PRELIMINARY STUDIES ON DEFENSINS EXPRESSION IN LIVER OF RABBITS EXPERIMENTALLY INFECTED WITH *LAGOVIRUS EUROPEUS* GI.1 and GI.1A

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ABSTRACT

Among innate immunity elements, defensins play a pivotal role in viral, bacterial and fungal infections and mechanisms of innate immunity are crucial in the pathogenesis of rabbit haemorrhagic disease caused in rabbits by *Lagovirus europeus*. Taking the above into the account, the main purpose of the study was to check the presence of defensin NP-4 in livers of rabbits infected experimentally with RHDV (*Lagovirus europeus* GI.1) and RHDVa (*Lagovirus europeus* GI.1a), as the first step in further analysis of involvement of those proteins in the course of this viral disease. The method used in the study is real time PCR. The presence of defensins has been confirmed in all tested samples infected with different virus strains with high accuracy. This is the first study on defensins in livers of rabbits infected with *Lagovirus europeus* and further experiments are needed.

Key words: defensin, *Lagovirus europaeus*, real-time PCR, rabbit, AMPs.

INTRODUCTION

Antimicrobial peptides (AMPs) are significantly involved in the innate immune response against various microorganisms and are a promising substitute for antibiotics to address the problem of microbial resistance (Pero et al., 2019). Defensins, formerly known as lysosomal cationic proteins, are one of the most conservative defense mechanisms of organisms, and are an important element of non-specific humoral immunity (Niedźwiedzka-Rystwej et al., 2008). These proteins are synthesized or induced by microbial components or inflammation (Nigro et al., 2015). The role of defensins in immunity against viruses, bacteria and fungi has been well described in mammals, mainly in humans (Caterino et al., 2015).

In rabbits defensins are represented by NP1, NP2, NP3a, NP-3b, NP4, NP5 (possibly corticostatin-6 according to GeneBank) (Selsted et al. 1985) and cryptidine -3 and -4 (Tunzi et al., 2000). According to Linde et al. (2008), defensins in rabbits are also represented by a peptide called NP1-3a, NP3b-5 and rNP1-2.4. It is accepted that defensins in laboratory animals, including rabbits, are important and major defensive elements in bacterial gastrointestinal infections (Linde et al., 2008). There are few studies on rabbit defensins in viral infections. Only the inhibitory effect of synthetic α-defensin of guinea pig, rabbit and rat on HIV and HSV replication has been described (Nakashima et al., 1993; Mattar et al., 2016). To date, no studies involving rabbit α-defensin have been reported in the course of *Lagovirus europaeus* GI.1 infection.

It has been proven that innate immunity elements are crucial in host defense of rabbits against *Lagovirus europeus*. With no doubt, although no studies have been conducted, defensins are present in neutrophils in peripheral blood of rabbits, and seem to be central cells of immune responses against *Lagovirus europeus* (Niedźwiedzka-Rystwej et al., 2010, 2013). As liver is the replication center of the virus, and pathogenicity of it depends on liver (Trzeciak-Ryczek et al., 2015), the aim of the study was to check the presence of defensin NP-4 in livers of rabbits experimentally infected with *Lagovirus europeus* GI.1 and GI.1a.

MATERIALS AND METHODS

Sample collection and preparation

For the experiment were used livers of rabbits infected with different strains of *Lagovirus europeus* GI.1: 237/04, V-411 and strains of *Lagovirus europeus* GI.1a: 72V/2003 and Erfurt. 3g slices were taken from each liver and homogenized.

Total RNA isolation

Total RNA was isolated from the prepared homogenates using the Total RNA Mini kit (A&A Biotechnology, Gdynia, Poland).

Reverse transcription

Reverse transcription of prepared RNA was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Holding AG, Basel, Switzerland).

Real-Time PCR

Real-Time PCR was performed using 2µl cDNA (50ng) with specific, *de* novo designed primers targeting NP-4 defensin gene: forward 5'-gCgAgACgAACgACggTA-3', reverse 5' gTAgTCggTAgggACTCACCTC -3'. 2µl of each primer were used, reaching the final concentration of 5mM per reaction probe. 2µl of LightCycler Fast Start Enzyme (LC Faststart DNA Master SYBR Green, Roche Holding AG, Basel, Switzerland) and 1,5mM of Mg^{2+} per probe were added. The final volume of the reaction probe was 18µl.
This way prepared tests were placed in a LightCycler 1.5 (Roche Holding AG, Basel, Switzerland).

RESULTS AND DISCUSSION

Results

As a positive result (indicating the presence of the rabbit α-defensin NP-4 in the analysed liver tissue collected from rabbits experimentally infected with different strains of *Lagovirus europaeus*), exponential increase in fluorescence was adopted (Figure 1), whereas specificity of amplified products was confirmed with melting curves (Figure 2).

