Food Control 84 (2018) 148–158

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Multiplex polymerase chain reaction-restriction fragment length polymorphism assay discriminates of rabbit, rat and squirrel meat in frankfurter products

Md. Eaqub Ali [a, b, c, *], Mohammad Nasir Uddin Ahamad [a], Asing [a], M.A. Motalib Hossain [a], Sharmin Sultana [a]

[a] Nanotechnology and Catalysis Research Center, Institute of Graduate Studies, University of Malaya, Kuala Lumpur 50603, Malaysia
[b] Institute of Halal Research University of Malaya, University of Malaya, Kuala Lumpur 50603, Malaysia
[c] Centre for Research in Biotechnology for Agriculture, University of Malaya, Kuala Lumpur 50603, Malaysia

ARTICLE INFO

Article history:
Received 30 April 2017
Received in revised form
7 July 2017
Accepted 23 July 2017
Available online 25 July 2017

Keywords:
Rabbitry
Tetraplex PCR-RFLP
Processed foods
Fingerprints
Restriction enzymes

ABSTRACT

The demands for rabbit meat are rapidly growing and Rabbitry is becoming a mean of livelihood for many youths. Rats and squirrels are very close relatives of rabbits, could be hunted freely or raised in domestic farming and so could be substituted in expensive rabbit meat. This study, for the first time, developed and validated a tetraplex polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay to identify and discriminate rabbit, rat and squirrel meat under raw and processed foods. Four sets of primes amplified 123, 108, 243, and 141 bp fragments from rabbit, rat, squirrel and all eukaryotes, respectively. Specificity was confirmed through sequencing and RFLP analysis. When PCR products were digested with *Bts*I*Mut*I and *Bts*CI enzymes, distinctive fingerprints (115 & 8 bp for rabbit; 64 & 44 bp for rat and 176 & 67 bp for squirrel) were obtained. The detection limit of the assay was 0.1% meat in frankfurter formulation.

1. Introduction

Ensuring food safety and quality from farm to fork requires regulatory laws, public awareness and monitoring systems working side by side (Ali, Sultana et al., 2016; Ali. Al Amin et al., 2016). Various food safety regulatory agencies have made it illegal to mix undeclared animal materials in any food products; the US Federal Meat Inspection Act (FMIA) and the European Parliament Regulation (EC) No. 178/2002 strictly prohibit meat and other animal material adulteration in food chain (FMIA, 2016; European Parliament, 2002). However, survey reports reveal the practice is going on unfettered across all continents; 68% meat products in South Africa, 19.4% in the USA, 33% in the Gulf countries, 22% in Turkey, and 8% in the UK are reported to have mislabeled animal materials (Hossain et al., 2016).

Rabbitry is a growing industry and rabbit (*Oryctolagus cuniculus*) meat has already got popularity in many European and African countries, such as, Malta, Cyprus, Italy, Czech Republic, Spain, Belgium, Luxembourg, Portugal, France, Egypt and Algeria (FAOSTAT, 2010). American restaurants are also experiencing larger and larger demands for rabbit meat (Lanz & Margoli, 2013) and it is also permissible in most religions and cultures. Rabbit meat is also appreciated as a functional food because of its lower contents of fat, cholesterol and sodium but high concentrations of digestible proteins (Dalle Zotte, 2002; Maertens & Coudert, 2006). Thus, rabbit meat is sold at a significantly higher price than those of other regular meats such as chicken, goat, beef and pork. In the last 50 years, the world's production of rabbit meat has increased by more than 2.5 fold accounting to 1.6 million tons in 2009. China is currently the world's leading producer of rabbit meat (700,000 t/year), followed by Italy (230,000 t/year), Spain (74,161 t/year) and France (51,400 t/year) (FAOSTAT, 2010).

