Potravinarstvo, vol. 8, 2014, no. 1, p. 216-220
doi:10.5219/390
Received: 14 June 2014. Accepted: 16 June 2014.
Available online: 28 July 2014 at www.potravinarstvo.com

ISSN 1337-0960 (online)

DETERMINATION OF THE SPECIES SPECIFICITY OF THE PRIMERS FOR THE DETECTION OF CHICKEN AND TURKEY MEAT BY REALTIME PCR METHOD

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ABSTRACT

The aim of this work was to use TaqMan Real-Time PCR for quantitative authentication of chicken and turkey meat. To meet this purpose, a specific pair of primers and TaqMan probe was used. The test was aimed at identifying the reaction cycle of turkey and chicken meat using by two sets of primers. With first set of primer designed for chicken we obtained the following results: Cp = 16.18 for 100% chicken DNA Cp = 29, 18 100% turkey DNA It was also amplified DNA of pig that exceeded the detection threshold fluorescence intensities in the 31.07 cycle (Cp = 31.07). Using primers designed for turkey we obtained the following results Cp = 31.16 for 100% CHDNA, Cp =16.18 100% TDNA. It was also amplified the 100% DNA of rabbit in 31.63 cycle (Cp = 31.63) and deer in cycle 32 (Cp = 32). The DNA of all other animal species was amplificated after more than 35 cycles (Cp >35). It follows that the second detection primer pair is specific enough to unrelated species of animals by 30 cycles of the reaction. Species authentication based on DNA analysis from this perspective overcomes all the shortcomings of proteins. At present, DNA analysis use different types of PCR. Is the most progressive Real-time PCR, which is suitable for the specific use of detection (primers and TaqMan probe). The TaqMan Real-time PCR is within the sensitivity and specificity, clearly one of the best methods for identifying the species of chicken and turkey meat. The specificity of this method, however, depends primarily on the specificity of the primers and TaqMan probe. The 30 cycle reaction was chosen by us as the threshold for specificity using primers for authentication chicken and turkey meat.

Keywords: primer; chicken meat; turkey meat; PCR; TaqMan

INTRODUCTION

For food producers is particularly attractive for economic reasons to replace expensive components with cheaper. Therefore, they falsify especially expensive products or products that are produced in large volumes due to higher profits **(Lees, 2003; Peris and Escuder-Gilabert, 2009)**.

Popelka et al. (2002) the adulteration of food is associated with the deteriorating quality of food.

In Slovakia is the necessary verification of genuineness of certain products as a necessary part of a comprehensive examination of quality of goods with regard to consumer protection, together with the fight against counterfeiting of products in the package itself or directly for sales **(Takáčová and Bugarský, 2010)**.

At present is more and more used PCR method allowing the direct quantification of PCR products during the amplification reaction – Real-Time PCR. Quantification of nucleic acid molecules is important in determining the amount of target DNA in the samples analysed **(Šmarda et al., 2008; Španová et al., 2005)**.

Haider et al. (2012), these techniques largely overcome the shortcomings of other methods, therefore, are promising and reliable tool for species identification of meat.

As an authentication marker, in this case, is use of nuclear genes or mitochondrial DNA. The DNA molecules are present in each cell, and in addition, in comparison with protein markers are more thermo stable **(Rojas et al. 2012)**.

Králová et al. (2007) indicate that a very important requirement for a successful reaction is to design appropriate primers so as to ensure the specificity of the reaction, the PCR is necessary to know the sequence of at least the border sections fragment to be amplified.

The method uses the properties of a DNA polymerase, such as the ability to synthesize the complementary strand by single-stranded template and primers need to initialize the polymerization **(Omelka et al., 2001)**.

The aim of this work is to evaluate the determination of species specificity of primers for detection of turkey and chicken.

