

## Bacteriological status and detection of adulteration with donkey meat by PCR in frozen beef meatballs

\*Abd-El-Malek, A. M.

Department of Food Hygiene (Meat Hygiene), Faculty of Veterinary Medicine, Assiut University, 71515 Assiut, Egypt

\*Corresponding author. Tel./Fax: +20-88-2366503 E-mail address: ashraf\_702001@yahoo.com

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**Abstract:** 50 packed frozen beef meatballs which commercially marketed in Assiut City were collected for the occurrence of *Aeromonas* spp., *Yersinia* spp., *Listeria* spp. and *Salmonella* spp. Other bacteriological aspects; aerobic plate count (APC), total *Staph. aureus*, psychrotrophic bacteria and anaerobic bacteria were also counted. In addition, adulteration of donkey meat in this product was determined by polymerase chain reaction (PCR). The achieved results revealed that the mean APC, total *Staph. aureus*, psychrotrophic count and anaerobic bacterial count of frozen beef meatballs were  $5 \times 10^6$ ,  $9 \times 10^2$ ,  $1 \times 10^6$  and  $6 \times 10^2$  CFU/g, respectively. 32%, 48%, 54 and 4% of samples were positive for the presence of *Aeromonas* spp., *Yersinia* spp., *Listeria* spp. and *Salmonella* spp., respectively. Assay of PCR revealed that the adulteration rate was 20% (2/10) for donkey meat in frozen meatballs. In conclusion, most of the examined frozen meatball samples revealed high levels of microbial contamination. Therefore, this product represent public health hazards considering the incidence of food poisoning microorganisms such as *A. hydrophila*, *Y. enterocolitica*, *L. monocytogenes*, *S. Typhimurium* and *S. Enteritidis*. Moreover, it was concluded that there was adulteration of meatballs, in which inferior quality and illegal donkey meat is mixed into beef. It was recommended that the generally acceptable microbial guideline value for psychrotrophic bacteria of frozen meatballs (kofta) set at  $<10^0$  cfu/g be adapted locally until more precise microbial criteria for Egyptian frozen meatballs are established. Also, it was recommended that applying PCR for fast, easy, and reliable control of adulteration and fraud in meat products in Egypt.

**Keywords:** Bacteriological status, frozen meatball, adulteration, donkey meat, PCR

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### I. Introduction

In recent years, the changes in individual's consumption habits and advances in food technology have caused an increase in the demand for different style convenience foods and greatly trend to fast food consumption by consumers. Meatball (Kofta) is one kind of the most preferred foods; especially in the Egyptian market because they represent quick easily prepared meat meals with low price for a large numbers of families with limited income.

Beef Meatballs can be defined as round meat foods obtained from the cattle meat that have been mashed and mixed with other additives in order to formulate a product that is better, affordable, more accessible, acceptable and healthy. In Egypt, meatball (kofta) prepared as "fingers" by blend of ground beef meat, rice, onions, garlic, parsley, salt, black pepper and other spices, which is mostly consumed after grilling.

Currently, *A. hydrophila*, *L. monocytogenes* and *Y. enterocolitica* has attracted attention primarily because of its ability to grow at chill temperatures, prompting the concern that any threat it might pose will increase with the increasing use of chilled foods (Adams and Moss, 2008). Moreover, most *Salmonella* serotypes can grow over the temperature range 7 – 48°C, but growth is slow at temperatures below 10°C. Nevertheless *Salmonella* is able to survive for extended periods in chilled and frozen foods.

In recent years, Egyptian society is disturbed by the issue of beef meatballs mixed with Donkey meat. Due to the higher price of beef, many sellers took the initiative to replace beef meat with Donkey meat, which is much cheaper than beef. The public unrest occurs, because Donkey meat is not halal for Muslims (Jakim, 2004). So, detection of undisclosed animal species in meat products is important not only for protecting the consumer from fraud but also to guarantee the respect of his religious beliefs and cultural preferences (Bourguiba-Hachemi and Fathallah, 2016).

Therefore, the present research was undertaken to assess the bacteriological aspects and to determine halal authentication of frozen meatballs purchased from different retail markets in Assiut City so that safety warranty of the consumer can be achieved.

## II. Materials And Methods

### Sampling

A total of fifty samples of frozen beef meatballs were collected from different supermarkets and shops in Assiut governorate, Egypt. Each packaged transferred to the laboratory in an ice box to be examined bacteriologically.

