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Short Communication

The C-reactive protein promoter polymorphism of selected rabbits

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The polymorphism of rabbit C-reactive protein promoter was investigated in two groups subjected to strict divergent selected rabbits according variation range of live-born kits at least after 3 litters. Difference in CRP plasma level between selected groups was observed on the basis of the sequence accession number NW_003159286 region: 13347320..13352360 available in GenBank, two oligonucleotide primers, rCRP_F and rCRP_R were designed and used to amplify a 501 bp-long fragment containing the CRP gene promoter, 5'UTR region and part of coding sequence was amplified by PCR and sequenced. A three single nucleotide polymorphism were detected. High resolution melting analysis was used for genotyping of P and F_1 generation. The results showed that HRM curve analysis was capable of detecting variations of 3 bp in PCR products of 501 bp. Results suggests that identified SNPs of rabbit CRP gene promoter may be relevant in the divergent selection of appropriate parental genotypes.

Keywords: C-reactive protein, CRP promoter, rabbit, high resolution melting analysis, single nucleotide polymorphism

1 Introduction

In multiparous species, such as the rabbit, maternal effect mediated by litter size and birth weight influences both growth and mortality in suckling and growing animals (Poigner et al., 2000). Researchers have demonstrated a marked maternal effect only up to 6 weeks of age, with a negligible effect afterwards (Blasco et al., 1983; Szendrö and Barna, 1984). It follows that the litter size weaned rabbits is correlated to genetic maternal predisposition, health status and overall breeding condition of the mother.

One of the main profile characteristics of health, immunological and genetic predispositions is C-reactive protein (CRP). The biological effect of CRP is its ability to bind phosphocholine, which makes CRP can recognize foreign pathogens and phospholipid components of damaged cells. From an immunological point of view CRP acts as an opsonin. After binding to the foreign particles and phagocytic cells activates the complement system via the classical pathway and the interactions with the humoral and cellular effector system of inflammation is triggered by removal of the target cells. The CRP rapid rise due to the induced stimulus suggests that CRP is part of the innate immune response (Peisajovich et al., 2008).

Xia and Samols (1997) showed that transgenic mice expressing high levels of rabbit CRP were partially protected from a lethal challenge of bacterial lipopolysaccharide compared with littermates in which CRP expression had been suppressed. Their results suggest that CRP functions in vivo by modulating host defense systems. In humans and rabbits, CRP is normally present in serum at concentrations of less than 0.5 μg ml^{-1}, with levels increasing as much as 1,000-fold following inflammatory stimuli.

Current evidence suggests that in humans CRP serum level is a complex trait, influenced by both clinical and genetic factors. In addition, heritability estimates for CRP levels have ranged from 27% to 40%, which suggests a role for DNA sequence variation in determining serum protein level. Genetic association studies using the finite set of CRP single nucleotide polymorphisms (SNPs) described in the present study could be used to clarify whether CRP is causally related to clinical manifestation. Whereas serum CRP level has been shown to predict a range of clinical phenotype (Kathiresan et al., 2006).

Several polymorphisms in the human CRP gene were identified and their influence on CRP level assessed (Brull et al., 2003; Carlson et al., 2005; Doumatey et al., 2012; Kaur et al., 2013).

High resolution melting analysis (HRMA) of amplicons of fewer than 200 bp have been used for detection of single nucleotide polymorphisms, mutation scanning, and genotyping (Lin et al., 2008). The use of larger amplicons has been reported to reduce sensitivity and specificity of HRMA (Chou et al., 2005; Krypuy et al., 2006). However, some studies have reported that the melting of larger

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amplicons, up to 1,000 bp, may produce multiple melting domains and can be used for genotyping (Jeffery et al., 2007; Price et al., 2007; Steer et al., 2009). The presence of more than one melting domain, as depicted by multiple peaks in the conventional melt curve profile, adds further variation to the normalized HRM-curve profile, thus increasing the power of the HRM-curve analysis technique for sequence differentiation and genotyping (Jeffery et al., 2007; Steer et al., 2009).

In the available literature, we could not find information about the rabbit´s C-reactive protein polymorphism. Therefore the objective of our study is to highlight the single nucleotide polymorphism of the rabbit partial sequence CRP promoter region and CRP plasma levels of selected does on the balance their of the litter size at birth and F_1 generation offsprings.

