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The occurrence and significance of *Pseudomonas aeruginosa* isolated from some meat products in Sohag city

Refaat Mahmoud Farghaly[1], Nahed Mahmoud Abdel-Aziz[1], Mohammed Hussain Mohammed[2*]

[1]Food Hygiene Department, Faculty of Veterinary Medicine, Sohag University, Egypt, [2]Food Hygiene Department, Animal Health Research Institute, Agriculture Research Center, Egypt.

Abstract

Contamination of meat and meat products with pathogenic and spoilage microorganisms is one of the most important challenges facing the meat industry that results in a range of human health problems and economic losses. This work aimed to identify the occurrence of *Pseudomonas* spp. especially *Pseudomonas aeruginosa* (*P. aeruginosa*) in some processed and ready-to-eat meat products in Sohag governorate. A total of 200 random meat product samples; minced beef meat, luncheon, burger, and sausage (50 of each) were purchased from different markets in Sohag governorate, Egypt over a period of 12 months from November 2020 to October 2021. *Pseudomonas* spp. was suspected in 32 (15%) of the meat products examined samples using the colony morphology on Cetrimide agar, represented as follows; 30%, 18%, 6%, and 10% in minced beef meat, luncheon burger, and sausage, respectively. Using the morphological and biochemical methods, *P. aeruginosa* was suspected in 12 isolates (37.5%) with an incidence of 12/200 (6%) of the total examined samples. The PCR results revealed that only 8/12 (66.7%) of the suspected isolates encoded the *16S rDNA* gene of *P. aeruginosa* with an incidence of 4% of the total examined samples, 4 (50%) of which were detected in the minced beef meat samples, 2 (25%) in the sausage samples while in the luncheon and burger *P. aeruginosa* was identified in only 1 sample (12.5%) for each.

Keywords:

16S rDNA gene, meat products, PCR, *Pseudomonas aeruginosa*.

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*Corresponding Author: M. Hussain Mohammed E-mail: mohammed.hussein@vet.sohag.edu.eg

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Introduction

Meat is a rich source of nutrients, including proteins, essential fatty acids, vitamins, iron, and other minerals. In Egypt, the meat products such as sausage, beef burgers, and luncheons are gaining popularity because they represent high nutritive value, have good taste, and are quickly prepared, easily served meat meals, also their reasonable prices, and able to alleviate the scarcity of high-cost fresh meat is out of reach for a large number of low-income families. On the other hand, meat and its products are highly susceptible to spoilage by microorganisms and could significantly raise the risk of infection and death, particularly in developing countries (Wickramasinghe et al. 2019; EFSA, 2016; Shawish and Al-Humam, 2016; Shawish and Tarabees, 2017; Lianou et al. 2017).

Meat products (such as minced meat, sausages, burgers, and pastırma) often contain different combinations of salt, additives, and spices. Several types of foodborne and spoilage microorganisms are commonly found in raw meat before the slaughter process, and they can invade the meat during the handling, carriage, packing, or meat processing, particularly for fresh products that are not subjected to heat treatments or other sanitization during their preparation. Microbial growth may occur post-processing, during storage, and during distribution which poses a threat to the safety of meat products and can influence public health worldwide. *Pseudomonas*, *Brochothrix, Acinetobacter,* and *Shewanella*, are among the important microbes contaminating meat and meat products and have been recovered from frozen meat. (Papadochristopoulos et al., 2021; Hazaa et al. 2019; Wickramasinghe et al., 2019; Doulgeraki et al. 2012; Al-Mutairi., 2011).

The genus *Pseudomonas* contains more than 140 species of Gram-negative, aerobic bacilli measuring 0.5 to 0.8, µm by 1.5 to 3.0 µm, and motile by a single polar flagellum most of which are saprophytic. While, more than 25 species are opportunistic human pathogens except for *P. mallei*, and *P. pseudomallei* cause specific human diseases: glanders and melioidosis. They are commonly found in soil, water, plants, and hospital environments. *P. aeruginosa* and *P. maltophilia* are responsible for approximately 80 % of pseudomonads recovered from clinical specimens. *P. aeruginosa* is receiving the most attention because it is the most frequently involved in human disease (Bhargava, 2020; Chatterjee et al., 2017; Moradali et al., 2017).