MATERIAL AND METHODOLOGY

As biological material we used muscles of domestic fowl (*Gallus gallus*) and domestic turkeys (*Meleagris gallopavo*), since turkey is the most commonly counterfeited with chicken. In the next section assess the specificity of the first and second detection kits examining cross-reaction with other species. We used 100% of the DNA of different species: pig (*Sus scrofa ferus*), ducks (*Anas platyrhynchos*), moufflon (*Ovis musimon*), deer (*Red deer*), wild boar (*Sus scrofa vittatus*), and rabbit (*Oryctolagus cuniculus*). DNA were isolated by phenol - chloroform extraction, preceded skiing individual samples (sample size was 1 mm) in 600 mL of lysis solution with

Table 1 Sequence of primers first detection kit reaction mixture 1

Primer	Bp	Sequence
Gallus F	27	5´-TCTCACTTACACTACTTGCCACATCTT-3´
Gallus R	23	5´-CGTGTGTGTCCTGTTTGGACTAG-3´
Gallus P	27	5´-(FAM) –CACTGCAACCTACAGCCTCCGCATAAC-(BHQ)-3

Table 2 Sequences of the primers of the second set of detection in the reaction mixture 2

Primer	Bp	Sequence
Mgal F	19	5' - CCGTAACCTCCATGCGAAT - 3'
Mgal R	22	5' - TAATATAGGCCGCGTCCAATGT - 3'
Mgal P	28	5' - (FAM)- CGCCTCATTCTTCTTCATCTGCATCTTC-(BHQ1) - 3'

the addition of 20 mL of the enzyme proteinase K. TaqMan Real-time PCR was carried out in the capillary reaction cycler LightCycler® 1.5 (Roche) and the results were evaluated with the help of the LightCycler software version 4.5 (Roche, Germany), which during the PCR reaction automatically creates a graph of the fluorescence intensity of the number of cycles.

Sets of primers and TaqMan probes first and second detection kit was designed according to **Jonker et al. (2008)** and all primers were synthesized by General Biotech (Czech Republic). Designed primers were derived from the sequences of a specific gene cyt b. The sequence of the primers and TaqMan probes of the first and second sets of detection are listed in Table 1 and Table 2.

The individual primers and TaqMan probes were supplied in lyophilized form. Dissolving the freeze-dried in ultrapure water (Milli-Q H_2O) were obtained 10x concentrated stock solutions of primers, which were stored at -20 °C. Primers from stock solutions were diluted working solutions so that their final concentration of 10 $pmol.\mu L^{-1}$. Working solutions were stored at 2-8 °C. Lyophilized TaqMan probe from a first and second detection kit was dissolved in ultrapure H_2O directly to a working concentration of 5 $pmol.\mu L^{-1}$. In a reaction mixture, we used the components necessary for optimum progress of the reaction: Colorless GoTaq® reaction buffer, $MgCl_2$, d NTP mix, individual primers and probes, and a dye ROX GoTaq ® Hot Start Polymerase. We used GoTaq® Hot Start polymerase having polymerase activity blocked. Restoring polymerase activity occurs at initiation denaturation at 94-95 °C for 2 minutes. This system eliminates nonspecific amplification and creating primer - dimer. Mastermix is added to the reference dye ROX, which is used for normalization of the reporter signal. The normalization of the signal is essential for the prevention of signal variations caused by the construction of the device frequently. Preparation of the reaction mixture was carried out in the UV-cleaner box (BioSan, Lithuania). Capillaries are adapted to the volume of the reaction mixture from 10 to 40 ml. After adding the desired amount of DNA we conclude capillaries and quickly spun on a centrifuge. After inserting the capillary into the rotary plate of thermo cycler (LightCycler 1.5) we recorded the intensity of the fluorescent signal after each cycle measured at a wavelength of 640 nm.

Temperature control PCR reactions for 1st and 2nd detection kit:

Initial denaturation	95 °C, 10 min.
Denaturation	95 °C, 10 sec.
Hybridzationa+ elongation	60 °C, 15 sec.
Cooling	40 °C, 30 sec.