Figure 1: Exemplary result of real-time PCR obtained for α-defensin NP-4 in *Lagovirus europaeus* infection

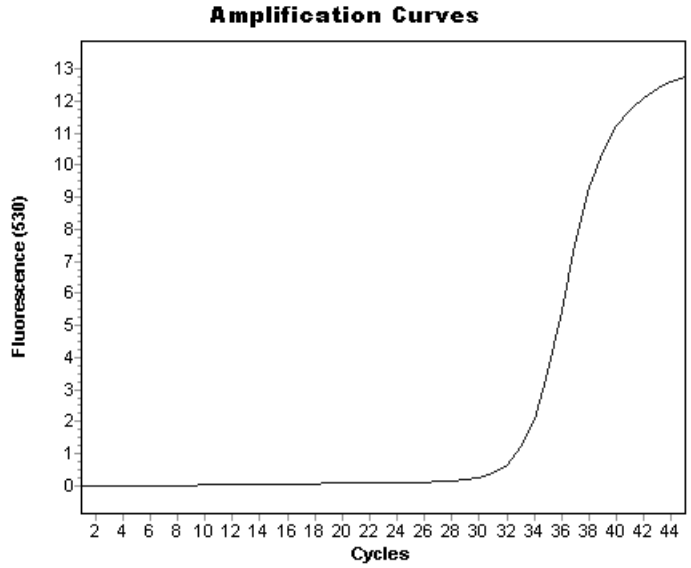


Table 1 shows the product melting temperature (Tm) and the number of reaction cycles in which fluorescence of the sample permeated to the logarithmic growth phase (Ct).

It was also noted that Ct values that increase before cycle 32 confirm the high efficiency of the reaction. The melting point of the product was also measured to eliminate the possibility of non-specific product formation as a result of impact with the SYBRGreen fluorescent dye, and it was shown that the melting point is similar for all tested *Lagovirus europaeus* strains, differing only by a maximum of 0.06 ^{o}C.

Table 1: Results obtained by detection with real-time PCR of rabbit α-defensin (NP-4) in the liver samples in the rabbits infected with different strains of *Lagovirus europaeus*

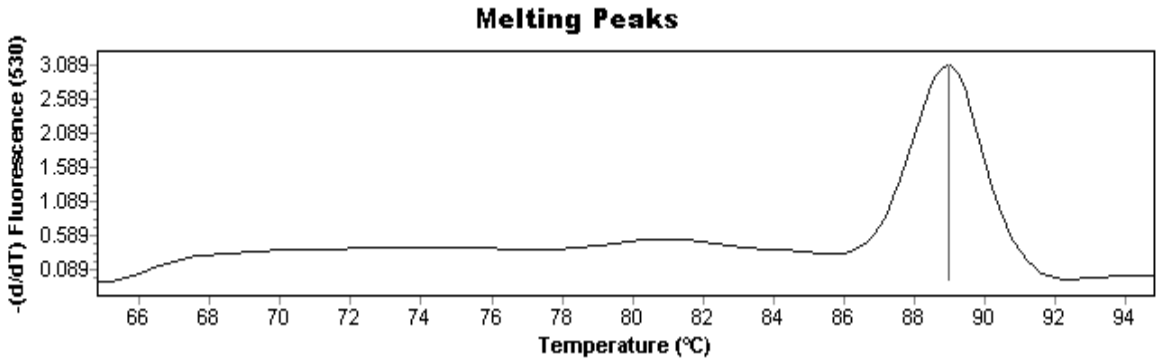
***Lagovirus europaeus* strain**	**Result**	**Tm (°C)**	**Ct**
237/04	+	88,88	33
V-411	+	88,83	33
72V/2003*	+	88,84	32
Erfurt*	+	88,82	32

Legend: + positive result; Tm – melting temperature; Ct – cycle threshold; * – antigenic variant

Discussion

The study shows that the real-time PCR method allows the diagnosis of NP-4 α-defensins in the liver tissue of rabbits infected with *Lagovirus europaeus*. Specific primers were used in the experiment, which were used for all tested samples. The primers were designed based on data obtained from the Gene Bank, based on the sequence of rabbit α-defensin NP-4 (Gene ID 100009135).The product melting temperature presents very similar values for all four strains (237/04, V-411, 72V/2003, Erfurt) *Lagovirus europaeus*, which proves the presence of the sought gene, high purity of the tested samples and allows to eliminate the formation of non-specific reaction products.

Figure 2: Exemplary analysis of melting curves and temperature for α-defensin NP-4 in *Lagovirus europaeus* infection



At the outset, it should be emphasized that logarithmic increase in fluorescence was considered a positive result of real-time PCR. This result was obtained (Table 1) for four strains, including two strains of *Lagovirus europaeus* GI.1 – 237/04 and V-411 and two strains of *Lagovirus europaeus* GI.1a – 72V/2003 and Erfurt. The presence of rabbit α-defensin NP-4 in the tested samples was obtained with high accuracy, as evidenced by similar moments of reaction entering the logarithmic increase in fluorescence (30 – 32 cycle) and the melting temperature of products, which fluctuates within 88,82 – 88,88°C.The obtained results confirm in 100% the presence and share of α-defensin NP-4 in infections caused by the *Lagovirus europaeus* virus. To date, no studies have been reported in rabbit defensins in the course of *Lagovirus europaeus* i

nfection.

CONCLUSIONS

Several mechanisms are employed in liver to protect it from infection, and the first line of protection is the epithelium with its mucus barrier, consisting of AMPs, like defensins. Their role is to inhibit microbial translocation to peripheral blood (Ostaff et al., 2013). Since liver is the replication centre in the pathogenesis of rabbit haemorrhagic disease, knowing the presence (and in the further studies) and the level of defensins in this organ may reflect the status of the host. Moreover, studies on defensins in human infected with haemorrhagic fevers show, that higher level of defensins may be a good life-prolonging factor (Aksoy et al. 2016).

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