1. INTRODUCTION

Meat and meat products are very important foods for human being due to their palatability and high nutritional value. They are important sources for protein, with good balance of essential amino acids, minerals, B-complex vitamins and other nutrients (Hu et al., 2001). The increased in demand for frozen meat arises resulting from Egypt’s new policy and development of international trade of foods to provide frozen meat at reduced affordable prices (GOVS, 2004). Thus, imported beef may be the main chance to bridge the gap between the consumption and domestic supply. Pseudomonas species are considered the most important Psychrotrophic microorganisms causing meat spoilage (Lu and Bi, 2007). Psychrotrophic bacteria grow well at or below 7°C and have their optimum temperature for growth between 20-30°C. Pseudomonas species are opportunistic Gram-negative pathogens that cause outbreak upon the change in normal environmental conditions to unhygienic one (Roberts, 2001). Massive use of antibiotics in food producing animals can develop antibiotic resistance in the intestinal bacteria. Further, the antibiotic resistance genes may transfer bacteria to disease causing bacteria resulting in antibiotic resistant infections for humans (Serrano, 2005).

Therefore, this study aimed to determine the incidence of pseudomonas species in frozen meat (cubic and minced) through estimation of Psychrotrophic counts, isolation and identification of Pseudomonas species in frozen meat. In addition, determination of their antibiotic susceptibility was kept in consideration.

2. MATERIAL AND METHODS

2.1. Collection of meat samples:
A total of 100 random samples of imported frozen meat (n=100) (50 cubic meat and 50 minced meat) were randomly collected from different retail shops and supermarkets in El-Menoufia Governorate, Egypt to be bacteriologically examined for detection of phenotypic and genotypic of Pseudomonas species.

2.2. Preparation of samples:
The examined samples were thawed at refrigerator under complete aseptic conditions. After that, 25 g of each examined sample were weighed aseptically by sterile scissor and forceps from the central part after surface sterilization by hot spatula then placed into separate sterile polyethylene bag to which, 225 ml of 0.1% sterile buffered peptone water were aseptically added to the content of bag. Thereafter, each sample was homogenized in a blender at 2000 rpm for 1-2 minutes to provide a homogenate of 1/10 dilution. One ml from the original dilution was transferred by sterile pipette to another sterile tube having 9ml sterile buffered peptone water 0.1% then mixed well to make the next dilution, to make tenfold decimal serial dilutions.

2.3. Determination of Psychrotrophic count (FAO, 1992):
Accurately 0.1 ml from each of the previously prepared serial dilution was spread over dry surface of plate count agar medium plates (CM0463-OXOID) by sterile bent glass spreader. Then inoculated and control plates were incubated
at 7 °C for 10 days. Then all colonies were counted, and the average number of colonies was recorded. And the total psychrotrophic bacterial count (cfu/g) was calculated.

2.4. Isolation and Identification of Pseudomonas:
The suspected colonies were purified and sub-cultural onto nutrient agar slopes (EB0336-OXOID) and incubated at 37°C for 24hrs. The purified colonies were subjected for further identification microbiologically as following Gram stain (Cruickshank et al., 1975), Motility test (MacFaddin 2002) and biochemically according to (Collins and Lynne 1984 and MacFaddin 2002) as following:

1. Pigment formation on nutrient agar (Collins and Lynne, 1984).
2. Catalase test (Kiss, 1984)
3. Sugar fermentation test (MacFaddin, 1976)
4. Nitrate reduction test (Kiss, 1984)
5. Indol production test (Bialy and Scott, 1978)
6. Methyl red test (Cruick Shank et al., 1975)
7. Voges – proskauer test (Cruick Shank et al., 1975)
8. Cytochrome oxidase test.
9. Growth at 42 °C and 4 °C (Collins and Lynne, 1984)
10. Arginine hydrolysis (Collins and Lynne, 1984)
11. 10% lactose agar test (Washington, 1981)
12. Starch hydrolysis test (Kiss, 1984)
13. Oxidation and fermentation test (Cruickshank et al., 1975)

2.5. Antibiotic susceptibility test of Pseudomonas isolates:
Five isolates of each pseudomonas species were submitted for antibiotic sensitivity test. Antibiotic susceptibility was performed by Disc diffusion method according to (Schreckenberger and Binnicker, 2011).