Of the genus *Pseudomonas*, *P. fluorescens*, *P. fragi*, *P. lundensis*, *P. migulae*, and *P. putida* are frequent species found in chilled meat. *P. aeruginosa* grows rapidly on the food surface leading to the formation of oxidized products and mucous substances. It is one of the leading foodborne pathogens, with important concerns in food safety since it is involved in food spoilage, foodborne infection and antimicrobial resistance in humans, biofilms production in water systems, and food contact surfaces. *Pseudomonas* is an adaptable microorganism in the food processing environment (Wickramasinghe et al., 2019; Bantawa et al. 2018; Stellato et al. 2017).

It has been isolated from food in hospitals, canteens, and schools (Urgancı et al., 2022; Xu et al., 2019). Also, it was isolated from processed food indicating that the food was either undercooked or subjected to post-cooking contamination, which may have occurred due to improper handling, prolonged storage, inappropriate chilling, or several other conditions (Mengistu et al., 2020).

Even with the use of modern methods of meat preservation, microbial contamination of meat and meat products remains a major threat. Psychrotrophic

(cold-tolerant) *Pseudomonas* species are one of the significant food spoilage microbes that lead to putrefaction in chilled and refrigerated meat, chicken meat, eggs, fish, shellfish, dairy products, and seafood as well as thawed frozen meat and thawed and refrozen meat under aerobic conditions. Water systems can be the source of contamination in the food and beverage industry. They cause discoloration and slime production stickiness, and rancidity on the meat surface. Among these species, *P. aeruginosa* is the most important species in the food industry. It limits the shelf life of meat stored in the cold by causing slipperiness and unpleasant odors on the surface. Colony and bacterial membranous layer formation in meat facilitate their identification (Caldera et al., 2016; Raposo et al., 2017; Franzetti and Scarpellini, 2007; Akan, 2009; Motoyama et al., 2010; Siegrist, 2007).

Although the microbial spoilage caused by *Pseudomonas* affects the appearance, structure, and sensory properties of foods, it can also be the cause of serious and fatal diseases. In addition to posing a great risk for humans and animals because it frequently causes secondary infections (Høiby et al., 2010; Lopez et al., 2015). *P. aeruginosa* are opportunistic pathogens that might result in mild to severe infections if they reach sterile sites of the body, such as blood, lungs, stomach, urinary tract, tendons, pressure sores, wounds, and burns In addition, the digestive tract infection with signs of headache, diarrhea, and fever which is often a sign of severe *Pseudomonas* infection (Sapkota, 2021).

P. aeruginosa can grow at 4-42°C and pH range of 5.6 and 8.0. It is resistant to high salt and dye concentrations, weak antiseptics, and many antibiotics and is characterized by its great ability to withstand difficult environmental conditions, which leads to the prevention of the growth of other microorganisms (Elbehiry et al., 2022).

Molecular methods, including analysis of 16S rRNA gene sequences, and other highly conserved "housekeeping" genes, have accelerated the step of taxonomic reorganization (Anzai et al.,2000; Palleroni, 2008).

Materials and methods

Sample collection:

A total of 200 meat product samples including; minced beef meat, luncheon, burger, and sausage (50 for each). Samples were collected from different shops and supermarkets in Sohag city, over a period of 12 months from November 2020 to October 2021. Each sample was collected in a separate sterile plastic bag and identified and then directly transferred to the laboratory in an ice- box within one hour to be examined bacteriologically for the presence of *P. aeruginosa.*

Sample preparation (AOAC, 1990)

10 g of each type of products samples were homogenized with 90 ml sterile buffered peptone water, then incubated at 37^{O}C for 24 hours.

