ISSN 0101-2061 (Print)
ISSN 1678-457X (Online)
Food Science and Technology

DOI: https://doi.org/10.1590/fst.11520

Bacteriological assessment and multiplex-PCR test for the detection of meat adulteration of different animal species

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Abstract

Meat samples of various animal species have been obtained from Giza Governorate's butcher shops, common retail markets, veterinary faculties, Zoo and hospitals. Physical, bacteriological, and molecular analysis was carried out on the meat samples. Physical examination showed that typical and untypical meat differed. Bacteriological examination showed the highest TCC was found in horse meat while the least was in goat meat. The total count of *S. aureus* was the highest in donkey and dog whilst the least in pork meat. As for *E.coli*, donkey meat was the highest while buffalo and mutton meat were the lowest. The highest total *Listeria monocytogenes* and *Salmonella* Species count were in horse, donkey, rat and dog meat but zero in the other species examined. Multiplex-PCR targeting partial-length of cytochrome b (*cyt b*) gene of mitochondrial DNA (mtDNA) was used for the discovery of adulteration of beef meat with chicken, pork, dog, cat and rat tissue. It showed high specificity in differentiating the six animal species meat. The study points out the importance of taking serious steps to control species meat adulteration that may lead to transmission of severe foodborne diseases and more studies need to be implemented to apply new and easy meat adulteration detection protocols.

Keywords: multiplex PCR; meat adulteration; bacteriological; Egypt.

Practical Application: Species-specific PCR for distinguishing five different animal species in raw beef meat to prevent meat adulteration and to show the severity of meat adulteration in food-borne pathogens transmission in Egypt.

1 Introduction

Meat is considered as an outstanding source of high-quality protein, fat, carbohydrates, vitamins and minerals and is delicious, palatable or easy to digest (Sharma & Bist, 2011). Raw meat eaten by more than 80% of the population is one of the main sources of foodborne diseases (Hassan Ali et al., 2010), meat is also considered to be an ideal medium for bacterial growth due to available favorable environmental factors (pH, temperature, minerals and other growth factors) (Russell, 2001).

Enterobacteriaceae group are often of global concern and is very difficult due to its close association with both raw and processed meat contamination. *E. coli, Proteus Salmonella* and *Klebsiella* sp. has always been chief species in all food poisoning circumstances linked to some meat products (Food and Agriculture Organization, 2000). *L. monocytogenes* is responsible for several listeriosis outbreaks related to meat product consumption. Initially, *L* monocytogenes are present in small quantities in foodstuffs, and can multiply at varying rates during chilled storage depending on the type of food product, both under aerobic and anaerobic conditions, adapt to disinfectants and adhere to different surfaces (Meloni, 2015).

Fraudulent or accidental mislabeling of food products is still widespread worldwide, which could not be identified using traditional techniques Adulteration may also include the use of low-priced meat like chicken meat as a high-priced meat like beef meat. Therefore, these consumer groups need methods of detecting meat species (dog, cat, pork, etc.) in the food (Haunshi et al., 2009). Developments in molecular biology have facilitated high precision identification of plant, bacteria, and animal species (Sasazaki et al., 2004). Polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), and random amplified polymorphic DNA (RAPD) techniques were widely used to classify meat species (Arslan et al., 2005). Accordingly, the present study examined raw meat samples from various animal species randomly collected from street vendors and famous Giza Governorate retail markets. Samples were examined microbiologically and species-specific multiplex PCR methods were created for the detection and identification of pork, cat, dog, chicken and rat or mouse tissues.

2 Materials and methods

2.1 Meat samples

Raw meat samples from 11 different animal species were collected randomly from Giza Governorate's butcher shops, common retail markets, veterinary faculties, Zoo and hospitals.