Samples were processed for culture and sensitivity pattern for Pseudomonas colonies were picked up (using a sterile loop) to tube containing 5 ml of Muller-Hinton broth. The broth culture was incubated at 37°C for 24 hours. Then the turbidity was adjusted to the turbidity of the 0.5 McFarland standards. After that about 1ml of the broth was inoculated on the surface of Muller-Hinton agar (CM0337-OXOID) and spread evenly over the entire surface of the agar plates by a sterile bended glass rod. The antibiotic discs of Amikacin, Cefepime, Ceftazidime, Ceftriaxone, Gentamicyn, Ciprofloxacin, Imipenem, Oxacillin and Tobramycin were applied on the inoculated plates. Then, the plates were placed in an incubator at 37°C for 18 h in inverted position. After 18 hours of incubation, the plates were examined and the diameters of zone of inhibition were measured in mm.

### Table 1 The used antimicrobial agents in the antibiotic sensitivity test

<table>
<thead>
<tr>
<th>Antimicrobial agents</th>
<th>Concentration</th>
<th>Company</th>
<th>Catalog number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin (AK)</td>
<td>30µg</td>
<td>Oxoid™</td>
<td>C80017B</td>
</tr>
<tr>
<td>Cefepime (FEP)</td>
<td>30µg</td>
<td>Oxoid™</td>
<td>C80015B</td>
</tr>
<tr>
<td>Ceftazidime (CAZ)</td>
<td>30µg</td>
<td>Oxoid™</td>
<td>C80417B</td>
</tr>
<tr>
<td>Ceftriaxone (CRO)</td>
<td>30µg</td>
<td>Oxoid™</td>
<td>C80442B</td>
</tr>
<tr>
<td>Ciprofloxacin (CIP)</td>
<td>5µg</td>
<td>Oxoid™</td>
<td>C804425B</td>
</tr>
<tr>
<td>Gentamicyn (CN)</td>
<td>10µg</td>
<td>Oxoid™</td>
<td>C80024B</td>
</tr>
<tr>
<td>Imipenem (IPM)</td>
<td>10µg</td>
<td>Oxoid™</td>
<td>C80455B</td>
</tr>
<tr>
<td>Oxacillin (OX)</td>
<td>1µg</td>
<td>Oxoid™</td>
<td>C80159B</td>
</tr>
<tr>
<td>Tobramycin (TOB)</td>
<td>10µg</td>
<td>Oxoid™</td>
<td>C80056B</td>
</tr>
</tbody>
</table>

2.6. Antibiotic resistance genes

PCR was carried on a T100 thermal cycler (Bio-rad) with temperature and time conditions shown in Table (2) according to (References of primers) and Dream Taq green Master Mix (Thermo scientific™, K1081).

### Table 2 Sequences, target and expected amplicon size for forward and reverse primer pairs used in the study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Amplified product</th>
<th>Reference</th>
</tr>
</thead>
</table>
| RpoB   | F: CAGTCATCGGAACGACAAACCGG  
R: ACCTTGTTGATCCGCGTGCCT | 759 | Benez CK et al. (2017) |
| OprL   | F: ATGGCAAATCGAAAATGCGC  
R: CTTCTTCTGCCTACGACGCG | 396 | Mohammed Gihan et al. (2013) |
| ToxA   | F: GAGAACGGGCCACGATCACGACC  
R: CGCTGGCCCATTCGCTCCAGCG | 504 |         |

### Table 3 Total Psychrotrophic bacterial count in the examined frozen meat (cfu/g) (n=50 of each).

<table>
<thead>
<tr>
<th>Type of meat</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Mean value ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frozen cubic</td>
<td>$6 \times 10^4$</td>
<td>$1.9 \times 10^5$</td>
<td>$2.44 \times 10^5 \pm 3.56 \times 10^3$</td>
</tr>
<tr>
<td>Frozen milled</td>
<td>$7 \times 10^4$</td>
<td>$9.0 \times 10^5$</td>
<td>$1.7 \times 10^6 \pm 3.07 \times 10^3$</td>
</tr>
</tbody>
</table>

S.E = standard error of mean.

### Table 4 Incidence of pseudomonas species isolated from examined frozen cubic and milled meat samples (50 of each).

<table>
<thead>
<tr>
<th>Meat sample</th>
<th>No. of examined samples</th>
<th>No. of positive samples</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frozen cubic meat</td>
<td>50</td>
<td>35</td>
<td>70%</td>
</tr>
<tr>
<td>Frozen milled meat</td>
<td>50</td>
<td>40</td>
<td>80%</td>
</tr>
</tbody>
</table>

Results in relation to No. of samples for each type of meat (n=50). ** Percentage in relation to No. of samples (n=100).
Ps. aeruginosa were sensitive to Gentamycin, Tobramycin, Cefepime, Ceftazidime, Imipenem, Akmacin and ciprofloxacin. Ciprofloxacin is the most potent drug available for the treatment of Ps. aeruginosa infections. Ps. aeruginosa were resistant to Ceftriaxone and Oxacillin. Ps. aeruginosa is considered to be one of the most dangerous species as it forms biofilms, which aids in colonization of food spoilage and increases resistant to antibiotics, disinfectants and antiseptics.

