universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

Echovirus 30: Tracing an enteric virus in an aquatic environment

angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag. rer. nat.)

Verfasserin / Verfasser:	**Armin Löckher**
Matrikel-Nummer:	**0103933**
Studienrichtung / Studienzweig (lt. Studienblatt):	**Diplomstudium Ökologie**
Betreuerin / Betreuer:	**Ao.Univ.Prof. Branko Velimirov** **Ao.Univ.Prof. Sylvia Hagemann**

Wien, im November 2010

Für meine Schwester.

Danksagung

An dieser Stelle möchte ich mich bei jenen bedanken, die mich maßgeblich bei der Fertigstellung dieser Diplomarbeit unterstürtzt haben, insbesondere bei:

Den beiden aus Linz, Herta und Dagmar, die mich zu diesem Studium ermuntert und es mir durch ihre vielfältige, geduldige Unterstützung auch in einer schwierigen Zeit ermöglicht haben, meinen Traum zu leben. Ohne sie wäre diese Arbeit nicht geschrieben worden.

Den beiden am Steuerruder, meinen Betreuern Ao.Univ.Prof.Dr. Sylvia Hagemann und Ao.Univ.Prof.Dr. Branko Velimirov, die mich mit kompetenter, ruhiger Hand über die Hürden in dieser Arbeit geführt haben, stets ein offenes Ohr für meine Vorschläge und Probleme hatten und mir die Mittel ihres Labors für die Beantwortung meiner Fragestellung zur Verfügung stellten. Ganz besonderer Dank geht dabei an Sylvia, die die Betreuung in einer kritischen Phase mit auf sich genommen und die mir mit ihrem Engagement sehr geholfen hat.

Den beiden Sonnenstrahlen im Labor, Ingrid Gerstl und Mag. Susanne Fister, für all die kleinen Rat- und Vorschläge, für die helfenden Hände bei der Laborarbeit und dafür, dass ihr die wohl amüsantesten Laborpartner seid, die ich je kennengelernt habe und ein Arbeitsklima geprägt habt, wie man es sich besser nicht wünschen kann.

Den fünfen im Hintergrund, die mit Rat und Tat immer hinter mir gestanden haben, für lange Jahre der Freundschaft und den Ausgleich, den sie mir zu meinem Studium und meiner Arbeit bieten.

Und last but not least...

Der einen, die mich nach bester Kräften während der heißen Phasen meiner Diplomarbeit entlastete, dabei in den letzten zwei Jahren auf viel gemeinsame Zeit verzichten musste und dann oft abends mit Ruhe und Geduld meine Laune ertragen hat.

Ihnen, und allen, die hier nicht genannt, aber ganz bestimmt nicht vergessen sind, ein von Herzen kommendes

Danke!

Table Of Contents

1 Introduction 1

1.1 Viruses – The Fascination Of The Unknown 1

1.1.1 Influence on aquatic ecosystems 2

1.1.2 Viruses as human pathogens 3

1.1.3 RNA Viruses 5

1.2 Enteroviruses – Knowing Your Enemy 6

1.2.1 The enterovirus genome 7

1.2.2 Enteroviruses as human pathogens 9

1.2.3 Enteroviruses as indicators for water quality 11

2 Aims And Objectives 12

3 Material And Methods 13

3.1 The Study Site 13

3.2 Sampling 13

3.2.1 Freshwater sampling 13

3.2.2 Sediment sampling 14

3.3 Sample Concentration and RNA Isolation 15

3.3.1 Freshwater samples 15

3.3.2 Sediment samples 16

3.4 Primer Design 17

3.5 cDNA Synthesis 21

3.6 Polymerase Chain Reaction (PCR) 22

3.6.1 Primary PCR 24

3.6.2 Secondary PCR 25

3.7 Agarose Gel Electrophoresis 26
3.8 Gel Elution 27
3.9 Ligation 27
3.10 Transformation 29
3.11 Blue/White Selection 30
3.12 Miniprep 31
3.13 Restriction 31
3.14 Sequencing 32
3.15 Sequence Analysis 32
3.16 Suppliers Of Used Chemicals And Material 33
4 Results 35
4.1 PCR 35
4.1.1 Sediment 35
4.1.2 Waterbody 37
4.2 Restriction Analysis 39
4.2.1 Sediment 39
4.2.2 Waterbody 41
4.3 Sequences and BLAST Results 42
4.3.1 August 2009 Alte Donau sediment 42
4.3.2 October 2009 Alte Donau sediment 42
4.3.3 August 2009 Alte Donau water column 42
4.4 Alignments 47
4.5 Pylogenetic Tree 53
5 Discussion 54
5.1 The Sediment 54
5.2 The Waterbody 55
5.3 A Significant Threat To Human Health? 56

5.4 Conclusion.....58

6 References 59

Figures and Tables.....63

Table Of Abbreviations 65

1 Introduction

1.1 Viruses – The Fascination Of The Unknown

Long time, virus abundances and distribution have been seriously underestimated, due to the fact that most viruses are non-cultivable. Culture independent methods revealed that the impact of viruses on global processes is much higher than previously estimated:
Nowadays we know that viruses are the most abundant biological entities on the planet, especially in aquatic environments. Breitbart et al. (2005) estimated the total number of viruses on earth on 10^{31}. There are approximately 10 to 100 million viruses per ml in marine and fresh waters, which makes them about 5-10 fold more abundant than prokaryotes (Chénard et al., 2008). Corinaldesi et al. (2010) indicate, that viral production rates in pelagic ecosystems range from 10^{5} to 10^{9} viruses l^{-1} h^{-1}, respectively from 10^{6} to 10^{8} viruses g^{-1} h^{-1} in benthic ecosystems, with correlated decay rates. This makes viruses also the largest reservoir of genetic diversity on Earth, for the most part still unexplored.
Suttle (2007) pointed out, that viruses, due to their lack of size, comprise only 5% of the microbial biomass, but about 94% of the nucleic-acid-containing particles in the oceans. (Fig. 1).

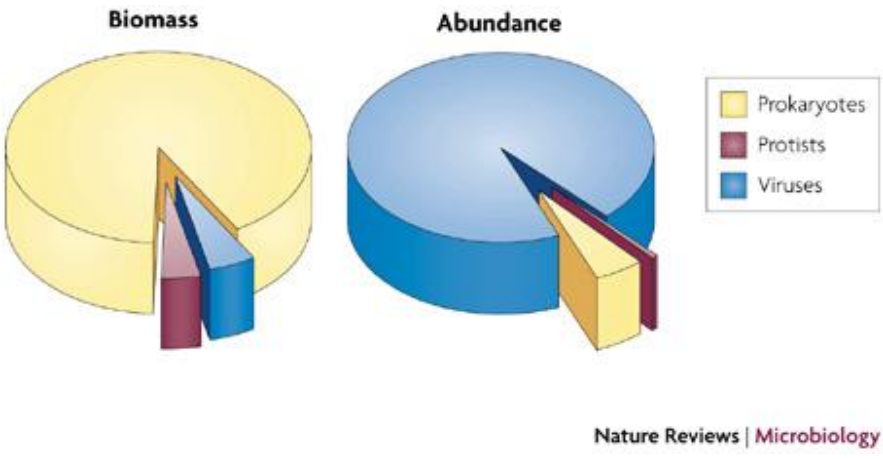


Fig. 1: Biomass and abundance of viruses (http://www.nature.com/nrmicro/journal/v5/n10/images/nrmicro1750-f1.jpg)

When thinking of viruses, one also has to bear in mind, that they have extreme evolutionary capacities. Viruses parasitize all known groups of organisms and can rapidly adapt to a large number of host species within a kingdom. Especially viruses with an RNA genome are known to replicate at a high level, resulting in highly diverse replicating populations.

1.1.1 Influence on aquatic ecosystems

A life form with such high abundance and distribution, with such a high genetic diversity is very likely to play a very important role in any ecosystem on Earth. In fact, viruses exercise influence on the abundance of organisms on all trophic levels of an aquatic food web. Virus-induced mortality and the release of cell lyses products into the environment directly affects global nutrient cycles by enhancing dissolved organic matter recycling, modulating primary production, decreasing transfer of carbon to higher trophic levels and exporting particulate organic carbon from the photic zone (Wommack et al., 2007).
Recent studies (Corinaldesi et al., 2010) showed, that 98% of the viruses in the sediment do not infect prokaryotic cells, but enter the benthic food web or the biogeochemical cycles, resulting in a huge pool of organic carbon. The exact fate of this pool is not known to date. As marine deep sea sediments are covering about 50% of the world's surface (Danovaro and Serresi, 2000), these results indicate, that viral decay also plays an important role in global biogeochemical cycles and trophodynamics.

Viruses have large impact on bacterial and algal biodiversity and genetic transfer. Virus infections may significantly alter the structure of microbial or algal host communities, changing species diversity and evenness by selective infection and decimation or removal of dominant host taxa from specific niches, e.g. by "killing the winner". Viruses also have direct influence on their host's phenotype through genetic exchange, either by specific or generalized transduction. (Wommack et al., 2007)

1.1.2 Viruses as human pathogens

While our knowledge about the genetic diversity of viruses in general is very limited, the diversity and pathology of viruses infecting humans are usually well studied, due to medical research. However, sources, transmission routes and sinks of viral pathogens are still widely unknown.

Viruses are a major cause for waterborne disease outbreaks. The incorporation risk of water related pathogenic viruses is not only given by drinking water or water used for recreational activities. Contaminated water used for agricultural purposes such as crop irrigation or food processing can also lead to a virus-caused outbreak. Also, fish grown in polluted water is a well known cause of gastroenteritis and hepatitis (Bosch et al., 2008).

Very few virus diseases are responding to a specific therapy. Most antibiotics have no effect on viruses and drugs with specifically antiviral effect are restricted to some very specific virus infections. The most effective therapy against virus infections is still prevention, thus vaccination: producing immunity to a disease via administration of antigens.

Most waterborne viruses that affect human health have RNA genomes (Table 1).

Genus (genome)	Disease(s) caused
Enterovirus (ssRNA)	Herpangina, meningitis, paralysis, fever, respiratory disease, hand-foot-and-mouth disease, myocarditis, heart anomalies, rush, pleurodynia, gastroenteritis
Hepatovirus (ssRNA)	Hepatitis
Reovirus (segmented dsRNA)	Unknown
Rotavirus (segmented dsRNA)	Gastroenteritis
Norovirus (ssRNA)	Gastroenteritis
Sapovirus (ssRNA)	Gastroenteritis
Hepevirus (ssRNA)	Hepatitis
Masamostrovirus (ssRNA)	Gastroenteritis
Coronavirus (ssRNA)	Gastroenteritis, respiratory disease, SARS
Orthomyxovirus (segmented ssRNA)	Influenza, respiratory disease
Parvovirus (ssDNA)	Gastroenteritis
Mastadenovirus (dsDNA)	Gastroenteritis, respiratory disease, conjunctivitis
Polyomavirus (dsDNA)	Progressive multifocal leucoencephalopathy, diseases of urinary tract
Circovirus (ssDNA)	Unknown, hepatitis

Table 1: Human viruses found in water environment (Bosch et al., 2008)

1.1.3 RNA Viruses

Virus genomes consist of either RNA or DNA. RNA viruses are divided into single-stranded (ss-) and double-stranded (ds-) RNA viruses. Most waterborne viruses that affect human health have ssRNA genomes (Table 1). ssRNA viruses can be further divided into positive stranded (+) and negative stranded (-) viruses: in +ssRNA viruses the genomic RNA serves as template for both RNA replication and translation, whereas –ssRNA viruses need to generate a positive copy of their genome in order to replicate. The majority of all virus species consist of a –ssRNA genome.

According to the International Committee on Taxonomy of Viruses, +ssRNA viruses split into two orders: Picornavirales and Nidovirales. The genus Enterovirus, which is part of the Picornavirales, was chosen as the main subject for this study.

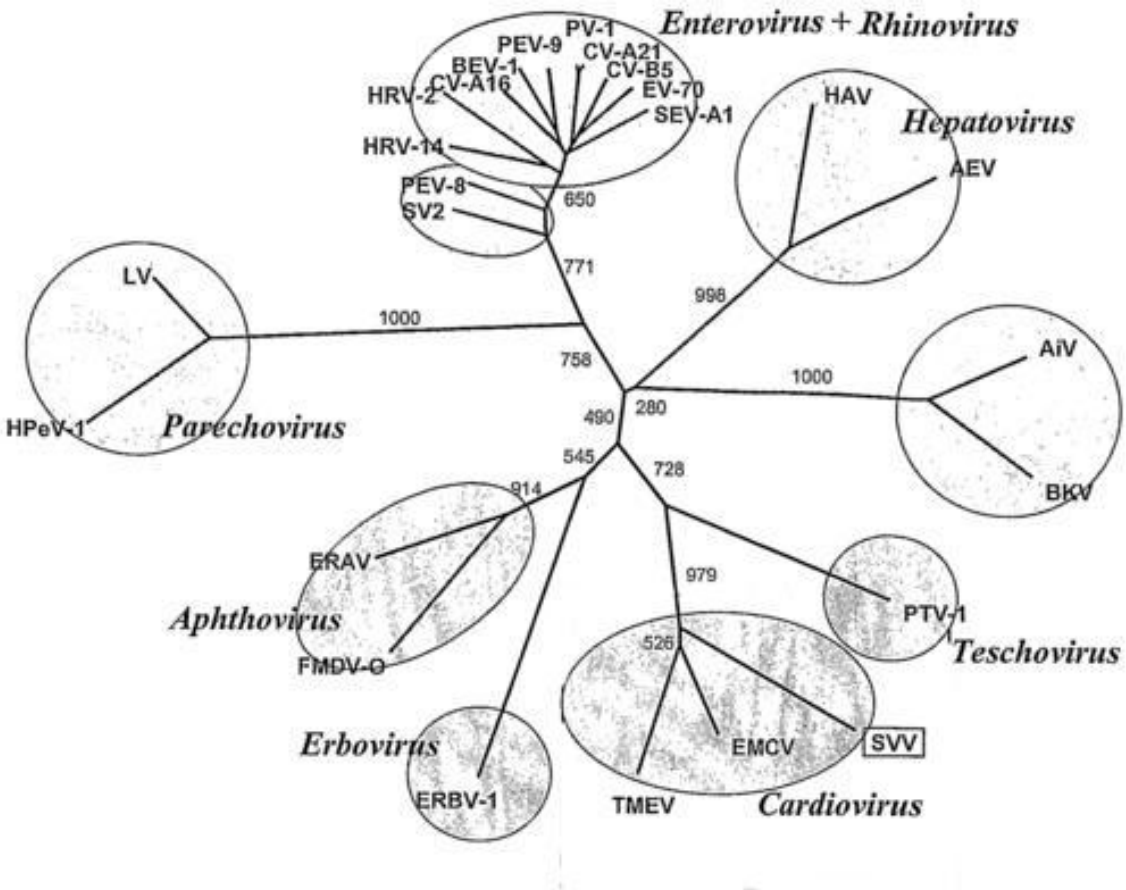


Fig. 2: The Picornaviridae family (adapted from http://www.wipo.int/pctdb/images/PCT-IMAGES/26042007/US2006039523_26042007_gz_en.x4-b.jpg)

1.2 Enteroviruses – Knowing Your Enemy

Enteroviruses (family *Picornaviridae*, genus *Enterovirus*) are among the most common viruses infecting humans. The genus consists of polioviruses, coxsackieviruses, enteroviruses and a large number of echovirus serotypes.

Enteric viruses transit easily through water environments and are usually present in insufficiently treated drinking water, ground water, rivers and seas, but can also be transported through aerosols emitted from sewage treatment plants (Rajtar et al., 2008; Fong and Lipp, 2005). They are most commonly introduced through various human activities, such as leaking sewage and septic systems and/or urban or agricultural runoff (Fong and Lipp, 2005).

1.2.1 The enterovirus genome

The genome of enteroviruses is a positive sense, single-stranded RNA molecule, approximately 7550 bp long. It consists of one single large open reading frame (ORF), which is flanked by a 5' and a 3' untranslated region (5'- and 3' UTR). The ORF is encoding for 3 polypeptides, namely P1, P2 and P3 (Fig 3).

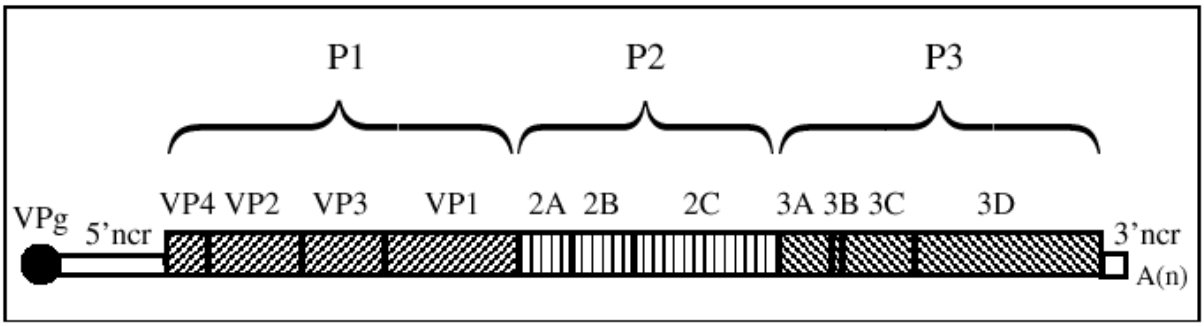


Fig. 3: The enterovirus genome (http://ethesis.helsinki.fi/julkaisut/mat/bioti/vk/airaksinen/fig3.gif)

P1 is cleaved by virus-encoded proteases to give the viral capsid proteins (VP1, VP2, VP3,VP4). P2 and P3 undergo cleavage into 7 nonstructural proteins. P2 is cleaved into 2A, 2B and 2C, P3 into 3A, 3B, 3C and 3D; the intermediate cleavage products are also functional. The proteins 2A, 3C and 3CD are proteases, 3D is the RNA-dependent RNA-polymerase. 2C is a helicase, and 2B, 2BC, 3A and 3AB play a role in the replication of viral RNA (Fig 4).

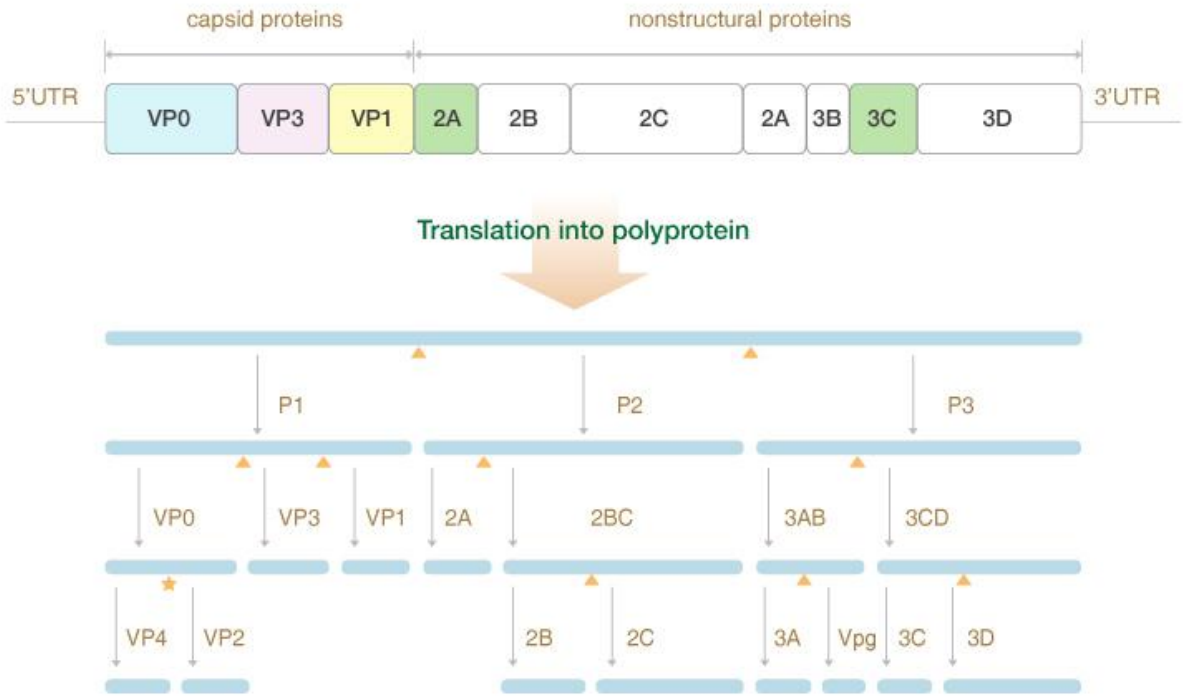


Fig. 4: Polyprotein translation of the enterovirus genome (http://kcdc.labkm.net/vsd/images/entero.jpg)

The 5'-UTR is 600-1200 bp long, which is about 10% of the entire genome. This part of the genome contains a "cloverleaf" secondary structure which is referred to as the Internal Ribosome Entry Site (IRES) (Pelletier and Sonenberg, 1988). Translation is initiated by binding ribosomes to the IRES, making the 5'-UTR essential for translation.

The 3'-UTR is shorter, 50-100 bp long. It is involved in negative strand synthesis.

Both ends of the genome are modified. The 3'-UTR is followed by a poly-A-tail as in eukaryotic mRNAs, whereas a basic protein (VPg) is attached to the 5' end of the genome.

The Enterovirus capsid is icosahedral, with a diameter ranging from 20-30 nm.

1.2.2 Enteroviruses as human pathogens

Enteroviruses can be a serious threat to human health. Of 89 known enterovirus serotypes, 64 are infective to humans (Oberste et al., 2001).
They are responsible for a wide spectrum of diseases such as respiratory infections, meningitis, hepatitis A, gastroenteritis, encephalitis and paralytic poliomyelitis as well as common colds (Nijhuis et al., 2002). Coxsackievirus infections are often connected with human heart diseases (Kocwa-Haluch, 2001). The enterovirus subgroup most isolated from clinical samples are echoviruses, which cause numerous subclinical and common cold-like illnesses, but have also been linked to serious diseases like meningitis, especially in children (Siafakas el al., 2005).

The pathogenesis is usually started by transmission of enteric viruses via the oral-fecal route. They multiply in faucal and intestinal lymphatic tissues and spread within the body by circulation of blood, affecting different organs (Fig 5). Enterovirus infections are type specific. Because of the multitude of enterovirus serotypes, control of infections and the development of an effective vaccine are very difficult.