2 Material and methods

2.1 Animals

The trial was performed on the experimental farm at the National Agricultural and Food Centre – Research Institute for Animal Production Nitra, Slovakia and was conducted on clinically healthy does crossbreed rabbit line based on New Zealand white rabbits. The does of parental (P) generation were subject to strict divergent selection, which was followed by mating with the aim of creating F_1 group of animals with higher weaning viability. The basic selection criterion for the creation of two groups of females was variation range of live-born kits at least after 3 litters. Females (E15, E35, F27) with 7–10 kits per litters were assigned to the group CRP1. The average whole CRP plasma level in this group was 2.548 ±0.173 mg l^{-1}. The females (E17, E77) with higher frequency variability in individual litters of kits (1–15 kits per litter) were assigned to the group CRP2 and average whole CRP plasma level in group CRP2 was 4.091 ±0.284 mg l^{-1}.

The females of both groups were inseminated with male (M16) of known CRP promoter genotype (selected after sequencing analysis).

After HRMA genotyping of F_1 offspring, ten animals of each CRP1 (L) and CRP2 (H) group with known genotype were analysed for whole CRP plasma level at age of 62 days.

Animals were individually housed in wire cages, arranged in flat-decks on one level. Cages were equipped with a hopper for food. The rabbits were fed with a commercial diet (pellets of 3 mm in diameter). All animals were given access to the feed *ad libitum*. Drinking water was provided with nipple drinkers *ad libitum*. A cycle of 16 h of light and 8 h of dark (minimum light intensity of 80 lux) was used throughout the trial. Temperature and humidity in the building were recorded continuously by a digital thermograph positioned at the same level as the cages. Heating and forced ventilation systems allowed the building temperature to be maintained within 17–21 °C throughout the trial. Relative humidity was about 60 ±5%.

In this animal study, institutional and national guidelines for the care and use of animals were followed, and all experimental procedures involving animals were approved by ethical committee.

2.2 Blood samples processing

Peripheral blood (1 ml) from each experimental rabbit was taken from the vena *auricularis centralis*. Within 30 minutes after collection, the blood was centrifuged for 15 minutes at 1000 × g at 4 °C to obtain the blood plasma for the subsequent challenge testing. The levels of rabbit fragment C-reactive protein in the blood plasma were quantified with a commercial rabbit ELISA kit (SunRed Bio, Shanghai, China,). The detection range of fragment CRP is 50–1000 µg l^{-1} and a microtiter spectrophotometer XS PowerWave at a wavelength of 450 nm was used. The values of whole CRP protein were obtained after conversion according instructions of manufacturer (personal communication with SunRedBio).

The buffy coat after blood centrifugation was used for isolation of DNA using a Maxwell 16 Magnetic Particle Processor and Maxwell Blood DNA purification kit (Promega, USA) following the manufacturer´s instructions.

2.3 Primers design and PCR amplification

Primers were designed from GenBank (NCBI) sequence of *Oryctolagus cuniculus* breed Thorbecke inbred chromosome 13 genomic scaffold, OryCun2.0, whole genome shotgun sequence accession number NW_003159286 region: 13347320..13352360 GPS_000400798.

A 501 bp-long fragment containing the CRP gene promoter and 5'UTR region was amplified by PCR using forward rCRP_F: 5´-CTGTCAGCTTGGCTCTGTCA -3´ and reverse rCRP_R: 5´-GATCAGGAAACACCACAGCA -3´ primers using Qiagen Multiplex PCR Kit (Qiagen). The PCR profile included an initial denaturation step at 95 °C for 15 min, 35 cycles of 95°C (20 s), 62 °C (30 s), 72 °C (30 s) and a final extension step of 10 min at 72 °C in C1000 Thermal cycler (Biorad).

2.4 Sequencing and nucleotide sequence analyses

PCR amplicons were checked by agarose-gel electrophoresis, purified from gel, and sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit in both directions, using the same primers as for PCR on

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ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The nucleotide sequences were compared with each other and with those available in GenBank using BLAST program (NCBI).