Received 12 Mar., 2020
Accepted 20 Apr., 2020
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Meat samples were preserved at –20 °C before physically analyzed (detection of differences in appearance, odor, texture and color of fat) (Lattuada & Dey, 1998), pH determination in meat (Korkeala et al., 1986). Bacteriological analysis followed by meat species identification was carried out using species-specific primers in multiplex PCR for common animal species.

2.2 Preparing samples for microbiological experiments (Datta et al., 2012)

Ten grams of each sample were added for 2-4 minutes to 90 mL of 0.1 per cent sterile peptone water each collected homogeneous and stood at room temperature for about 5 minutes, then tenfold serial dilutions were used to count microorganisms under complete aseptic conditions.

2.3 Microbiological examinations (Datta et al., 2012):

Complete bacterial count was performed using standard plate count agar medium. Under aseptic conditions, one ml of each of the previously prepared serial dilutions was inoculated in duplicate plates and incubated for 24-48hrs at 37 °C. Then the colonies numbered were measured and registered as cfu/g (cfu colony forming unit).

2.4 Determination of total E.coli, Staphylococcus aureus, Salmonella species and Listeria monocytogenes count (Datta, et al., 2012; Abuelnaga et al., 2017)

Total counts of *E.coli, Staphylococcus aureus, Salmonella*, and *Listeria monocytogenes* were completed. One ml of each of the serial dilutions previously prepared was inoculated in duplicate plates of E.M.B, MacConkey, Mannitol salt, Salmonella shigella agar plates incubated at 37°C and Listeria species was isolated in compliance with International Organization for Standardization (2004). A portion of 0.1 ml of primary enrichments in peptone water was transferred to 10 ml of buffered Listeria enrichment broth with Listeria selective enrichment supplement (with cicloeximide) (Oxoid) and 24 hour incubated at 30 °C. Secondary enrichments were streaked to Palcam agar with Palcam selective supplement (Oxoid) then incubated for 24 h at 37 °C. Colonies were measured and these colonies were classified with the API 20E kit (Bio Merieux) to detect the biochemical profile of the isolated species according to the manufacturer's instructions.

2.5 Multiplex-polymerase chain reaction on common animal meat species

Meat samples

Muscle tissue samples from randomly selected species represent large and small animals (pork, dog, cat, cow, chicken, and mouse) were used as positive control alongside meat samples randomly collected from Giza Governorate's butcher shops, common retail markets, veterinary faculties, Zoo and hospitals.

DNA extraction from meats samples

DNA was extracted from meat samples using the GF-1 Tissue DNA Extraction Kit (Cat.no. GF- TD 050, Vivantis Technologies, Malaysia) Following manual instructions with some modifications where 50 mg of tissue samples are used and DNA was eluted in 50 µL of the preheated Elution Buffer included in the kit.

Primer design

Species specific PCR primers for the amplification of Pig, dog, cat, cattle, chicken and mouse meat have been developed as shown in Table 1. All primers were obtained from Vivantis Technologies, Malaysia.

Polymerase Chain Reaction (PCR)

The 50 µL reaction mixture was prepared in an eppendorf tube containing 25µl of 2X ViRed Taq Master Mix (Cat. no.CLMM01, Vivantis Technologies, Malaysia), 20 pmol of each primer, and 5 µL of target DNA. Two multiplex PCR were performed, the first one was targeting bovine, dog, cat and pork meat while the second one was targeting chicken and mouse meat. The PCR cycles begins with Initial Denaturation at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 45 sec followed by annealing for 45 sec at 58 °C for the first m-PCR and 54°C for the 2nd m-PCR followed by extension at 72 °C for 45 Sec and single final extension at 72 °C for 5min.Electrophoresis was performed on agarose gel (1.5%) at 100V for 2h on a 15 µL portion of the

Table 1. The primer pairs used in specific PCR identification of bovine, horse, and donkey meats.