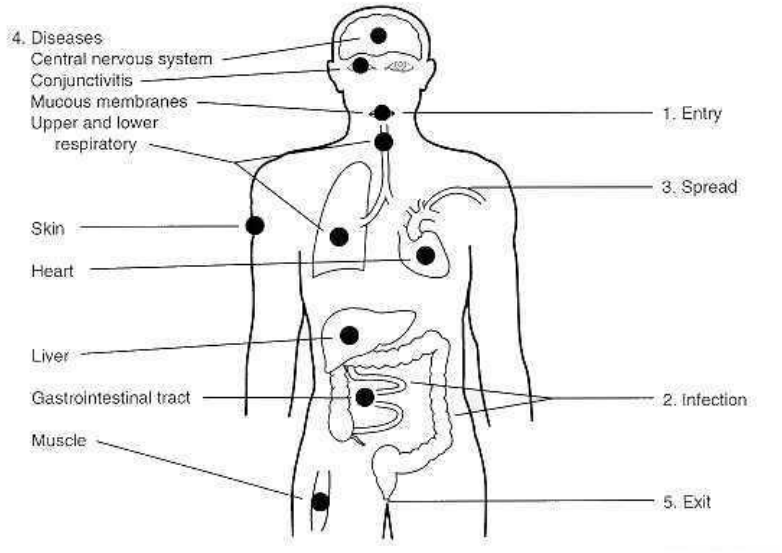


Fig. 5: Pathogenesis of enterovirus infections (http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=mmed&part=A2862)

There is a continual low-level transmission of potentially pathogenic enteroviruses through the environment (Fig. 6). Infections are often silent and remain unrecognized until secondary

spread, making predictions of outbreaks and tracking of the environmental pathways of virus serotypes almost impossible.

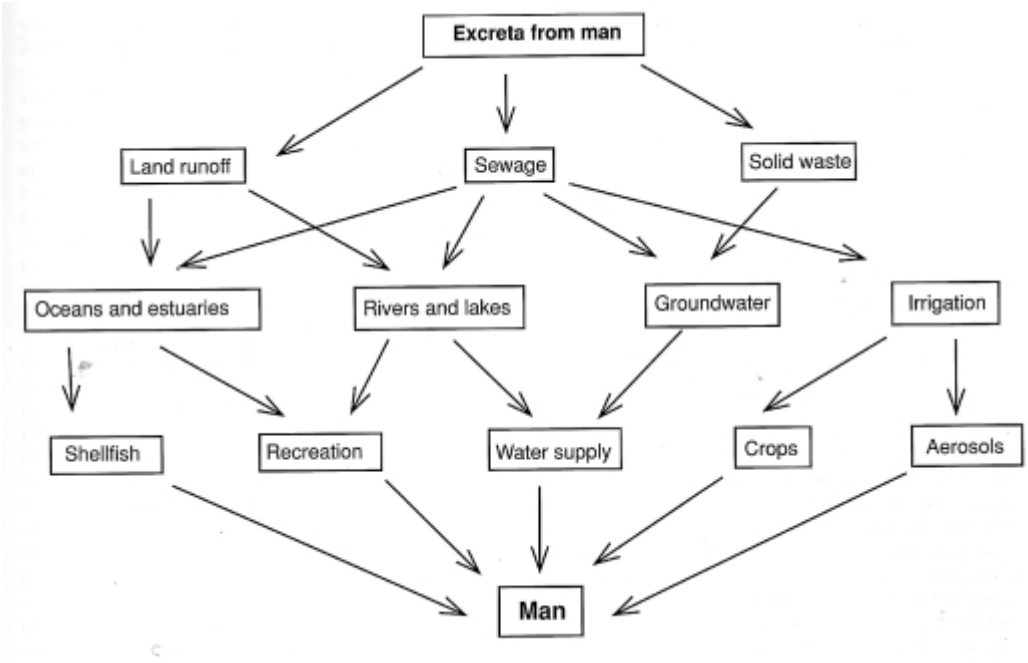


Fig 6: Transmission routes of human enteric viruses in the water environment. (Bosch et al., 1998)

Like other virus pathogens, enteric virus infections can cause a dangerous cascade effect. Various studies have reported that contamination of drinking water due to inadequate sewage treatment can lead to insufficient removal of viruses and therefore outbreaks of waterborne diseases (Boccia et al., 2002; Brown et al., 2001). As a consequence of an outbreak a massive secondary spread of viruses could contaminate additional surface water, leading to a repeatedly occurring cycle of viral contamination (Lodder et al., 2005; Lee and Lee, 2008)

The above mentioned facts would be a very good reason for surveillance of water systems regarding pathogenic viruses and especially enteroviruses; however the role of waterborne viruses as indicator for the microbiological quality of a water body has become more and more a focus of research (Rose et al., 2006; Colford et al., 2007; Arnone and Walling, 2007).

1.2.3 Enteroviruses as indicators for water quality

In most countries including the European Union and the United States, hygiene and health regulators rely only on bacterial indicators (enterococci, fecal coliform or total coliform bacteria) to measure the quality of water. However, these indicators do not always show the true risk of contaminated water, as they do not reflect the threat of other pathogens such as viruses or non-cultivable bacteria. In fact, a relationship between current bacterial indicator detection and the presence of specific viruses has not been demonstrated (Borchardt et al., 2003), and there have been numerous virus-related outbreaks linked to ingestion of waters that met fecal coliform standards (Fong and Lipp, 2005). Coliphages showed strong association with indicator bacteria and enterovirus, but weak association with other enteric viruses (Espinosa et al., 2009). Rose et al. (2006) also report, that there was no significant correlation between thermotolerant coliform densities and enterovirus levels in their study about the hygienic situation of Venice, Italy.

Additionally, bacterial indicators do not have a high survivability compared to the persistence of viral indicators, especially in marine waters (Bordalo et al., 2002). Viruses are even able to survive standard wastewater treatment such as chlorination (Fong and Lipp, 2005). This makes viruses a much more significant alternative for setting up a hygiene standard.

They are nowadays regarded as a most promising tool for determining the microbiological quality of water because of their host specifity and high resistance to environmental conditions. Detecting the presence of human enteric viruses in a water sample could help a lot in preventing waterborne diseases.

2 Aims And Objectives

According to recent research (Fister, 2009), enteric viruses are present in sediments of the oxbow lakes Alte Donau and Wasserpark, both located within the city of Vienna. The results of this study suggest, unlike other studies, that there is also an enterovirus population in the sediment during the winter months. With the primer-pairs employed in the study of 2009, Fister could only verify the presence of a single enterovirus type, namely Echovirus 30.

Based on these data and Fisters recommendations concerning experimental conditions – especially the change of employed primers – we wanted to expand the study in terms of finding other enteroviruses in the sediment and also investigating the presence of enteroviruses in the watercolumn.

In summary, this study was designed to answer the following questions:

- Are there other enteroviruses than Echovirus 30 in the sediment of the Alte Donau?
- Are there enteroviruses in the water body of the Alte Donau?
- Do these enteroviruses represent a (significant?) threat as human and/or animal pathogens?

3 Material And Methods

3.1 The Study Site

The study site was set up at the Alte Donau, an urban shallow oxbow lake within the city of Vienna with an area of about 1.6 km², a volume of 3.7 × 106 m³ and a depth of 3.5 m.

The Alte Donau is heavily used for public recreational purposes. The area counts numerous sailing schools and public baths, including one of Vienna's most popular ones, the Gänsehäufel. It can be concluded that anthropogenic factors exert a dominating influence on the water body of the Alte Donau, in particular the input of viruses into the microbial environment. Therefore, it seems a very good sampling site for a study aiming at viruses that show a coincidence between their occurrence and human use of the corresponding water body.

3.2 Sampling

3.2.1 Freshwater sampling:

The results of several studies (Chambon et al., 2001; Nairn and Clements, 1999) indicate, that enterovirus infections are most common in the months of summer and early fall, suggesting that virus density in the environment is highest at this time. Other studies report enterovirus detection especially in summer and early fall in temperate climates (Khetsuriani et al., 2006), which may be due to increased human recreational activity. According to these studies, our samples were taken in July to October 2009, in order to gain maximum yield of enteric viruses.

Freshwater samples were taken in July, August, September and October 2009 (Table 2). A sample taken in April at the nearby Wasserpark acted as cold-water reference. Each sample consisted of 15-30 liters freshwater, taken from different depths.

3.2.2 Sediment sampling:

Sediment samples were taken in August and October 2009. Samples were stored at -70°C until further processing.

Freshwater

Date	Time	Water temperature	Location
03.04.2009	10.30 am	12°C	Wasserpark
28.07.2009	09.30 am	23°C	Alte Donau
06.08.2009	10.30 am	21°C	Alte Donau
03.09.2009	09.00 am	23°C	Alte Donau
14.10.2009	10.00 am	16°C	Alte Donau

Sediment

Date	Time	Water temperature	Location
06.08.2009	10.30 am	21°C	Alte Donau
14.10.2009	10.00 am	16°C	Alte Donau

Table 2: Sampling Data

3.3 Sample Concentration and RNA Isolation

3.3.1 Freshwater samples:

Virus concentration was done using a modified protocol of Culley et al. (2006).

Material:
GF/C glass microfiber filter (Whatman)
0,22 µm pore-size DURAPORE GV membrane (Millipore)
30.000 kDa molecular-weight cutoff filter cartridge (Millipore)
peqGOLD TriFast™FL (peqlab)

Procedure:
Each freshwater sample consisted of 15-30 liters, taken from different depths. Each sample was filtered through a GF/C glass microfiber filter (Whatman) for fine particle retention, and afterwards through a 0,22 µm pore-size DURAPORE GV membrane (Millipore) for elimination of particles bigger than the expected size of enteric viruses. To get a better initial volume for RNA isolation, all samples were concentrated to approx. 15 - 20 ml by tangential flow filtration, using a 30.000 kDa molecular-weight cutoff filter cartridge (Millipore), resulting in a 100-200-fold concentration. These virus concentrates were stored at 4°C until further processing.

Concentration of water samples took about a day, and there was no possibility to fixate the samples before concentration. Although Enteroviruses can survive for days at room temperature, and can remain infective for months in river-, marine- and ground water at low temperatures (Kocwa-Haluch, 2001), several studies (Ward et al., 1986; Hurst et al., 1989) report a dramatic increase in virus inactivation due to water temperatures above 27°C. As presented in Table 2, water temperature was already 23°C during sampling in July and September 2009; this may have further increased during tangential flow filtration, as we could not cool the samples during concentration.

RNA was isolated from freshwater concentrates using peqGOLD TriFast™FL (peqlab) following the manufacturer's instructions. Various other methods were tried out but did not achieve results.

3.3.2 Sediment samples:

RNA was isolated from sediment by the method described by Ahmed and Sörensons (1995). Virus concentration was achieved using a modified protocol of Monpoeho et al. (2001). Both methods were adapted as recommended by Fister (2009).

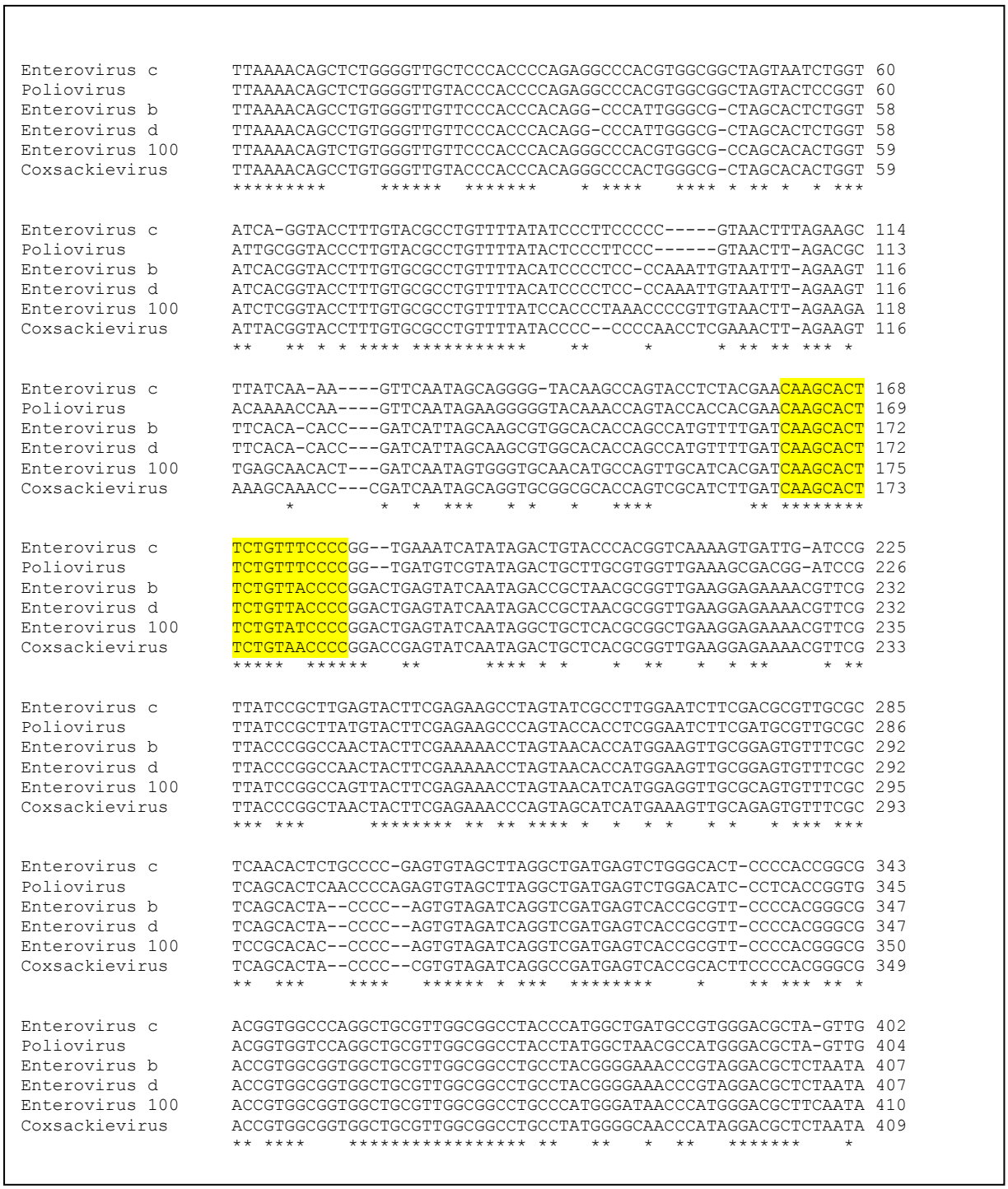
Material:
10% Beef extract (10% Lab-Lemco pH=9.0)
0,15M Na_3PO_4 buffer
PEG6000
RNA PowerSoil™ Total RNA Isolation Kit (MOBIO)
Trichlormethane ($CHCl_3$)

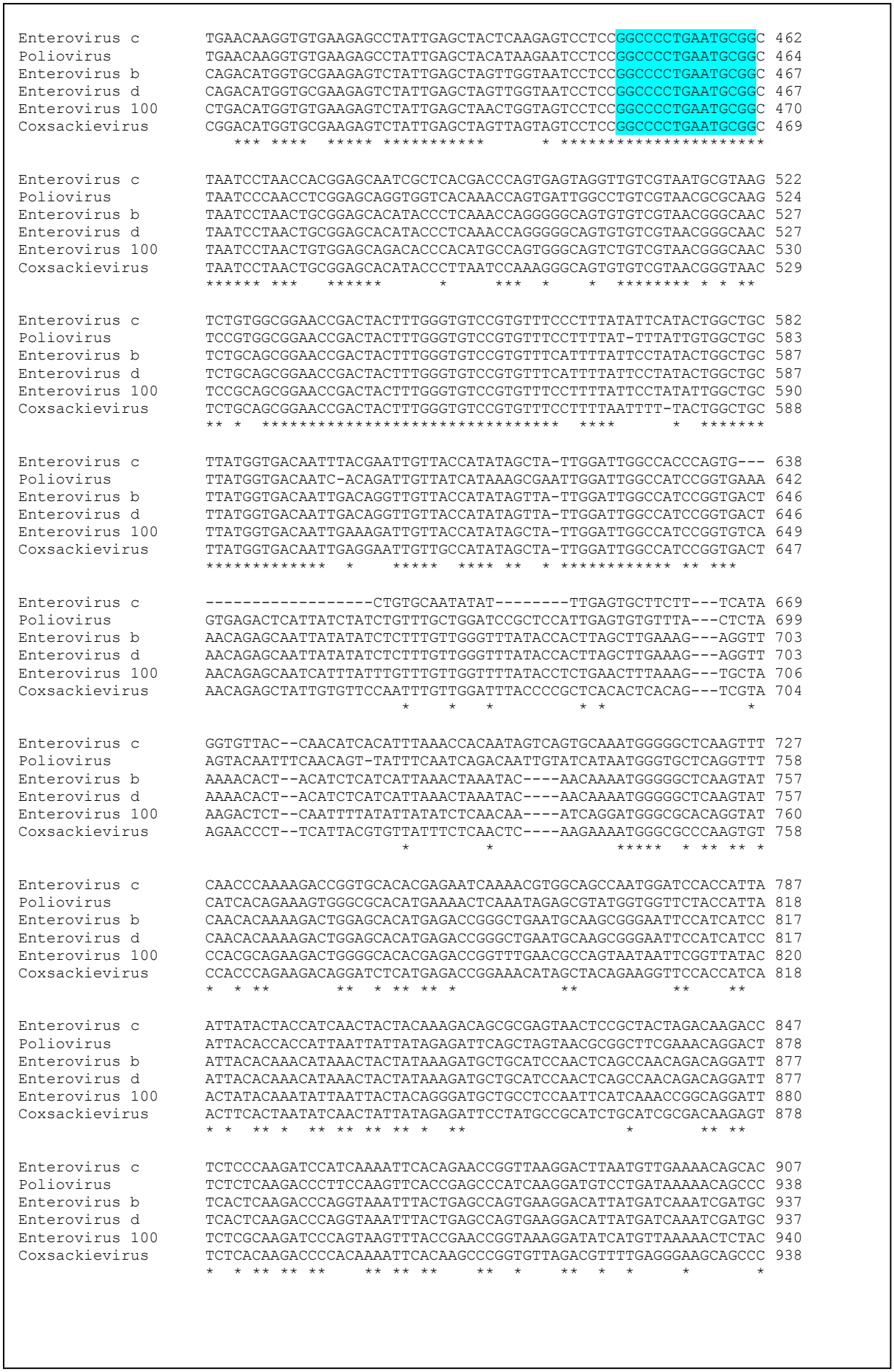
Procedure:
5g of sediment and 45ml 10% beef extract were stirred at 500rpm for 30min and sonicated on ice for 5min with 1min bursts. Afterwards the sample was mixed for 5 min on a "vortexer" and centrifuged at 5.000 x g at 4°C for 1h. The supernatant was adjusted to pH 7.2 using 32% HCl. 25ml 8% (w/v) PEG6000 was added for virus precipitation done at 4°C overnight. Then centrifugation was carried out at 10.000 x g for 90min at 4°C. The supernatant was discarded and the pellet was resuspended in 5ml phosphate buffer. It was decontaminated by adding 500µl trichlormethane and vortexing for 2 minutes. After another centrifugation period at 10.000 x g for 15 minutes at 4°C, the supernatant was transferred into 5 tubes.
RNA-isolation from the concentrate was done using RNA PowerSoil™ Total RNA Isolation Kit (MOBIO). 2 ml of the virus concentrate were added to the Bead Tube of the kit; in all other aspects RNA-isolation followed the kit protocol.

With the use of 3µl of RNA, total RNA concentration and purity were determined by spectral photometry at 260 and 280 nm, respectively. All extracts were stored at -80°C.

3.4 Primer Design

For primer design, sequences of several enteric viruses were aligned (Fig. 7) using ClustalW multiple sequence alignment software (http://www.ebi.ac.uk/Tools/clustalw/index.html) in order to determine the most suitable regions for primer binding.