Also, the obtained results indicated that Ps. aeruginosa are more resistant to Amikacin, Oxacillin, Cefepime and Ceftazidime Gentamicin and Ciprofloxacin. Actually Ps. diminuta was more sensitive to Amikacin, Ceftazidime and Tobramycin, however, it was resistant to Cefepime, Ciprofloxacin, Gentamicin, Imipenem and Oxacillin. Ps. diminuta was resistant to Amikacin, Oxacillin, and also was sensitive to Cefepime, Ceftazidime, Ciprofloxacin, Gentamicin, Imipenem, Ceftriaxone and Tobramycin. Furthermore, the results showed that Ps. fragi was more resistant to Oxacillin, Amikacin, Ceftazidime, Gentamicin and Ceftriaxone and Tobramycin. While it was sensitive to Cefepime, Ceftriaxone, Imipenem, Ciprofloxacin and Gentamycin.

The recorded virulence genes for some Ps. aeruginosa strains isolated from the examined frozen meat samples by PCR (n: 1-4 frozen meat and 3-8 minced meat) is shown in table (7) and fig. (1).

Table 7 Virulence genes for Ps. aeruginosa strains isolated from the examined frozen meat samples by PCR (n: 1-4 frozen meat and 5-8 minced meat)

<table>
<thead>
<tr>
<th>Isolation No</th>
<th>rpoB</th>
<th>Oprl</th>
<th>TonaA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Ps. aeruginosa were sensitive to Gentamycin, Tobramycin, Cefepime, Ceftazidime, Imipenem, Akmacin and ciprofloxacin. Ciprofloxacin is the most potent drug available for the treatment of Ps. aeruginosa infections. Ps. aeruginosa were resistant to Ceftriaxone and Oxacillin. Ps. aeruginosa is considered to be one of the most dangerous species as it forms biofilms, which aids in colonization of food spoilage and increases resistant to antibiotics, disinfectants and antiseptics.

Also, the obtained results indicated that Ps. aeruginosa are more resistant to Amikacin, Oxacillin, Cefepime and Ceftazidime Gentamicin and Ciprofloxacin. Actually Ps. diminuta was more sensitive to Amikacin, Ceftazidime and Tobramycin, however, it was resistant to Cefepime, Ciprofloxacin, Gentamicin, Imipenem and Oxacillin. Ps. diminuta was resistant to Amikacin, Oxacillin, and also was sensitive to Cefepime, Ceftazidime, Ciprofloxacin, Gentamicin, Imipenem, Ceftriaxone and Tobramycin.

Furthermore, the results showed that Ps. fragi was more resistant to Oxacillin, Amikacin, Ceftazidime, Gentamicin and Ceftriaxone and Tobramycin. While it was sensitive to Cefepime, Ceftriaxone, Imipenem, Ciprofloxacin and Gentamycin.

The results showed that the psychrotrophic bacterial count (cfu/g) in the examined samples of frozen minced meat ranged from (7 × 10³) to (9 × 10⁵) with mean value (1.7 × 10⁴). Accordingly, all examined samples were accepted according to EOS (2005) which reported that the maximum limit for psychrotrophic bacterial count in imported frozen meat shouldn’t exceed 10⁶ cfu/g. Higher results were obtained by Karaboz and Dincer (2002), Badawi (2008), Ibrahim et al. (2011) and Ali (2016) in the examined frozen meat those found that average count (2.18 × 10⁴ ± 0.1 × 10⁴ cfu/g, .43 × 10³ cfu/g with mean value (9 × 10³ ± 6.1 × 10³) sample from Alex Seaport, (9.9 × 10² to 4.2 × 10⁶) with mean value (3.7 × 10³ ± 7.9 × 10³) cfu/g and (9.9 x10² to 4.2 × 10⁶) cfu/g with mean value (3.7 × 10³ ± 7.9 × 10³) cfu/g, respectively. Also Abd El-Hady (2014) reported that psychrotrophic count in imported frozen beef chunk and ribs were 1 × 10⁵ to 1.8 × 10⁵ with mean value 2.78 × 10⁴±2.54.24 × 10³ cfu/g, 1.7 × 10³ to 7.9 × 10³ with mean value 3.87 x 10³±4.35 x 10³ cfu/g, respectively. With incidence of 24 (96%), 24 (96%) respectively.