2.5 Genotyping of offsprings by HRMA

Offsprings of F_1 generation were genotyped using HRMA. Amplification of target sequence was carried out using a Rotor-Gene 6000 thermal cycler (Corbett Research) and Type-it HRM PCR kit (Qiagen) according to the manufacturer's recommendations. A 20µl reaction mix contained 0.7µM (final concentration) of each forward rCRP_F and reverse rCRP_R primer and 20–50 ng of DNA. One cycle of 5min at 95 °C was followed by 45 amplification cycles of 5 s at 95 °C, 30 s at 62 °C and 30 s at 72 °C. Optical measurements in green channel were recorded during the extension step. HRM-curve analysis was performed using increasing temperatures from 70 °C to 95 °C at intervals (ramps) of 0.1 °C with a hold of 2 s at each step. The conventional melt curves were generated automatically and analysis performed using Rotor-Gene 6000 series software version 1.7. Genotyping was further performed using the normalized HRM data.

Genotypes were defined by selecting a representative sequenced sample for each genotype. The software then auto-called the genotype of each sample and provided confidence percentages (C%) as an integrity check.

3 Results and discussion

On the basis of the sequence *Oryctolagus cuniculus* breed Thorbecke inbred chromosome 13 genomic scaffold, OryCun2.0, whole genome shotgun sequence accession number NW_003159286 region: 13347320..13352360 GPS_000400798 available in GenBank, two oligonucleotide primers, rCRP_F and rCRP_R designed

Table 1 Detected SNPs in does of CRP1 (F-E15, F-E35, F-F27) and CRP2 (F-E17, F-E77) groups and 7 rabbit males (M1-M16) by sequencing of PCR products

Nucleotide position	-366	-363	-119
Sample			
F-E15	T/C	T/C	G/T
F-E35	T/C	T/C	G/T
F-F27	T/C	T/C	G/T
F-E17	C	C	T
F-E77	C	C	T
M1	C	C	T
M2	T/C	T/C	G
M4	C	C	T
M10	C	C	T
M12	T	T/C	G/T
M15	T	T/C	G
M16	T/C	T/C	G/T
CRP	T	T	G

CRP – reference sequence from Genbank: *Oryctolagus cuniculus* breed Thorbecke inbred chromosome 13 genomic scaffold, OryCun2.0, whole genome shotgun sequence accession number NW_003159286 region: 13347320 13352360 GPS_000400798.

Figure 1 Sequenced fragment of 501 bp (-390 to +110), and positions of identified SNPs (-366, -363, -119) of *Oryctolagus cuniculus* CRP promoter (+1 – start of transcription, ATG – initiation codon). Primers sequences rCRP_F and rCRP_R (upper case, bold)

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and used to amplify a 501 bp-long fragment containing the CRP gene promoter, 5'UTR region and part of coding sequence was amplified by PCR and sequenced from selected rabbits (Figure 1). Detected SNPs in parental rabbits are summarized in Table 1.
All females in CRP1 group were heterozygous in indicated nucleotide position with T/C T/C G/T genotype. On the other hand females in CRP2 group were homozygous C/C, C/C, T/T in indicated nucleotide. The male M16 used for mating experiments for production of F_1 generation offsprings was heterozygous in indicated nucleotide position with T/C T/C G/T genotype. Six other analysed males with indicated genotypes are referred in Table 1.

Genomic DNA of sequenced samples was analysed by HRMA to obtain melting and HRM curve characteristic for each known genotype. Offsprings of F_1 generation were genotyped using HRMA. Amplification of target sequence was carried out and sequenced samples were used as the standards of each genotype. PCR products from each CRP group were subjected to HRM curve analysis as described in Material and methods. Sequenced samples in group CRP1 generated a two major peaks melting between 83.25 and 83.78 °C, and samples in group CRP2 a three peaks melting between 84.0 °C–84.52 °C, 84.97–85.02 °C, and 86.50–86.70 °C (Figure 2 a, b). Analyses of the normalized curves (Figure 2 c, d) revealed distinct profiles also represented distinct HRM genotypes. Similarly, samples of F_1 generation also produced the same profiles as sequenced samples CRP1 and CRP2 group, respectively. Several HRM runs (performed on different days) using at least one representative from each genotype resulted in only minor variations in melting temperatures of the peaks, and following normalization of data, the profiles were consistent with those described above.

The results showed that HRM curve analysis was capable of detecting variations of 1 bp in PCR products of 501 bp.