Enterovirus c TGAACAAGGTGTGAAGAGCCTATTGAGCTACTCAAGAGTCCTCCGGCCCCTGAATGCGGC 462
Poliovirus TGAACAAGGTGTGAAGAGCCTATTGAGCTACATAAGAATCCTCCGGCCCCTGAATGCGGC 464
Enterovirus b CAGACATGGTGCGAAGAGTCTATTGAGCTAGTTGGTAATCCTCCGGCCCCTGAATGCGGC 467
Enterovirus d CAGACATGGTGCGAAGAGTCTATTGAGCTAGTTGGTAATCCTCCGGCCCCTGAATGCGGC 467
Enterovirus 100 CTGACATGGTGTGAAGAGTCTATTGAGCTAACTGGTAGTCCTCCGGCCCCTGAATGCGGC 470
Coxsackievirus CGGACATGGTGCGAAGAGTCTATTGAGCTAGTTAGTAGTCCTCCGGCCCCTGAATGCGGC 469
TAATCCTAACCACGGAGCAATCGCTCACGACCCAGTGAGTAGGTTGTCGTAATGCGTAAG 522
TAATCCCAACCTCGGAGCAGGTGGTCACAAACCAGTGATTGGCCTGTCGTAACGCGCAAG 524
TAATCCTAACTGCGGAGCACATACCCTCAAACCAGGGGGCAGTGTGTCGTAACGGGCAAC 527
TAATCCTAACTGCGGAGCACATACCCTCAAACCAGGGGGCAGTGTGTCGTAACGGGCAAC 527
TAATCCTAACTGTGGAGCAGACACCCACATGCCAGTGGGCAGTCTGTCGTAACGGGCAAC 530
TAATCCTAACTGCGGAGCACATACCCTTAATCCAAAGGGCAGTGTGTCGTAACGGGTAAC 529
TCTGTGGCGGAACCGACTACTTTGGGTGTCCGTGTTTCCCTTTATATTCATACTGGCTGC 582
TCCGTGGCGGAACCGACTACTTTGGGTGTCCGTGTTTCCTTTTAT-TTTATTGTGGCTGC 583
TCTGCAGCGGAACCGACTACTTTGGGTGTCCGTGTTTCATTTTATTCCTATACTGGCTGC 587
TCTGCAGCGGAACCGACTACTTTGGGTGTCCGTGTTTCATTTTATTCCTATACTGGCTGC 587
TCCGCAGCGGAACCGACTACTTTGGGTGTCCGTGTTTCCTTTTATTCCTATATTGGCTGC 590
TCTGCAGCGGAACCGACTACTTTGGGTGTCCGTGTTTCCTTTTAATTTT-TACTGGCTGC 588
TTATGGTGACAATTTACGAATTGTTACCATATAGCTA-TTGGATTGGCCACCCAGTG--- 638
TTATGGTGACAATC-ACAGATTGTTATCATAAAGCGAATTGGATTGGCCATCCGGTGAAA 642
TTATGGTGACAATTGACAGGTTGTTACCATATAGTTA-TTGGATTGGCCATCCGGTGACT 646
TTATGGTGACAATTGACAGGTTGTTACCATATAGTTA-TTGGATTGGCCATCCGGTGACT 646
TTATGGTGACAATTGAAAGATTGTTACCATATAGCTA-TTGGATTGGCCATCCGGTGTCA 649
TTATGGTGACAATTGAGGAATTGTTGCCATATAGCTA-TTGGATTGGCCATCCGGTGACT 647
----------CTGTGCAATATAT--------TTGAGTGCTTCTT---TCATA 669
GTGAGACTCATTATCTATCTGTTTGCTGGATCCGCTCCATTGAGTGTGTTTA---CTCTA 699
AACAGAGCAATTATATATCTCTTTGTTGGGTTTATACCACTTAGCTTGAAAG---AGGTT 703
AACAGAGCAATTATATATCTCTTTGTTGGGTTTATACCACTTAGCTTGAAAG---AGGTT 703
AACAGAGCAATCATTTATTTGTTTGTTGGTTTTATACCTCTGAACTTTAAAG---TGCTA 706
AACAGAGCTATTGTGTTCCAATTTGTTGGATTTACCCCGCTCACACTCACAG---TCGTA 704
GGTGTTAC--CAACATCACATTTAAACCACAATAGTCAGTGCAAATGGGGGCTCAAGTTT 727
AGTACAATTTCAACAGT-TATTTCAATCAGACAATTGTATCATAATGGGTGCTCAGGTTT 758
AAAACACT--ACATCTCATCATTAAACTAAATAC----AACAAAATGGGGGCTCAAGTAT 757
AAAACACT--ACATCTCATCATTAAACTAAATAC----AACAAAATGGGGGCTCAAGTAT 757
AAGACTCT--CAATTTTATATTATATCTCAACAA----ATCAGGATGGGCGCACAGGTAT 760
AGAACCCT--TCATTACGTGTTATTTCTCAACTC----AAGAAAATGGGCGCCCAAGTGT 758
CAACCCAAAAGACCGGTGCACACGAGAATCAAAACGTGGCAGCCAATGGATCCACCATTA 787
CATCACAGAAAGTGGGCGCACATGAAAACTCAAATAGAGCGTATGGTGGTTCTACCATTA 818
CAACACAAAAGACTGGAGCACATGAGACCGGGCTGAATGCAAGCGGGAATTCCATCATCC 817
CAACACAAAAGACTGGAGCACATGAGACCGGGCTGAATGCAAGCGGGAATTCCATCATCC 817
CCACGCAGAAGACTGGGGCACACGAGACCGGTTTGAACGCCAGTAATAATTCGGTTATAC 820
CCACCCAGAAGACAGGATCTCATGAGACCGGAAACATAGCTACAGAAGGTTCCACCATCA 818
ATTATACTACCATCAACTACTACAAAGACAGCGCGAGTAACTCCGCTACTAGACAAGACC 847
ATTACACCACCATTAATTATTATAGAGATTCAGCTAGTAACGCGGCTTCGAAACAGGACT 878
ATTACACAAACATAAACTACTATAAAGATGCTGCATCCAACTCAGCCAACAGACAGGATT 877
ATTACACAAACATAAACTACTATAAAGATGCTGCATCCAACTCAGCCAACAGACAGGATT 877
ACTATACAAATATTAATTACTACAGGGATGCTGCCTCCAATTCATCAAACCGGCAGGATT 880
ACTTCACTAATATCAACTATTATAGAGATTCCTATGCCGCATCTGCATCGCGACAAGAGT 878
TCTCCCAAGATCCATCAAAATTCACAGAACCGGTTAAGGACTTAATGTTGAAAACAGCAC 907
TCTCTCAAGACCCTTCCAAGTTCACCGAGCCCATCAAGGATGTCCTGATAAAAACAGCCC 938
TCACTCAAGACCCAGGTAAATTTACTGAGCCAGTGAAGGACATTATGATCAAATCGATGC 937
TCACTCAAGACCCAGGTAAATTTACTGAGCCAGTGAAGGACATTATGATCAAATCGATGC 937
TCTCGCAAGATCCCAGTAAGTTTACCGAACCGGTAAAGGATATCATGTTAAAAACTCTAC 940
TCTCACAAGACCCCACAAAATTCACAAGCCCGGTGTTAGACGTTTTGAGGGAAGCAGCCC 938

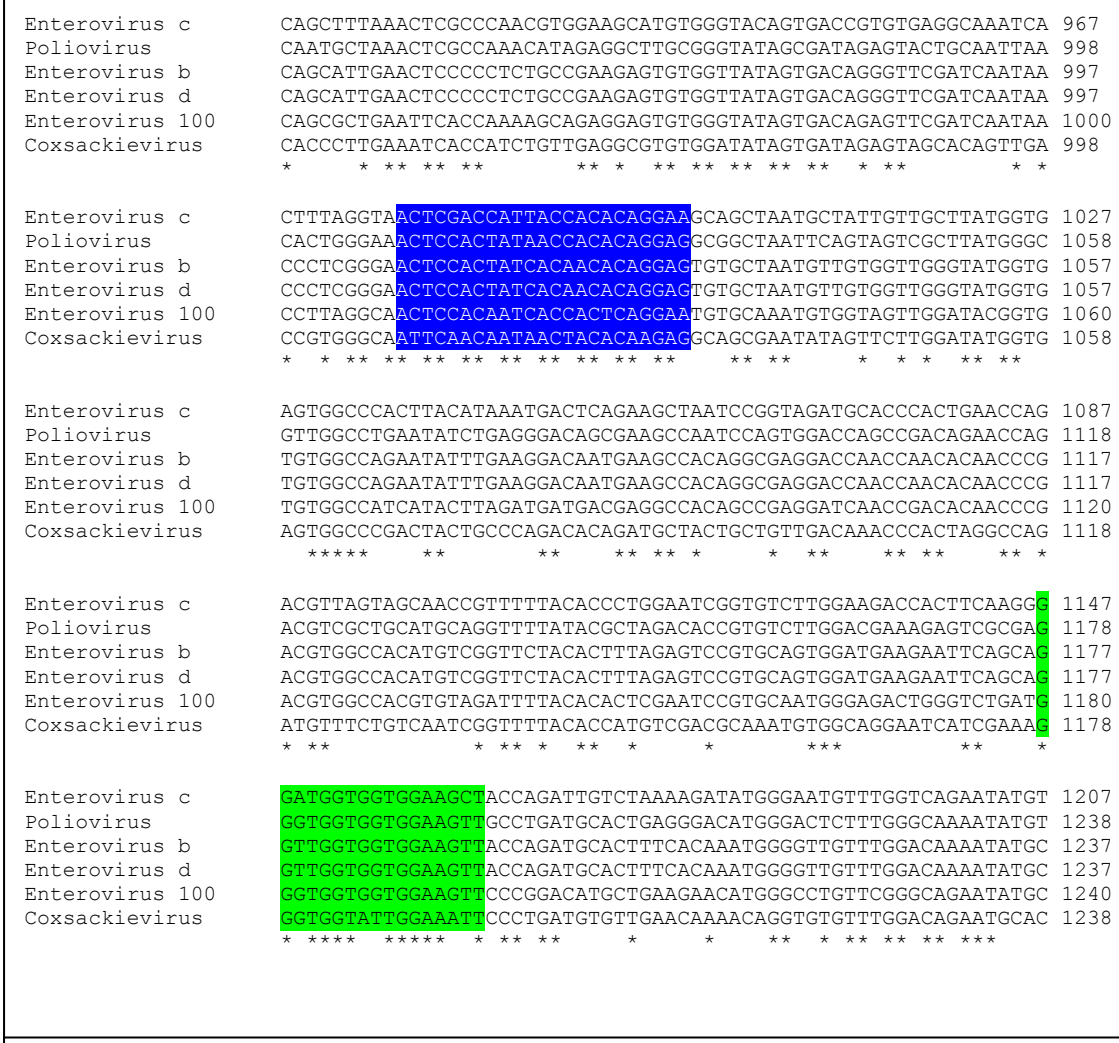


Fig .7: Alignment of human Enterovirus b, Enterovirus c, Enterovirus d, Enterovirus 100, Poliovirus and Coxsackievirus

Light green:	Binding site of EV1196_Rev primer
Yellow:	Binding site of EV168mod_Fw primer
Light blue:	Binding site of EV455_Fw primer
Dark blue:	Binding site of EV1031_Rev primer

Primer sequences were selected from highly conserved parts of the 5'-NCR and tested using "Primer3", a PCR primer designing program available via internet (http://frodo.wi.mit.edu/primer3/). Two primer pairs were selected – the primer pair A for primary PCR, the primer pair B for the following nested PCR.

Primer pair A (for primary PCR):

EV168mod_Fw: CAAGCACTTCTGTATCCCC
EV1196_Rev: AACTTCCACCACCACCC

EV168mod_Fw was a modified version of a primer published earlier by Zoll et al. (1992).

This primer pair includes approximately 1 kbp and has an annealing temperature of 52°C.

Primer pair B (for secondary, nested PCR):

EV455_Fw: GGCCCCTGAATGCGG
EV1031_Rev: TCCTGAGTGGTGATTGTGGAGT

This primer pair includes a 540bp region and its annealing temperature is 54°C.

3.5 cDNA Synthesis

Sample RNA as well as positive control RNA was transcribed into cDNA for further use in PCR application.

Material:

Primer

5x AMV RT buffer (Fermentas)

RiboLock ™ RNase Inhibitor (Fermentas)

dNTP Mix 10mM each

AMV Reverse Transcriptase (Fermentas)

Procedure:

For First Strand cDNA synthesis AMV Reverse Transcriptase (Fermentas) was used. Following the Fermentas protocol, following components were mixed on ice:

5 ng RNA template (5-7 µl)

1 µl EV1196_Rev

DEPC-treated Water topped up to 13 µl

As the RNA-template was expected to contain secondary structures (Oberste et al, 1998), it was incubated at 65°C for 5 minutes before adding following components:

4 µl 5x AMV RT buffer (Fermentas)

0,5 µl RiboLock ™ RNase Inhibitor (Fermentas)

2 µl dNTP Mix (10mM dATP, dCTP, dTTP, dGTP)

0,5 µl AMV Reverse Transcriptase (Fermentas)

The resulting mixture was incubated at 54°C for 60 minutes. The reaction was terminated by heating the mixture at 85°C for 5 minutes. cDNA was stored at -20°C until further processing.

3.6 Polymerase Chain Reaction (PCR)

PCR is used for amplifying small amounts of DNA so as to get enough material for further analyses. This technique requires that at least the flanking regions of the DNA sequence of interest are known in order to design the complementary single stranded primers - oligonucleotides of 15 to 30 bases.

The PCR technique consists of 3 steps (Fig. 8):

1. *Denaturation:*

The DNA is heated briefly to high temperature (94-96°C). The temperature change causes the hydrogen bonds between complementary bases to disrupt, resulting in single strands of DNA.

2. *Annealing:*

After lowering the temperature to 50-60°C, the primers are attached to the single strands of DNA. Annealing does only work when primer and target DNA sequences match closely to one another; otherwise no stable hydrogen bonds are formed. The polymerase binds to the primer/template complex.

3. *Extension:*

At this step the reaction is heated up to the working temperature of the heat-stable polymerase. By adding nucleotides to the 3'-end of the primer, the polymerase synthesizes a new strand of DNA complementary to the original one. At each extension step, the amount of DNA in the reaction is approximately doubled.

These steps are repeated, resulting in a cyclical reaction that amplifies the target DNA exponentially (Fig. 9). The reaction is terminated by a final elongation step to ensure that any remaining single stranded DNA is fully extended.

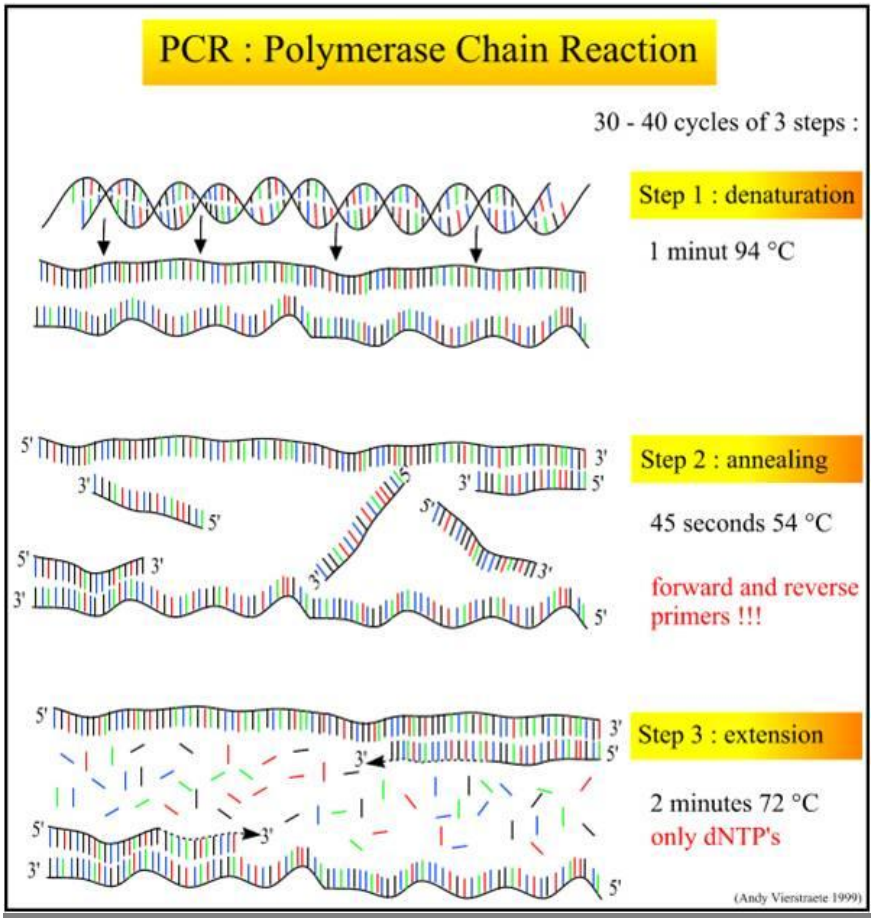


Fig. 8: Steps of the PCR technique (http://www.le.ac.uk/ge/genie/vgec/images/PCR_000.jpg)

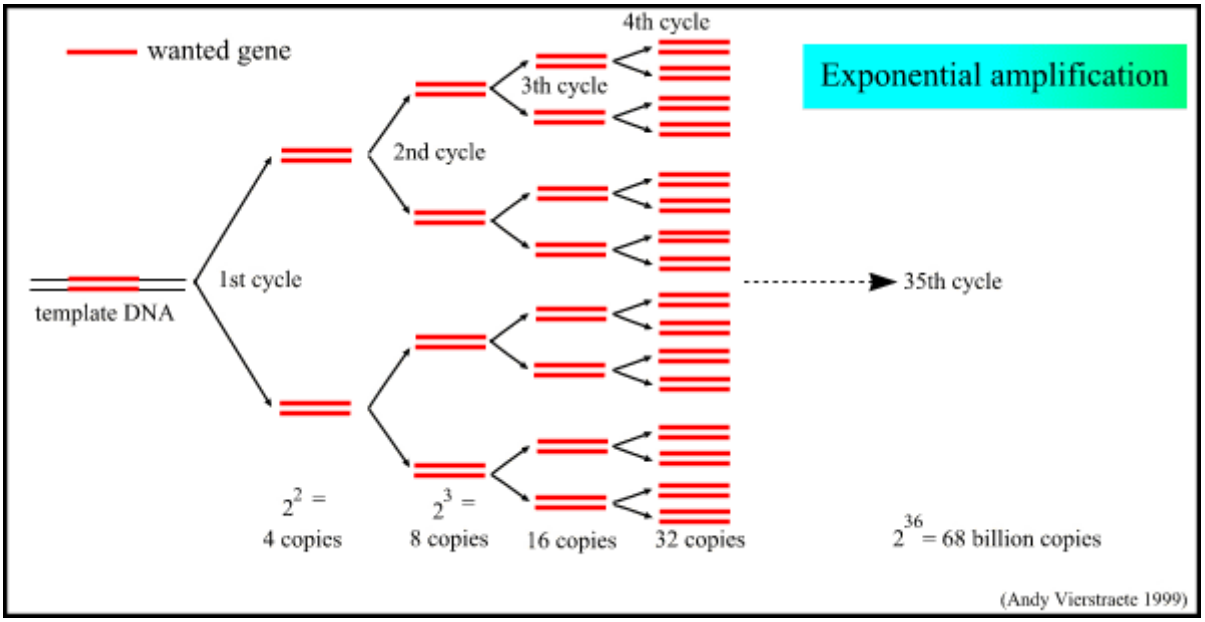


Fig. 9: Exponential amplification of a target DNA sequence. (http://users.ugent.be/~avierstr/principles/pcrcopies.gif)

Material:

Primer

dNTP-Mix (50x conc.): 10mM

$MgCl_2$ (10x conc.)

Taq-buffer (10x conc.)

Taq-Polymerase

3.6.1 Primary PCR:

For primary PCR, primer pair A was used.

For 25 µl reaction mixture:

1 µl	cDNA
0,25 µl	FW-Primer (50pM/ µl)
0,25 µl	RV-Primer (50pM/µl)
2.5 µl	$MgCl_2$ (2.5mM final concentration)
2.5 µl	buffer (10x concentrated)
0.5 µl	dNTPs (0.2mM final concentration)
0.63 µl	Taq-Polymerase (1.25u/50µl final concentration)
17.37 µl	A. dest.

For this PCR application, following parameters were used:

- Denaturation at 94°C for 5 min
- 40 cycles:
    - Denaturation at 94°C for 30 sec
    - Annealing at 52°C for 30 sec
    - Polymerisation at 72°C for 30 sec
- Final polymerization at 72°C for 10 min

3.6.2 Secondary PCR:

Using primer pair B, a nested PCR was performed after the first PCR application to increase the specificity of DNA amplification by decreasing the amount of mispriming during amplification. 1 µl of the first PCR reaction was taken as template for nested PCR.

For 25 µl reaction mixture:

1 µl	Primary PCR reaction
0,25 µl	FW-Primer (50pM/ µl)
0,25 µl	RV-Primer (50pM/µl)
2.5 µl	$MgCl_2$ (2.5mM final concentration)
2.5 µl	buffer (10x concentrated)
0.5 µl	dNTPs (0.2mM final concentration)
0.63 µl	Taq-Polymerase (1.25u/50µl final concentration)
17.37 µl	A. dest.

For this PCR application, following parameters were used:

- Denaturation at 94°C for 5 min
- 40 cycles:
    - Denaturation at 94°C for 30 sec
    - Annealing at 54°C for 30 sec
    - Polymerisation at 72°C for 30 sec
- Final polymerization at 72°C for 10 min

All experiments included a positive control (Echovirus 30 cDNA) and a negative control (A. dest.)

3.7 Agarose Gel Electrophoresis

PCR products were analyzed by Agarose gel electrophoresis. As DNA molecules are negatively charged, they migrate from the negative to the positive pole, with the gel working as a molecular sieve: Smaller fragments migrate faster than large ones, thus the DNA fragments are separated according to their size. The use of an appropriate marker allows the determination the size of the fragment.

Material:

Agarose
1xTAE-Buffer (0,04x Trisacetat, 0.001M EDTA)
Ethidium bromide (10mg/ml A.dest.)
6x Loading Dye (Fermentas)
Low Range Marker (Fermentas)

Procedure:

Agarose was melted in 1x TAE buffer by boiling. Under consideration of the estimated size of the product, 2% agarose was used. This made separation more precise than in a 1% agarose gel. The gel was cooled to approximately 50°C and was poured into a gel rack with a comb. After full polymerization the gel was put into a tank containing 1xTAE-Buffer.

The samples loaded on the gel consisted of 5 µl PCR product and 1 µl 6x Loading Dye (Fermentas); a 80-1031bp low range marker (Fermentas) was used. The gel was run at approximately 10 V/cm.

For documentation, the gel was stained with ethidium bromide solution (0,0005% final concentration) for 15 minutes, making DNA bands fluorescent on an UV screen.

3.8 Gel Elution

Material:

illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare)

Procedure:

Resulting DNA bands in with an appropriate fragment size were cut out and eluted from the agarose gel using illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare), following the manufacturer's instructions. In the final step, DNA was eluted to 10 µl volume. DNA was stored at -20°C until further processing.

3.9 Ligation

The PCR products were ligated into the plasmid pGEM-T-Easy, which is adapted for PCR products. The Taq-polymerase used in this study creates an A-nucleotide overhang at the 3' end of the amplification products. The pGEM-T-Easy plasmid is a linearized vector with a single 3´-terminal thymidine at both ends, therefore being perfectly adapted for a sticky-end ligation with the PCR product.

The vector pGEM-T-Easy carries a resistance gene for ampicillin and a multiple cloning site (MCS). An MCS is a DNA section within a vector containing numerous restriction sites for various restriction endonucleases. Two EcoR1 restriction sites are flanking the MCS of this specific vector allowing the release of the insert by using only one enzyme.

Another sequence within the vector is part of the lacZ gene encoding for the enzyme beta-galactosidase. The MCS of the vector lies within this sequence; therefore insertion of a DNA fragment into the vector causes the disruption of the reading frame of the beta-galactosidase-gene – thus beta-galactosidase is not expressed when an insert is ligated.

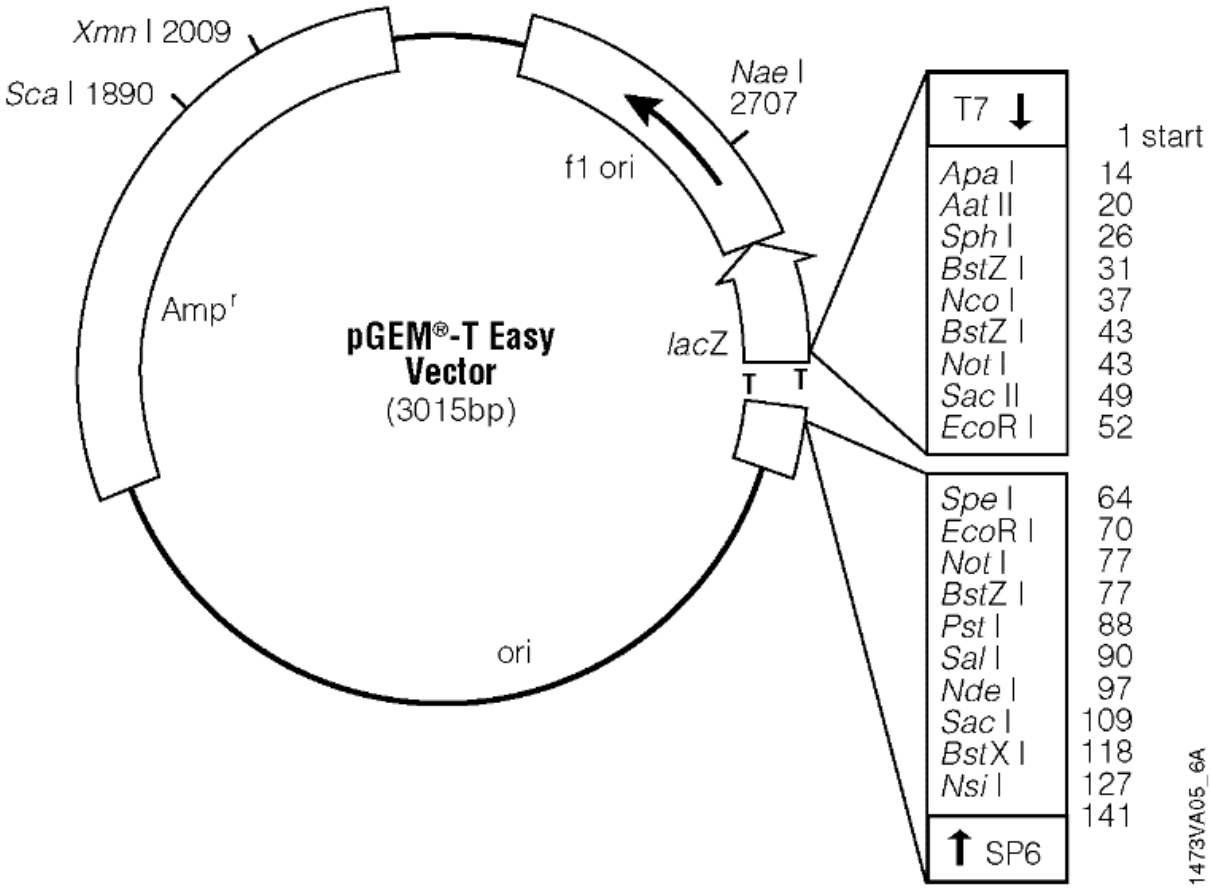


Fig. 10: The pGEM-T Easy Vector (http://ymbc.ym.edu.tw/est/1473vaw4.gif)

Material:

Eluted DNA

T4 DNA Ligase

pGEM T-Easy vector (linearized)

Ligation buffer (2xconc.): 60mM Tris-HCl (pH=7.8), 20mM MgCl2, 20mM DTT, 2mM ATP, 10% Polyethylene glycol (MW 8000)

Procedure:

Following components were mixed on ice:

3 µl template

5 µl 2x Ligation buffer

1 µl pGEM-T-Easy vector (50 ng/ µl)

1 µl T4 DNA Ligase

All mixtures were incubated at 4°C overnight.