### Sample preparation

Ten g of meatball samples were taken into plastic bags under aseptic conditions and 90 ml of trypticase soya broth (TSB) was added and stirred for 3 min after which serial distillations were prepared. The prepared samples were subjected to the following bacteriological examination.

### Bacteriological analysis

Determination of APC using Plate Count Agar (PCA) medium (USDA, 2011). Determination of Staphylococcal count (FDA, 2002). Determination of Pshycrotrophic count: The same technique of APC was used for Pshycrotrophic bacteria except the inoculated agar plates were incubated at 5°C for 7 days. Determination of Anaerobic bacterial count: into tubes of freshly prepared cooked meat broth medium. All tubes were incubated upright position anaerobically in the anaerobic jar at 37°C for 48 h. Then Thioglycollate agar (Britania, BO214006) was inoculated and incubated in an anaerobic jar at 37°C for 24-48 h. BBLGasPak<sup>R</sup> anaerobic chamber with BBL GasPak Co<sub>2</sub> gas packs (Becton Dickinson Microbiology System, Boston, MA) was used to create an anaerobic environment for evaluation (FDA, 2001). Isolation of *Aeromonas* spp. (Ashiru *et al.*, 2011): on trypticase soya broth (Biolife, CP4712) then streaked onto *Aeromonas* selective agar (Biolife, CN0801). Isolation of *Yersinia* spp. (Siriken, 2004): Pre-enrichment (cold) in tripticase soy broth (Biolife, CP4712) then streaked onto Cefsulodin-Irgasan-Novobiocin (CIN) agar (Himedia-M 843). *Listeria* spp. was isolated through methods carried out by Hudson, *et al.* (1994). The suspect *Listeria* colonies were confirmed according to ISO 11290-1 (1996). *Salmonella* isolation conducted as method described by ISO-6579 (2002).

### Determination of adulteration of meatballs with donkey meat by PCR

Ten selected samples of frozen meatballs were subjected to PCR for detection of adulteration with donkey meat.

#### 1. Primer sequences of species specific gene used for PCR identification system:

Application of PCR for identification of cytochrome b (*cyt b*) gene for detection of equine meat was performed essentially by using primers (Pharmacia Biotech) as shown in the following Table:

Target gene	Oligonucleotide sequence (5' → 3')	Product Size (bp)	References
<i>cyt b</i> (F)	5'CCTCCAGCTCCATCAAACATCTCATCTTGATGA AA'3	439	Matsunaga, <i>et al.</i> (1999)
<i>cyt b</i> (R)	5' CTCAGATTCACTCGACGAGGGTAGTA '3		

#### 2. DNA extraction from the examined meatballs (Obrovská *et al.*, 2002):

Mitochondrial DNA was extracted from the samples. Accurately, 500 mg of muscle tissue was pulverized in liquid N<sub>2</sub> and ground to powder, and mixed with 1 ml Lysis buffer-ST 50 mM Tris-HCl (pH-8.0), 10 mM EDTA (pH-8.0), 100 mM NaCl, 150 µg/ml proteinase-K and SDS to make final concentration to 2%. The samples were then incubated overnight at 55°C ensuring homogeneity. Incubated lysate was transferred to a clean tube; equal volume of tris-saturated phenol was added and mixed gently for 10 min, followed by centrifugation for 10 min at 10000 rpm at 15°C. The aqueous phase was then re-extracted once with half the volume of tris-saturated phenol and twice with chloroform: isoamyl alcohol in the ratio of 24:1. The aqueous phase from the final chloroform/isoamyl alcohol extraction was transferred to a new tube and then precipitated by adding 1/20th volume of 3M sodium acetate (pH-5.5) and equal volume of isopropyl alcohol. The precipitated mitochondrial DNA was washed with 70% ethanol, dried and dissolved in 500 µl of TE (10 M Tris-HCl, 1 mM EDTA, pH 8.0). The quality and purity of DNA were checked on agarose gel electrophoresis and quantitation done by UV-spectrophotometry.