Phenotypic identification of *Pseudomonas* species (Quinn et al., 2002)

A loopful of inoculated nutrient broth was streaked firstly onto the surface of Cetrimide agar then one colony on nutrient agar and blood agar for purification and conformation.

Microscopy and Gram stain properties

All purified strains were sub-cultured again for Microscopic identification of *Pseudomonas* species using the Gram staining technique (Becerra et al. 2016).

Enzymatic activities

The enzymatic activities of *Pseudomonas* species were evaluated using certain biochemical tests, including oxidase, citrate utilization, and indole tests (LaBauve and Wargo 2012). The positive

oxidase activity is indicated by the appearance of a purple color. The capacity of *Pseudomonas* spp. to utilize citrate as a source of energy was carried out by streaking a freshly purified colony onto a Simmons citrate agar (OXOID, CM0155) slant and incubated at 37 °C for 5–7 successive days. Citrate utilization was indicated by the appearance of a sky-blue color. Indole test is performed by inoculation of the suspected organism into1% peptone water (OXOID, CM0509), then incubating at 37 °C for a couple of days, thereafter a few drops of xylene, followed by 0.5 ml of Kovac's indole reagent were added, a red-violet color appeared, indicating positive results, while a yellow color indicates negative results.

Molecular identification of *P. aeruginosa* isolated from meat products:

DNA extraction

DNAs of *P. aeruginosa* suspected strains were extracted using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) following the manufacturer's recommendations. Into the bottom of a 1.5 ml microcentrifuge tube, 200 µl of the bacterial suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56°C for 10 min. After incubation, 200 µl of ethanol (96%) was added to the lysate and mixed again by pulse vortex for 15 seconds. The mixture was carefully applied to the QIAamp mini spin column (in a 2ml collecting tube) the cap was closed, and centrifugated at 8000 rpm for 1 min. The nucleic acid was eluted with 100 µl of AE elution buffer provided in the kit. The QIAamp mini spin column was incubated at room temperature (15-25˚C) for 1 min, and then centrifugated at 8000 rpm for 1 min.

Polymerase chain reaction (PCR) amplification using oligonucleotide primers

Primers used were supplied from Metabion (Germany), as shown in Table 1. in a 25 µl PCR tube, the following mixture was added; 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of the forward primer (20 pmol), 1 µl of the Reverse primer (20 pmol). 5 µl of template DNA and complete up to 25 µl with nuclease-free water (5.5 µl), and. PCR tubes were cycled using an applied biosystem thermal cycler and the PCR conditions were performed according to Spilker et al.(2004) as the following; initial denaturation at 95˚C for 5 minutes, 35 cycles at 94˚C for 30 sec., then 52˚C for 30 sec., 72˚ C for 50 sec. and a final extension step for 10 minutes 72˚C.

Analysis of the PCR products

The products of PCR were examined in 1.5 % agarose gel electrophoresis (Applichem, Germany) with ethidium bromide and run in 1× TBE buffer at room temperature using gradients of 1-5V/cm of the tank length. The wells were loaded with the PCR products (15 µl) and 6 µl of a 100 bp DNA Ladder (Qiagen, Germany, GmbH) was loaded into the gel slot to determine the product sizes. The run was stopped after about 30 min, and the gel was transferred to the UV cabinet. The gel was photographed by a light transilluminator (Biometra) using a gel documentation system (Alpha Innotech, Biometra). *P. aeruginosa* (ATCC 27853) was used as positive control and distilled water (Merck, Germany) was used as a negative control in all PCR reactions.