3.10 Transformation

The recombinant vector is introduced into competent cells of the Escherichia coli strain JM109.

Material:

SOC-medium (100ml):	2g Trypton, 0.5g Yeast extract, 1ml 1M NaCl, 0.25ml 1M KCl, ad 100ml with A.dest. and pH value is adjusted to 7; After autoclaving and cooling down 1ml 2M sterile Mg-solution and 1ml glucose solution were added
Mg solution (2M):	4.07g $MgCl_2$ x 6H2O and 4.93g $MgSO_4$ x 7H2O are dissolved in 10ml A. dest., filter sterilized
Glucose solution (2M):	3.96g dissolved in 10ml A. dest., filter sterilized
IPTG (0,1M):	23.83mg IPTG dissolved in 10ml in A. dest., filter sterilized
X-Gal 2%:	100mg X-gal dissolved in 5ml N,N`dimethylformamide and sterile filtered
LB-Amp plates:	1l: 10g Trypton, 10g NaCl, 5g Yeast extract, 15g Agar; pH-value is adjusted to 7.2-7.6; after autoclaving and cooling down to <60°C 1µl Amp (50mg/ml) /ml medium is added and plates are casted.

Procedure:

100 µl competent cells (JM109) were thawed on ice and gently mixed with 2 µl of ligation reaction. The mixture was incubated on ice for 20 minutes. After a heat shock (42°C for 45 sec) the sample was placed on ice for 2 minutes, then 950 µl SOC-medium were added, and the sample was incubated at 37°C for 90 min, shaking at 150 rpm.

For each transformation reaction, 3 ampicillin containing LB-plates were treated with 40 µl of IPTG solution and 40 µl X-Gal solution each for enabling blue/white selection. 150-300 µl of the transformation reaction were added per plate, afterwards they were incubated overnight at 37°C.

3.11 Blue/White Selection

Only bacteria which carry a pGEM-T-Easy plasmid can grow on the ampicillin-containing LB-plates, which is due to the presence of an ampicillin resistance gene on the plasmid. To distinguish between recombinant and non recombinant clones the blue/white selection system was established.

As mentioned before, the pGEM-T-Easy vector carries a segment of the lacZ-gene (Fig. 10), that encodes the amino terminal fragment of beta-galactosidase. The MCS of the vector is located within the sequence coding for this enzyme.
As long as the segment of the lacZ-gene is not interrupted by an insert, beta-galactosidase is expressed. The enzyme splits X-Gal to galactose and 5-Brom-4-chlor-indoxyl. The latter is oxidated by aerial oxygen and turns the colony expressing beta-galactosidase from white to blue.
If there is an insert, the LacZ gene is interrupted, beta-galactosidase is not expressed, X-Gal is not processed and the colony remains white.

This way, recombinant clones can be distinguished from non-recombinant by color.

IPTG supports the beta-galactosidase system. IPTG works as an activator for the lac-operon by binding the repressor LacI and prohibiting its interaction with the operator of the gene. By adding a sufficient concentration of IPTG, it can be assured that the lac-operon is activated if it is not interrupted by an insert.

White colonies were transferred to fresh ampicillin-containing LB-plates and incubated overnight at 37°C.

3.12 Miniprep

The Miniprep technique is used for quick isolation of small amounts of plasmid DNA from bacteria while simultaneously limiting contamination by genomic DNA and proteins. There are different procedures and kits on the market. The procedure used in this study is based on alkaline lyses: Bacterial cells are lysed under alkaline conditions, which denatures both DNA and proteins. When the sample is neutralized by a buffer solution, supercoiled chromosomal DNA fragments and proteins cannot renature correctly due to their size and precipitate. In contrast, plasmids renature and stay in solution, separating them from chromosomal DNA.

Material:

Invisorb® Spin Plasmid Mini Two (Invitek GmbH Germany)

Procedure

For Miniprep Invisorb® Spin Plasmid Mini Two (Invitek GmbH Germany) was used according to the manufacturer's instructions.

3.13 Restriction

The pGEM-T-Easy has two EcoR1 restriction sites flanking the MCS. Therefore, the insert can be cut out of the vector easily using EcoR1. This was done mainly for control purpose, to assure that the insert had the expected size.

Material

10x buffer H (Roche)

10x RNase (Roche)

EcoR1 (12-16 units; Roche)

Procedure:

For each restriction reaction, following components were put together (on ice) and mixed gently:

2 µl plasmid DNA
1 µl 10x buffer H (Roche)
1 µl 10x RNase (Roche)
1 µl EcoR1 (12-16 units; Roche)
5 µl A. dest.

The reaction was incubated at 37°C for 2-4 hours to ensure complete restriction. Afterwards, a gel electrophoresis was run to determine whether the plasmid had an insert of the expected size or not. Samples with an appropriate insert and sufficient concentration were selected for sequencing.

3.14 Sequencing

Sequencing was done by 4base-lab GmbH (Germany) using the primer M13-40 (GGT AAC GCC AGG GTT TTC C).

3.15 Sequence Analysis

For analysis, sequences were examined using BLAST (Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi), more precisely the program megablast. BLAST compares nucleotide sequences to sequence databases and calculates the statistical significance of possible matches. Megablast limits the results of this search to only highly similar sequences.

Alignments and phylogenetic trees were created with Clustalw (http://www.ebi.ac.uk/Tools/clustalw/index.html) and the program PAUP. Before the sequences were aligned the primer sequences were removed.

3.16 Suppliers Of Used Chemicals And Material

A	Agar-agar	Merck GmbH
	Agarose	Promega
	Ampicillin	Carl Roth GmbH
	AMV Reverse Transcriptase + buffer	Fermentas
D	T4-DNA ligase	Promega
	dNTPs	Fermentas
	Durapore®Membrane Filters 0.22µm GV	Millipore
E	EcoRI + Puffer H	Roche Diagnostics GmbH
	Ethanol absolute	Merck GmbH
	Ethidium bromide (10mg/ml)	Carl Roth GmbH
	Ethylene diaminetetraacetic acid disodium salt dehydrate (EDTA, $C_{10}H_{14}O_6$)	Sigma-Aldrich Chemie GmbH
F	N,N`dimethylformamide	Fisher Scientific UK Limited
G	GF/C Glass Microfibre Filters 47mmØ	Whatman® International Ltd
	α-D(+)-glucose, anhydrous ($C_6H_{12}O_6$)	Fluka Biochemika AG
H	Hydrochloric acid 32% and 37% (HCl)	Carl Roth GmbH
I	Isoamylalcohol ($C_5H_{12}O$)	Carl Roth GmbH
	IPTG (Isopropyl-beta-D-thioglactopyransoide, $C_9H_{18}O_5S$)	Carl Roth GmbH
	Isopropanol (C_3H_7OH)	Fluka Chemie AG
J	JM 109	Promega
L	Lab-Lemco Powder	Oxoid LTD.
	Low Range Marker	Fermentas
M	Magnesium chloride $MgCl_26H_2O$	Carl Roth GmbH
	Magnesium sulfate $MgSO_4x7H_20$	Merck

	Minimate^{TM} TFF Capsule (Omega ^{Tl}30K Membrane)	Pall Life Sciences
P	PEG: Polyethylene glycol 6000 ($C_{2n+2}H_{4n+6}O_{n+2}$)	Carl Roth GmbH
	peqGOLD Trifast™FL	Peqlab Biotechnologie GmbH
	pGEM T-Easy vector	Promega
	Phenol (C_6H_5OH)	Carl Roth GmbH
	2-Propanol (C_3H_8O)	Carl Roth GmbH
	Potassium chloride (KCl)	Merck
R	RiboLock™ RNase Inhibitor	Fermentas
	RNase, Dnase-free	Roche Diagnostics GmbH
S	Sodium acetate anhydrous ($C_6H_3NaO_2$)	Merck KgaA
	Sodium chloride (NaCl)	Carl Roth GmbH
	(di-) Sodium hydrogen phosphate dihydrate (Na_2HPO_4 $2H_2O$)	Carl Roth GmbH
T	Taq DNA Polymerase + Buffer	Fermentas
	Trichloromethane ($CHCl_3$)	Carl Roth GmbH
	Tris ($C_4H_{11}NO_3$)	Carl Roth GmbH
	Triton X 100 ($C_{33}H_6O_{10}$)	Carl Roth GmbH
	Trypton	Carl Roth GmbH
U	Urea (CH4N2O)	Carl Roth GmbH
X	X-Gal ($C_{14}H_{15}BrCINO_6$)	Carl Roth GmbH
Y	Yeast extract	Carl Roth GmbH

Kits:

- Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare UK Limited Amersham Place, UK)
- Invisorb® Spin Plasmid Mini Two (Invitek GmbH Germany)
- RNA PowerSoil^{TM} Total RNA Isolation Kit (Molbio Laboratories, Inc.)

4 Results

4.1 PCR

All negative controls were negative. Positive controls showed one band in nested, but two bands in primary PCR. Experimentation proved that this could be eliminated by adjusting the annealing temperature in the Re-PCR. However, as this was not substantial for the results, we decided not to change the experimental design. PCR products were only obtained in Re-PCR, primary PCR never gave bands in any sample.

4.1.1 Sediment

4.1.1.1 August 2009

An approximately 500 bp long PCR product was obtained from Re-PCR, matching the fragment size of the positive control. The band of the fragment was very feint, but was nevertheless used for cloning.

4.1.1.2 October 2009

Again, a 500 bp long PCR product was obtained from Re-PCR. The band was far less faint than the one of the August sample, suggesting the presence of more enterovirus RNA in this sample.

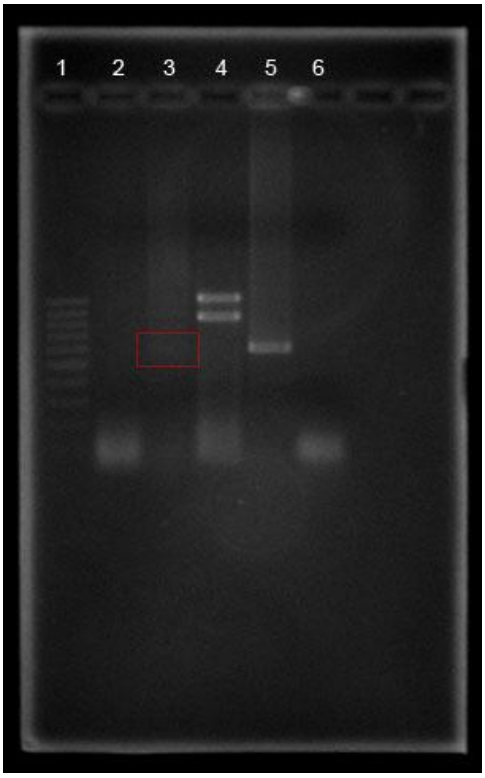


Fig. 11: PCR of August 2009 sediment sample

1 Low Range Marker
2 Sample Primary PCR
3 Sample Re-PCR
4 Positive Control Primary PCR
5 Positive Control Re-PCR
6 Negative Control

Red framed band was used for cloning.

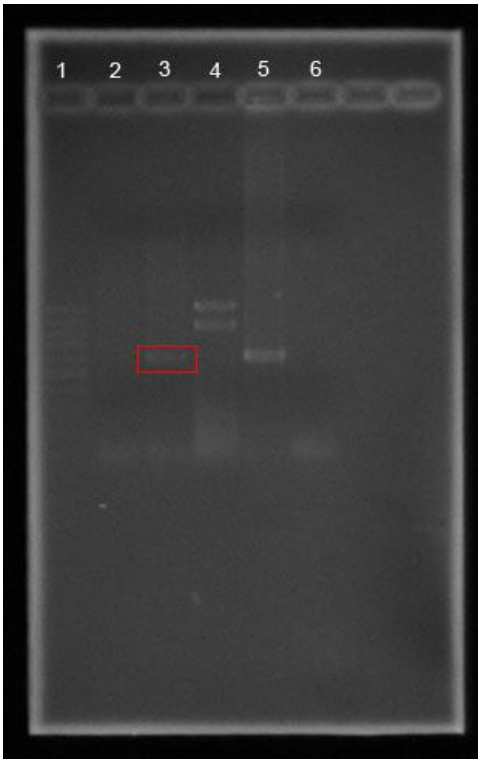


Fig. 12: PCR of October 2009 sediment sample

1 Low Range Marker
2 Sample Primary PCR
3 Sample Re-PCR
4 Positive Control Primary PCR
5 Positive Control Re-PCR
6 Negative Control

Red framed band was used for cloning.

4.1.2 Waterbody:

Except the positive control, the PCR of the Alte Donau water body samples did not show any bands in July, September and October. However, a strong band was detected in the Re-PCR of the August sample. The fragment was about 500 bp long and matched the size of the positive control fragment. The band was cut out and used for cloning.

We could not obtain any PCR product from the cold water reference sample taken in April 2009 from the Wasserpark water column. Only a positive control band was observed.

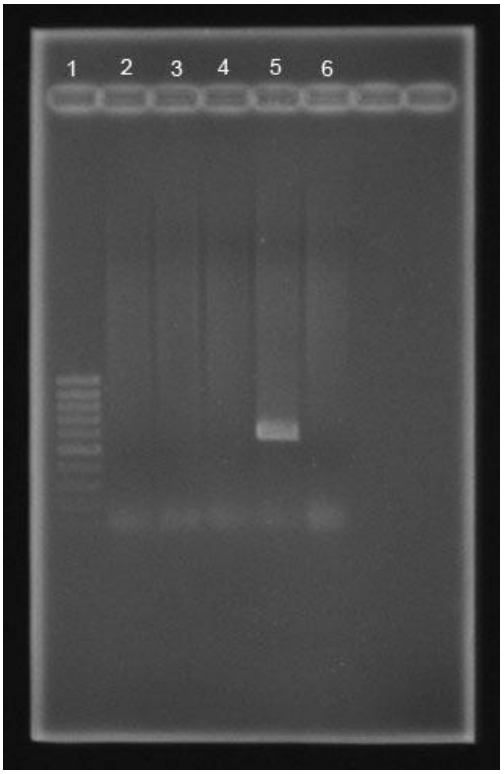


Fig 13: PCR of July, September and October 2009 Alte Donau water column samples

1	Low Range Marker
2	July 2009 watercolumn sample Re-PCR
3	September 2009 watercolumn sample Re-PCR
4	October 2009 watercolumn sample Re-PCR
5	Positive Control Re-PCR
6	Negative Control

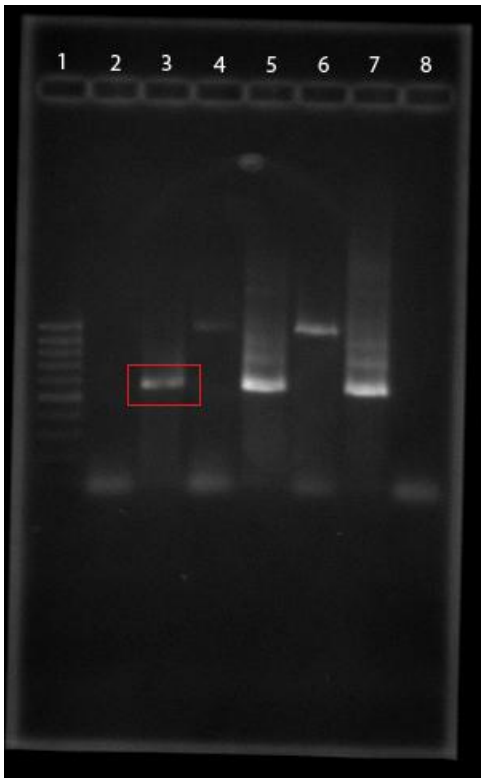


Fig. 14: PCR of August 2009 Alte Donau water column sample

1 Low Range Marker
2 August 2009 sample primary PCR
3 August 2009 sample Re-PCR
4 August 2009 sample spiked primary PCR
5 August 2009 sample spiked Re-PCR
6 Positive Control primary PCR
7 Positive Control Re-PCR
8 Negative Control

Red framed band was used for cloning.

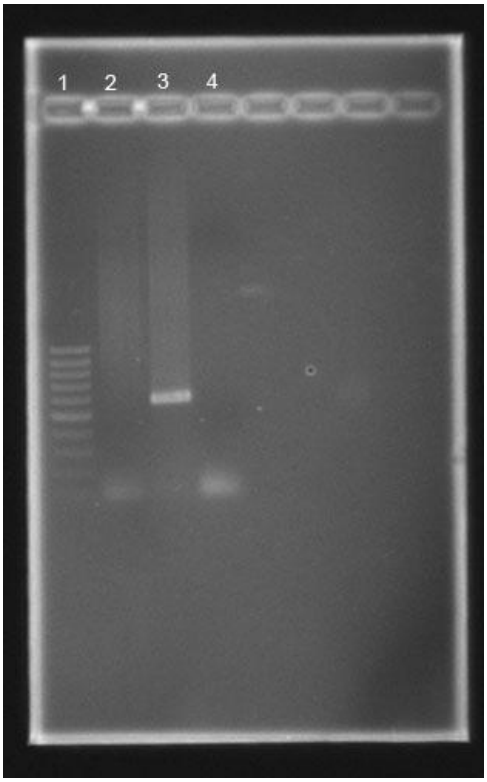


Fig. 15: PCR of April 2009 Wasserpark water column sample

1 Low Range Marker
2 Sample Re-PCR
3 Positive Control Re-PRC
4 Negative Control

4.2 Restriction Analysis

4.2.1 Sediment

4.2.1.1 August 2009

10 clones were used for restriction analysis (Fig. 16). In clones seau3, seau4, seau7 and seau10 an insert of the expected size was found. The insert of clones seau3, seau4 and seau10 had a length of approximately 600 bp. The insert of clone seau7 was 500 bp long. The other clones were either empty or showed a restriction pattern that could not be interpreted. To obtain a better resolution the relevant clones were electrophorized again (Fig. 17).

4.2.1.2 October 2009

7 clones were used for restriction analysis. All clones showed an insert of 500 bp length although the insert of Seok6 is hardly to be seen due to the little DNA yield of this preparation (Fig. 18).

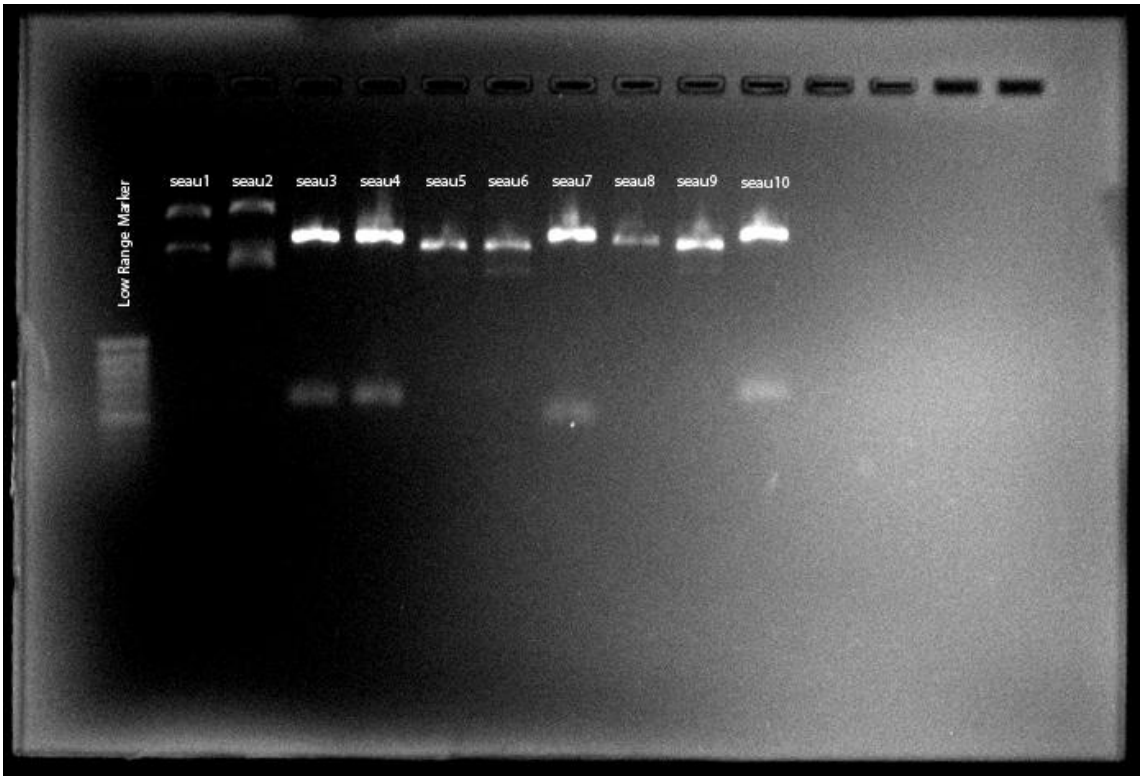


Fig 16: Restriction of Alte Donau August 2009 sediment clones

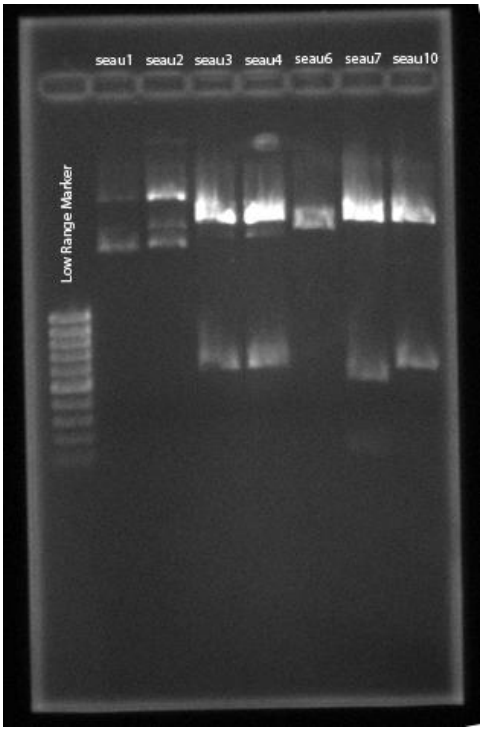


Fig. 17: Restriction of Alte Donau August 2009 sediment clones

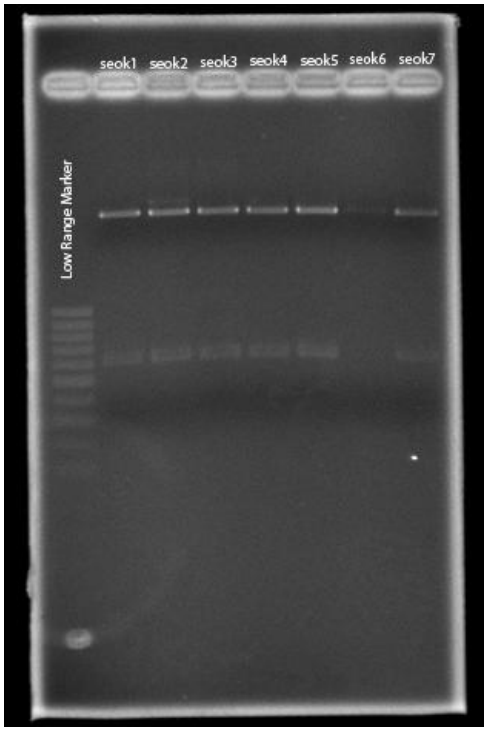


Fig 18: Restriction of Alte Donau October 2009 sediment clones

4.2.2 Waterbody

12 clones were used for the restriction analysis of the August 2009 Alte Donau water column sample. All clones (wcau1-12) showed an about 500 bp insert (Fig. 19).