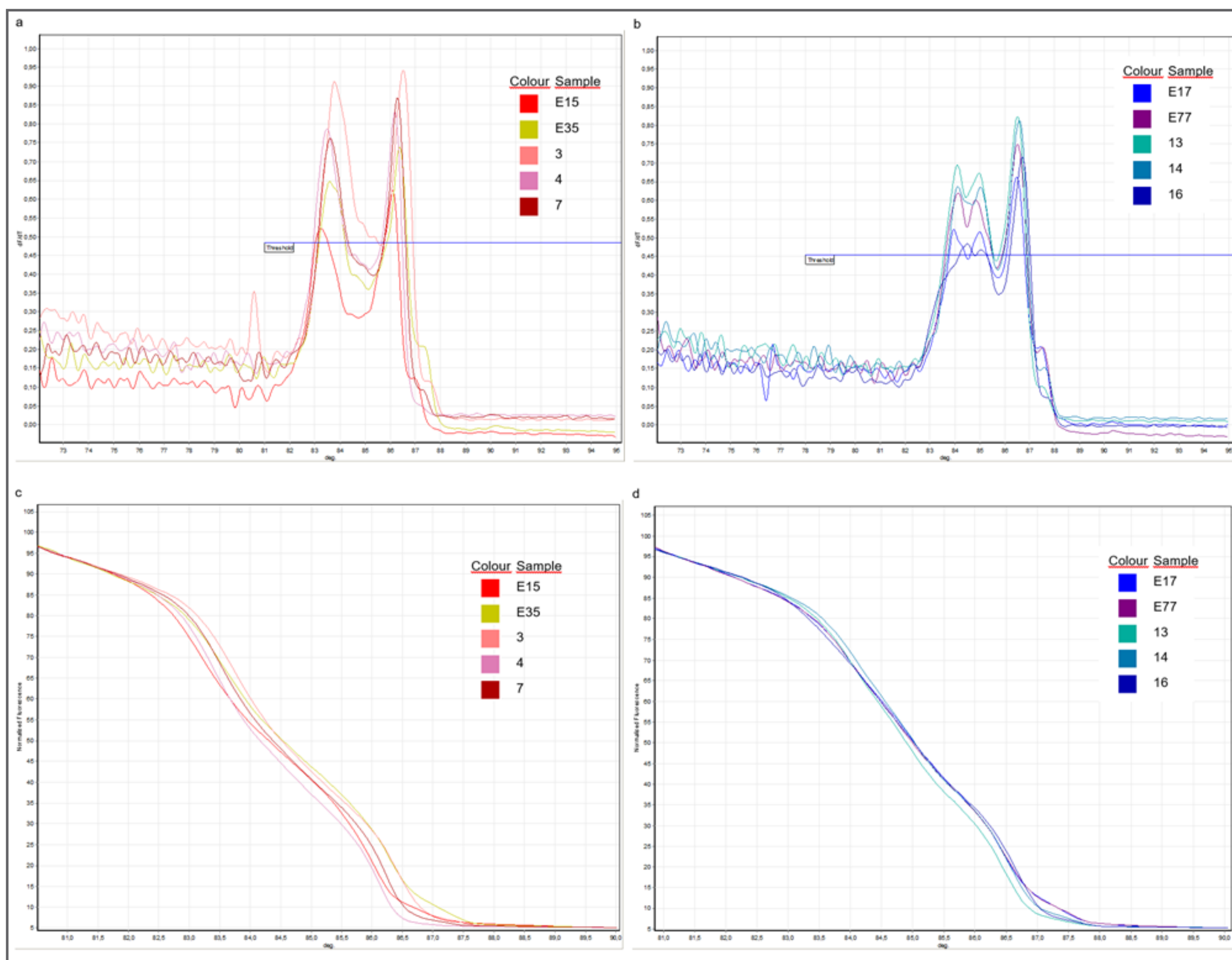

Figure 2 Representative results of melting curves and normalized HRM graph of genotyped samples
a – results of melting curves of group CRP1 (E15, E35) and F_1 generation offsprings of females with low (L) frequency variability in individual litters (with 7–10 kits per litter), b – results of melting curves of samples of group CRP2 (E17, E77) and F_1 generation offspring of females with higher (H) frequency variability in individual litters of kits (1–15 kits/litter, c – results of normalized HRM graph of genotyped samples as in a, d – results of normalized HRM graph of genotyped samples as in b