Table 1. Oligonucleotide primers sequences for *16S rDNA gene of P. aeruginosa*

Gene	Primer sequence (5'-3')	Product size	Reference
16S rDNA	GGGGGATCTTCGGACCTCA	956 bp	Spilker et al. (2004)
	TCCTTAGAGTGCCACCCG		

Results

Identification of *P. aeruginosa* (Quinn et al., 2002):

Fig. 1 and Table 2 showed that the culture characters of *P. aeruginosa* on Cetrimide agar medium were observed as opaque green colonies with grape-like odor, non-lactose fermenters on MacConkey agar, and showed hemolysis on blood agar. Gram-negative motile rods showed a positive reaction for oxidase, catalase, citrate, and gelatin liquefaction.

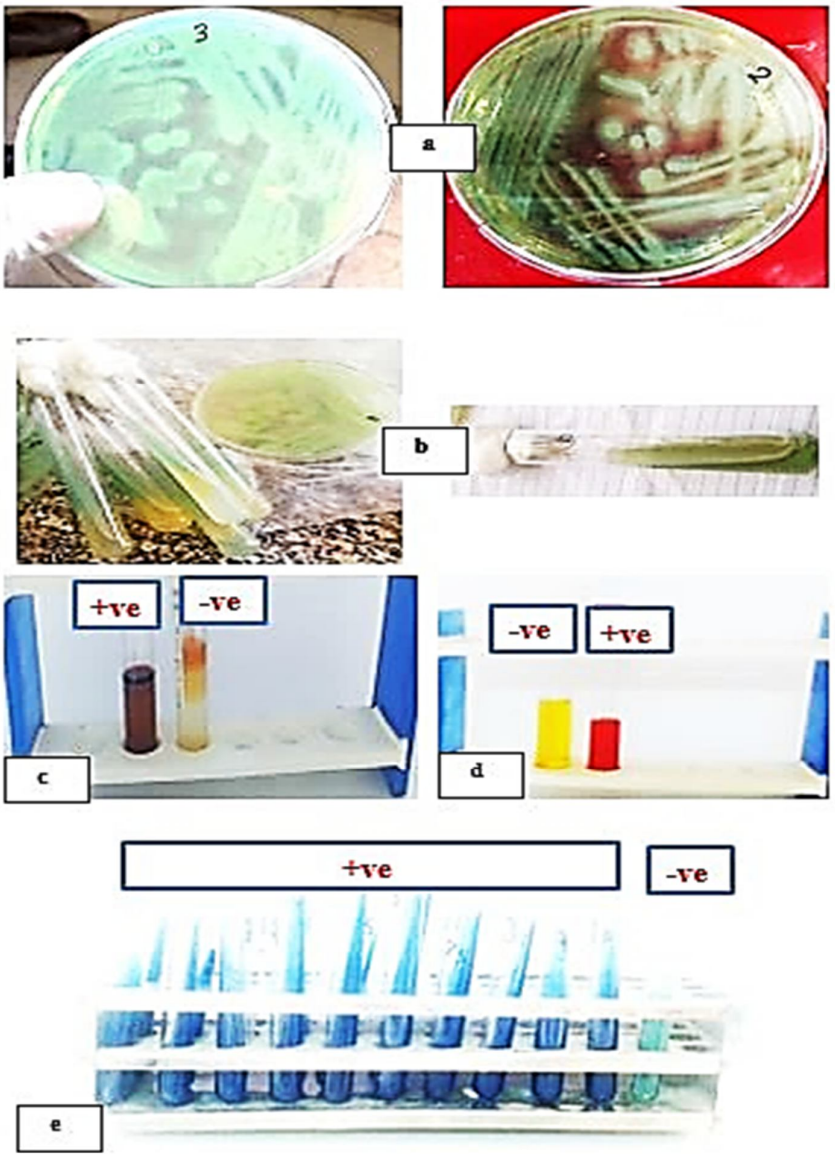

Fig. 1. Morphological and biochemical reactions of *P. aeruginosa* isolated from some meat products: (a) colony characters and pigment production on Cetrimide agar. (b) pigmentation of nutrient agar plates and nutrient agar slope. (c) Indole test; tube on the left showed +ve reaction (pink ring formation) and tube on the right -ve indole for *P.aerugenosa*. (d) Methyle red test; tube on the left showed -ve reaction (yellow color) for *P.aeruginosa* and tube on the right +ve pink color.(e) Citrate test; tubes on the left showed +ve reaction (blue color) for *P.aerugenosa* and tube on the right -ve (no change of green color).