Species	Sequence	PCR product	Reference
Bovines	5'- GCCATATACTCTCCTTGGTGACA-3' 5'- GTAGGCTTGGGAATAGTACGA- 3'	271bp	Ilhak & Arslan (2007)
Dog	5'- GATGTGATCCGAGAAGGCACA-3' 5'- TTGTAATGAATAAGGCTTGAAG-3'	322bp	Ilhak & Arslan (2007)
Cat	5'- CATGCCTATCGAAACCTAACATAA-3' 5'- AAAGAAGCTGCAGGAGAGTGAGT-3'	274bp	Ilhak & Arslan (2007)
Chicken	5'- CTCGCCCTACTTGCCTTCC-3' 5'- TAGGACGCAACGCAGGTGT-3'	256bp	Haunshi et al. (2009)
Pork	5'- CCCAGCCCCCTCAAACATCTCA-3' 5'- ATGTACGGCTGCGAGGGCGGTAA-3'	525bp	Kairalla et al. (2005)
Mouse	5'- AATCCAACTTATATGTGAAAATTCATTGT-3' 5'- TGGGTCTTAGCTATCGTCGATCAT-3'	96bp	Martín et al. (2007)

amplified DNA fragments with the using of 50bp ladder plus (Cat No. M7115 BIOMATIK, Canada). The resultant gel was treated with ethidium bromide (0.5 µg/mL), visualized and filmed using a UV transilluminator. Dog and cat were tested in a single step using multiplex PCR and also Chicken and Mouse.

3 Results

3.1 Physical and pH examination of the various species of meat

Physical characteristics (colour, smell and texture) of various animal species where the usual color of meat varies from dark red to reddish depending on age and species (Table 2).

3.2 Bacteriological examination

Specificity of the species-specific primers

For the identification of bovine, dog, cat, pig, pork and mouse tissues in the meat product, specificity of the species-specific primers and optimum PCR conditions were planned. The primers provided unique species specific fragments of 271, 322, 274,256,525 and 96bp for bovine, dog, cat, chicken, pork and mouse tissue, respectively. PCR amplification of bovine, dog, cat, chicken, pork and mouse genomic DNA with each prime set confirmed the specificity of each species-specific priming pair. These produced PCR products that were engineered only from the DNA extracted species and displayed no cross-reactivity with the DNA from the other species (Figures 1, 2). PCR products with any of the species-specific priming sets were not obtained for samples with negative controls.

Monitoring trials for more than one species in a single step (Multiplex PCR) were successful for cat dogs and also for mouse chicken.

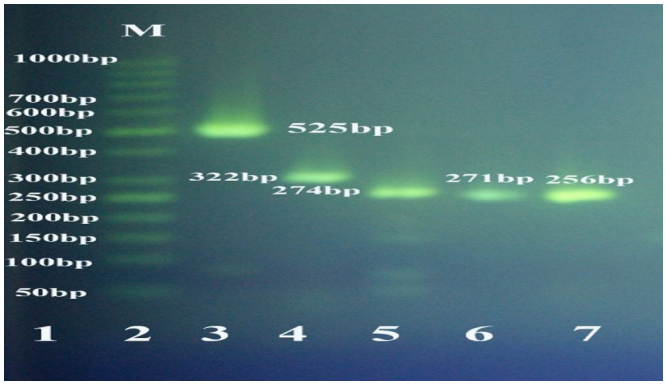

Figure 1. Agarose gel analysis of PCR product amplified with species specific primers. 1: negative control; 2: molecular marker (50 bp); 3: pork meat; 4: dog meat; 5: cat meat; 6: bovine meat; 7: chicken meat; M: Marker.