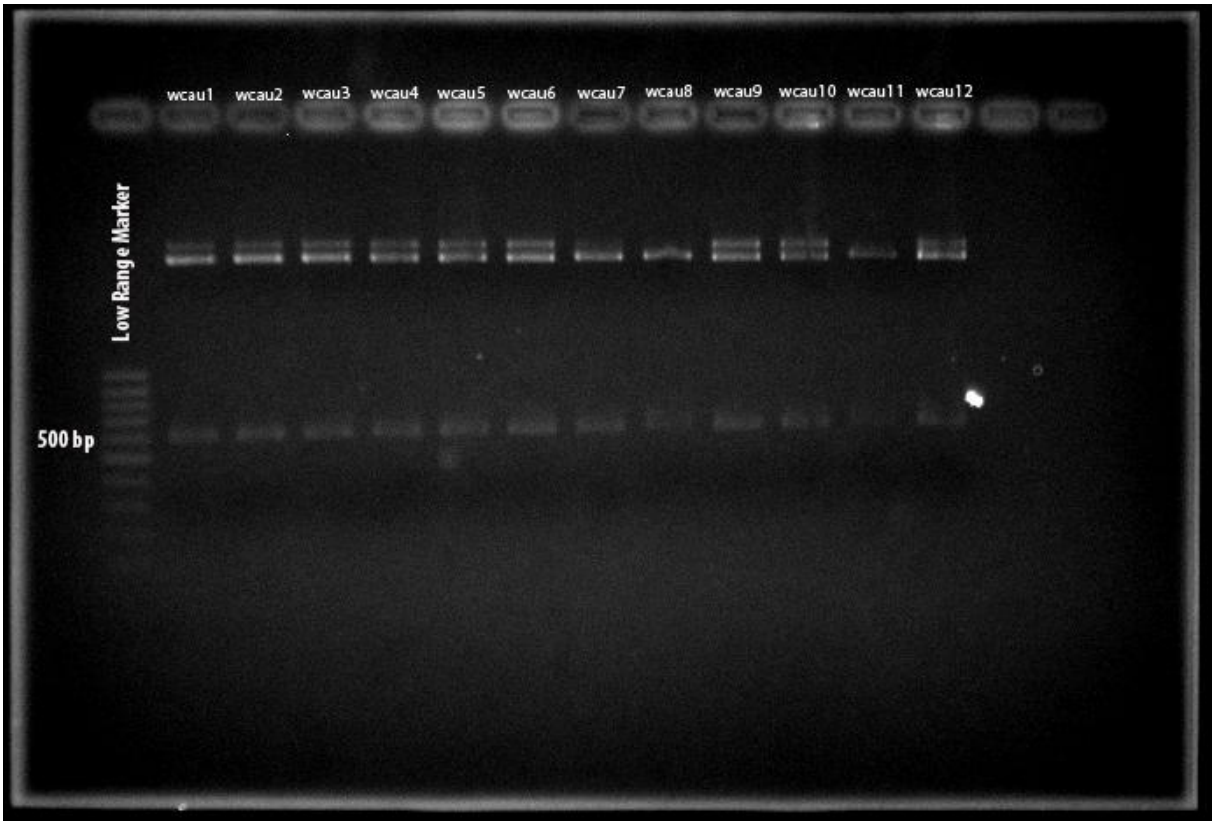


Fig. 19: Restriction of Alte Donau August 2009 water body clones

4.3 Sequences and BLAST Results

Only clones with the expected insert size of 500-600bp were sequenced

4.3.1 August 2009 Alte Donau sediment

The clones seau3, seau4, seau7 and seau10 were commercially sequenced. seau3, seau4 and seau10 matched to Echovirus30. The insert of clone seau7 turned out to be a vector homologous one.

4.3.2 October 2009 Alte Donau sediment

seok 1-5 and seok7 were selected for sequencing. Complete insert sequences were obtained from seok 1, seok2 and seok 4; these 3 inserts were homologous to Echovirus30. Seok5 and seok7 also matched to Echovirus 30 but were incomplete.
The sequence of seok3 was also incomplete. BLAST hits obtained by using the program "Somewhat similar sequences (blastn)" showed similarities to Echovirus11 and EnterovirusB sequences as well as Echovirus30 sequence.

4.3.3 August 2009 Alte Donau water column

wcau4, wcau6, wcau7 and wcau10 were completely sequenced and matched to Echovirus30.

Complete sequences in detail.

Fig. 20 shows the sequences of all viral inserts of all samples.

Fig. 20A: sequence of seau3 insert

GAATTCGATTGGCCCCTGAATGCGGCTAATCCTAACTGCGGAGCAGATACCCACACGCCAGTGGGCAGTCTGTCGTAA
CGGGCAACTTCGCAGCGGAACCGACTACTTTGGGTGTCCGTGTTTCCTTTTATTTCTATACTGGCTGCTTATGGTGACA
ATTGAGAGATTGTTGCCATATAGCTATTGGATTGGCCATCCGGTGTCCAACAGAGCGATCATTTATTTGTTTGTTGGAT
TCGTACCCTTGAGTCACAAAGCACTTGTAACACTTGAATACATTATAGACCTCAACACAACAAAATGGGGGCCCAGGT
ATCAACACAAAGAACTGGAGCTCATGAGACCGGCTTGAATGCTAGTGGAAACTCCATTATACATTACACAAATATTAAT
TACTACAAGGACTCTGCTTCTAACTCATTGAACCGGCAAGACTTTACCCAGGATCCCAGTAAATTCACAGAACCGGTGA
AGGACGTGATGATTAAGACCTTGCCCACTTTGAATTCACCTACTGTAGAAGAATGCGGCTTCAGTGACCGAGTGCGGT
CGATCACCTTGGGGAACTCCACAATCACCACTCAGGAAATCACTAGTGAATTC

Fig. 20B: sequence of seau4 insert

GAATTCGATTGGCCCCTGAATGCGGCTAATCCTAACTGCGGAGCAGATACCCACACGCCAGTGGGCAGTCTGTCGTAA
CGGGCAACTTCGCAGCGGAACCGACTACTTTGGGTGTCCGTGTTTCCTTTTATTTCTATACTGGCTGCTTATGGTGACA
ATTGAGAGATTGTTGCCATATAGCTATTGGATTGGCCATCCGGTGTCCAACAGAGCGATCATTTATTTGTTTGTTGGAT
TCGTACCCTTGAGTCACAAAGCACTTGTAACACTTGAATACATTATAGACCTCAACACAACAAAATGGGGGCCCAGGT
ATCAACACAAAAAACTGGAGCTCATGAGACCGGCTTGAATGCTAGTGGAAACTCCATTATACATTACACAAATATTAAT
TACTACAAGGACTCTGCTTCTAACTCATTGAACCGGCAAGACTTTACCCAGGATCCCAGTAAATTCACAGAACCGGTGA
AGGACGTGATGATTAAGACCTTGCCCGCTTTAAATTCGCCTACTGTAGAAGAATGCGGCTTCAGTGACCGTGTGCGGT
CGATCACCTTGGGGAACTCCACAATCACCACTCAGAAATCACTAGTGAATTC

Fig. 20C: sequence of seau10 insert

GAATTCGATTGGCCCCTGAATGCGGCTAATCCTAACTGCGGAGCAGATACCCACACGCCAGTGGGCAGTCTGTCGTA
ACGGGCAACTTCGCAGCGGAACCGACTACTTTGGGTGTCCGTGTTTCCTTTTATTTCTATACTGGCCGCTTATGGTGAC
AATTGAGAGATTGTTGCCATATAGCTATTGGATTGGCCATCCGGTGTCCAACAGAGCGATCATTTATTTGTTTGTTGGA
TTCGTACCCTTGAGTCACAAAGCACTTGTAACACTTGAATACATTATAGACCTCAACACAACAAAATGGGGGCCCAGG
TATCAACACAAAAAACTGGAGCTCATGAGACCGGCTTGAATGCTAGTGGAAACTCCATTATACATTACACAAATATTA
ATTACTACAAGGACTCTGCTTCTAACTCATTGAACCGGCAAGACTTTACCCAGGATCCCAGTAAATTCACAGAACCGGT
GAAGGACGTGATGATTAAGACCTTGCCCGCTTTAAACTCACCTACTGTAGAAGAATGCGGCTACAGTGACCGAGTGC
GGTCGATCACCTTGGGGAACTCCACAATCACCACTCAGAAATCACTAGTGAATTC

Fig. 20D: sequence of seok1 insert

GAATTCGATTGGCCCCTGAATGCGGCTAATCCTAACTGCGGAGCAGATACCCACACGCCAGTGGGCAGTCTGTCGTA
ACGGGCAACTTCGCAGCGGAACCGACTACTTTGGGTGTCCGTGTTTCCTTTTATTTCTATACTGGCTGCTTATGGTGAC
AATTGAGAGATTGTTGCCATATAGCTATTGGATTGGCCATCCGGTGTCCAACAGAGCGATCATTTATTTGTTTGTTGGA
TTCGTACCCTTGAGTCACAAAGCACTTGTAACACTTGAATACATTATAGACCTCAACACAACAAAATGGGGGCCCAGG
TATCAACACAAAAAACTGGAGCTCATGAGACCGGCTTGAATGCTAGTGGAAACTCCATTATACATTACACAAATATTA
ATTACTACAAGGACTCTGCTTCTAACTCATTGAACCGGCAAGACTTTACCCAGGGTCCCAGTAAATTCACAGAACCGGT
GAAGGACGTGATGATTAAGACCTTGCCCGCTTTAAATTCACCTACTGTAGAAGAATGCGGCTTCAGTGACCGAGTGCG
GTCGATCACCTTGGGGAACTCCACAATCACCACTCAGGAAATCACTAGTGAATTC

Fig. 20E: sequence of seok2 insert

GAATTCGATTGGCCCCTGAATGCGGCTAATCCTAACTGCGGAGCAGATACCCACACGCCAGTGGGCAGTCTGTCGTA
ACGGGCAACTTCGCAGCGGAACCGACTACTTTGGGTGTCCGTGTTTCCTTTTATTTCTATACTGGCTGCTTATGGTGAC
AATTGAGAGATTGTTGCCATATAGCTATTGGATTGGCCATCCGGTGTCCAACAGAGCGATCATTTATTTGTTTGTTGGA
TTCGTACCCTTGAGTCACAAAGCACTTGTAACACTTGAATACATTATAGACCTCAACACAACAAAATGGGGGCCCAGG
TATCAACACAAAAAACTGGAGCTCATGAGACCGGCTTGAATGCTAGTGGAAACTCCATTATACATTACACAAATATTA
ATTACTACAAGGACTCTGCTTCTAACTCATTGAACCGGCAAGACTTTACCCAGGGTCCCAGTAAATTCACAGAACCGG
TGAAGGACGTGATGATTAAGACCTTGCCCGCTTTAAATTCACCTACTGTAGAAGAATGCGGCTTCAGTGACCGAGTGC
GGTCGATCACCTTGGGGAACTCCACAATCACCACTCAGGAAATCACTAGTGAATTC

Fig. 20F: sequence of seok3 insert

GAATTCGATTGGCCCCTGAATGCGGCTAATCCTAACTGCGGAGCAGATACCCACACGCCAGTGGGCAGTCTGTCGTA
AC

Fig. 20G: sequence of seok4 insert

GAATTCGATTGGCCCCTGAATGCGGCTAATCCTAACTGCGGAGCAGATACCCACACGCCAGTGGGCAGTCTGTCGTAA
CGGGCAACTTCGCAGCGGAACCGACTACTTTGGGTGTCCGTGTTTCCTTTTATTTCTATACTGGCTGCTTATGGTGACA
ATTGAGAGATTGTTGCCATATAGCTATTGGATTGGCCATCCGGTGTCCAACAGAGCGATCATTTATTTGTTTGTTGGAT
TCGTACCCTTGAGTCACAAAGCACTTGTAACACTTGAATACATTATAGACCTCAACACAACAAAATGGGGGCCCAGGT
ATCAACACAAAAAACTGGAGCTCATGAGACCGGCTTGAATGCTAGTGGAAACTCCATTATACATTACACAAATATTAA
TTACTACAAGGACTCTGCTTCTAACTCATTGAACCGGCAAGACTTTACCCAGGATCCCAGTAAATTCACAGAACCGGTG
AAGGACGTGATGATTAAGACCTTGCCCGCTTTAAATTCACCTACTGTAGAAGAATGCGGCTTCAGTGACCGAGTGCGG
TCGATCACCTTGGGGAACTCCACAATCACCACTCAGGAAATCACTAGTGAATTC

Fig. 20H: sequence of seok5 insert

GAATTCGATTGGCCCCTGAATGCGGCTAATCCTAACTGCGGAGCAGATACCCACACGCCAGTGGGCAGTCTGTCGTAA
CGGGCAACTTCGCAGCGGAACCGACTACTTTGGGTGTCCGTGTTTCCTTTTATTTCTATACTGGCTGCTTATGGTGACA
ATTGAGAGATTGTTGCCATATAGCTATTGGATTGGCCATCCGGTGTCCAACAGAGCGATCATTTATTTGTTTGTTGGAT
TCGTACCCTTGAGTCACAAAGCACTTGTAACACTTGAATACATTATAGACCTCAACACAACAAAATGGGGGCCCAGGT
ATCAACACAAAAAACTGGAGCTCATGAGACC

Fig. 20I: sequence of seok7 insert

GAATTCGATTGGCCCCTGAATGCGGCTAATCCTAACTGCGGAGCAGATACCCACACGCCAGTGGGCAGTCTGTCGTAA
CGGGCAACTTCGCAGCGGAACA

Fig. 20J: sequence of wcau4 insert

GAATTCGATTGGCCCCTGAATGCGGCTAATCCTAACTGCGGAGCAGATACCCACACGCCAGTGGGCAGTCTGTCGTAA
CGGGCAACTTCGCAGCGGAACCGACTACTTTGGGTGTCCGTGTTTCCTTTTATTTCTATACTGGCTGCTTATGGTGACA
ATTGAGAGGTTGTTGCCATATAGCTATTGGATTGGCCATCCGGTGTCCAACAGAGCGATCATTTATTTGTTTGTTGGAT
TCGTACCCTTGAGTCACAAAGCACTTGTAACACTTGAATACATTATAGACCTCAACACAACAAAATGGGGGCCCAGGT
ATCAACACAAAAAACTGGAGCTCATGAGACCGGCTTGAATGCTAGTGGAAACTCCATTATACATTACACAAATATTAA
TTACTACAAGGACTCTGCTTCTAACTCATTGAACCGGCAAGACTTTACCCAGGATCCCAGTAAATTCACAGAACCGGTG
ACGGACGTGATGATTAAGACCTTGCCCGCTTTAAATTCGCCTACTGTAGAAGAATGCGGCTTCAGTGACCGTGTGCGG
TCGATCACCTTGGGGAACTCCACAATCACCACTCAGGAAATCACTAGTGAATTC

Fig. 20K: sequence of wcau6 insert

GAATTCGATTGGCCCCTGAATGCGGCTAATCCTAACTGCGGAGCAGATACCCACACGCCAGTGGGCAGTCTGTCGTAA
CGGGCAACTTCGCAGCGGAACCGACTACTTTGGGTGTCCGTGTTTCCTTTTATTTCTATACTGGCTGCTTATGGTGACA
ATTGAGAGATTGTTGCCATATAGCTATTGGATTGGCCATCCGGTGTCCAACAGAGCGATCATTTATTTGTTTGTTGGAT
CCGTACCCTTGAGTCACAAAGCACTTGTAACACTTGAATACATTATAGACCTCAACACAACAAAATGGGGGCCCAGGT
ATCAACACAAAAAACTGGAGCTCATGAGACCGGCTTGAATGCTAGTGGAAACTCCATTATACATTACACAAATATTAA
TTACTACAAGGACTCTGCTTCTAACTCATTGAACCGGCAAGACTTTACCCAGGATCCCAGTAAATTCACAGAACCGGTG
AAGGACGTGATGATTAAGACCTTGCCCGCTTTAAATTCACCTACTGTAGAAGAATGCGGCTTCAGTGACCGAGTGCGG
TCGATCACCTTGGGGAACTCCACAATCACCACTCAGGAAATCACTAGTGAATTC

Fig. 20L: sequence of wcau7 insert

GAATTCGATTGGCCCCTGAATGCGGCTAATCCTAACTGCGGAGCAGATACCCACACGCCAGTGGGCAGTCTGTCGTAA
CGGGCAACTTCGCAGCGGAACCGACTACTTTGGGTGTCCGTGTTTCCTTTTATTTCTATACTGGCTGCTTATGGTGACA
ATTGAGAGATTGTTGCCATATAGCTATTGGATTGGCCATCCGGTGTCCAACAGAGCGATCATTTATTTGTTTGTTGGAT
TCGTACCCTTGAGTCACAAAGCACTTGTAACACTTGAATACATTATAGACCTCAACACGACAAAATGGGGGCCCAGGT
ATCAACACAAAAAACTGGAGCTCATGAGACCGGCTTGAATGCTAGTGGAAACTCCATTATACGTTACACAAATATTAA
TTACTACAAGGACTCTGCTTCTAACTCATTGAACCGGCAAGACTTTGCCCAGGATCCCAGTAAATTCACAGAACCGGTG
AAGGACGTGATGATTAAGACCTTGCCCGCTTTAAATTCGCCTACTGTAGAAGAATGCGGCTTCAGTGACCGTGTGCGG
TCGATCACCTTGGGGAACTCCACAATCACCACTCAGGAAATCACTAGTGAATTC

Fig.20M: sequence of wcau10 insert

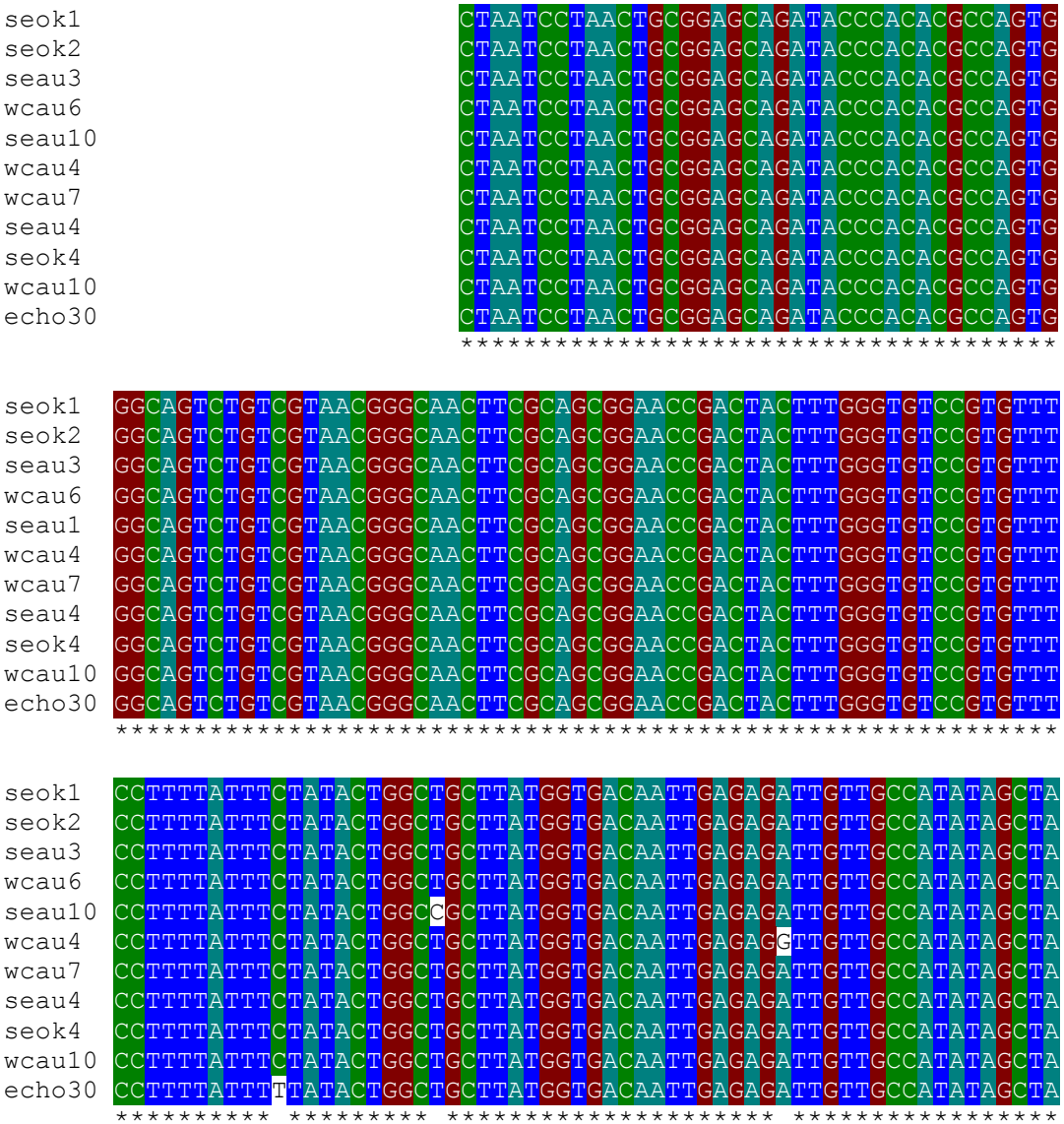
GAATTCGATTGGCCCCTGAATGCGGCTAATCCTAACTGCGGAGCAGATACCCACACGCCAGTGGGCAGTCTGTCGTAA
CGGGCAACTTCGCAGCGGAACCGACTACTTTGGGTGTCCGTGTTTCCTTTTATTTCTATACTGGCTGCTTATGGTGACA
ATTGAGAGATTGTTGCCATATAGCTATTGGATTGGCCATCCGGTGTCCAACAGAGCGACCATTTATTTGTTTGTTGGAT
TCGTACCCTTGAGTCACAAAGCACTTGTAACACTTGAATACATTATAGACCTCAACACAACAAAATGGGGGCCCAGGT
ATCAACACAAAAAACTGGAGCTCATGAGACCGGCTTGAATGCTAGTGGAAACTCCATTATACATTACACAAATATTAAT
TACTACAAGGACCCTGCTTCTAACTCATTGAACCGGCAAGACTTTACCCAGGATCCCAGTAAATTCACAGAACCGGTGA
AGGACGTGATGATTAAGACCTTGCCCGCTTTAAATTCACCTACTGTAGAAGAATGCGGCTTCAGTGACCGAGTGCGGT
CGATCACCTTGGGGAACTCCACAATCACCACTCAGGAAATCACTAGTGAATTC