#### 3. DNA amplification of *cyt b* gene for equine meat (Jain *et al.*, 2007):

The amplification was performed on a Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany). PCR amplification was performed in a 25 µl reaction volume containing 50 mM KCl, 1.5 mM MgCl<sub>2</sub>,

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200 µM dNTP mix, primer mix (4-60 pmol each), 1.25 unit Taq DNA polymerase and 2 µl (90 ng template DNA). Species-specific oligonucleotide primers consisting of common forward primer SIM for equine meat were adopted. Amplification conditions consisted of an initial denaturation at 94°C followed by 31 cycles at 94°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 30 sec and final extension at 72°C for 10 min using a Thermal Cycler. Molecular size markers were indicated on each gel. The products of PCR amplification were analyzed by agarose gel electrophoresis. Amplified DNA fragments were analyzed by 4% of agarose gel electrophoresis (Applichem, Germany, GmbH) in 5 µl /100 ml TBE buffer stained with ethidium bromide and captured as well as visualized on UV transilluminator. Electrophoretic separation of DNA fragments was done at 100 V for 60 min. A 100 bp plus DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes.

**III. Results And Discussion**

**Table 1. Mean values of microbiological profile (CFU/g) and acceptability of the examined meatballs samples in comparison to ES (2005)**

Microorganisms	Positive samples		Min.	Max.	X <sup>-</sup>	SE	Accepted	Unaccepted	ES (2005)
	No.	%							
APC	44	88	1x10 <sup>3</sup>	2x10 <sup>8</sup>	5x10 <sup>6</sup>	2x10 <sup>6</sup>	88	12	1x10 <sup>6</sup>
Total Staph. count	34	68	<10	8x10 <sup>3</sup>	9x10 <sup>2</sup>	3x10 <sup>2</sup>	76	24	1x10 <sup>2</sup>
Psychrotrophic count	47	94	2x10 <sup>3</sup>	3x10 <sup>7</sup>	1x10 <sup>6</sup>	6x10 <sup>5</sup>	-	-	-
Total anaerobic count	32	64	<10	7x10 <sup>3</sup>	6x10 <sup>2</sup>	2x10 <sup>2</sup>	80	20	1x10 <sup>2</sup>

Min.= Minimum  
 Max.= Maximum  
 X<sup>-</sup>= Mean  
 SE= Standard Error

**Table 2. Incidence of *Aeromonas* spp. in the examined meatballs samples**

Samples	No.	<i>Aeromonas</i> spp.		<i>A. hydrophila</i>		<i>A. caviae</i>		<i>A. sobria</i>	
		No.	%	No.	%	No.	%	No.	%
Frozen meatballs	50	16	32	13	26	1	2	2	4

**Table 3. Bioyping of isolated *Y. enterocolitica* and other *Yersinia* spp. recovered from the examined meatballs samples (no=50)**

Samples	<i>Yersinia</i> spp.		<i>Y. enterocolitica</i>						<i>Y. intermedia</i>	<i>Y. kristensini</i>	<i>Y. fredreksini</i>	<i>Y. massiliensis</i>				
			biotype 1		biotype 4		biotype 5									
	No.	%	No.	%	No.	%	No.	%								
Frozen meatballs	24	48	2	4	4	8	4	8	9	18	3	6	1	2	1	2

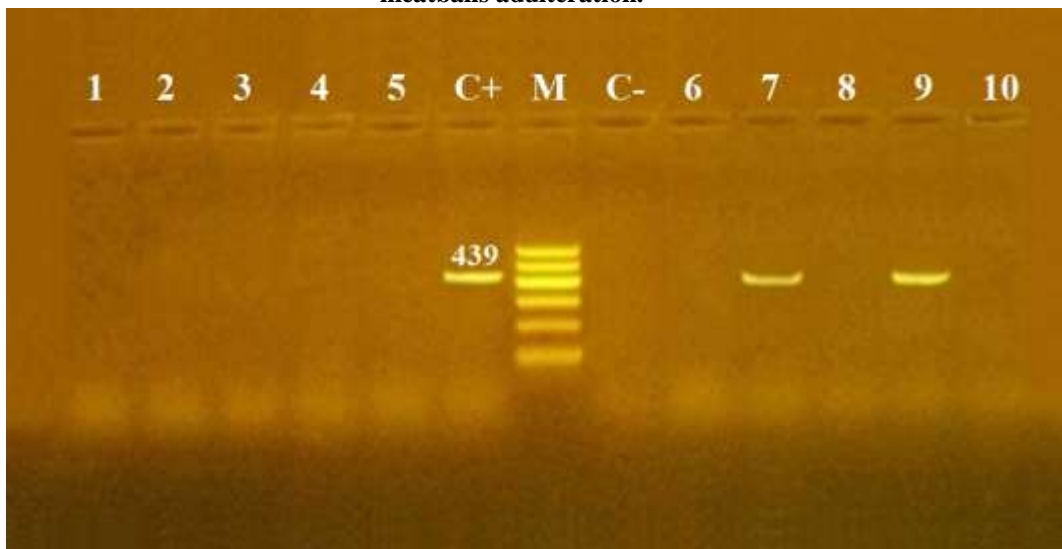
**Table 4. Incidence of different types of *Listeria* spp. in the examined meatballs samples (no=50)**