Table 2. Morphological and biochemical identification of *P. aeruginosa* isolated from some meat products

Samples	Tested isolates	Gram -ve rods/ Motile	Oxidase +ve	Indole -ve	MR -ve	Citrate +ve	Urease -ve	Gelatin hydrolysis +ve	Pigment
Minced meat	15	12	4	9	6	4	4	4	6
Luncheon	9	8	5	6	5	4	4	4	3
Burger	3	3	3	3	3	2	2	2	1
Sausage	5	5	4	4	3	2	2	2	2
Total	32	28	17	22	17	12	12	12	12

The incidence of *P. aeruginosa* isolated from some meat products:

As shown in Fig. 2 and Table 3, *Pseudomonas* spp. was identified in 32 (16%) of the examined meat product samples with the highest incidence in minced beef meat 30 % followed by 18 %, 10 %, and 6 % in luncheon, sausage, and burger, respectively.

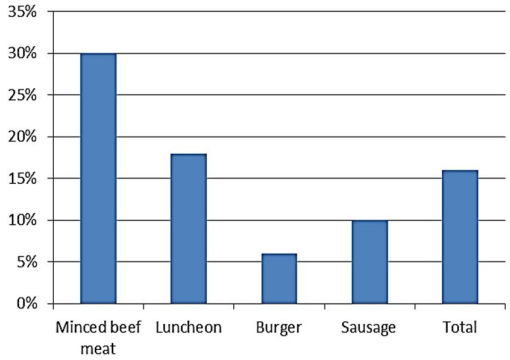

Fig. 2. The incidence of *Pseudomonas* species isolated from some meat products.

Table 3. The incidence of *Pseudomonas* spp. isolated from some meat products

Meat product samples	No. of examined Samples	Positive samples	
		No	%
Minced beef meat	**50**	**15**	**30**
Luncheon	**50**	**9**	**18**
Burger	**50**	**3**	**6**
Sausage	**50**	**5**	**10**
Total	**200**	**32**	**16**

Identification of *P. aeruginosa* isolated from meat products using c-PCR:

Table 4 and Fig. 3 and Fig. 4 revealed the incidence of PCR- confirmed strains of *P. aeruginosa* 8 of 12 PCR-tested isolates were confirmed as *P. aeruginosa* using the *16S rDNA* gene-specific primer (the sequence as described by Spilker et al.(2004) was shown in Table 1. *P. aeruginosa* was detected in 8 samples with an incidence of 4% of the total examined meat product samples; the highest incidence was for the minced beef meat 4(50%) of the confirmed isolates followed by sausage 2 isolates (25%) while Luncheon and burger showed an incidence of 12.5% for each.

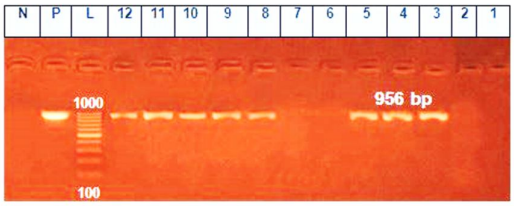

Fig. 3. PCR results for *P. aeruginosa 16S rDNA* gene, Lane L: 100 bp DNA marker; Lane P: Control positive; Lane N: Control negative; Lanes 1, 2: negative results for *16S rDNA* gene of *P. aeruginosa* strains isolated from luncheon. Lanes 3: positive results for *16S rDNA* gene of *P. aeruginosa* strains isolated from luncheon. Lanes 4& 5: positive results for *16S rDNA* gene of *P. aeruginosa* strains isolated from sausage. Lanes 6 &7: negative results for *16S rDNA* gene of *P. aeruginosa* strains isolated from minced meat. Lanes 8, 9, 10 & 11: positive results for *16S rDNA* gene of *P. aeruginosa* strains isolated from minced meat. Lane 12: positive results for *16S rDNA* gene of *P. aeruginosa* strains isolated from burger.