However, like other meat items, rabbit meat is also not free from adulteration risks. Recently, one ton of fresh and frozen carcasses of cat were seized by the Chinese Police in an enforcement operational raid at Shunjiang in China, where they were being sold as rabbit meat (Fang & Zhang, 2016). Definitely, it is a strong piece of evidence that adulteration of such meat is really taking place. The

* Corresponding author. Nanotechnology and Catalysis Research Center, Institute of Graduate Studies, University of Malaya, Kuala Lumpur 50603, Malaysia.
E-mail addresses: eaqubali@gmail.com, eaqubali@um.edu.my (Md.E. Ali).

http://dx.doi.org/10.1016/j.foodcont.2017.07.030
0956-7135/

rabbit species belongs to the lagomorph family which was previously classified under rodents (Fiedler, 1990). Rats and squirrels are prominent rodent species widespread in all continents; they could be hunted free of charge or domestically raised in small farms without much investment but they do not have any market demands; these have made them a lucrative adulterant for substitution in rabbit and other meat items. In 2013, Chinese police detained more than 900 people to unmask a ring involved in the chemical transformation of rat, mink and fox meat into mutton like appearances and selling them as mutton (The Guardian, 2013).

Commonly black, red, brown and cane rat species are found in the south-east Asian regions and in most of the cases they are considered as an agricultural pest (Anonymous, 2016a). The black rats (*Rattus rattus)* are especially ubiquitous because of their high reproductive rates and very strong adaptation power in most environments (BPCA, 2016; IUCN, 2016). Despite having rejection from most of the societies, some rat species are domesticated and consumed in certain communities because of their greater carcass yield (ca. 65%), purported nutritional values, soft bones and taste like bird's meat (Ajayi & Olawoye, 1974; Odebode et al., 2011). Wild rats are frequently traded for consumption along road sides shops in many African countries (Redhead & Boelen, 1990); Anonymous, 2016b). About, 80 million pieces of cane rats are hunted per year only in western Africa, with a yield of 300,000 metric tons of meat (Hoffman & Cawthorn, 2012). However, rat meat consumption is greatly risky for public health because they are the potential careers of many infectious microbes, such as *Leptospira* spp, *Listeria* spp, *Yersinia enterocolitica*, *Pasturella* spp, *Pseudomonas* spp, *Yersinia pestis* and *Hantavirus,* which can cause several life threating listeriosis, yersiniosis, pasturellosis, meilioidosis and plague (Anonymous, 2016c). The Indian outbreak of plague in 1994 that took at least 60 lives was linked to the rat meat consumption (Deutsch & Murakhver, 2012). Plague outbreak in Vietnam in 1967 was also implicated to rat meat consumption (Hauck, Hanks, & Sudsaneh, 1959). Rat meat adulteration in common meat items is also a very sensitive and serious issue because it is a taboo in most societies and not permissible in most of the religious foods (Doosti, Dehkordi, & Rahimi, 2014).

On the other hand, squirrel species, especially the plantain squirrels (*Callosciurus notatus)* are widespread across the Penisular Malaysia, Singapore, Thailand, Indonesia and Myanmar (Anonymous, 2016d; Mohd, 1998). Certain Southeast Asian and African countries consume squirrel meat as a source of animal proteins as well as exotic dishes (Davis, Cheng, Lassar, & Weintraub, 1990). Certain attributes such as the distinctive flavor, high proteins, low fat, less cholesterol and the absence of health-threatening anabolic steroids in bush meat also encourage the hunting of squirrel species (Redhead & Boelen, 1990). Squirrel dishes are also frequently sold in exotic restaurants in the UK and USA (Anonymous, 2016e). However, like rat, squirrels are also a potential carrier of *Salmonella, Borrelia burgdorferi, Francisella tularensis, Leptospir* that may cause life threating Creutzfeldt-Jakob disease (CJD) syndrome (Neurodegenerative disease) (Anonymous, 2016f) and Lyme disease (Anonymous, 2016g).