Samples	<i>Listeria</i> spp.		<i>L. monocytogenes</i>		<i>L. innocua</i>		<i>L. welshimeri</i>		<i>L. ivanovii</i>		<i>L. grayi</i>	
	+ve	%	+ve	%	+ve	%	+ve	%	+ve	%	+ve	%
Frozen meatballs	27	54	6	12	12	24	1	2	0	0	8	16

**Table 5. *Salmonella* serovars from the examined meatballs samples**

Samples	No.	<i>Salmonella</i> spp.		<i>S. Typhimurium</i>		<i>S. Enteritidis</i>	
		No.	%	No.	%	No.	%
Frozen meatballs	50	2	4	1	2	1	2

**Fig. 1. Agarose gel electrophoresis of PCR of *cyt b* gene for equine meat (439 bp) for detection of meatballs adulteration.**



**Lane M:** 100 bp ladder as molecular size DNA marker.  
**Lane C+:** Control positive for *cyt b* gene of equine meat at 439 bp.  
**Lane C-:** Control negative.  
**Lanes 7 & 9:** Positive equine meat.  
**Lanes 1, 2, 3, 4, 5, 6, 8 & 10:** Negative equine meat.

The bacteriological status of the examined frozen meatballs recorded in Table 1 pinpoint that the examined samples had APC varied from  $1 \times 10^3$  to  $2 \times 10^8$  with a mean value of  $5 \times 10^6$  cfu/g. According to the safe permissible limit stipulated by ES (2005) No (1973-2005) for APC in frozen meatballs (which not exceed  $10^6$  cfu /g), it was indicated that 88% of 50 meatballs samples investigated were suitable to the standards where 12% were not (Table 1). In a related study, Bouzid et al. (2015) recorded lower counts ( $5 \times 10^4$  cfu/g). The high rate of APC in the examined frozen meatballs may be due to contaminant materials (ex: packaging), bad conditions of storage and source of untreated water (Bouzid *et al.*, 2015). Furthermore, high initial numbers of different groups of meatballs can be attributed to the grinding process, which contributes to the increase of total viable counts of meat (Skandamis and Nychas, 2001). Generally, higher APC usually relates to poorer quality and a reduced shelf-life (Siriken, 2004).

Presented data in Table (1) revealed that *Staph. aureus* was detected in 34 (68%) samples and total *Staph. aureus* count varied from  $<10$  to  $8 \times 10^3$  with a mean value of  $9 \times 10^2$  cfu/g in the examined meatballs. Coagulase positive Staphylococcus is considered as an indicator of poor hygiene/handling procedures. The results indicated that 76% of samples fell into the guideline categories of "Accepted" and 24% classified as "Unaccepted" for positive Staphylococci according to Egyptian Standards (ES, 2005).

According to the results recorded in Table 1, it is evident that psychrotrophic bacteria were existed in the examined frozen meatballs samples in number varied from  $2 \times 10^3$  to  $3 \times 10^7$  with a mean value of  $1 \times 10^6$  cfu/g. Egyptian standards (ES, 2005) don't specify regulation for psychrotrophic count in frozen meatballs. The ICMSF (1986) set a standard of  $10^6$  to  $10^7$  CFU/g as the end of shelf live in meats for aerobic psychrotrophic microorganisms. Based on this, in the present study the limit set as the end of shelf life in beef meatballs was  $10^6$  CFU/g. The 84% of frozen meat samples examined according to the ICMSF (1986) for psychrotrophic bacteria were found to be suitable to the standards whereas the 16% weren't (Table 1). Although aerobic psychrotrophic bacteria are generally non pathogenic to man, they are important to the hygienists because they are almost common cause of refrigerated food spoilage.