Fluorescence intensity was measured at the end of each cycle of hybridization and elongation. After completion of the PCR results were evaluated in the LightCycler 5.4 using the tool "Absolute Quantification". This function determines the value of the threshold cycle (Cp), under which the samples were evaluated and used to determine the actual number of copies of the unknown sample in comparison to a standard curve. Of Cp value is based absolute quantification of the product, since it is inversely proportional to the logarithm of the initial template copy number **(Ciglenečki et al., 2008)**. Cp value is the lower, the higher the number of copies of template in the sample before the start of the reaction **(Yilmaz et al., 2012)**.

RESULTS AND DISCUSSION

We watched the specificity of the first and second detection kit for the screening and cross-react with other species. 100% of the DNA of different species: pig (*Sus scrofa ferus*), ducks (*Anas platyrhynchos*), mouflon (*Ovis musimon*), deer (*Red deer*), wild boar (*Sus scrofa vittatus*), rabbit (*Oryctolagus cuniculus*).

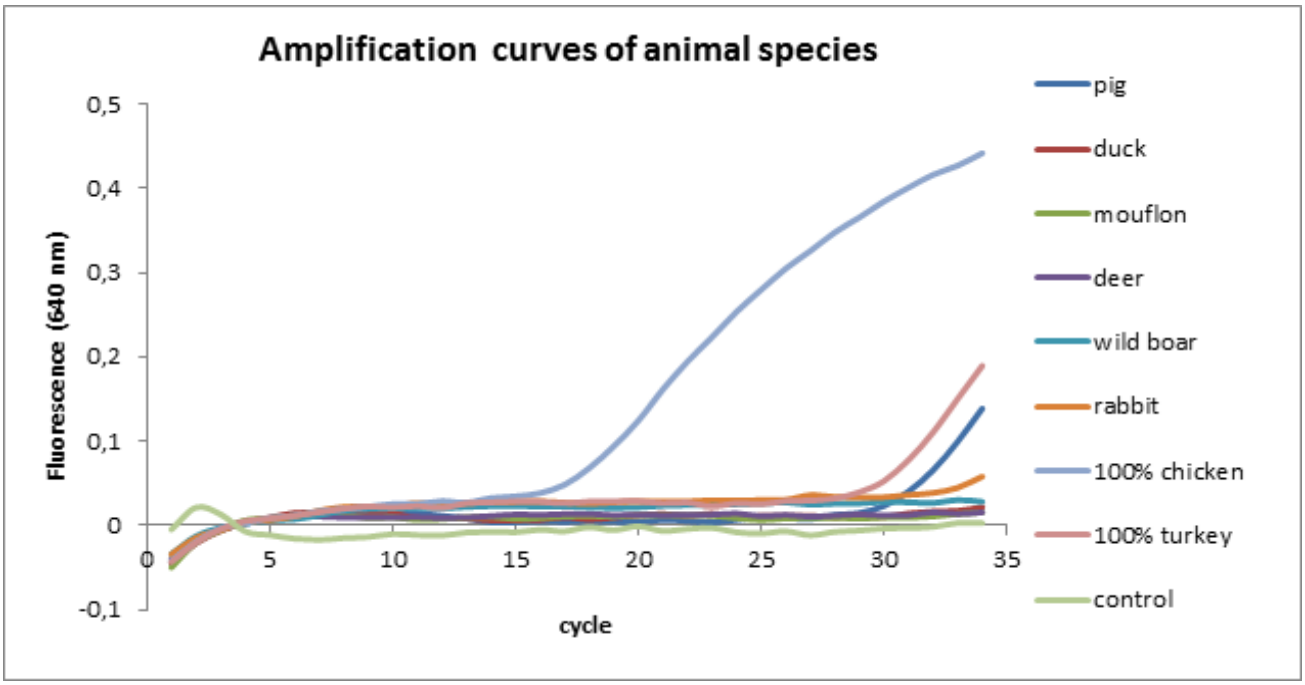

Figure 1 Amplification curves of species (first detection set): pig (*Sus scrofa ferus*), ducks (*Anas platyrhynchos*), mouflon (*Ovis musimon*), deer (*Red deer*), wild boar (*Sus scrofa vittatus*), rabbit (*Oryctolagus cuniculus*) 100% chicken and 100% turkey DNA, using the first detection kit (Gal F, R primers, TaqMan probe)