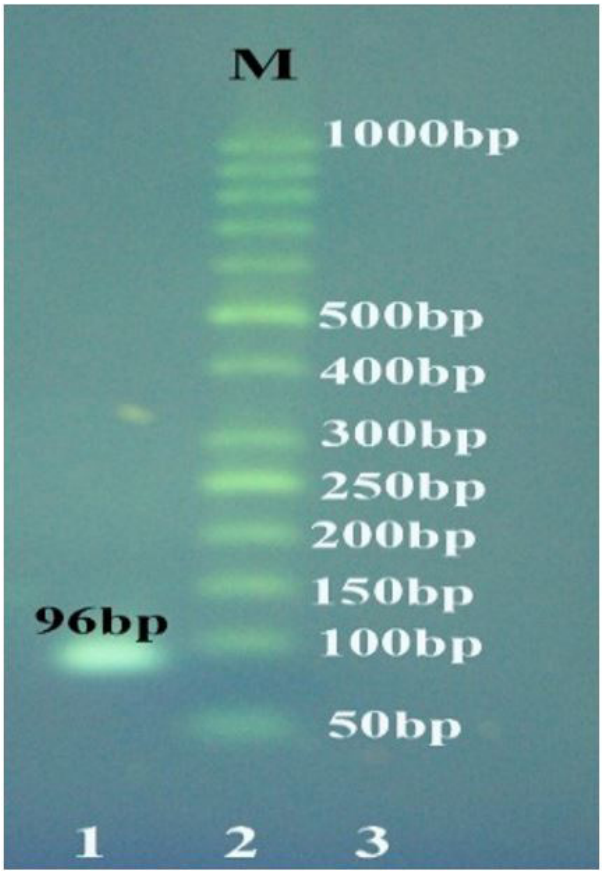

Figure 2. Agarose gel analysis of PCR product amplified with mouse specific primers. 1: mouse meat; 2: molecular marker (50 bp); 3: negative control; M: Marker.

Table 2. Physical and pH examination of meat from different species.

Species	pH	Color	Texture	Odor	fat
Sheep	6.8-7.2	reddish	soft	Sheep odor	Hard white
Goat	7.1	Rosy red	soft	normal	Hard white
Cow	6.3	Dark red, rosy red according to age	Soft (betlo) Hard(kandoz)	normal	Yellow white
Buffalo	7.3	red	Course bundles	normal	white
Camel	6.6-7.1		Course bundles	normal	Yellow white
Pig	6.4-6.7	Reddish grey	soft	normal	Yellow white inside muscles
Donkey	7.1	red	course	Stable odor urine odor	yellowish
Horse	6.8	Dark red	course	Stable odor urine odor	yellowish
White rat	7.5	Reddish white like chicken	soft	Abnormal odor	yellowish
Dog	6.5	Rosy red	soft	Abnormal odor	yellowish
Cat	6.7	Reddish white	soft	Abnormal odor	yellowish

4 Discussion

One of the most serious problems facing meat consumers is meat species adulteration, which affects public health and microbial contamination of the meat consumed, which can be increased by species adulteration, leading to increased spread of foodborne diseases, especially bacterial contamination, which can contribute to the spread of antibiotic foodborne bacteria, So in this research we examined different meat animal species physically and pH, bacteriologically and by PCR. In Table 2 physical examination (colour, smell and texture) of meat of various animal species reveals the natural color of meat ranging from dark red to reddish by age and species, where many authors (Singh, 2008a, b, 2010; Sachan & Singh, 2010; Singh & Sachan, 2010) showed that we usually do physical techniques to identify different meat species for general appearance. It is a combined experience of colour, texture, odor and appearance of other parts of the body as well as meat. It gives the primary idea about the meat species based on the meat quality features. Meat texture also varies from bundles of soft to course, as decided by Singh (2008a, b) and Sachan & Singh (2010) who believes that we can easily identify the meat species to which it belongs, on the basis of the anatomical structure of different animal species used for meat production.