Total anaerobic bacterial count in the examined meatballs varied from  $<10$  to  $7 \times 10^3$  with a mean value of  $6 \times 10^2$  cfu/g. According to Egyptian Standards (ES, 2005) out of 50 examined frozen meatballs samples, 10 samples (20%) were considered "Unaccepted" (unfit for human consumption), while 40 samples (80%) were classified as "Accepted" (Table 1). In vacuum-packaged meat, psychrotrophic facultative anaerobic and anaerobic bacteria can grow and cause different types of spoilage. These foods are expected to have a long shelf life, some up to 100 days. Thus, even an initial population of anaerobic or facultative anaerobic psychrophilic / psychrotrophic bacteria of  $10^1$  / g or less can multiply and achieve numbers that could cause deterioration or make the food insecure (Ray, 2000).

In general, Meatball (kofta) is prepared by mince which is a quite suitable environment for microbial growth. The quality of mince and other additives used in preparation of meatballs determines its quality. Studies on the microbiological quality of food shows that meatballs is a medium rich in nutrients required for the growth of pathogenic microorganisms (Norman and Gravani, 2006).

As shown in Table 2, *Aeromonas* spp. was found in 16 out of 50 samples of frozen meatballs with incidence of 32%, in which 13 (26%) were classified as *A. hydrophila*, 2 (4%) as *A. sobria* and 1 (2%) as *A. caviae*. On the contrary, higher incidence (46.4%) were recorded by Gobat and Jemmi (1993) who reported that out of 28 samples of frozen minced meat examined, 13 were contaminated with *Aeromonas* spp. Most *Aeromonas* spp. isolates are psychrotrophic and can grow and produce toxins in refrigerated conditions (Praveen *et al.*, 2016) indicating that refrigeration cannot be effective enough to control this pathogen (Kirov, 1993).

Regarding *Yersinia*, It could be isolated with percent 48% and the most commonly found species was *Y. enterocolitica*, which was isolated from 20% of all collected samples, followed by *Y. intermedia* (18%), *Y. kristensenii* (6%) *Y. frederiksenii* (2%) and *Y. massiliensis* (2%). The most common *Y. enterocolitica* bioserotype was biotype 4 and biotype 5 (8%) followed by *Y. enterocolitica* biotype 1 (4%) as presented in Table (3). In a related study conducted in Turkey by Siriken (2004), *Yersinia* spp. were detected in 20 of 61 (32.8%) samples of ground beef. Among the positive samples, 17 (27.9%) were identified as *Y. enterocolitica*, 2 (3.3%) as *Y. intermedia* and 1 (1.6%) as *Y. frederiksenii*.

*Yersinia enterocolitica* can grow in properly refrigerated foods and survive in frozen foods for long periods; infection is most often acquired by eating contaminated food, especially raw or undercooked products (Mauro *et al.*, 2008). Because of its psychrotrophic nature, a cold temperature does not prevent growth, but only retards it.

The results in Table 4 showed that out of total 50 samples, 27 (54%) were found to be contaminated with *Listeria* spp. the isolation rate was as follows: 6 *L. monocytogenes* (12%), 12 *L. innocui* (24%), 8 *L. grayi* (16%) and 1 *L. welshimeri* (2%). In contrast, Pao and Ettinger (2009) reported that 29% of frozen beef patties sold in Virginia, USA were contaminated with *L. monocytogenes*. Higher incidence (75%) of *L. monocytogenes* in imported frozen beef was recorded by Hassan, *et al.* (2001). Because of its ability to survive and proliferate at refrigeration temperature, *L. monocytogenes* may cause disease through frozen foods (Schillinger *et al.*, 1991). Some authors report that method of food packaging (vacuum packing or packaging at high CO<sub>2</sub> concentration) does not influence the growth of *L. monocytogenes* and *Y. enterocolitica*, which grow very well in both conditions (Barakat and Harris, 1999; Nissen *et al.*, 2000). Moreover, Rhoades, *et al.* (2009) argued that extra handling and ingredients during processing of meat products give rise to the *Listeria* contamination.