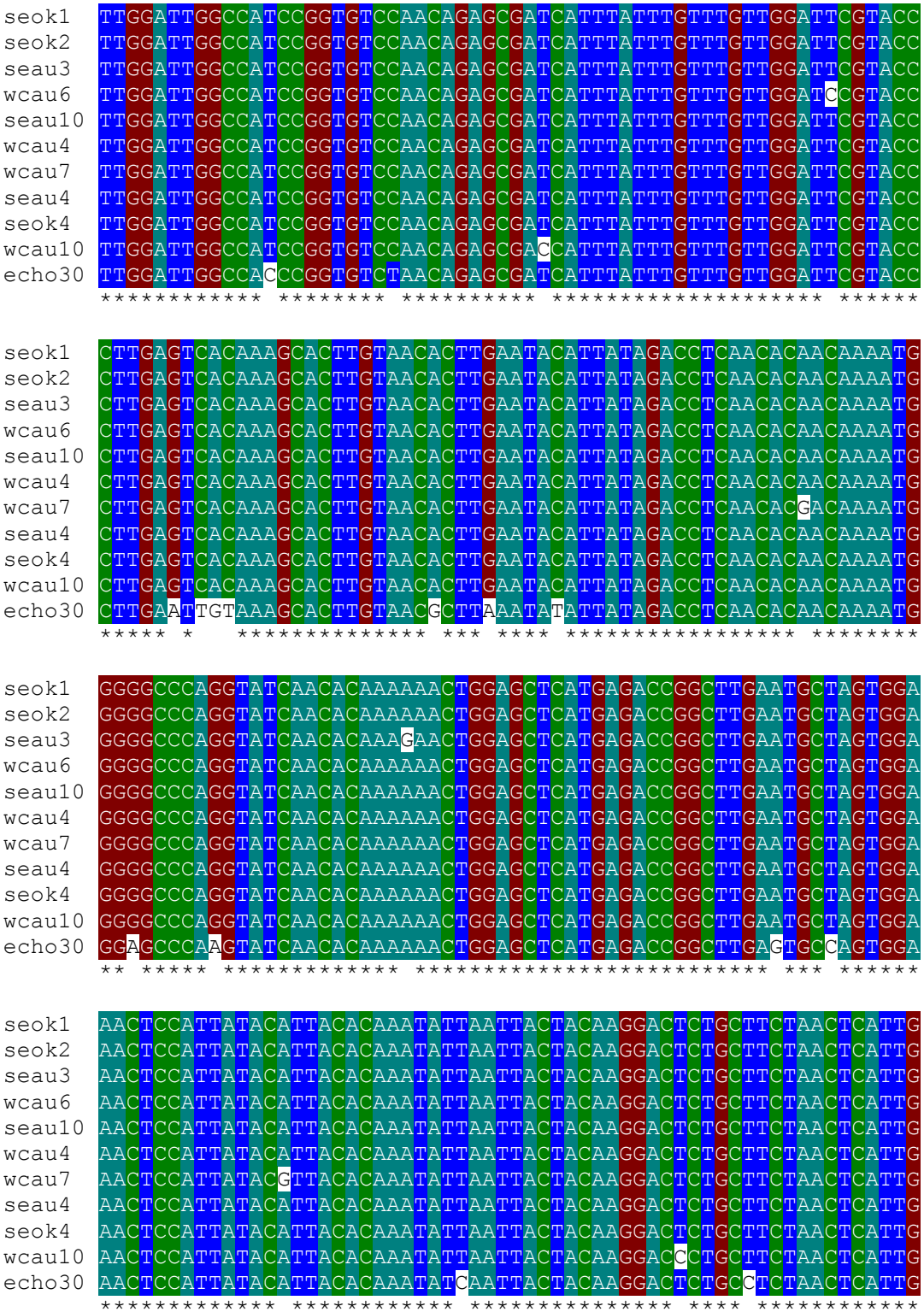
Fig. 20: Sequences of all samples

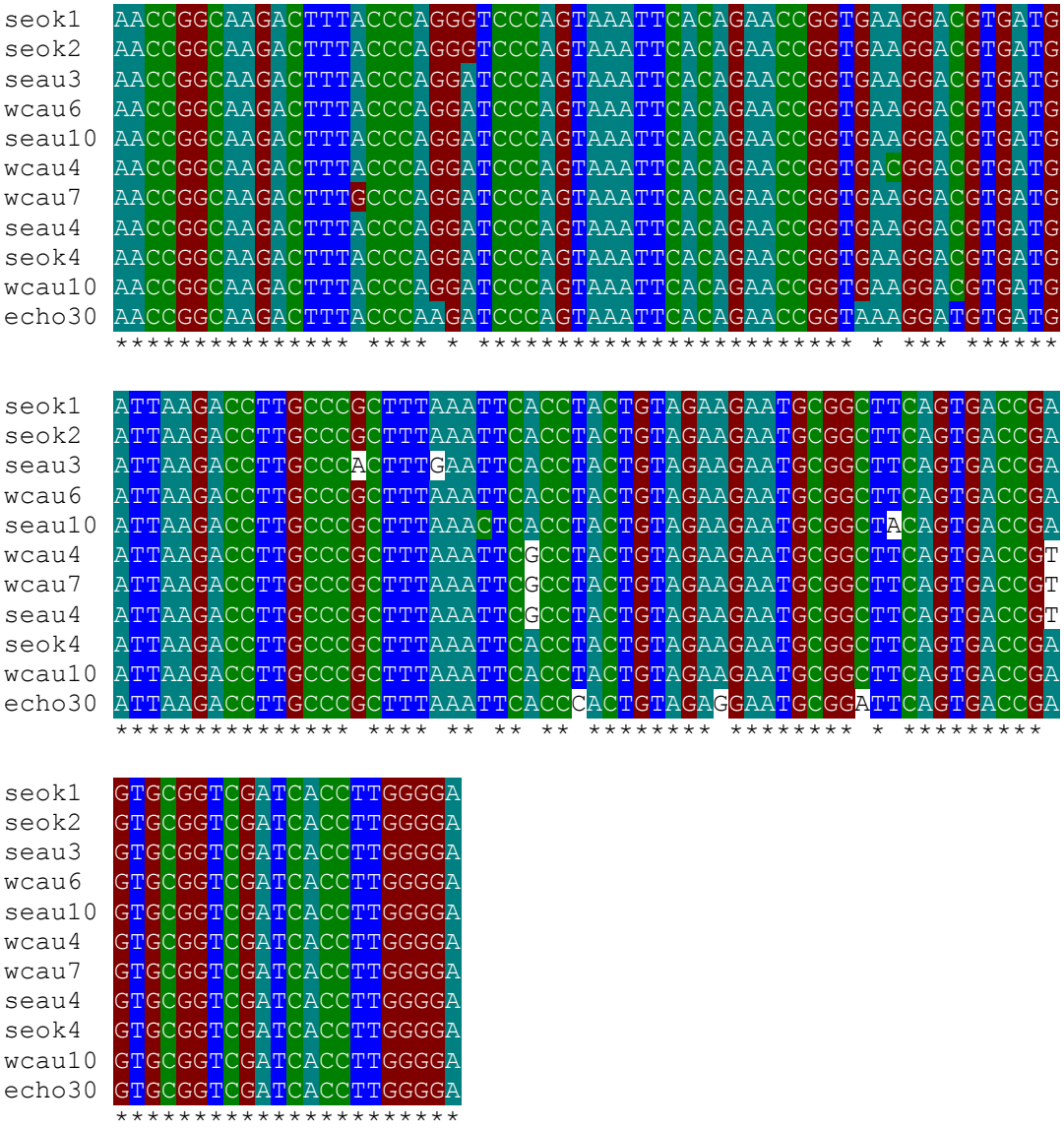
Light green:	EcoR1 Restriction sites
Dark green:	Vector sequences
Light blue:	EV455_fw primer sequence or part of it
Dark blue:	EV1031_rev primer sequence or part of it
Red:	Not part of original vector or primer sequence

4.4 Alignments

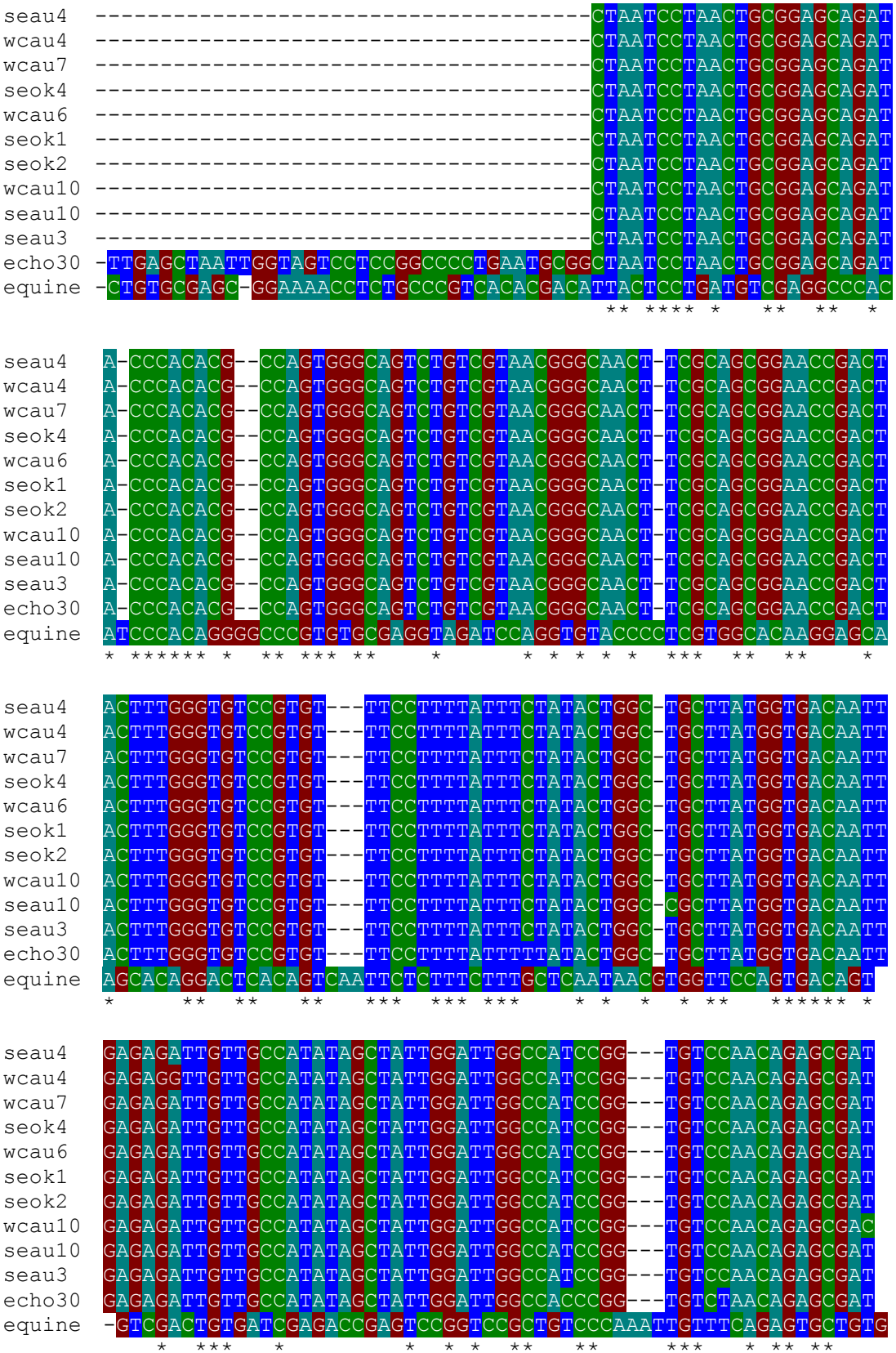
Complete sequences were aligned using ClustalW alignment software. An Echovirus30 sequence available at the NCBI homepage (gi|110350434) was used as reference.
All found sequences were highly similar to each other and to Echovirus30:

