International Food Research Journal 24(1): 159-163 (February 2017)
Journal homepage: *http://www.ifrj.upm.edu.my*

Development and utility of a tri-primer PCR for simultaneous detection of cattle, sheep and pig in meat products

[1]Chaobo, X., [2]Feng, G.., [1]Pingya, W., [2]Jin, Z. and [2*]Yuanyuan, L.

[1]Zhoushan Institute for Food and Drug Inspection and Testing, 316021 Zhoushan, P. R. China
[2]College of Life Sciences, China Jiliang University, 310018 Hangzhou, P. R. China

Article history

Received: 9 December 2015
Received in revised form: 18 March 2016
Accepted: 4 April 2016

Keywords

Tri-primer multiplex PCR
Species identification
Cattle
Sheep
Pig

Abstract

In this study, a tri-primer multiplex PCR method was developed for simultaneous detection of pig, sheep and cattle DNA from meat products. Three primers were designed in mitochondrial DNA region, included one universal downstream primer and two upstream specific primers. PCR reactions generated 570 bp, 549 bp and 420 bp length fragments from sheep, cattle and pig, respectively. This assay had no cross-reaction with isolated DNA from chicken, duck, hare, horse, rat, donkey and fish. This method proved to be sensitive in detecting the presence of low DNA levels at 10 pg in 20 µl PCR mixture. The method was also successfully used for identification of commercial mutton, beef and pork products. It suggested that this method could be used as a reliable routine screening assay for the presence of sheep, cattle and pig ingredients in foodstuffs.

Introduction

The consumption of meat and meat products is increasing every year. On the other hand, food frauds especially meat adulteration becomes a worldwide incident that would affect people health and meat trade, even the religious belief. As a typical case, in 2013, the European meat industry was cast into the spotlight when the Food Safety Authority of Ireland (FSAI) announced that horse meat was discovered in several beef burgers (Nau, 2013). In China, fox or mouse meat was even detected in mutton products, and pork was mixed in many mutton or processed beef products, because beef, mutton and pork were the most meat products in China and the pork was the cheapest one. Moreover, pork or any derived ingredients were forbidden in halal foods. The increasing occurrence of food frauds suggests that species identification should be part of food authentication. In this regard, development of reliable and simple analytical method to evaluate common meat authenticity is very critical.

Constant developments of technique for animal and meat identification are being made. In the large number of identification methods, many approaches based on DNA assay have been developed in order to exploit residual DNA content of fresh or processed meat products for speciation and authentication purposes (Hopwood *et al*., 1999; Murugaiah *et al*., 2009; Wang *et al.*, 2010). Currently, several multiplex PCR assays were developed and used because they had some advantages over conventional simplex PCR, such as decreasing cost and testing time (Ali *et al*., 2015; Hou *et al*., 2015; Iwobi *et al*., 2015; Stefanova *et al.*, 2015). Agrimonti *et al.* (2015) reported a multiplex PCR method using species specific primer pair of each animal that could simultaneous identifying beef, mutton, pork and chicken. Safdar and Junejo (2016) developed a hexaplex PCR for identification of five meat and one plant species in foodstuffs, including pig, sheep, poultry, horse and cow. But multiplex PCR had its disadvantages, such as difficulty for PCR conditions optimization, low priming efficiency and limited detection number of animal species (Edwards and Gibbs, 1994; Bottero and Dalmasso, 2011; Sint *et al*., 2012; Pan *et al*., 2014; Wang *et al*., 2014). With the increasing of the number of primer, the negative effects on PCR amplification are getting more and more sensible. In general, the number of detection was limited in four or five in a multiplex PCR reaction (Bottero and Dalmasso, 2011). Furthermore, mutton roll and beef roulade were popular with consumers, these products became the most easily adulterated meat products in Chinese retail markets. The present study was aim to develop a multiplex PCR based on conventional PCR platform for simultaneous detection of sheep, cattle and pig with the minimum number of primers, and to provide another choice for researchers in meat

*Corresponding author.
Email: *guanfengzgjl@163.com, yyluo@cjlu.edu.cn*

species identification.

Materials and Methods

Samples collection and DNA extraction

The samples of known species were collected from slaughterhouses or presented by other laboratory. The collected samples included cattle, buffalo, sheep, rat, goat, pig, horse, donkey, rabbit, chicken, duck and fish. Furthermore, ten raw (five mutton rolls, five beef rolls) and eight processed meat products (three dried beef cubes, five dried pork jerky) were purchased from retail markets and hot pot restaurants in Hangzhou City, P.R China. All the samples were stored at -20°C. Genomic DNA was extracted from samples using Animal Tissue DNA Extraction Kit (Takara) in accordance with supplied instructions. DNA concentration was measured using a UV-Vis spectrophotometer (Nanodrop 2000, Thermo) and diluted to 10 µg/µl stored at 4°C for next use.

