Enteroviruses are members of *Picornaviridae* family, *Enterovirus* genus and are classified into class IV according to Baltimore classification system, since their genome consists of a positive sense single stranded RNA molecule. Enteroviruses are small, non-enveloped viruses, with an icosahedral protein capsid protecting their genetic material. Human Enteroviruses are classified into four groups: EV-A, EV-B, EV-C and EV-D and transmitted via fecal-oral route. They have been detected in clinical samples, so as well as in food and in the environment.

The aim of the present PhD thesis was the molecular monitoring of thermal inactivation efficiency of Enteroviruses strains Sabin 1 and Echo 12, which belong to EV-C and EV-B respectively, and reveal the impact of thermal inactivation on the integrity of viral genome and the subsequent aptitude of Sabin 1 and Echo 12 to infect their hosts and complete successfully their replication cycle. The selection of two viral strains that belong to different groups of the same genus was intended in order to identify similarities and differences in their thermal inactivation process.

At the initial part of the present study, we developed kinetics for each strain targeting firstly the positive strand in order to detect the Enterovirus genome and secondly the negative strand to detect their replicating activity and thus their capacity to successfully infect cell lines. For the detection of the negative strand, we designed a Stem-Loop Reverse Transcription Real-Time PCR assay that shows high sensitivity and specificity due to the thermodynamically stable form of the RT primer. Furthermore, the specific stem-loop primer used in Reverse Transcription assay was designed to target the 5'UTR of Enteroviruses which is a conserved region among *Enterovirus* genus. As a result, this molecular assay can be used to detect the negative strand in most Enterovirus strains. This assay allowed the detection of the negative strand (replicative strand) of Sabin 1 and Echo 12. Thus, we created one kinetic for replication activity of each strain in two virus concentrations, a high 10^6 $CCID_{50}$ and a lower concentration 10 $CCID_{50}$ in certain hours after the infection of a cell culture. Simultaneously, we created kinetics for the detection of the positive strand in certain hours after the infection of a cell culture. For the detection of the positive strand, we performed a conventional Real Time PCR assay by using the universal primer set ENV2/ENV1 which targets the 5'UTR of the positive strand of Enteroviruses. The results proved that both strains showed a similar pattern for the detection of their negative strand for both concentrations. For the 10^6 $CCID_{50}$ concentration the negative strand was detected 4 hours post infection of the cell culture for both strains, while for the lower concentration the

negative strand was detected 24 hours post infection of the cell culture. This practically means that an actively replicating Sabin 1 or Echo 12 strain concerning the 10^6 $CCID_{50}$ concentration can be detected 4 hours post infection of the cell culture and 24 hours post infection of the cell culture concerning the 10 $CCID_{50}$ concentration. These results compared with those which occurred from the CPE monitoring in a cell culture inoculation reveal that the Stem-Loop Reverse Transcription Real-Time PCR assay can detect the replicating activity of Enteroviruses faster than the cell culture. As for the positive strand, it was detected 2 hours post infection of the cell culture in the higher concentration, while for the lower concentration of Sabin 1 and Echo 12 strains it was detected 2 and 4 hours post infection respectively. The difference between the two strands is due to the fact that the positive strand exists in higher concentration levels (30-50 times) into the cells than the negative strand.

Moreover, the kinetics that were developed concerning the lower concentration (10 $CCID_{50}$) were implemented in 15 cerebrospinal fluid clinical samples (CSF), that were previously characterized as positive for Enteroviruses. In CSF, Enteroviruses exist in very low concentrations explaining why we decided to use the kinetics that were developed for the concentration of 10 $CCID_{50}$. It revealed that actively replicating Enteroviruses were detected only in 5 out of 15 CSF samples, while in all samples their positive RNA genome was detected. The strains that were positively identified for both strands were subjected to molecular cloning and sequencing. The results showed that 3/5 were identified as Echovirus 30, 1/5 was identified as Coxsackievirus B3 and 1/5 as Coxsackievirus B5, proving that through the present molecular assay we can detect the negative RNA strand in the most strains of *Enterovirus* genus, as the stem loop RT primer was designed to target the 5'UTR of Enteroviruses.

These kinetics can be used as a confirmatory method in order to test the effectiveness of inactivation of Enteroviruses in shorter time compared to cell cultures. In addition, they can be used in Enterovirus strains that cannot induce cytopathic effect in existing cell lines, such as some Coxsackievirus A strains.

Subsequently, to the next part of the present study we identified the appropriate inactivation temperatures for the strains S1 and E12 that were used in two different concentrations, a high (10^6CCID_{50}) and a low (10 $CCID_{50}$) by using an integrated system including cell culture and molecular assays. Moreover, we tested the impact of inactivation on viral genome targeting the most sensitive regions of viral genome (5'UTR, 3C, 3D and

3’UTR) for strand breaks. We determined that the most susceptible region to thermal inactivation is the 3’UTR, whereas the 5’UTR was determined as the most resistant. These results were also confirmed by calculating the GC content for both 5’UTR and 3’UTR regions. Furthermore, due to the importance of 5’UTR for Enterovirus detection, we decided to study this region more extensively in order to determine precisely the point of strand break after thermal processing at 82^{o}C, which was identified as the appropriate inactivation temperature for both strains that were used. By this way, the break in this region was determined to be located near to the annealing site of the universal UC53 primer (position 588-606nt).

The last goal of the present study was the development of an isothermal molecular assay (Real Time RT-LAMP) with high specificity and sensitivity for the detection of Enteroviruses, as an alternative approach in order to test the effectiveness of thermal inactivation of Enteroviruses targeting their 5’UTR region. Thus, we developed a Real Time RT-LAMP assay that can provide in one step (Reverse Transcription and amplification) the amplification of an RNA template under isothermal conditions in 60min. In this method 6 primers targeting the 5’UTR of Enteroviruses were designed and used. In the reaction mixture a specific Bst2.0 DNA polymerase with strand displacement activity and the WarmStartRTx Reverse Trancriptase were included. In the reaction mixture a fluorescent dye was included as well in order to provide results in real time during the reaction by measuring the fluorescence emission. The results validated that the present Real Time RT-LAMP assay can be used successfully in order to test the effectiveness of thermal inactivation of the strain Sabin 1 concerning the higher (10^{6}CCID$_{50}$) concentration, as they were in agreement with the results from the conventional Real Time PCR. Instead, for the Sabin 1 10 CCID$_{50}$ it was proved that this method is inappropriate to test the effectiveness of thermal inactivation due to its sensitivity limit. During thermal processing the viral titer is reduced significantly and as a result it cannot be detected. In conclusion, for concentrations below 10 CCID$_{50}$ the conventional Real Time PCR is the appropriate method in order to test the effectiveness of thermal inactivation. Same results occurred for the strain Echo 12 as well.

Summarizing, we proved that thermal inactivation affects the integrity of Enterovirus genome as it causes breaks in many regions, with the 3’UTR identified to be the most sensitive region, whereas the 5’UTR determined as the most resistant in thermal inactivation. It was also revealed that there is a correlation between the integrity of viral genome and viral infectivity. So, in order to test the effectiveness of thermal inactivation it is

essential to use an integrated system including cell culture for 2 or 3 passages combined with molecular assays testing for breaks on 3'UTR and 5'UTR and detecting the positive but mainly the negative strand of Enteroviruses, while the detection of their positive RNA does not reflect their ability to replicate and infect their hosts.