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Table 2 The C-reactive protein levels in blood plasma of F_1 generation offsprings at age 62 days

Animal	Genotype -366 -363 -119	L – F_1 (CRP in mg l^{-1})	Animal	Genotype -366 -363 -119	H – F_1 (CRP in mg l^{-1})
1	T/C T/C G/T	0.776	11	C/C C/C T/T	1.091
2	T/C T/C G/T	0.963	12	C/C C/C T/T	3.075
3	T/C T/C G/T	1.257	13	C/C C/C T/T	2.822
4	T/C T/C G/T	1.045	14	C/C C/C T/T	2.888
5	T/C T/C G/T	1.070	15	C/C C/C T/T	2.760
6	T/C T/C G/T	0.889	16	C/C C/C T/T	2.844
7	T/C T/C G/T	1.128	17	C/C C/C T/T	2.192
8	T/C T/C G/T	1.032	18	C/C C/C T/T	1.301
9	T/C T/C G/T	0.902	19	C/C C/C T/T	1.424
10	T/C T/C G/T	1.032	20	C/C C/C T/T	1.059
Mean ±sd		1.009 ±0.135	Mean ±sd		2.146 ±0.834
t-test					$P = 0.0002$; $t – 4{,}25^{+++}$

L – offsprings of F_1 generation of females with low frequency variability in individual litters (with 7–10 kits per litter); H – offsprings of F_1 generation of females with higher frequency variability in individual litters of kits (1–15 kits per litter)

Nucleotide sequencing is now available at a relatively low cost. However, nucleotide sequencing is time-consuming procedure and require skill for interpretation of results. In contrast, the HRM curve analysis is rapid and convenient, and all relevant procedures including PCR and melting-curve analysis can be performed in a single tube.

From all genotyped F_1 generation ten offspring animals with genotype correspond with parental genome females of CRP1 group with T/C T/C G/T genotype and ten F_1 animals of CRP2 group homozygous C/C, C/C, T/T for indicated nucleotides, were analysed for whole CRP plasma level at age of 62 days with a commercial rabbit ELISA kit.

The results showed that in animals of F_1 generation have been statistically significant difference of C-reactive protein plasma levels between groups assigned according genotype in indicated nucleotides (Table 2).

CRP1 does (E15, E35, F27, the number of monitored litters $n = 4$, $n = 3$, $n = 6$) which were heterozygous (T/C, T/C, G/T) in the CRP promoter, reaching a low variability of offspring at birth from 7.87% to 14.53%, with weaning their kits (42 days old) without a single death. On the other hand, CRP2 does (E17, E77, the number of monitored litters $n = 5$, $n = 9$) were homozygous (C/C, C/C, T/T) the in the CRP promoter, achieving a higher variability of offspring at birth from 48.73% to 58.03%, with simultaneous recording of deaths for their offspring to weaning (day 42 age) ranging from 11.1% to 30%.

The results suggests that identified SNPs of rabbit CRP gene promoter may be relevant in the divergent selection of appropriate parental genotypes. Further animal analyzes are in progress.

4 Conclusions

The difference in CRP plasma level between groups subjected to strict divergent selected rabbits according variation range of live-born kits was observed. The single nucleotide polymorphisms of rabbit C-reactive protein promoter was investigated. On the basis of the sequence accession number NW_003159286 region: 13347320.13352360 available in GenBank, oligonucleotide primers, rCRP_F and rCRP_R were designed and used to amplify a 501 bp-long fragment containing the CRP gene promoter, 5'UTR region and part of coding sequence was amplified by PCR and sequenced. High resolution melting analysis was used for genotyping of *P* and F_1 generation. The results showed that HRM curve analysis was capable of detecting variations of 3 bp in PCR products of 501 bp. The combination of PCR and HRM curve analysis is a rapid and specific technique for animal genotyping. The entire process including extraction of DNA, PCR and HRM curve analysis can be completed within 3.5 h. Obtained results suggests that identified SNPs of rabbit CRP gene promoter may be relevant in the divergent selection of appropriate parental genotypes.

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References

BLASCO, A. et al. (1983) Análisis fenotipico de caracteres productivos en el conejo para carne. *Arch. Zootech.,* vol. 32, pp. 111–130.