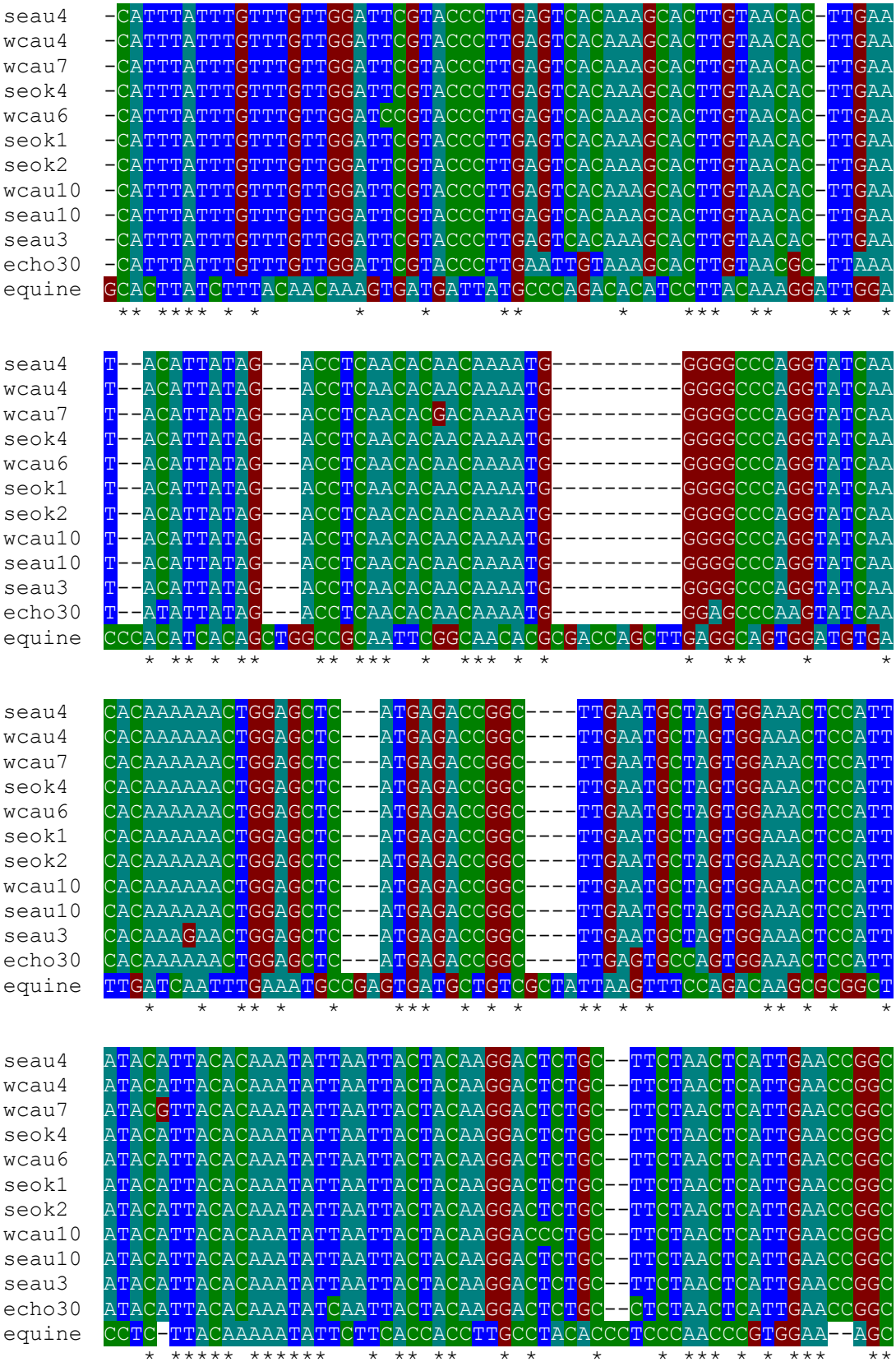


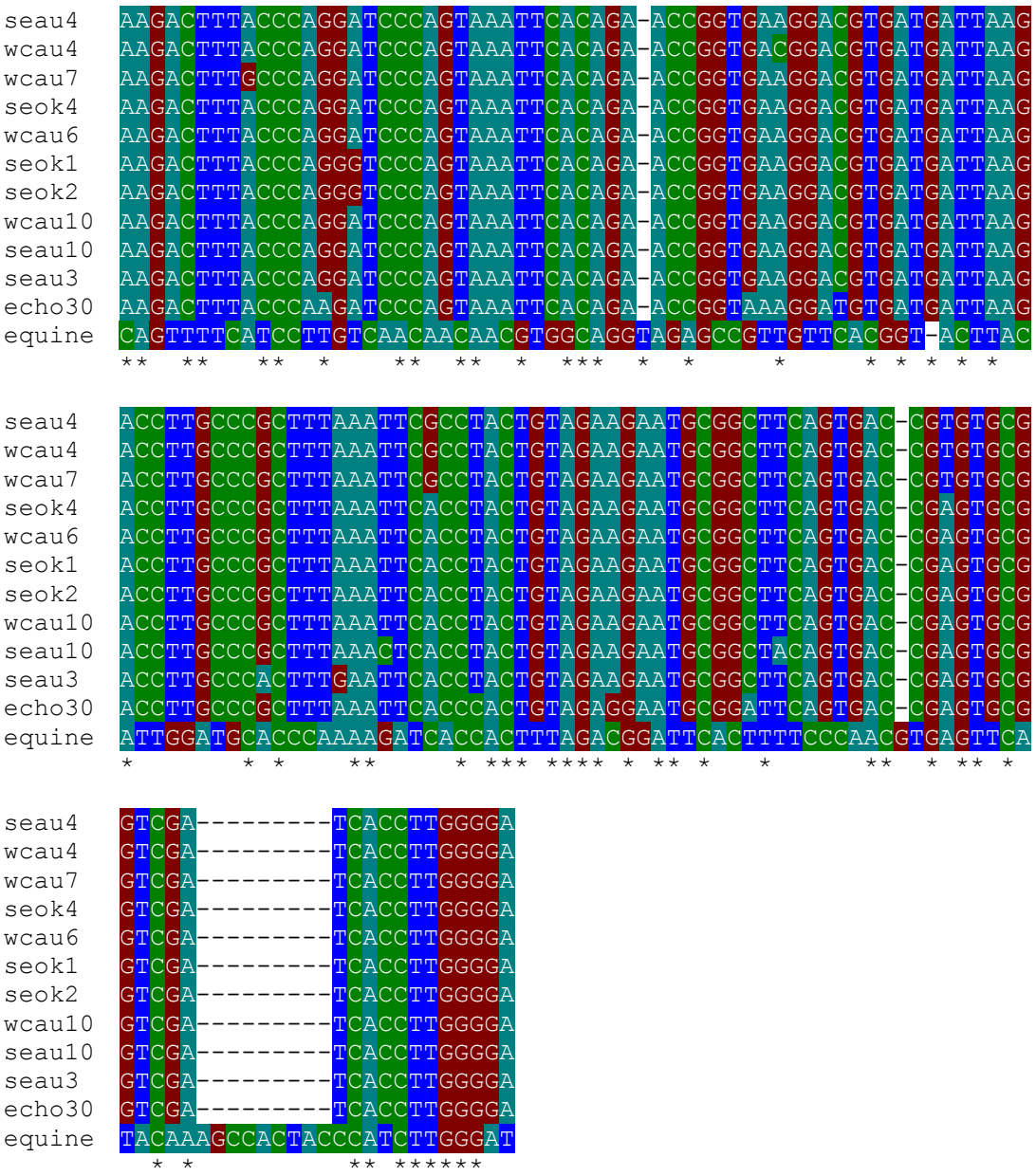

seok1 TTGGATTGGCCATCCGGTGTCCAACAGAGCGATCATTTATTTGTTTGTTGGATTCGTACC
seok2 TTGGATTGGCCATCCGGTGTCCAACAGAGCGATCATTTATTTGTTTGTTGGATTCGTACC
seau3 TTGGATTGGCCATCCGGTGTCCAACAGAGCGATCATTTATTTGTTTGTTGGATTCGTACC
wcau6 TTGGATTGGCCATCCGGTGTCCAACAGAGCGATCATTTATTTGTTTGTTGGATCCGTACC
seau10 TTGGATTGGCCATCCGGTGTCCAACAGAGCGATCATTTATTTGTTTGTTGGATTCGTACC
wcau4 TTGGATTGGCCATCCGGTGTCCAACAGAGCGATCATTTATTTGTTTGTTGGATTCGTACC
wcau7 TTGGATTGGCCATCCGGTGTCCAACAGAGCGATCATTTATTTGTTTGTTGGATTCGTACC
seau4 TTGGATTGGCCATCCGGTGTCCAACAGAGCGATCATTTATTTGTTTGTTGGATTCGTACC
seok4 TTGGATTGGCCATCCGGTGTCCAACAGAGCGATCATTTATTTGTTTGTTGGATTCGTACC
wcau10 TTGGATTGGCCATCCGGTGTCCAACAGAGCGACCATTTATTTGTTTGTTGGATTCGTACC
echo30 TTGGATTGGCCACCCGGTGTCTAACAGAGCGATCATTTATTTGTTTGTTGGATTCGTACC
********** ******** ********** ******************** ******
seok1 CTTGAGTCACAAAGCACTTGTAACACTTGAATACATTATAGACCTCAACACAACAAAATG
seok2 CTTGAGTCACAAAGCACTTGTAACACTTGAATACATTATAGACCTCAACACAACAAAATG
seau3 CTTGAGTCACAAAGCACTTGTAACACTTGAATACATTATAGACCTCAACACAACAAAATG
wcau6 CTTGAGTCACAAAGCACTTGTAACACTTGAATACATTATAGACCTCAACACAACAAAATG
seau10 CTTGAGTCACAAAGCACTTGTAACACTTGAATACATTATAGACCTCAACACAACAAAATG
wcau4 CTTGAGTCACAAAGCACTTGTAACACTTGAATACATTATAGACCTCAACACAACAAAATG
wcau7 CTTGAGTCACAAAGCACTTGTAACACTTGAATACATTATAGACCTCAACACGACAAAATG
seau4 CTTGAGTCACAAAGCACTTGTAACACTTGAATACATTATAGACCTCAACACAACAAAATG
seok4 CTTGAGTCACAAAGCACTTGTAACACTTGAATACATTATAGACCTCAACACAACAAAATG
wcau10 CTTGAGTCACAAAGCACTTGTAACACTTGAATACATTATAGACCTCAACACAACAAAATG
echo30 CTTGAATTGTAAAGCACTTGTAACGCTTAAATATATTATAGACCTCAACACAACAAAATG
***** * ********** *** **** ********** ********
seok1 GGGGCCCAGGTATCAACACAAAAAACTGGAGCTCATGAGACCGGCTTGAATGCTAGTGGA
seok2 GGGGCCCAGGTATCAACACAAAAAACTGGAGCTCATGAGACCGGCTTGAATGCTAGTGGA
seau3 GGGGCCCAGGTATCAACACAAAGAACTGGAGCTCATGAGACCGGCTTGAATGCTAGTGGA
wcau6 GGGGCCCAGGTATCAACACAAAAAACTGGAGCTCATGAGACCGGCTTGAATGCTAGTGGA
seau10 GGGGCCCAGGTATCAACACAAAAAACTGGAGCTCATGAGACCGGCTTGAATGCTAGTGGA
wcau4 GGGGCCCAGGTATCAACACAAAAAACTGGAGCTCATGAGACCGGCTTGAATGCTAGTGGA
wcau7 GGGGCCCAGGTATCAACACAAAAAACTGGAGCTCATGAGACCGGCTTGAATGCTAGTGGA
seau4 GGGGCCCAGGTATCAACACAAAAAACTGGAGCTCATGAGACCGGCTTGAATGCTAGTGGA
seok4 GGGGCCCAGGTATCAACACAAAAAACTGGAGCTCATGAGACCGGCTTGAATGCTAGTGGA
wcau10 GGGGCCCAGGTATCAACACAAAAAACTGGAGCTCATGAGACCGGCTTGAATGCTAGTGGA
echo30 GGAGCCCAAGTATCAACACAAAAAACTGGAGCTCATGAGACCGGCTTGAGTGCCAGTGGA
** ***** ********** ************************** *** ******
seok1 AACTCCATTATACATTACACAAATATTAATTACTACAAGGACTCTGCTTCTAACTCATTG
seok2 AACTCCATTATACATTACACAAATATTAATTACTACAAGGACTCTGCTTCTAACTCATTG
seau3 AACTCCATTATACATTACACAAATATTAATTACTACAAGGACTCTGCTTCTAACTCATTG
wcau6 AACTCCATTATACATTACACAAATATTAATTACTACAAGGACTCTGCTTCTAACTCATTG
seau10 AACTCCATTATACATTACACAAATATTAATTACTACAAGGACTCTGCTTCTAACTCATTG
wcau4 AACTCCATTATACATTACACAAATATTAATTACTACAAGGACTCTGCTTCTAACTCATTG
wcau7 AACTCCATTATACGTTACACAAATATTAATTACTACAAGGACTCTGCTTCTAACTCATTG
seau4 AACTCCATTATACATTACACAAATATTAATTACTACAAGGACTCTGCTTCTAACTCATTG
seok4 AACTCCATTATACATTACACAAATATTAATTACTACAAGGACTCTGCTTCTAACTCATTG
wcau10 AACTCCATTATACATTACACAAATATTAATTACTACAAGGACCCTGCTTCTAACTCATTG
echo30 AACTCCATTATACATTACACAAATATCAATTACTACAAGGACTCTGCCTCTAACTCATTG
********** ********** ********** **** **********


seok1 AACCGGCAAGACTTTACCCAGGGTCCCAGTAAATTCACAGAACCGGTGAAGGACGTGATG
seok2 AACCGGCAAGACTTTACCCAGGGTCCCAGTAAATTCACAGAACCGGTGAAGGACGTGATG
seau3 AACCGGCAAGACTTTACCCAGGATCCCAGTAAATTCACAGAACCGGTGAAGGACGTGATG
wcau6 AACCGGCAAGACTTTACCCAGGATCCCAGTAAATTCACAGAACCGGTGAAGGACGTGATG
seau10 AACCGGCAAGACTTTACCCAGGATCCCAGTAAATTCACAGAACCGGTGAAGGACGTGATG
wcau4 AACCGGCAAGACTTTACCCAGGATCCCAGTAAATTCACAGAACCGGTGACGGACGTGATG
wcau7 AACCGGCAAGACTTTGCCCAGGATCCCAGTAAATTCACAGAACCGGTGAAGGACGTGATG
seau4 AACCGGCAAGACTTTACCCAGGATCCCAGTAAATTCACAGAACCGGTGAAGGACGTGATG
seok4 AACCGGCAAGACTTTACCCAGGATCCCAGTAAATTCACAGAACCGGTGAAGGACGTGATG
wcau10 AACCGGCAAGACTTTACCCAGGATCCCAGTAAATTCACAGAACCGGTGAAGGACGTGATG
echo30 AACCGGCAAGACTTTACCCAAGATCCCAGTAAATTCACAGAACCGGTAAAGGATGTGATG
ATTAAGACCTTGCCCGCTTTAAATTCACCTACTGTAGAAGAATGCGGCTTCAGTGACCGA
ATTAAGACCTTGCCCGCTTTAAATTCACCTACTGTAGAAGAATGCGGCTTCAGTGACCGA
ATTAAGACCTTGCCCACTTTGAATTCACCTACTGTAGAAGAATGCGGCTTCAGTGACCGA
ATTAAGACCTTGCCCGCTTTAAATTCACCTACTGTAGAAGAATGCGGCTTCAGTGACCGA
ATTAAGACCTTGCCCGCTTTAAACTCACCTACTGTAGAAGAATGCGGCTACAGTGACCGA
ATTAAGACCTTGCCCGCTTTAAATTCGCCTACTGTAGAAGAATGCGGCTTCAGTGACCGT
ATTAAGACCTTGCCCGCTTTAAATTCGCCTACTGTAGAAGAATGCGGCTTCAGTGACCGT
ATTAAGACCTTGCCCGCTTTAAATTCGCCTACTGTAGAAGAATGCGGCTTCAGTGACCGT
ATTAAGACCTTGCCCGCTTTAAATTCACCTACTGTAGAAGAATGCGGCTTCAGTGACCGA
ATTAAGACCTTGCCCGCTTTAAATTCACCTACTGTAGAAGAATGCGGCTTCAGTGACCGA
ATTAAGACCTTGCCCGCTTTAAATTCACCCACTGTAGAGGAATGCGGATTCAGTGACCGA
GTGCGGTCGATCACCTTGGGGA
GTGCGGTCGATCACCTTGGGGA
GTGCGGTCGATCACCTTGGGGA
GTGCGGTCGATCACCTTGGGGA
GTGCGGTCGATCACCTTGGGGA
GTGCGGTCGATCACCTTGGGGA
GTGCGGTCGATCACCTTGGGGA
GTGCGGTCGATCACCTTGGGGA
GTGCGGTCGATCACCTTGGGGA
GTGCGGTCGATCACCTTGGGGA

For phylogenetic analysis, the obtained sequences were aligned with Echovirus 30 and Equine Rhinovirus 3 sequences, both acquired from the NCBI database (gi|110350434 and gi|15192761).




seau4 -CATTTATTTGTTTGTTGGATTCGTACCCTTGAGTCACAAAGCACTTGTAACAC-TTGAA
wcau4 -CATTTATTTGTTTGTTGGATTCGTACCCTTGAGTCACAAAGCACTTGTAACAC-TTGAA
wcau7 -CATTTATTTGTTTGTTGGATTCGTACCCTTGAGTCACAAAGCACTTGTAACAC-TTGAA
seok4 -CATTTATTTGTTTGTTGGATTCGTACCCTTGAGTCACAAAGCACTTGTAACAC-TTGAA
wcau6 -CATTTATTTGTTTGTTGGATCCGTACCCTTGAGTCACAAAGCACTTGTAACAC-TTGAA
seok1 -CATTTATTTGTTTGTTGGATTCGTACCCTTGAGTCACAAAGCACTTGTAACAC-TTGAA
seok2 -CATTTATTTGTTTGTTGGATTCGTACCCTTGAGTCACAAAGCACTTGTAACAC-TTGAA
wcau10 -CATTTATTTGTTTGTTGGATTCGTACCCTTGAGTCACAAAGCACTTGTAACAC-TTGAA
seau10 -CATTTATTTGTTTGTTGGATTCGTACCCTTGAGTCACAAAGCACTTGTAACAC-TTGAA
seau3 -CATTTATTTGTTTGTTGGATTCGTACCCTTGAGTCACAAAGCACTTGTAACAC-TTGAA
echo30 -CATTTATTTGTTTGTTGGATTCGTACCCTTGAATTGTAAAGCACTTGTAACGC-TTAAA
equine GCACTTATCTTTACAACAAAGTGATGATTATGCCCAGACACATCCTTACAAAGGATTGGA
seau4 T--ACATTATAG---ACCTCAACACAACAAAATG----------GGGGCCCAGGTATCAA
wcau4 T--ACATTATAG---ACCTCAACACAACAAAATG----------GGGGCCCAGGTATCAA
wcau7 T--ACATTATAG---ACCTCAACACGACAAAATG----------GGGGCCCAGGTATCAA
seok4 T--ACATTATAG---ACCTCAACACAACAAAATG----------GGGGCCCAGGTATCAA
wcau6 T--ACATTATAG---ACCTCAACACAACAAAATG----------GGGGCCCAGGTATCAA
seok1 T--ACATTATAG---ACCTCAACACAACAAAATG----------GGGGCCCAGGTATCAA
seok2 T--ACATTATAG---ACCTCAACACAACAAAATG----------GGGGCCCAGGTATCAA
wcau10 T--ACATTATAG---ACCTCAACACAACAAAATG----------GGGGCCCAGGTATCAA
seau10 T--ACATTATAG---ACCTCAACACAACAAAATG----------GGGGCCCAGGTATCAA
seau3 T--ACATTATAG---ACCTCAACACAACAAAATG----------GGGGCCCAGGTATCAA
echo30 T--ATATTATAG---ACCTCAACACAACAAAATG----------GGAGCCCAAGTATCAA
equine CCCACATCACAGCTGGCCGCAATTCGGCAACACGCGACCAGCTTGAGGCAGTGGATGTGA
seau4 CACAAAAAACTGGAGCTC---ATGAGACCGGC----TTGAATGCTAGTGGAAACTCCATT
wcau4 CACAAAAAACTGGAGCTC---ATGAGACCGGC----TTGAATGCTAGTGGAAACTCCATT
wcau7 CACAAAAAACTGGAGCTC---ATGAGACCGGC----TTGAATGCTAGTGGAAACTCCATT
seok4 CACAAAAAACTGGAGCTC---ATGAGACCGGC----TTGAATGCTAGTGGAAACTCCATT
wcau6 CACAAAAAACTGGAGCTC---ATGAGACCGGC----TTGAATGCTAGTGGAAACTCCATT
seok1 CACAAAAAACTGGAGCTC---ATGAGACCGGC----TTGAATGCTAGTGGAAACTCCATT
seok2 CACAAAAAACTGGAGCTC---ATGAGACCGGC----TTGAATGCTAGTGGAAACTCCATT
wcau10 CACAAAAAACTGGAGCTC---ATGAGACCGGC----TTGAATGCTAGTGGAAACTCCATT
seau10 CACAAAAAACTGGAGCTC---ATGAGACCGGC----TTGAATGCTAGTGGAAACTCCATT
seau3 CACAAAGAACTGGAGCTC---ATGAGACCGGC----TTGAATGCTAGTGGAAACTCCATT
echo30 CACAAAAAACTGGAGCTC---ATGAGACCGGC----TTGAGTGCCAGTGGAAACTCCATT
equine TTGATCAATTTGAAATGCCGAGTGATGCTGTCGCTATTAAGTTTCCAGACAAGCGCGGCT
seau4 ATACATTACACAAATATTAATTACTACAAGGACTCTGC--TTCTAACTCATTGAACCGGC
wcau4 ATACATTACACAAATATTAATTACTACAAGGACTCTGC--TTCTAACTCATTGAACCGGC
wcau7 ATACGTTACACAAATATTAATTACTACAAGGACTCTGC--TTCTAACTCATTGAACCGGC
seok4 ATACATTACACAAATATTAATTACTACAAGGACTCTGC--TTCTAACTCATTGAACCGGC
wcau6 ATACATTACACAAATATTAATTACTACAAGGACTCTGC--TTCTAACTCATTGAACCGGC
seok1 ATACATTACACAAATATTAATTACTACAAGGACTCTGC--TTCTAACTCATTGAACCGGC
seok2 ATACATTACACAAATATTAATTACTACAAGGACTCTGC--TTCTAACTCATTGAACCGGC
wcau10 ATACATTACACAAATATTAATTACTACAAGGACCCTGC--TTCTAACTCATTGAACCGGC
seau10 ATACATTACACAAATATTAATTACTACAAGGACTCTGC--TTCTAACTCATTGAACCGGC
seau3 ATACATTACACAAATATTAATTACTACAAGGACTCTGC--TTCTAACTCATTGAACCGGC
echo30 ATACATTACACAAATATCAATTACTACAAGGACTCTGC--CTCTAACTCATTGAACCGGC
equine CCTC-TTACAAAAATATTCTTCACCACCTTGCCTACACCCTCCCAACCCGTGGAA--AGC


seau4 AAGACTTTACCCAGGATCCCAGTAAATTCACAGA-ACCGGTGAAGGACGTGATGATTAAG
wcau4 AAGACTTTACCCAGGATCCCAGTAAATTCACAGA-ACCGGTGACGGACGTGATGATTAAG
wcau7 AAGACTTTGCCCAGGATCCCAGTAAATTCACAGA-ACCGGTGAAGGACGTGATGATTAAG
seok4 AAGACTTTACCCAGGATCCCAGTAAATTCACAGA-ACCGGTGAAGGACGTGATGATTAAG
wcau6 AAGACTTTACCCAGGATCCCAGTAAATTCACAGA-ACCGGTGAAGGACGTGATGATTAAG
seok1 AAGACTTTACCCAGGGTCCCAGTAAATTCACAGA-ACCGGTGAAGGACGTGATGATTAAG
seok2 AAGACTTTACCCAGGGTCCCAGTAAATTCACAGA-ACCGGTGAAGGACGTGATGATTAAG
wcau10 AAGACTTTACCCAGGATCCCAGTAAATTCACAGA-ACCGGTGAAGGACGTGATGATTAAG
seau10 AAGACTTTACCCAGGATCCCAGTAAATTCACAGA-ACCGGTGAAGGACGTGATGATTAAG
seau3 AAGACTTTACCCAGGATCCCAGTAAATTCACAGA-ACCGGTGAAGGACGTGATGATTAAG
echo30 AAGACTTTACCCAAGATCCCAGTAAATTCACAGA-ACCGGTAAAGGATGTGATGATTAAG
equine CAGTTTTCATCCTTGTCAACAACAACGTGGCAGGTAGAGCCGTTGTTCACGGT-ACTTAC
** ** ** * ** ** * *** * * * * * * * *
seau4 ACCTTGCCCGCTTTAAATTCGCCTACTGTAGAAGAATGCGGCTTCAGTGAC-CGTGTGCG
wcau4 ACCTTGCCCGCTTTAAATTCGCCTACTGTAGAAGAATGCGGCTTCAGTGAC-CGTGTGCG
wcau7 ACCTTGCCCGCTTTAAATTCGCCTACTGTAGAAGAATGCGGCTTCAGTGAC-CGTGTGCG
seok4 ACCTTGCCCGCTTTAAATTCACCTACTGTAGAAGAATGCGGCTTCAGTGAC-CGAGTGCG
wcau6 ACCTTGCCCGCTTTAAATTCACCTACTGTAGAAGAATGCGGCTTCAGTGAC-CGAGTGCG
seok1 ACCTTGCCCGCTTTAAATTCACCTACTGTAGAAGAATGCGGCTTCAGTGAC-CGAGTGCG
seok2 ACCTTGCCCGCTTTAAATTCACCTACTGTAGAAGAATGCGGCTTCAGTGAC-CGAGTGCG
wcau10 ACCTTGCCCGCTTTAAATTCACCTACTGTAGAAGAATGCGGCTTCAGTGAC-CGAGTGCG
seau10 ACCTTGCCCGCTTTAAACTCACCTACTGTAGAAGAATGCGGCTACAGTGAC-CGAGTGCG
seau3 ACCTTGCCCACTTTGAATTCACCTACTGTAGAAGAATGCGGCTTCAGTGAC-CGAGTGCG
echo30 ACCTTGCCCGCTTTAAATTCACCCACTGTAGAGGAATGCGGATTCAGTGAC-CGAGTGCG
equine ATTGGATGCACCCAAAAGATCACCACTTTAGACGGATTCACTTTTCCCAACGTGAGTTCA
* * * ** * *** **** * ** * * ** * ** *
seau4 GTCGA---------TCACCTTGGGGA
wcau4 GTCGA---------TCACCTTGGGGA
wcau7 GTCGA---------TCACCTTGGGGA
seok4 GTCGA---------TCACCTTGGGGA
wcau6 GTCGA---------TCACCTTGGGGA
seok1 GTCGA---------TCACCTTGGGGA
seok2 GTCGA---------TCACCTTGGGGA
wcau10 GTCGA---------TCACCTTGGGGA
seau10 GTCGA---------TCACCTTGGGGA
seau3 GTCGA---------TCACCTTGGGGA
echo30 GTCGA---------TCACCTTGGGGA
equine TACAAAGCCACTACCCATCTTGGGAT
* * ** ******

4.5 Pylogenetic Tree

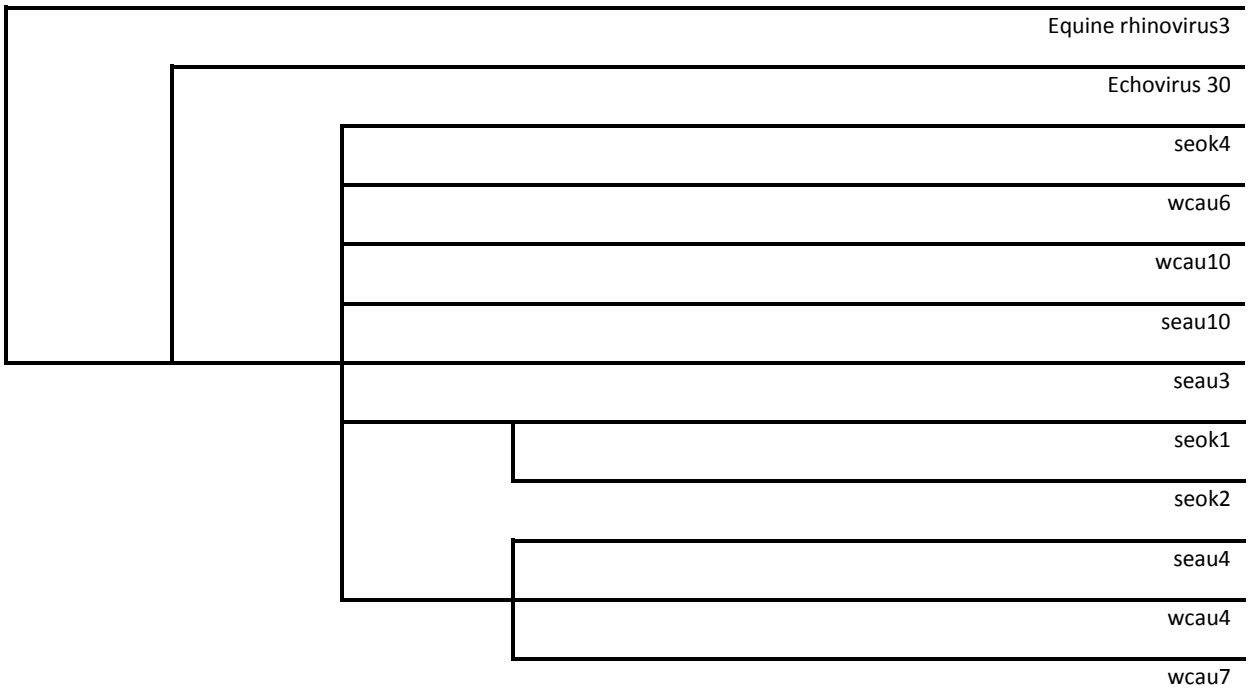


Fig. 21: Phylogenetic tree

According to the alignment and the phylogenetic tree, seok1 and seok2 have identical sequences. Seau4, wcau4 and wcau7 are also completely identical to each other. All clones share more similarities with each other than with the original Echovirus30 sequence.

5 Discussion

Despite the importance of enteric viruses in means of human health, methods for direct surveillance in fresh water generally have not yet been taken into account due to difficult and expensive execution. This study consolidates the results of Fister (2009) reporting the presence of Echovirus 30 in the sediment of the Alte Donau and suggests a low level of Echovirus contamination of the water body at the same location.

5.1 The Sediment

The results of Fister (2009) prove that there are Enteroviruses in the sediment of the Alte Donau. Fister (2009) did not find evidence for the presence of other enteric viruses than Echovirus 30. In this study, primers were adjusted to be able to find a higher diversity of enteric viruses in the Alte Donau. However, the results achieved with these primers were even more Echovirus 30 specific, confirming the presumption that this Echovirus serotype is actually the dominant enterovirus in the sediment of the Alte Donau.

The main explanation for this may be the fact that Echovirus 30 is one of the most common enteroviruses around the world. In France in the year 2000, 41% of the 1262 enteroviruses identified were Echovirus 30 (Noah and Reid 2002). McWilliam Leitch et al. (2009) report high frequencies of Echovirus 30 detection in surveillance programs throughout Europe in 2000 and 2001, and again in 2005 to 2006. The results of Lee and Lee (2008) indicate that also in Korean surface waters Echovirus 30 is the predominant enterovirus species.