In this study, *Salmonella* spp. were recovered from 4 % of the examined frozen meatballs samples. *S. Enteritidis* and *S. Typhimurium* were identified as the predominant serovars at the rates of 2% for each (Table 5). On the contrary, contamination with salmonella reached 20 % of frozen minced beef samples in a related study conducted in Assiut city (Hassanein *et al.*, 2011). Contamination of beef meatballs with salmonella is regarded the major problem in food hygiene. Furthermore, resistance of salmonella to freezing is well known in the literature; in the stored meatballs for 21 days at -20°C (Roberts *et al.*, 1980). *Salmonella* is also a common type of bacteria that contaminates refrigerated foods and may cause serious illness. Certain strains or serotypes of *Salmonella* spp. may also grow slowly under refrigeration conditions.

#### **Adulteration with donkey meat**

In the current study, the presence of donkey meat was determined in 2 samples of frozen meatballs (20%) as demonstrated in Fig. 1. Donkey meat is not halal for Muslims, so that the presence of Donkey meat in any food is a crucial issue. This is likely related to the fact that the Islamic religion bans the consumption of both pork and horsemeat.

Comparatively, lower incidence (7%) of tested 105 samples of imported raw and processed food products marketed in the Arabian Gulf region showed the presence of horse DNA (Bourguiba-Hachemi and Fathallah, 2016). Also, Kyřová, *et al.* (2015) recorded the presence of horse meat in 7 samples (9.2%) of beef meat products. Kane and Hellberg (2016) analysed 48 samples of ground meat products sold on the U.S. commercial market for potential mislabeling. Ten samples were found to be mislabeled. Horse meat which was

illegal to sell on the U.S. commercial market, detected in two of the samples acquired from on-line specialty meat distributors.

On the other hand, in 2013, in Istanbul, Ozpinar, *et al.* (2013) sampled 73 meat products including meatballs. Real-time PCR as well as DNA based LCD array showed that out of 73 samples, 39 (53.4%) contained undeclared meat species. The products with the highest prevalence of undeclared meats were meatballs. No horse DNA was detected in any of the samples tested.

Meat adulteration in ground and comminuted products has been a wide spread problem in retail markets. The replacement of beef meatballs with donkey's meat can be categorized, as adulteration that appears to be due to reasons such as intentional mixing of lower-cost (inferior and cheaper) meat species into higher cost products (Bourguiba-Hachemi and Fathallah, 2016).

The adulteration/substitution of meat has always been a concern for various reasons such as public health, religious purposes (Islam don't permit the consumption of donkey meat), wholesomeness, and unhealthy competition in meat market. Consumer should be protected from these malicious practices of meat adulterations by quick, precise, and specific identification of meat animal species (Kumar *et al.*, 2015). Thus, several analytical techniques such as PCR have been suggested for the identification of meat species either in individual or in mixed samples to protect consumers from the fraudulent and bad habits of marketing (Farag *et al.*, 2015).

Generally, the usual reasons of meat adulteration are economic reasons. The high price of meat and passiveness of consumer safety warranty further encourage the Small and Medium-sized Enterprises (SMEs) and traditional market sellers to substitute beef with other kind of meat in the making of meatballs (Roostita *et al.*, 2014).

#### IV. Conclusion

In conclusion, this study showed that some frozen meatballs samples analyzed presented unsatisfactory bacteriological quality according to the Egyptian Standards. The status of harboring some foodborne pathogenic bacteria, particularly *A. hydrophila*, *Y. enterocolitica*, *L. monocytogenes*, *S. Typhimurium* and *S. Enteritidis* raise concerns regarding the hygienic quality of raw material and the handling process of frozen meatballs and its potential hazards for public health. Moreover, there was adulteration of frozen beef meatballs, in which cheaper, inferior quality and illegal donkey meat is mixed into beef.

While there is still no microbial guideline value for psychrotrophic bacteria of Egyptian frozen meatballs (kofta), the adoption of the generally accepted psychrotrophic bacteria guideline value of  $<10^6$  cfu/g of food sample may be appropriately used until more comprehensive psychrotrophic bacteria guideline values for Egyptian frozen meatballs are established.

Also, it was recommended that applying PCR for fast, easy, and reliable control of adulterated meat products. This technique could prove useful for inspection programs intended to assess the species identity of meat products in Egypt.

Finally, to minimize contamination of meatballs, GHP or GMP and HACCP systems that are specific to the control of the pathogenic and spoilage bacteria at all stages of manufacture, storage, transport and retail could be applied.

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