were considered negative. Samples are listed according to Cluster number.

Figure 6: Sequence alignment of RNA-samples

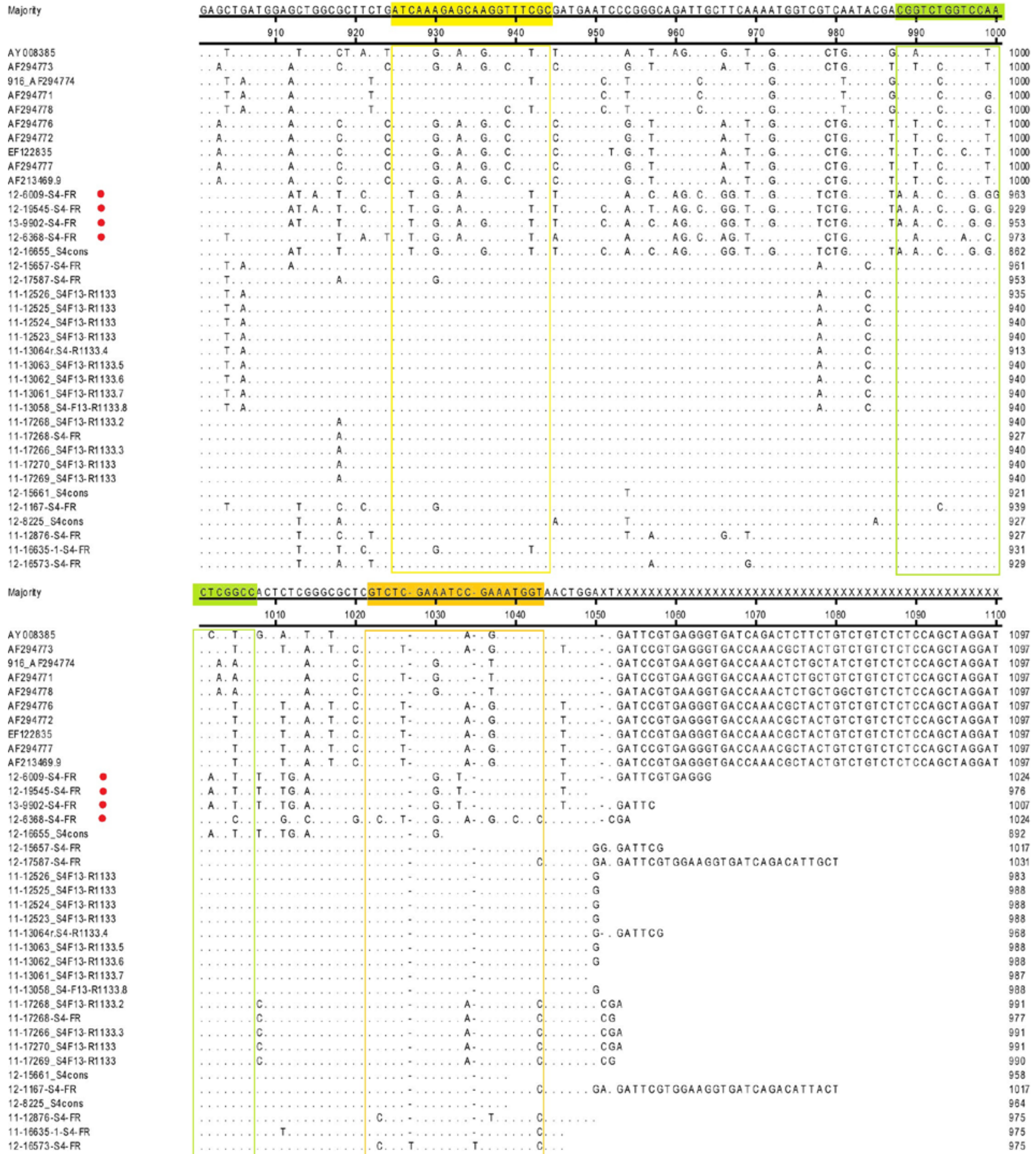


Figure 4: Sequence alignment of RNA-samples. The positions of the primers and probe are depicted. Yellow: position forward primer S4-rt-F1. Orange: position reverse primer S4-rt-R1. Green: position probe S4-rt-P1.