BRULL, D.J. et al. (2003) Human CRP gene polymorphism influences CRP levels: implications for the prediction and pathogenesis of coronary heart disease. Arterioscler. *Thromb. Vasc. Biol.,* vol. 23, pp. 2063–2069

CARLSON, C.S. et al. (2005) Polymorphisms within the C-reactive protein (CRP) promoter region are associated with plasma CRP levels. *Am. J. Hum. Genet.,* vol. 77, pp. 64–77. doi: http://dx.doi.org/dx.doi.org/10.1086/431366

CHOU, L. S. et al. (2005) A comparison of high resolution melting analysis with denaturing high-performance liquid chromatography for mutation scanning: cystic fibrosis transmembrane conductance regulator gene as a model. *Am. J. Clin. Pathol.,* vol. 124, pp 330–338.
doi: http://dx.doi.org/10.1309/BF3MLJN8J527MWQY

DOUMATEY, A.P. et al. (2012) C-reactive protein (CRP) promoter polymorphisms influence circulating CRP levels in a genome-wide association study of African Americans. *Human Molecular Genetics,* vol. 21, pp. 3063–3072.
doi: http://dx.doi.org/10.1093/hmg/dds133

JEFFERY, N. et al. (2007) Classification of *Mycoplasma synoviae* strains using single-strand conformation polymorphism and high-resolution melting-curve analysis of the *vlhA* gene single-copy region. *Microbiology,* vol. 153, pp. 2679–2688. doi: http://dx.doi.org/10.1099/mic.0.2006/005140-0

KATHIRESAN, S. et al. (2006) Contribution of Clinical Correlates and 13 C-Reactive Protein Gene Polymorphisms to Interindividual Variability in Serum C-Reactive Protein Level. *Circulation,* vol.113, pp.1415–1423.

KAUR, R. et al. (2013) C-reactive protein +1059 G>C polymorphism in type 2 diabetes and coronary artery disease patients. *Meta Gene,* vol. 1, pp. 82–92.
doi: http://dx.doi.org/10.1016/j.mgene.2013.10.012

KRYPUY, M. et al. (2006) High resolution melting analysis for the rapid and sensitive detection of mutations in clinical samples: KRAS codon 12 and 13 mutations in non-small cell lung cancer. *BMC Cancer,* vol. 6, pp. 295.
doi: http://dx.doi.org/10.1186/1471-2407-6-295

LIN, J. H. et al. (2008) Rapid differentiation of influenza A virus subtypes and genetic screening for virus variants by high-resolution melting analysis. *J. Clin. Microbiol.,* vol. 46, pp. 1090–1097. doi: http://dx.doi.org/10.1128/JCM.02015-07

PEISAJOVICH, A. et al. (2008) C-reactive protein at the interface between innate immunity and inflammation. *Expert Rev Clin Immunol,* vol. 3, pp. 379–390.
doi: http://dx.doi.org/10.1586/1744666X.4.3.379

POIGNER, J. et al. (2000) Effect of birth weight and litter size on growth and mortality in rabbits. *World Rabbit Science,* vol. 8, pp. 17–22. doi: http://dx.doi.org/10.4995/wrs.2000.413

PRICE, E. P. et al. (2007) High-resolution DNA melt curve analysis of the clustered, regularly interspaced short-palindromic-repeat locus of *Campylobacter jejuni*. *Appl. Environ. Microbiol.,*vol. 73, pp. 3431–3436.
doi: http://dx.doi.org/10.1128/AEM.02702-06

SZENDRŐ, Z. and BARNA, J. (1984) *Some factors affecting mortality of suckling and growing rabbits.* 3rd World Rabbit Congress. Rome, pp. 166–173.

STEER, P.A. e al. (2009) Classification of fowl adenovirus serotypes by use of high-resolution melting-curve analysis of the hexon gene region. *J Clin Microbiol.,* vol. 47, pp. 311–321. doi: http://dx.doi.org/10.1128/JCM.01567-08

XIA, D. and SAMOLS, D. (1997) Transgenic mice expressing rabbit C-reactive protein are resistant to endotoxemia. *Proc. Natl. Acad. Sci.* USA, vol. 94, pp. 2575–2580. Retrieved January 12, 2016 from http://www.pnas.org/content/94/6/2575.full.pdf

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