Primer design and PCR amplification

In this study, three primers were designed for development of this method. A piece of universal downstream primer termed Seq1 that matched with mtDNA sequences of sheep (NC001941), pig (DQ534707) and cattle (JQ437479). The primer Seq2 was specific for cattle and sheep, primer Seq3 was specific for pig. The primers of Seq1 and Seq2 generated two different length fragments for cattle and sheep. The primer sequences were as follows: Seq1: 5′-TGGCTGGCACGAGATTTA-3′, Seq2: 5′-GCTGGACTTAACTGCATC-3′, Seq3: 5′-ACGCCAATCTACCACAAA-3′.

The PCR was conducted on MJ PTC-200 PCR system. PCR reactions were carried out in a total volume of 20 µl mixture, and the 20 µl PCR mixture was prepared as follows: 2 µl 10x PCR buffer, 1.2 µl $MgCL_2$ (25 mM), 0.2 µl dNTP mixture (2.5 mM), 0.2 µl *Taq* polymerase (1 U), 2 µl primer Seq1 (10 µM), 0.2 µM of each primer Seq2 and Seq3 (10 µM), 3.0 µL DNA template, added ddH_2O to 20 µl. Amplification was carried out with program as follows: initial denaturation at 94°C for 10 min and 30 cycles at 94°C for 30 sec, 53.2°C for 30 sec, and 72°C for 30 sec followed by 72°C for 5 min. The 5.0 µl PCR product obtained was analyzed using 2% agarose gel in 1X TAE buffer stained with 4S Red as a visualizing agent and run for 40 min at 80V. The PCR reactions were done in triplicates on three different days and new isolation DNA samples were used in each of experiment.

Specificity and sensitivity test

The specificity of the developed method was tested against the backdrop of referenced animal species. Cross species testing was performed in a PCR run using DNA templates from known DNA samples. Furthermore, obtained PCR products were purified and sequenced, and then the sequences were analyzed using the BLAST local alignment tool to confirm the specificity.

The sensitivity was tested using DNA samples from pig, sheep and cattle by serially diluting, the concentration was measured using Nanodrop 2000 spectrophotometer, and until the PCR product could not be visualized over 2.0% agarose gel, the detection level of DNA in a PCR reaction was regarded as the PCR sensitivity.

Commercial samples test

The developed PCR assay was used for the detection of target meat in eighteen commercial meat products labeled as pork, beef and mutton, respectively.

Results

In the present study, all the genomic DNA samples isolated from collected samples could be amplified by tri-primer PCR. The spectrophotometric assessment results showed the concentration of extraction DNA varied between 20.2-200.5 ng/µl, and the purity (A_{260}/A_{280}=1.72-1.98) was suitable for PCR amplification.

The tri-primer PCR reaction system was optimized for PCR conditions and reagents. The optimal PCR conditions could successfully amplify genomic DNA from cattle, sheep and pig separately or jointly. PCR reactions generated 570 bp, 549 bp and 420 bp length fragments for sheep, cattle and pig, respectively. In addition, goat DNA sample was amplified and a 570 bp fragment was obtained. The same results were obtained for all the repeat experiments. The electrophoresis pattern for PCR products from pig, sheep and cattle DNA samples were showed in Figure 1. These three animal species ingredients can be identified clearly based on the electrophoresis pattern.

The specificity was tested using DNA from chicken, horse, rat, buffalo, donkey, rabbit, duck and fish, and results showed that there had no cross reaction with these DNA samples (Figure 2). The PCR products were purified and directly sequenced, and the BLAST results showed that the obtained sequences were identical to the expected sequences. The sensitivity test was conducted using serially

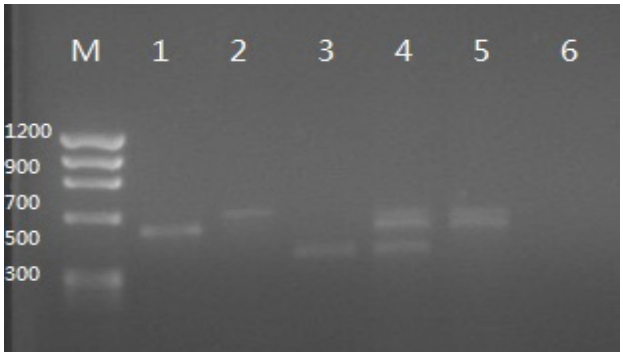

Figure 1. Tri-primer PCR for cattle, sheep and pig species identification

Note: Note: M: DNA maker; 1: cattle; 2: sheep; 3: pig; 4: cattle, sheep and pig; 5: cattle and sheep; 6: negative control