As authentication is concerned, squirrels were subjected to phylogeographical (Finnegan, Edwards, & Rochford, 2008) and cross-chromosome painting for genome organizations (Li et al., 2004) as well as sequencing for structuring, hybridization and population fragmentation analyses (Barratt, Gurnell, Malarky, Deaville, & Bruford, 1999; Spiridonova et al., 2005). Recently, a real-time PCR has been proposed for the differentiation of red and gray squirrels (O'Meara, Turner, Coffey, & O'Reilly, 2012). On the other hand, several molecular detection schemes have been proposed for the identification of rat (Fang & Zhang, 2016; Rahmania & Rohman, 2015) and rabbit species (Amaral, Santos, Melo, Oliveira, & Mafra, 2014; Hanapi, Desa, Ismail, & Mustafa, 2015) (Rafayova, Lieskovska, Trakovicka, & Kovacik, 2009). However, these methods are mostly based on a single species target and long DNA marker that breaks down under food processing treatments, compromising reliability and making the analysis costlier (Ali, Razzak, et al., 2015). The recent evolution of multiplex polymerase change reaction (mPCR) assays are especially promising because multiple target oligos could be identified in a single assay platform, saving both analytical cost and time (Ali, Razzak, et al., 2015; Hossain et al., 2016). Recently, mPCR assays have been reported for pig, dog, cat, rat and monkey species (Ali, Razzak, et al., 2015); beef, pork, horse and sheep species (Köppel, Ruf, & Rentsch, 2011); beef, pork, lamb, chicken, ostrich and horse species (Kitpipit, Sittichan, & Thanakiatkrai, 2014) and cattle, buffalo and porcine species (Hossain et al., 2016). The species-specific PCR-restriction fragment length polymorphism (PCR-RFLP) assays are furthermore interesting because they can authenticate the amplified PCR product through restrictive digestion using one or more restriction enzymes (REs) (Rashid et al., 2015). Using the existing sequence variation within a defined region of DNA, the differentiation of even closely related species has been done (Hsieh & Hwang, 2004); cattle, yak, and buffalo (Chen, Liu, & Yao, 2010); cattle-buffalo and sheep-goat (Girish et al., 2005); swine and wild boar (Mutalib et al., 2012) and various fish species (Nebola, Borilova, & Kasalova, 2010) have been successfully discriminated by applying PCR-RFLP techniques. However, to the best of our knowledge, no multiplex PCR or PCR-RFLP assays have been reported for rabbit, rat and squirrel authentication. In this study, these gaps were addressed for the first time through the development and validation of a tetraplex PCR-RFLP assay with short-lengths of amplicon for the simultaneous identification and discrimination of rabbit, rat and squirrel materials under real food matrices, such as frankfurter formulation.

2. Materials and methods

2.1. Sample collection

Fresh muscle tissues or specimens were obtained from rabbit (*Oryctolagus cuniculus*), squirrel (*Calloscious notatus*), chicken *(Gallas gallus*), beef (*Bos Taurus*), buffalo (*Bubalus bubalis*), sheep (*Ovis aries*), goat (*Capra hiscus*), pig (*Sus scrofa*), duck (*Anas platyrnychos*), pigeon (*Columba livia*), crocodile (*Crocodylus niloticus*), donkey (*Equus asinus*), amboina box turtle (*Cuora amboinensis*), chinese edible frog (*Hoplobatracus rugulosus*), deer (*Cervus nippon yesoensis*), dog (*Canis lupus familiaris*), cat (*Felis catus*), tuna (*Thunnus orientalis*), salmon (*Salmo salar*) and plant species, namely, wheat (*Triticum aestivum*), cucumber (*Cucumis sativus*), onion (*Allium cepa*), and chili (*Capsicum Capsicum annuum*). Where available, meat, fish and plant spices specimens were collected from commercial wet (Pudu Raya) and super markets (Aeon, Tesco and Giant) at Kuala Lumpur on three different days to increase the genetic diversity of the collected samples. Deer (*Cervus nippon*) meat was procured in triplicates from the Faculty of Veterinary Sciences at the University of Putra Malaysia, located at Serdang in Selangor. Stray dog *(Canis lupus familiaris)*, cat (*Felis catus*) and rat (*Rattus rattus*) muscle were donated by Kuala Lumpur City Hall (KLCH/DBKL) at Air Panas in Kuala Lumpur. Monkey (*Macaca fascicularis* sp*)* meat was a gift from the Department of Wildlife and National Park Malaysia (DWNPM/PERHILITAN) at Cheras in Kuala Lumpur. It is worthy to note that DBKL routinely kills rats, cats and stray dogs for population control and public security purposes in the town area; so no animals were killed for this study purposes but sufficient amount of muscle tissues were taken from the already killed animals following institutional and country laws. Details information of all the collected samples is given in Table 1. The