Table 4. PCR results of 16S rDNA gene of *P. aeruginosa* isolated from meat products

Sample	Tested isolates	+ve isolates	% *
Burger	**1**	**1**	**12.5**
Luncheon	**3**	**1**	**25**
Sausage	**2**	**2**	**25**
Minced meat	**6**	**4**	**50**
Total	**12/200**	**8/200**	**8/12**
Total %	**6%**	**4%**	**67.7**

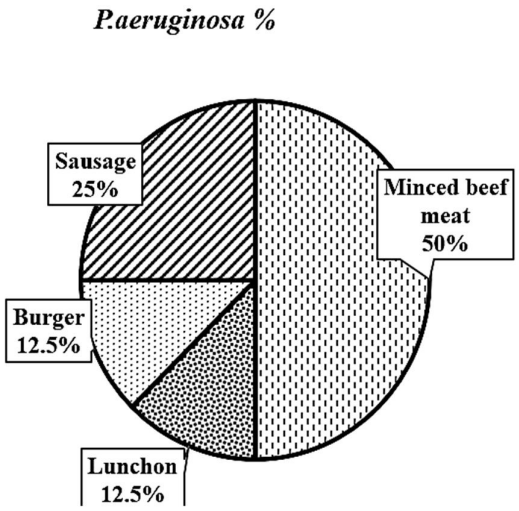

Fig. 4. The incidence of PCR- confirmed *P. aeruginosa* isolated from some meat products in Sohag governorate.

Discussion

The prevalence of foodborne illness and food-borne pathogens was increasing day to day life. Due to food products getting contaminated while handling, harvesting, and processing equipment and transportation. Food products can be polluted at any point along the food chain, from cultivation or manufacture to consumer consumption (Virupakshaiah and Hemalata, 2016).

Minced beef is widely used all over the world as a basic ingredient of various food preparations such as; burger patty formulations and sausage (Djordjević et al., 2018).

Table. 3 and Fig. 2 showed that *Pseudomonas* spp. was detected in 15 out of 50 examined minced beef meat with an incidence of 30 %. Our findings are consistent with a previous study by (Samson et al., 2000), who found *Pseudomonas* spp. (*P. aeruginosa*, *P. fluorescens*, and *P. putida*) in various meat samples. The grinding process causes the leakage of tissue fluids that represent a rich nutritional component for a wide range of microorganisms, promoting a rapid microbial growth, also when it is immediately wrapped and chilled (Djordjević et al., 2018).

The results of morphological and microscopical identification of the bacterial isolates showed that out of 50 selected minced meat samples, only 15 strains have been predicted to be *Pseudomonas* spp., a similar result (30%) was also reported by (Qasim et al., 2019) in minced meat from the local markets in Baghdad governorate. Higher incidences were reported by Algayar et al., (2014) and Khalafallah et al. (2020) who detected *Pseudomonas* spp. in 32 of 45 tested samples (71.11%), and 40/50 (80%) of minced meat and frozen minced meat samples, respectively. A lower incidence was reported by Tassew et al., (2010) isolated *Pseudomonas* in 9 samples (5.5%).

Our results showed that 9 (18 %) luncheon samples harbored *Pseudomonas* spp. This is corresponding with (Ahmed et al., 2017) who isolated *Pseudomonas* spp. from luncheon with an incidence of

18.33%. While in the burger samples the presumptive incidence of *Pseudomonas* spp. was 6%. A lower incidence was reported by Ahmed et al. (2017) and Sofy et al. (2017) who found *Pseudomonas* spp. in 2% and 1%, respectively. While Algayar et al., (2014) isolated *Pseudomonas* by a percentage of (26.67%). On the other side, 10 % of the sausage samples were positive for *Pseudomonas* spp., this is higher than 8.3% and 5%reported by (Ahmed et al., 2017) and Sofy et al. (2017) in burger samples.