5.2 The Waterbody

The river Danube is known to contain enteric viruses (Simkova and Cervenka 1981, Walter et al. 1990) Walter et al. reported the presence of Cocksackievirus B and Echovirus 3, 7, 11, 30 and 33 strains whereas Simkova and Cervenka found Poliovirus, Cocksackievirus and Echovirus strains. However, these results were obtained in the 1980s. Since then the water quality of the river Danube has greatly improved due to better sewage treatment.

Based on the results of Fister (2009), the presence of enteric viruses in the waterbody of the Alte Donau seemed a logical consequence. The sediment of a waterbody is generally considered a sink for viruses, due to the fact, that harmful environmental effects such as UV radiation do not influence this habitat, and others like pH value or temperature do not fluctuate as much as in the water above. However, sediment can be resuspended, for example by bioturbation or wind induced water movement. According to Brookes et al. (2004), pathogen resuspension events from the sediment are common when turbulent velocity fluctuations at the benthic boundary reach a certain level, which is "attributable to internal waves or wave action of leeward shores". One reason for these resuspension events are anthropogenic stresses (Tragaou et al., 2005).

As stated before, the Alte Donau is heavily used for recreational purposes. The Gänsehäufel, the biggest lido of the area, counted a record of 30.000 users per day in 2007. Other bathing resorts at the Alte Donau have capacities of another 15.000 users per day. (http://www.wien-konkret.at/reisen/ausflugsziele/alte-donau/) This is a huge impact on a 1,6 km^2 large ecosystem. The facts that human enteroviruses are transmitted by contaminated water via the faecal-oral route, and that the Alte Donau is used for recreation in such a substantial degree, indicate that enteric viruses must be present in the Alte Donau to a certain extent.

Contrary to this conclusion, enterovirus levels in the water column of the Alte Donau were generally too low for detection. The titer was only in August 2009 high enough to give positive results on Echovirus 30.

All obtained sequences were similar to Echovirus 30. The fact that only Echovirus 30 strains were found correlates well with the data collected from the Alte Donau sediment.

Furthermore, the analogy between sequences obtained from the sediment and the waterbody at that time (Fig. 21) suggest a close relationship between those strains. It is an indication for a resuspension event, during which the viruses were released from the sediment to the waterbody.

The results achieved in this study suggest that the presence of Echovirus 30 in the waterbody of the Alte Donau is assumed to be a consequence of a resuspension effect.

5.3 A Significant Threat To Human Health?

Echovirus 30 is known to be responsible for large epidemic outbreaks, followed by a quiescent period that may last several years. “The quiescence is most probably due to the development of population immunity that occurs in a high infection-rate epidemic. The virus may cause only sporadic cases until a large cohort of non-immune individuals has developed, often over a period of several years, setting the stage for another large epidemic.” (Oberste et al., 1999)

In Europe, Echovirus 30 was responsible for major outbreaks in France, Iceland, Kosovo, and the Netherlands in 2000, and in England and Wales, Scotland, Germany, and Ireland in 2001 (Noah and Reid 2002). An Echovirus 30 outbreak was observed in northwestern Austria in 2000, matching the time of the trans-European epidemic (Ortner et al., 2009). The virus was also linked to an outbreak of aseptic meningitis in France, 2005 (Brunel et al., 2008)

Echovirus 30 was also the most frequently identified enterovirus in a study of 61 children with enteroviral meningitis (Henquell et al., 2001). Generally, Echovirus 30 was the most common cause of aseptic meningitis in Austria from 1999 to 2007, being isolated in 48 out of 65 recorded cases of Echovirus-induced meningitis, with a peak in the year 2000 (Ortner et al., 2009).

Briefly, Echovirus 30 is a virus with a high potential risk. It is present in the sediment and to some extent also in the water column of the Alte Donau. Although Brookes et al. (2004) indicate that there need not necessarily be a link between the presence of a pathogen within a

reservoir and a risk to human health, and the pathogen survival timeframe does play an important role in risk assessment, Enteroviruses are known to have a high persistence under various harmful environmental conditions including changes of pH value, temperature or salinity (Ley et al., 2002) and can remain infective in river water for a time ranging from weeks to several months. The problem with enteric viruses is the fact, that if they contaminate the environment in numbers high enough to represent a threat for human health, these numbers are still low enough to make detection difficult.

With the experimental design used in this study, no statement about the infectivity of the found Echovirus 30 can be made, due to the lack of cell culture experiments. However, the results of a study comparing RT-PCR and cell culture side by side (Donaldson et al., 2002) suggest, that 55 viral particles in a sample equate to one infectious particle. When taken into account that in this study Echovirus was only detected after heavy concentration of samples and only by using nested PCR after regular PCR, the concentration of infectious Echovirus particles is assumedly very low.

The responsible authorities of Vienna attest bathing quality to the water of the Alte Donau. This is based on bacterial parameters only (Table 3).

Bathing site	Date	E. coli MPN/100 ml	enterococci KBE/100 ml	Bathing quality
Angelibad	27.04.2010	<15	20	yes
Strandbad	27.04.2010	30	8	yes
Gänsehäufel Weststrand	27.04.2010	30	10	yes
Gänsehäufel Oststrand	27.04.2010	30	82	yes
Gänsehäufel Südstrand	27.04.2010	<15	4	yes
Kaiserwasser	27.04.2010	15	8	yes
Untere Alte Donau	27.04.2010	<15	4	yes

Table 3: Water quality at different bathing sites of the Alte Donau (http://www.wien.gv.at/forschung/laboratorien/ifum/biologie/badewasserqualitaet/index.html)

5.4 Conclusion

The results obtained in this study consolidate the assumptions made by S. Fister in 2009 concerning the enterovirus contamination of the Alte Donau. Echovirus 30 is the predominant enterovirus species in both the sediment and the water body of the Alte Donau. However, the contamination level seems to be very low, especially in the water body. The presence of Echovirus 30 in the water body seems to be due to resuspension from the sediment.

6 References

Ahmed A.U., D.L. Sörenson, 1995: Kinetics of pathogen destruction during storage of dewatered biosolids. *Water Environ. Res. 67: 143- 150.*

Arnone R.D., J.P. Walling, 2007: Waterborne pathogens in urban watersheds. *J. of Water & Health 05.1: 149-162.*

Boccia D., A.E. Tozzi, B. Cotter, C. Rizzo, T. Russo, G. Buttinelli, A. Caprioli, M.L. Marziano, E.M. Ruggeri, 2002: Waterborne outbreak of Norwalk-like virus gastroenteritis at a tourist resort, Italy. *Emerg. Infect. Dis. 8: 563-568.*

Bordalo A.A., R. Onrassami, C. Dechsakulwatana, 2002: Survival of faecal indicator bacteria in tropical estuarine waters (Bangpakong River, Thailand). *J. Appl. Mikrobiol. 93: 864–871.*

Bosch A., 1998: Human enteric viruses in the water environment. *Int Microbiol 1: 191-196.*

Bosch A., S. Guix, D. Sano, R. Pintó, 2008: New tools for the study and direct surveillance of viral pathogens in water. *Curr. Opinion in Biotechnology 19: 295-301.*

Breitbart, M., F. Rohwer, 2005: Here a virus, there a virus, everywhere the same virus?. *Trends in Microbiology 13(6): 278-284.*

Brookes, J.D., J. Antenucci, M. Hipsey, M.D. Burch, N.J. Ashbolt, C. Ferguson, 2004: Fate and Transport of pathogens in lakes and reservoirs. *Environ. Intern. 30: 741-759.*

Brown, C.M., J.W. Cann, G. Simmons, R.L. Fankhauser, W. Thomas, U.D. Parashar, M.J. Lewis, 2001: Outbreak of Norwalk virus in a Caribbean island resort: application of molecular diagnostics to ascertain the vehicle of infection. *Epidemiol. Infect. 126: 425-432.*

Brunel D., N. Lévêque, J. Jacques, F. Renois, J. Motte, L. Andréoletti, 2008: Clinical and virological features of an aseptic meningitis outbreak in North-Eastern France, 2005. *J Clin Virol 4, 225–228.*

Chambon M., C. Archimbaud, J. Bailly, C. Hemquell, C. Regagnon, F. Charbonné, H. Peigue-Lafeuille, 2001: Circulation of enteroviruses and persistence of meningitis cases in the winter of 1999-2000. *Jour. Med. Viro 65: 340-347.*

Chénard, C., A.C. Suttle, 2008: Phylogenetic diversity of sequences of cyanophage photosynthetic gene *psba* in marine and fresh waters. *Appl. Environ. Microbiol. 74/17: 5317-5324.*

Colford, J. M. Jr.,T.J. Wade, K.C. Schiff, C.C. Wright, J.F. Griffith, S.K. Sandhu, S. Burns, M. Sobsey, G. Lovelace, S.B. Weisberg, 2007: Water Quality Indicators and the Risk of Illness at Beaches With Nonpoint Sources of Fecal Contamination. *Epidemology 18: 27-35.*

Corinaldesi C., A. Dell'Anno, M. Magagnini, R. Danovaro, 2010: Viral decay and viral production rates in continental-shelf and deepsea sediments of the Mediterranean Sea. *FEMS Microbiol Ecol 72: 208-218.*

Culley, A.I., A.S. Lang, C.A. Suttle, 2006: Metagenomic analysis of costal RNA virus communities. *Sci 312:1795- 1798.*

Danovaro R., M. Serresi, 2000: Viral density and virus-to-bacteria ratio in deep-sea sediments of the eastern Mediterranean. *Appl. Environ. Microbiol 66/5: 1857-1861.*

Donaldson K.A., D.W. Griffin, J.H. Paul, 2002: Detection, quantitation and identification of enteroviruses from surface waters and sponge tissue from the Florida Keys using real-time RT–PCR. *Water Research 36(10): 2505-2514.*

Espinosa A.C., C.F. Arias, S. Sánchez-Colón, M. Mazari-Hiriart, 2009: Comparative study of enteric viruses, coliphages and indicator bacteria for evaluating water quality in a tropical high-altitude system. *Environ.Health 8:49.*

Fister S., 2009: RNA viruses in aquatic systems. Diploma Thesis.

Fong, T.-T., E. K. Lipp. 2005: Enteric viruses of humans and animals in aquatic environments: Health risks, detection, and potential water quality assessment tools. *Appl. Environ. Microbiol. 69(2): 357-371.*

Henquell C, M. Chambon, J.L. Bailly, 2001: Prospective analysis of 61 cases of enteroviral meningitis: interest of systematic genome detection in cerebrospinal fluid irrespective of cytologic examination results. *J Clin Virol. 21: 29–35.*

Hurst C.J., W.H. Benton, K.A. McClellan, 1989: Thermal and water source effects upon the stability of enteroviruses in surface freshwaters. *Can J Microbiol. 35(4): 474-480.*

Khetsuriani N, Lamonte-Fowlkes A, Oberst S, Pallansch MA 2006: Centers for Disease Control and Prevention: Enterovirus surveillance – United States, 1970-2005. *MMWR Surveill Summ, 55: 1-20.*

Kocwa-Haluch R, 2001: Waterborne Enteroviruses as a Hazard for Human Health. *Pol. Jour. Environm. Stud.10/6: 485-487.*

Lee, G., C. Lee. 2008: Molecular detection and characterization of human enteroviruses in Korean surface water. *J. Microbiol. 46(3): 319-324.*

Ley, V., J. Higgins, R. Fayer. 2002: Bovine enteroviruses as indicators of fecal contamination. *Appl. Environ. Microbiol. 68(7): 3455- 3461.*

Lodder, W.J., A.M. De Roda Husman, 2005: Presence of noroviruses and other enteric viruses in sewage and surface waters in the Netherlands. *Appl. Environ. Microbiol 71: 1453-1461.*

McWilliam Leitch E.C., J. Bendig, M. Cabrerizo, J. Cardoza, T.Hyypiä, O.E. Ivanova, A. Kelly, A.C.-M. Kroes, A. Lukashev, A. MacAdam, P. McMinn, M. Roivainen, G. Trallero, D.J. Evans, P. Simmons, 2009: Transmission Networks and Population Turnover of Echovirus 30. *Jour. Virol. 83/5: 2109-2118.*

Monpoeho, S., A. Maul, B. Mignotte-Cadiergues, L. Schwartzbrod, S.Billaudel, V.Ferré. 2001: Best viral elution method available for quantification of enteroviruses in sludge by both cell culture and reverse transcription-PCR. *Appl. Environ. Microbiol. 67(6): 2484-2488.*

Nairn, C., and G. B. Clements. 1999: A study of enterovirus isolations in Glasgow from 1977 to 1997. *J. Med. Virol. 58: 304-312.*

Nijhuis, M, N. Van Maarseveen, R. Schuurman, S. Verkuijlen, M. De Vos, K. Hedriksen, A. M. Van Loon. 2002: Rapid and sensitive routine detection of all members of the genus enterovirus in different clinical specimens by real-time PCR. *J. Clin. Microbiol. 40: 3666-3670.*

Noah N., F. Reid, 2002: Recent increases in incidence of echoviruses 13 and 30 around Europe. *Euro Surveill. 2002; 6(7): 2029.*

Ortner B., C. Huang, D. Schmid, I. Mutz, G. Wewalka , F. Allerberger, J. Yang, H. P. Huemer, 2009: Epidemiology of enterovirus types causing neurological disease in Austria 1999-2007: Detection of clusters of echovirus 30 and enterovirus 71 and analysis of prevalent genotypes. *J. Med. Virol. 81:317-324.*

Oberste, M. S., K. Maher, M. A. Pallansch. 1998: Complete sequence of echovirus 23 and its relationship to echovirus 22 and other human enteroviruses. *Vir. Res 56/2: 217-223.*

Oberste M.S., K. Maher, M. L. Kennett, 1999: Molecular epidemiology and genetic diversity of echovirus type 30 (E30): genotypes correlate with temporal dynamics of E30 isolation. *J Clin Microbiol 37: 3928–3933.*

Oberste M.S., D. Schnurr, K. Maher, S. al-Busaidy, M. A. Pallansch, 2001: Molecular identification of new picornaviruses and characterization of a proposed enterovirus 73 serotype. *Jour. Gen. Vir. 82: 409–416.*

Pelletier J., Sonenberg N., 1988: Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. *Nature 334: 320-325.*

Rajtar, B., Majek M., Polański L., Polz-Dacewicz M., 2008: Enteroviruses in water environment – a potential threat to public health. *Ann Agric Environ Med 15: 199–203.*

Rose, M.A., A.K. Dhar, H.A. Brooks, F. Zecchini, R.M. Gersberg, 2006: Quantitation of hepatitis A virus and enterovirus levels in the lagoon canals and Lido beach of Venice, Italy, using real-time RT-PCR. *Water Research 40: 2387-2396.*

Siafakas N., D. Papaventsis, S. Levidiotou-Stefanou, N.C. Vamvakopoulos, P. Markoulatos, 2005: Classification and Structure of Echovirus 5‘-UTR Sequences. *Virus Genes 31: 293–306.*

Simkova A., J. Cervenka, 1981: Coliphages as ecological indicators of enteroviruses in various water systems. *Bulletin of the World Health Organization, 59 (4): 611-618.*

Suttle C.A., 2007: Marine viruses - major players in the global ecosystem. *Nature Reviews Microb. 5: 801-812.*

Tragou E, V. Zervakis, E. Papageorgiou, S. Stavrakakis, V. Lykousis, 2005: Monitoring the Physical Forcing of Resuspension Events in the Thermaikos Gulf-NW Aegean during 2001-2003. *Cont. Shelf Res., v25: 2315-2331.*

Walter, R., R. Dumke, J. Dürkop, S. Guyer, U. Krämer. 1990: Virological investigations of the river Danube. *Acta hydrochim. hydrobiol. 18(3): 333-343.*

Ward R.L., D.R. Knowlton, P.E. Winston, 1986: Mechanism of Inactivation of Enteric Viruses in Fresh Water. *Appl. Environ. Microbio 52/3: 450-459.*

Wommack, K.E., K.E. Williamson, M. Radosevich, 2003: Sampling natural viral communities from soil for culture-independent analyses. *Appl. Environ. Microbiol 69/11: 6628-6633.*

Wommack K.E., S.R. Bench, T.E. Hanson, K.E. Williamson, D. Ghosh, M. Radosovich, K. Wang, 2007: Metagenomic Characterization of Chesapeake Bay Virioplankton. *Appl. Environ. Microbiol. 73/23: 7629-7641.*

Zoll, G. J., W. J. G. Melchers, H. Kopecka, G. Jambroes, H. J. A. Van der Poel, J. M. D. Galama. 1992: General primer-mediated polymerase chain reaction for detection of enteroviruses: Application for diagnostic Routine and persistent infections. *J. Clin. Microbiol. 30(1): 160-165.*

Figures and Tables

Introduction

Fig. 1: Biomass and abundance of viruses 1

Tab. 1: Human viruses documented to be found in water environment 4

Fig. 2: The Picornaviridae family 5

Fig. 3: The enterovirus genome 7

Fig. 4: Polyprotein translation of the enterovirus genome 8

Fig. 5: Pathogenesis of enterovirus infections 9

Fig. 6: Transmission routes of human enteroviruses in the environment 10

Material and Methods

Tab. 2: Sampling data 14

Fig .7: Alignment of human enterovirus b, c, d, 100, poliovirus and coxsackievirus 19

Fig. 8: Steps of the PCR technique 23

Fig. 9: Exponential amplification of a target DNA sequence 23

Fig. 10: The pGEM-T Easy Vector 24

Results

Fig. 11: PCR of August 2009 Sediment Sample 31

Fig. 12: PCR of October 2009 Sediment Sample 31

Fig 13: PCR of July, September and October 2009 Alte Donau water column samples 32

Fig. 14: PCR of August 2009 Alte Donau water column sample 33

Fig. 15: PCR of April 2009 Wasserpark water column sample 33

Fig. 16: Restriction of Alte Donau August 2009 sediment clones 35

Fig. 17: Restriction of Alte Donau August 2009 sediment clones 35

Fig. 18: Restriction of Alte Donau October 2009 sediment clones 35

Fig. 19: Restriction of Alte Donau August 2009 water body clones 36

Fig. 20: Sequences of all samples 38- 41

Fig. 21: Phylogenetic tree 48

Discussion

Tab 3: Water quality at different bathing sites of the Alte Donau 52

Table Of Abbreviations

A. dest.	distilled water
BLAST	Basic Local Alignment and Search Tool
bp	base pair
cDNA	complementary DNA
cm	centimeter
dNTP	2'-desoxy-nucleoside-5'-triphosphate
dsRNA	double-stranded RNA
DTT	Dithiothreitole
EDTA	Ethylene diamine tetraacetic acid
e.g.	example given
g	gramme
h	hour
IPTG	Isopropyl-beta-D-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
l	liter
LB	Luria-Bertani (medium)
M	mole, molar
max	maximal
MCS	multiple cloning site
min	minute
ml	milliliter
mm	millimeter
mM	millimole, millimolar
mRNA	messenger RNA
ng	nanogramme
µl	Microlitre
µg	Microgramme
NaAc	Sodium acetate

nm	Nanometre
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
PEG	Polyethylene glycol
pM	picomole
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT	room temperature
SDS	Sodium dodecylsulphate
sec	second
ssRNA	single-stranded RNA
U	unit
UTR	untranslated region
UV	ultraviolet
V	volt
Vol	volume
X-gal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranosid

Abstract

Enteric viruses are common waterborne pathogens that can cause numerous diseases in humans, sometimes with serious or even fatal consequences. Enteroviruses infecting humans are found worldwide and humans are the only known natural hosts, although the viruses have been detected in water, soil, vegetables and shellfish. This study aims to provide further evidence for the occurrence of enteric viruses in the sediment and the waterbody of the Viennese oxbow lake Alte Donau as previous studies document the presence of an enteric virus, namely Echovirus 30, in the local sediment.

Water sampling was done in July, August, September and October 2009; sediment samples were taken in August and October 2009. Sample concentration was done via tangential flow filtration (freshwater samples) or precipitation (sediment samples). After RNA isolation, the RNA was transcribed into cDNA. Amplification of a part of the 5'UTR was carried out using PCR and semi-nested PCR, and PCR products were cloned and sequenced. Sequence data were analyzed computer-based.

Positive results were obtained from all sediment samples. Freshwater samples only gave positive results in August 2009, all other samples were negative. All obtained sequences were similar to Echovirus 30.

The results achieved in this study show that there is a low level presence of Echovirus 30 in the sediment and the waterbody of the Alte Donau. The sequences obtained from waterbody samples are highly similar to those obtained from sediment samples at the same time, suggesting that the presence of Echovirus 30 in the waterbody of the Alte Donau is due to a resuspension event.

Zusammenfassung

Enteroviren sind häufige, über Wasser verbreitete Pathogene, die verschiedene Krankheiten bei Menschen auslösen können, manche davon mit ernsten oder sogar tödlichen Folgen. Humanpathogene Enteroviren kommen weltweit vor und der Mensch ist der einzige bekannte natürliche Wirt, obwohl sie auch schon in Wasser- und Bodenproben, Gemüse und in Schalentieren nachgewiesen wurden. In dieser Arbeit wurde das Vorkommen von Enteroviren im Sediment und Freiwasser der Alten Donau in Wien untersucht, nachdem vorherige Arbeiten bereits das Auftreten eines Enterovirus, nämlich Echovirus 30, im Sediment des Gewässers dokumentiert haben.

Wasserproben wurden im Juli, August, September und Oktober 2009 entnommen, Sedimentproben im August und Oktober 2009. Die Proben wurden via "tangential-flow-filtration" (Wasserproben) oder Ausfällung (Sedimentproben) konzentriert. Nach der RNA-Isolation wurde die RNA in cDNA umgeschrieben. Ein Teil der 5`-UTR wurde mittels PCR und "semi-nested" PCR amplifiziert, danach kloniert und sequenziert. Die Sequenzdaten wurden computergestützt analysiert.

Beide Sedimentproben lieferten positive Ergebnisse. Bei den Freiwasserproben konnte nur im August 2009 ein positives Resultat erzielt werden, alle anderen Proben waren negativ. Alle erhaltenen Sequenzen wiesen starke Ähnlichkeit zu Echovirus 30 auf.

Die in dieser Arbeit erzielten Resultate zeigen das Vorhandensein einer niedrigen Konzentration von Echovirus 30 im Sediment und Freiwasser der Alten Donau. Die Sequenzen, die aus dem Sediment extrahiert wurden, waren jenen aus dem Freiwasser sehr ähnlich, was den Schluss nahe legt, dass das Vorhandensein von Echovirus 30 im Wasserkörper auf Resuspension aus dem Sediment zurückzuführen ist.

Curriculum Vitae

Personal details

Name:	Armin Löckher
Address:	Sechshauserstraße 81/15, 1150 Wien
Email:	armin.loeckher@gmx.at

Education

December 2008 – present:	Diploma thesis "Echovirus 30 – tracing an enteric virus in an aquatic environment" Department of Virology, Microbiology and Molecular Biology, Medical University of Vienna, Austria
Since 2001:	Studies in Biology (Ecology) Main subject: Marine Biology Minor subject: Microbial Ecology University of Vienna, Austria
2000-2001:	Military service
1992-2000:	Grammar school
1988-1992:	Elementary school