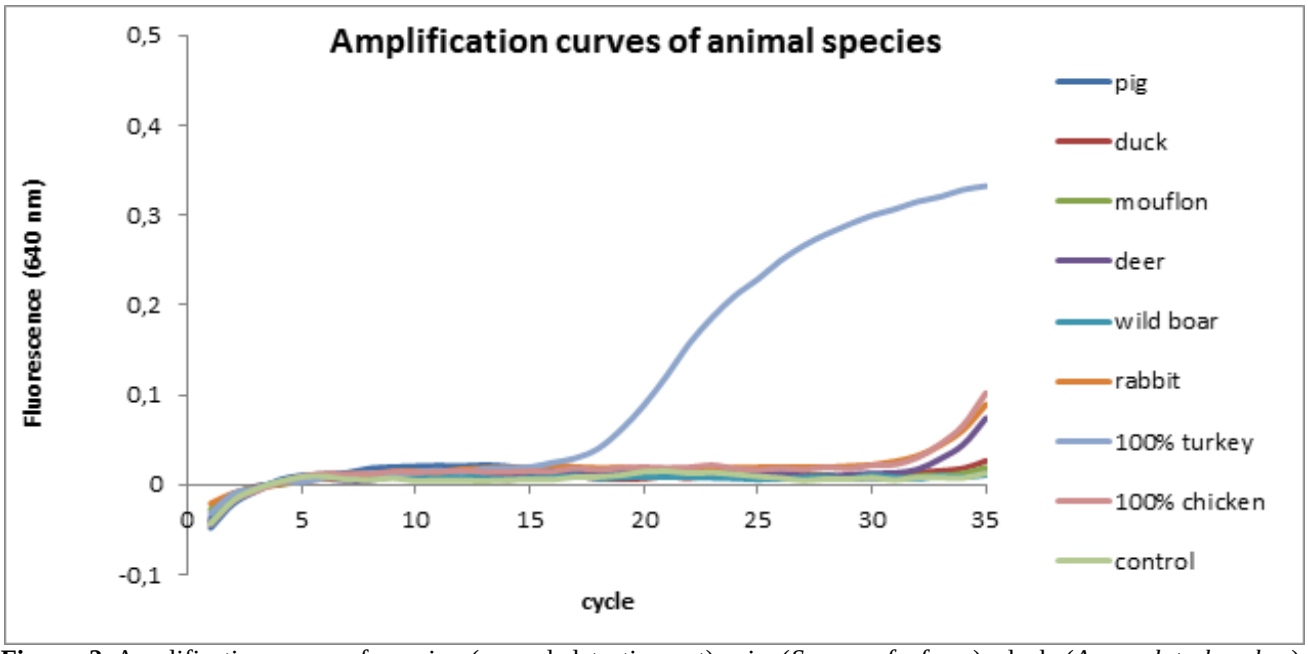

Figure 2 Amplification cures of species (second detection set): pig (*Sus scrofa ferus*), duck (*Anas platyrhynchos*), mouflon (*Ovis musimon*), deer (*Red deer*), wild boar (*Sus scrofa vittatus*), rabbit (*Oryctolagus cuniculus*), 100% turkey a 100% chicken DNA, using the second detection kit (Mgal F, R primers, TaqMan probe)