Contaminated meat is one of the main sources of foodborne disease and death from agents that enter the body by ingestion (World Health Organization, 2007). Foodborne diseases are diseases that result from ingestion of bacteria, toxins and cells produced by food-borne micro-organisms (Okonko et al., 2010). Bacteria that may cause disease in humans, such as *Salmonellae* species, *Staphylococcus aureus*, *Listeria monocytogenes*, *Campylobacter* species, and *Escherichia coli* O157: H7, are generally recognized as the most important food-borne hazards from fresh meat. The main sources of pollution are the slaughtered animals themselves, the workers and the work environment and, to a lesser extent, air contamination through aerosols and carcass dressing water (Birhanu et al., 2017).

In our study, on bacteriological examination of different animal species presented in Table 3 the highest TCC was observed in Horse (6×10^5cfu) accompanied by donkey (5×10^5 cfu), dog (2×10^5 cfu), cow (2×10^4 cfu), Buffalo, sheep, pig, Rat, cat the same TCC (2×10^3 cfu), camel (4×10^2 cfu) and Goat the least TCC (3×10^2 cfu). The existence of high bacteria may be associated with poor hygienic and sanitary activities at the Abattoir, butcher shop and during transportation. This agree with Pius (2013) and Haileselassie et al. (2013) Who reported high levels of meat contamination with these pathogens due to Lack of good manufacturing and handling standards along the meat production chain and of sanitary standard operating procedures. Also Bersisa et al. (2019) revealed that the poor hygienic condition of the abattoir and butcher shops resulted in a high bacterial load than the acceptable standard limit.

The total number of *Staphylococcus aureus* was the highest in donkey and dog (3×10^3, 2×10^3 cfu) followed by the rest of the species tested (2×10^2 cfu), and the highest number of *E.coli* was in donkey (4×10^3 cfu), cow and goat (2×10^3 cfu), horse (5×10^2 cfu), camel and pig (3×10^2 cfu); While the slightest amount of *E.coli* was found in Buffalo, sheep, dog, cat and rat (2×10^5 cfu) and the presence of *E. coli* in all meat species is evident and this is accepted . Bersisa et al. (2019) who found among bacteria isolated The predominant organism was *E.coli* followed by *S. aureus* and *salmonella* Species with minimum load of objectively isolated and identified bacteria from meat from and butcher shops and abattoirs.

The largest number with Total *Salmonella* species was in horses (4×10^2 cfu), donkeys, rats and dogs (2×10^2 cfu), and none in other species examined. This agree with Saleh et al. (2013) who was unable to detect Salmonellae in both moutons and beef. Also, Datta et al. (2012) and Selvan et al. (2007) Which both did not recover *Salmonella* species from Raw meat samples and retail meat products.

The highest number of *Listeria monocytogenes* was in donkey (3×10^2 cfu), horse, rodent, and dog (2×102 cfu), and none in the other species tested. On the contrarily, Thévenot et al. (2006) addressing the existence of *L. monocytogenes* in raw pork meat, in the processing atmosphere and in the completed products and its presence is growing from the farm to the production plants and this is mainly due to cross-contamination.

Zerabruk et al. (2019) Found *Enterobacteriaceae, Staphylococcus* spp, and *Bacillus* spp were the dominant microflora of meat and surface contact samples and concluded some factors leading to beef meat contamination. Low awareness of hygienic

Table 3. Bacterial count of meat for different species.

Species	TCC	*S. aureus*	*E.coli*	*Salmonella*	*L. monocytogenes*
Sheep	2×10^3	2×10^2	2×10^2	0	0
Goat	3×10^2	2×10^2	2×10^3	0	0
Cow	2×10^4	2×10^2	2×10^3	0	0
Buffalo	2×10^3	2×10^2	2×10^2	0	0
Camel	4×10^2	2×10^2	3×10^2	0	0
Pig	2×10^3	2×10^2	3×10^2	0	0
Donkey	5×10^5	3×10^3	4×10^3	2×10^2	3×10^2
Horse	6×10^5	2×10^3	5×10^2	4×10^2	2×10^2
Rat	2×10^3	2×10^2	2×10^2	2×10^2	2×10^2
Dog	2×10^5	2×10^3	2×10^2	2×10^2	2×10^2
Cat	2×10^3	2×10^2	2×10^2	0	0