P. aeruginosa has 3 types of colonies; a large 2-3 mm in diameter, round, matt surface, raised in the middle, white in color and fluorescent, with blue-green pigments spread all over the medium result in the fried egg appearance; a smaller, fluffy, convex and irregular colonies, mostly isolated from natural sources and the R-type colonies, which have a mucoid appearance. Cultures of *P. aeruginosa* have a characteristic sweet, fruity, grape or trimethyl amine-like odor, the occurrence of metallic patches, and the tendency to form localized swarming from the edge of the colony (Virupakshaiah and Hemalata, 2016; Sapkota, 2021).

The results of morphological and biochemical identification are shown in Table. 3 and Fig. 2 illustrated that out of the 32 *Pseudomonas* suspected isolates, there were 12 (37.5%) were positive for pigment production, as the following; 6 (40 %), 3 (33.3 %), 1 (33.3 %), and 2 (40 %) of minced beef meat, luncheon, burger, and sausage, respectively. A higher result was reported by Sidhom (2007) who found that (41.1%) of *P. aeruginosa* isolates were pigment producers.

The conventional culture method for detecting *P. aeruginosa* in food is labor-intensive, expensive, and time-consuming. In addition depending on the green pigment produced by *P. aeruginosa* strains which will lead to wrong judgment in actual inspection because some strains of *P. aeruginosa* do not produce pigment, while *P. fluorescens* produces pigment similar to that of *P. aeruginosa* (chon et al. 2021; Junaid et al., 2021).

The results in Table 2, and Fig. 1 showed that out of 32 isolates from different meat products 12 (37.5%) isolates were suspected to be *P.aeruginosa.* A higher incidence 15 of 50 minced meat samples (30 %) was also reported by (Qasim et al., 2019) from the local markets in Baghdad governorate, also Virupakshaiah and Hemalata (2016) who identified *P. aeruginosa* that isolates from food. An incidence was reported by Elgayar et al., (2014) who detected *Pseudomonas* spp. in 32 of 45 tested samples (71.11%), and a lower incidence was reported by Tassew et al*.,* (2010) isolated *Pseudomonas* in 9 samples (5.5%). On contrast, Al-Naggar (2000) who isolated different *Pseudomonas* spp. from examined meat products such as beef burger, luncheon and Pasterma in variable incidences however, *P. aeruginosa* could not be detected.

The results of the amplification of the *P.aeruginosa 16S rDNA* gene-specific primers using the PCR technique are shown in Table 4, and Fig. 3,4, and 8 of the 12 examined isolates were confirmed as *P.aeruginosa* with an incidence of 66.7 %. This result is similar to those reported by Ibrahim et al.(2016) who indicated that using the PCR technique, all examined meat samples by conventional method show positive results by both PCR and conventional method for *Pseudomonas* species on another hand, a culture-identified *P. aeruginosa* isolate failed to be detected by the PCR technique.

Conclusion

P. aeruginosa is one of the most polluting microbes in meat products, to reduce the contamination of meat and meat products, proper detection and treatment are critical. Most *Pseudomonas* species have

the capacity to release different thermotolerant proteolytic and lipolytic enzymes that can seriously decrease the quality and shelf life of meat and its products. *P. aeruginosa* is the most important species due to its role as an opportunistic pathogen to humans and implicated with several disease conditions. Therefore, this study concentrated on identification and differentiation of *P. aeruginosa* using conventional cultural methods and molecular methods. The presence of *P. aeruginosa* contamination in some processed and ready to eat meat products even in such a low rate should be paid more attention to ensure the required safety levels and diminish the bad impacts on public health along with reducing product recall and spoilage. Application of good hygienic practice and HACCP plans throughout the production procedure can improve the product quality and prevent microbial spoilage and foodborne of diseases.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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