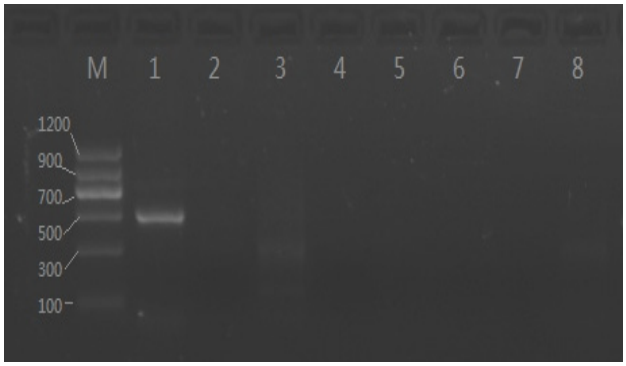

Figure 2. Specificity test for tri-primer multiplex PCR

As showed in this pattern, the tri-primer PCR had no cross-reaction with DNA from chicken, duck, horse, fish, rat, buffalo and rabbit.

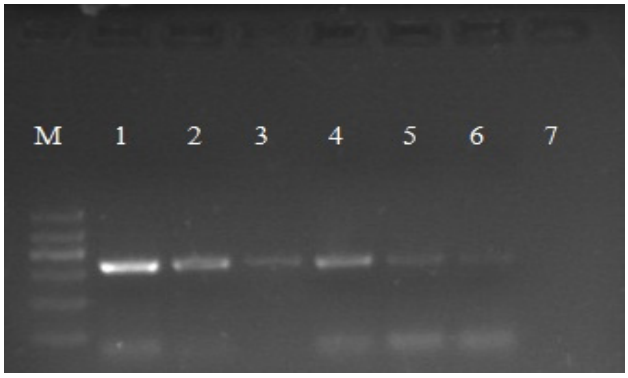

Figure 3. Detection limit of the tri-primer PCR for sheep DNA

Lanes 1-6 showed the PCR products with serially dilution of sheep DNA, the content of DNA was (ng): 50.0, 25.0, 10.0, 1.0, 0.1, 0.01, and lane 7 was negative control.

dilution of DNA, and the DNA band patterns on 2.0% agarose gel indicated that the minimum detection level of pig DNA was at about 6 pg in a 20 µl PCR system, sheep and cattle were at 10 pg and 10 pg, respectively. The pork was added to mutton or beef at 1% and it could be clearly detected. The sensitivity test for sheep genomic DNA was shown in Figure 3.

The test for commercial meat products showed that no mutton or beef ingredient was detected in five labeled pork products, but pork ingredient was detected in four mutton products (three quick-frozen lamb rolls, one raw mutton bunch), and even was not detected sheep DNA in one mutton product. Pork ingredient was detected in a beef cube, one of the most leisure foods from beef in Chinese market. The results suggested that the mutton and beef products were usually adulterated with pork in Chinese retail market.

Discussions

At present, meat and meat products are the most nutritious delicious diets for most families. Moreover, pork, mutton and beef are of the largest consumption meat in China. On the other hand, the rising price and decreasing availability of high quality meat steer the tendency of misrepresentation and adulteration of meat and meat products by some meat producers. The most common economic fraudulence widely spread in meat industry is adulteration or substitution of costlier meat with cheaper or inferior meat (Ali *et al*., 2015; Di Pinto *et al.*, 2015; Hou *et al*., 2015). In China, hang sheep head sell vinegar is a widely known proverb by people. In recent years, it was reported that pork was frequently adulterated into processed mutton and beef products (He *et al*., 2012), and even some leisure labeled mutton or beef products were completely replaced by pork. Under these conditions, meat identification is very critical. The developed tri-primer PCR was designed correspondingly to detect pork, mutton and beef, the most meats in Chinese daily life, yet the pork was forbidden in halal food.