TCC: Total colony count.

practices, regular handling of paper currency, broken cold chain, and poor butcher shop sanitation are among the prevailing factors that led to beef meat contamination and seriously compromised meat product quality. Species identification of animal's tissues in meat is a notable topic for protecting consumers from any fraud or abuse for political, religious and health reasons. Adulteration or misrepresentation of foodstuffs for greater profit is popular throughout (Shears, 2010; Doosti et al., 2014; Meira et al., 2017). Substitution to illegally used species is so difficult to identify by visual inspection after grinding and/or heat processing in such products (Abd El-Nasser et al., 2010). In the past, few specialists in Egypt had confirmed adulteration with donkey and horse meat (Mousa et al., 2017; Abd El-Razik et al., 2019).

The specificity of the species-specific primers and optimum PCR conditions for the identification of bovine, dog, cat, chicken, pork and mouse tissues in the meat product were developed in the present study. The primers provided unique species fragments of 271, 322, 274, 256, 525 and 96bp respectively for bovine, dog, cat, chicken, pork and mouse tissue.

Monitoring trials for more than one species in a single step (Multiplex PCR) were positive for bovine, dog and pork for the 1st m-PCR and also for mouse with chicken in the 2nd m-PCR. Animal tissue identification in meat products is an important issue for protecting the consumer from illicit and/or undesirable adulteration or fraudulent substitution; for cultural, religious and health reasons. Adulteration or misrepresentation of food products is a common practice worldwide for more financial gain. (Doosti et al., 2014). Visual observation after grinding and/or heat processing is very difficult to detect substitution with cheaper species in such products (Abd El-Nasser et al., 2010). Due to improper handling and the use of shared equipment, accidental cross contamination of meat products may also occur during processing (Ilhak & Arslan, 2007).

Abd El-Razik et al. (2019) pointed to the question of meat adulteration in Egypt by applying species-specific PCRs to identify donkey and horse tissue in beef meat and meat products without the need to incorporate RFLP or sequencing, and do not require expensive tools such as real-time PCR analyses.

In this study, the species-specific PCR was developed for the identification of pork, chicken, dog, cat and rat or mouse species in common cattle meat and meat products obtained from street vendors and common retails markets of Giza governorate with a single PCR reaction step.

Multiplex PCR seems to be one of the most realistic techniques of detecting more than one species in a quick, precise and simultaneous way. Multiplex PCR applications have already been recorded for the identification of species in meat samples (Dai et al., 2015).

In this study, trials to test more than one animal species tissue in a single step using multiplex PCR were successful with respect to bovine, dog and cat and pork tissue as well as with chicken and mouse tissue. This coincided with that of Hou et al. (2015).

The current extraction method was less time-consuming and technically less complex than the one of Di Pinto et al. (2005) mentioned above. The specificity of the PCR products suggests a high specificity of the PCR method, in line with the results obtained by Di Pinto et al. (2005).

In our investigation, however, the rate of contamination with donkey meat was higher than that reported in Assuit Governorate in Egypt by Abd El-Nasser et al. (2010) in minced meat (7%) and sausage (8%); this could be due to our stress on street vendors where food safety is powerless. Equines are not legally used for human feeding in Egypt. Its existence affirms adulteration as a point of benefit drive and thus indicates that meat has been treated in unhygienic conditions that present a conceivable risk to human health.

5 Conclusions

This study ensured the existence of high microbial counts in the meat samples tested from different animal species that were known to be a source of food-borne infection affecting public health. Therefore, further exposure to the correct meat treatment procedures is essential to reduce bacterial counts. Use species-specific PCR saves effort, time and more precision in detecting meat adulteration than other techniques do.

Acknowledgements

This work was supported by a grant (11020304) from the National Research Center (NRC) in Egypt.

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