In Figure 1 we follow the DNA amplification using by the first set of reaction kit with 100% chicken DNA at 16,18 cycle (Cp = 16.18) and 100% turkey DNA in cycle 29.18 (Cp = 29.18). It was also amplified DNA pig that exceeded the detection threshold fluorescence intensities in the 31.07 cycle (Cp = 31.07). Other species were amplified after more than 35 cycles, or at all. In this experiment was included as pig DNA in some products used, for example haemoglobin pork, pork fat, DNA boar as a close second to the pig and duck as another deputy of poultry. Also **Dooley et al. (2004)** developed a test for the detection of chicken, turkey, pork, beef and lamb. As authentication markers have chosen also species-specific regions of cyt b, which were amplified using appropriate species-specific primers. Detection of amplicons provides only two probes. The first was specific markers for mammalian meat and other markers for meat or birds. The test specifically targeting specificity chicken averages observed cross-

Table 3 Cross reaction of the first and second detection kits in various animal species.

	Concentration DNA (%)	Chicken primers (Gall)(Gall)		Turkey primers (Mgal)	
		Cp to 35 cycle of reaction	**Cross reaction to 30 cycle**	**Cp to 35 cycle of reaction**	**Cross reaction to 30 cycle**
Sus scrofa ferus	100	31.07	-	>35	-
Anas platyrhynchos	100	>35	-	>35	-
Ovis musimon	100	>35	-	>35	-
Red deer	100	>35	-	32	-
Sus scrofa vittatus	100	>35	-	>35	-
Oryctolagus cuniculus	100	>35	-	31.63	-
Gallus gallus	100	16.18	16.18	31.6	-
Meleagris gallopavo	100	29.18	29.18	16.85	-

reaction with DNA of all kinds. DNA was most intense with pork; amplification curve crossed the threshold of detection in 30.05 cycles. It's concluded that the threshold of 30 cycles is sufficient for qualitative authentication chicken. The gene for cytochrome b later used to authenticate the chicken also **Laube et al. (2007), Tanabe et al. (2007) and Jonker et al. (2008)**. **Kesmen et al. (2012)** point out the difficulty of developing a detection system to distinguish chicken from the turkey as it is a closely related species, which are characterized by a high degree of DNA homology.

Figure 2 display species specificity of the second screening kit. As can be seen there has been amplification of the 100% turkey DNA in 16.85 cycle (Cp = 16.85). Was also amplified a 100% chicken DNA 31.6 cycle (Cp = 31.6). Came amplification and the 100% rabbit DNA in 31.63 cycle (Cp = 31.63) and 32 deer in the cycleta (Cp = The DNA of all other animal species was amplificated after more than 35 cycles (Cp >35). It follows that the second detection primer pair is specific enough to unrelated species of animals by 30 cycles of the reaction. But we must take into account that it was not possible to determine the detection limit for a given set of primers.

As we shown in Table 3, using the two sets of detection kits occurring cross-reactions, but all up to 30 cycles of the reaction. The 30 cycle of reaction was chosen by us as the threshold for specificity using primers for authentication chicken and therefore can be considered a first screening set for species-specific and due to the presence of DNA species verified by us. A second set of screening can be considered a species-specific animal species verified by us but we have to take into account that it is not specific enough within the species. **Cammas et al. (2012)** summarized the results of studies published in 2012 that the generic authentication meat using Real-Time PCR using TaqMan probes. Found that many of them describe the cross-reactivity of primers and probes designed with other types of DNA, as well as the low efficiency of amplification of markers, especially in relation to the degradation of DNA in heat-treated foods. Effectiveness (efficacy) with the decline of efficiency, the number of amplicons generated in each cycle decreases, resulting in the generation of amplification curves of later cycles.

CONCLUSION

Currently, the use of TaqMan Real-Time PCR in food analysis focuses primarily on quantitative detection of materials of animal and plant origin in food with very complex texture. TaqMan real-time PCR is within the sensitivity and specificity clearly one of the best methods for identifying the species of chicken and turkey meat.

The specificity of this method, however, depends primarily on the specificity of the primers and TaqMan probes.

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Acknowledgments:
This project is being co-financed by the European Union, ITMS 26240220080. We support research activities in Slovakia

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