Many meat or animal species identification methods have been developed till date. Conventional methods used for identifying the meat species included sensory analysis, anatomical and histological differences and DNA hybridization (He *et al.*, 2012; Kumar *et al*., 2015). DNA assay become a widely used method in practice because it had many advantages over other analyses. The stability of mitochondrion DNA (mtDNA) was higher than genomic DNA and distributed in all the tissues, so mtDNA was selected as target gene for species identification in many methods (Maine *et al*., 2015; Shabani *et al*., 2015). Recently, several multiplex PCR assays were developed for simultaneously detecting two or more animal species (Saderi *et al*., 2013; Ali *et al*., 2015; Hou *et al.*, 2015; Stefanova *et al.*, 2015), which had greatly improved the test efficiency. But on the other hand, multiplex PCR had its disadvantages such as lower priming efficiency and limited detection number of animal species. The number of primers was an important factor that affects the multiplex PCR efficiency. So the present

study was aim to develop a multiplex PCR assay with minimum number of primers. This method combined the advantage of mtDNA stability with multiplex PCR, which was suitable for detection of three common meats sold in Chinese market. In fact, tri-primer PCR assay had been used for animal species identification (Michelini *et al*., 2007; Sha *et al*., 2011), gene mutation (Zheng *et al*., 2014), molecular diagnosis (Ciotti *et al*., 2004) and viral detection (Ishaq and Stoner, 1993). Michelini *et al.* (2007) developed a one-step triplex-PCR assay based on CytB gene to discriminate three tuna species, which was regarded as a method with a good accuracy, low cost, and with potential automation for large-scale high-throughput screenings in small in-house laboratories. Sha *et al.* (2011) developed a triplex-PCR assay using for animal species identifying but only cattle and pig ingredients. The development of tri-primer assays suggested that they had advantage over existed methods.

The developed tri-primer multiplex PCR test results indicated that it was a sensitive, specific and accurate method for simultaneous detection of pig, cattle and sheep DNA in raw and processed meat. Furthermore, this method was developed based on conventional PCR, which could be done in most laboratories. And the tri-primer multiplex PCR had no cross-reaction with common animals, such as duck, chicken, donkey and horse. Its ability to amplify a target DNA was even at a low level at about 10 pg in a 20 µl PCR mixture. The sensitivity of this assay is even the same high as that of real time PCR using genomic DNA. It suggests that this method can be used as a common screening method for meat fraud supervision in practice. And it provides another choice for meat identification except existed methods.

Conclusion

In conclusion, a tri-primer PCR method was established successfully for simultaneous detection of cattle, sheep and pig ingredients. This assay had been proven to be specific, sensitive and reliable in the identification of pig, cattle and sheep ingredients from meat and meat products, and it had a high potential as a molecular screen tool used in meat fraud inspection. It could be used as a reliable routine screening assay of various food products for the presence of cattle, pig and sheep ingredients in foodstuffs.

Acknowledgments

We gratefully acknowledge financial support from the Science and Technology Project of Zhejiang Food and Drug Administration (No. 2014020), the National Natural Science Foundation of China (No.31360540), the National Key Technology and Program of Ministry of Science and Technology of China (No. 2011AA100307).

References

Agrimonti, C., Pirondini, A., Marmiroli, M. and Marmiroli, N. 2015. A quadruplex PCR (qxPCR) assay for adulteration in dairy products. Food Chemidtry 187: 58-64.

Ali, M.E., Razzak, M.A., Hamid, S.B., Rahman, M.M., Amin, M.A., Rashid, N.R. and Asing. 2015. Multiplex PCR assay for the detection of five meat species forbidden in Islamic foods. Food Chemistry 177: 214-224.

Bottero, M.T.and Dalmasso, A. 2011. Animal species identification in food products: evolution of biomolecular methods. Veterinary Journal 190: 34-38.

Ciotti, P., Di Maria, E., Bellone, E., Ajmar, F. and Mandich, P. 2004. Triplet repeat primed PCR (TP PCR) in molecular diagnostic testing for Friedreich ataxia. Journal of Molecular Diagnostic 6: 285-289.

Di Pinto, A., Bottaro, M., Bonerba, E., Bozzo, G., Ceci, E., Marchetti, P., Mottola, A. and Tantillo, G. 2015. Occurrence of mislabeling in meat products using DNA-based assay. Journal of Food Science and Technology 52: 2479-2484.

Edwards, M.C. and Gibbs, R.A. 1994. Multiplex PCR: advantages, development, and applications. PCR Methods Application 3: S65-75.

He, W.L., Huang, M. and Zhang, C. 2012. Recent technological advances for identification of meat species in food products. Food Science 33: 304-307.

Hopwood, A.J., Fairbrother, K.S., Lockley, A.K. and Bardsley, R.G. 1999. An actin gene-related polymerase chain reaction (PCR) test for identification of chicken in meat mixtures. Meat Science 53: 227-231.

Hou, B., Meng, X., Zhang, L., Guo, J., Li, S. and Jin, H. 2015. Development of a sensitive and specific multiplex PCR method for the simultaneous detection of chicken, duck and goose DNA in meat products. Meat Science 101: 90-94.