The results showed that the incidence of Psychrotrophic bacteria were higher in frozen minced meat than frozen meat. The incidence of Pseudomonas species isolated from the examined samples was 76%, 89% in frozen cubic meat and minced meat, respectively. The incidence of Ps. aeruginosa, Ps. aeruginosa, Ps. diminuta, Ps. putid and Ps. fragi in frozen meat were 15(30%), 40(80%), 8(16%), 5(10%) and 8(16%), respectively. In addition, the incidence of Ps. aeruginosa, Ps. aeruginosa, Ps. diminuta, Ps. putid and Ps. fragi were 20(40%), 45(90%), 5(10%) 7(14%) and 8(16%), respectively. Lower results were obtained by El Nawawi et al. (2012), Hassan (2013), Abd Ela (2017) and also El-Dakrory
who found that *ps. aeruginosa* in imported frozen meat was (6%), (4%), (12.2%), (7.1%), respectively. While Rizk (2014) reported that the incidence of *ps. fluorescence* was (30%), while *Ps. aeruginosa* was not isolated. Higher results for isolation of *ps. fluorescence* were obtained by Abd Elaala –Mohiga (2017), El-Dakrory (2017) and Ibrahim, et al., (2016) those explained that the incidence of *ps. fluorescence* in imported frozen meat was 64.44%, 46.67% and 73.33%, respectively. Abd Elaala (2017) and Ibrahim et al. (2016) those reported that the incidence of *Ps. putida* in imported frozen meat was (8.34%) and (8.34%) respectively.

The results of Abd Elaala (2017) and El-Dakrory (2017) showed that the incidence of *Ps. fragi* in imported frozen meat was (8.34%) and (28.27%), respectively. The presence of pseudomonas species in food represents risk as they lead to spoilage of food (Jay, 2000). *Ps. fluorescence* and *Ps. aeruginosa* represented the major species which could be isolated due to its resistance to many stress factors as low temperature, water activity and inhibitory action of carbon dioxide. These finding agreed with El-Dakrory - Amira (2017).

Antibiotics are massively used in beef industry which increases the risk of developing antibiotic resistant microorganisms. Therefore, the isolated Pseudomonas species were submitted to antibiotic susceptibility testing. The results showed that *Ps. aeruginosa* were sensitive to Gentamycin, Tobramycin, Cefepime, Cefazidime, Imipenem, Amikacin, Tobramycin and ciprofloxacin which nearly results were approved by Gales et al. (2001), who reported that Ciprofloxacin is one of the most potent drug available for the treatment of *ps. aeruginosa* infections. *Ps. aeruginosa* was resistant to Ceftriaxone and Oxacillin. Also, the results showed that *Ps. fluorescence* are more resistant to Amikacin, Oxacillin, Cefepime and Cefazidime. Gentamycin and Ciprofloxacin which nearly results recorded by Morgan (2014).

*Ps. putida* was resistant to Amikacin, Oxacillin and Ceftriaxone. And also, it was sensitive to Cefepime, Cefazidime, Ciprofloxacin, Gentamycin, Imipenem and Tobramycin. These results were nearly similar to those reported by Muller (2011). In addition, the results showed that *Ps. fragi* was more resistant to Oxacillin, Amikacin, Cefazidime and Tobramycin. While it was sensitive to Cefepime, Ceftriaxone, Imipenem, Ciprofloxacin and Gentamycin.

Pseudomonas species have multiple intrinsic and acquired resistance genes, host several mobile genetic elements and exchange them with other families of Gram-negative bacilli. (Pfeifer et al., 2010). The reason for increased antimicrobial resistant was the overuse of antimicrobial drugs for preventing or treating infections in human and veterinarian (WHO, 2007).

PCR is rapid and reliable tool for identification of different bacterial species (Settanni and Corsetti, 2007). The PCR results for virulence genes of *ps. aeruginosa* for detection of (oprL, rpoB, ToxA) detected that oprL virulence gene were showed in 1 from 8 strains (12.5%) with 504 bp amplicon, toxA virulence gene were absence in 8 strains, rpoB virulence gene were showed in 4 from 8 strains (50%) with 759 bp. 2 samples in frozen meat and 2 samples from minced meat.

### 5. CONCLUSIONS

The examined imported frozen meat samples were highly contaminated with various species of pseudomonas reflecting unhygienic measures and unsuitable environmental condition during handling, transportation and storage. Pseudomonas species are the most psychrotrophic bacteria contaminated the examined samples. The examined samples were highly contaminated with *Ps. fluorescense* followed by *Ps. aeruginosa*. The rpoB virulence gene were present in 4 strains of *Ps. aeruginosa* (50%) then oprl virulence gene (12.5%) and absence of Toxa A virulence gene in 8 strains.

### 5. REFERENCES