Ishaq, M. and Stoner, G.L. 1993. Tri-primer PCR can distinguish free and cloned viral DNA in a single reaction. Journal of Virology Methods 42: 95-98.

Iwobi, A., Sebah, D., Kraemer, I., Losher, C., Fischer, G., Busch, U. and Huber, I. 2015. A multiplex real-time PCR method for the quantification of beef and pork fractions in minced meat. Food Chemistry 169: 305-313.

Kumar, A., Kumar, R.R., Sharma, B.D., Gokulakrishnan, P., Mendiratta, S.K. and Sharma, D. 2015. Identification of species origin of meat and meat products on the DNA basis: a review. Critical Review in Food Science and Nutrition 55: 1340-1351.

Saderi M., Saderi AH and Rahimi G. 2013. Identification

of bovine, ovine and caprine pure and binary mixtures of raw and heat processed meats using species specific size markers targeting mitochondrial genome. Iranian Journal of Veterinary Research 14: 29-34.

Maine, I.R., Atterbury, R. and Chang, K.C. 2015. Investigation into the animal species contents of popular wet pet foods. Acta Veterinar Scandinavia 57: 1-4.

Michelini, E., Cevenini, L., Mezzanotte, L., Simoni, P., Baraldini, M., De Laude, L. and Roda, A. 2007. One-step triplex-polymerase chain reaction assay for the authentication of yellowfin *(Thunnus albacares)*, bigeye *(Thunnus obesus)*, and skipjack *(Katsuwonus pelamis)* tuna DNA from fresh, frozen, and canned tuna samples. Journal of Agriculture Food and Chemistry 55: 7638-7647.

Murugaiah, C., Noor, Z.M., Mastakim, M., Bilung, L.M., Selamat, J. and Son, R. 2009. Meat species identification and Halal authentication analysis using mitochondrial DNA. Meat Science 83: 57-61.

Nau, J.Y. 2013. Horse meat: first lessons of a scandal. Rev Med Suisse 9(376): 532-533.

Pan, W., Byrne-Steele, M., Wang, C., Lu, S., Clemmons, S., Zahorchak, R.J. and Han, J. 2014. DNA polymaerase preference determines PCR priming efficiency. BMC Biotechnology 14(1): 41-50.

Saderi M., Saderi AH and Rahimi G. 2013. Identification of bovine, ovine and caprine pure and binary mixtures of raw and heat processed meats using species specific size markers targeting mitochondrial genome. Iranian Journal of Veterinary Research 14: 29-34.

Safdar, M. and Junejo. Y. 2016 The development of a hexaplex-conventional PCR for identification of six animal and plant species in foodstuffs. Food Chemistry 192: 745-749.

Sha, C., Yang, S., Liao, S. and Bo, Q. 2011. Idemtification for pig and cattle ingredients from meat products using triplex PCR. Livestock and Poultry Industry 272: 52-53.(In Chinese)

Shabani, H., Mehdizadeh, M., Mousavi, S.M., Dezfouli, E.A., Solgi, T., Khodaverdi, M., Rabiei, M., Rastegar, H. and Alebouyeh, M. 2015. Halal authenticity of gelatin using species-specific PCR. Food Chemistry 184: 203-206.

Sint, D., Raso, L. and Traugott, M. 2012. Advances in multiplex PCR: balancing primer efficiencies and improving detection success. Methods Ecolgy and Evolution 3: 898-905.

Stefanova, P., Angelova, G., Georgieva, T., Gotcheva, V. and Angelov, A. 2015. A novel Multiplex PCR Method for Simultaneous Detection of Genetically Modified Soybean Events. International Journal Current Microbiology and Applied Science 4: 256-268.

Wang, Q., Zhang, X., Zhang, H.Y., Zhang, J., Chen, G.Q., Zhao, D.H., Ma, H.P. and Liao, W.J. 2010. Identification of 12 animal species meat by T-RFLP on the 12S rRNA gene. Meat Science 85: 265-269.

Wang, X., Seo, D.J., Lee, M.H. and Choi, C. 2014. Comparison of conventional PCR, multiplex PCR, and loop-mediated isothermal amplification assays for rapid detection of Arcobacter species. Journal of Clinical Microbiology 52: 557-563.

Zheng, C.N., Wang, Q.Y., Huang, Y.X., Ye, G.H., Xu, C.C. and Yang, H.Y. 2014. Research on gout-related SNP genotyping by improved tri-primer allele gene amplification. Chongqing Medical Magazine 12:1476-1479. (In Chinese)