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Incidence of Pseudomonas species and effect of their virulence factors on milk and milk products.

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ABSTRACT

Pseudomonas species are mainly associated with contamination of dairy products due to their proteolytic activity. Therefore, the goal of the present study was to isolate *Pseudomonas spp* and then identify their virulence factors that have a bad impact on milk quality. One hundred samples of milk and milk products samples from (raw milk, kareish cheese, yoghurt and ice cream) divided to 25 samples from each one. They collected and examined to isolate that *Pseudomonas* and then identify their species using PCR technique. *Pseudomonas aeuroginosa* was present in 20 ,16 ,8 and 8 %of the examined raw milk, kareish cheese, yoghurt and ice cream samples, respectively while, *Pseudomonas fluorescence* present in 28, 20,12 and 8% respectively in examined samples, meanwhile *Ps. Putida* was present in 8 and 4% in the examined raw milk, karish cheese only. *Pseudomonas diminuta* and *Pseudomonas fragi* were detected with percentage of 12 and 8 while but not found in yoghurt and ice cream samples. The incidence percent of *Pseudomonas spp* in examined samples of raw milk, kareish cheese, yoghurt and ice cream 56 %, 54%, 12%, 20% and 20%, respectively. *Pseudomonas aeuroginosa* isolates were subjected to PCR technique to detect the alkaline protease gene *opr*l, opsl and txoA its virulence genes. The results revealed that proteolytic activity and presence of these virulent genes in the examined dairy products and their spoilage potential at different temperatures.

1. INTRODUCTION

Spoilage of milk due to presence of psychrotrophic microorganisms leads to significant losses for the food industry and is a particular pattern of the dairy industry (Dogan and Boor, 2003). Milk is kept at low temperatures for 2 to 5 days before heat treatment (De Jonghe *et al.*, 2011. During storage of milk at low temperatures, psychrotrophic microorganisms can grow and reduce the quality of raw milk (Lafarge *et al.*, 2004 and Xin *et al.*, 2017). *Pseudomonas* species are the most one of Gram-negative bacteria which may spoil dairy products producing heat-stable extracellular enzymes (Cousin *et al.*, 2001). These enzymes also can survive both pasteurization and UHT treatment (Bhunia, 2008). They can effect on the quality of dairy products giving bitter, rancid flavors and also changing the coagulation properties of milk (Richter and Vedamuthu, 2001).

Pseudomonas spp. Have high genetic diversity and metabolic ability, allowing them to survive in different environments, such as soil, water, and air. These characteristics allow them to survive on the equipment used in the dairy production chain, such as pipelines, bulk tanks, milking machines, and animal production environment (Simões, 2010).

Pseudomonas fluorescens, *Pseudomonas aeruginosa* , *and Pseudomonas putida* are the most common *Pseudomonas* species found in the dairy chain and are responsible for the production of proteolytic enzymes that are highly stable at high temperatures and causing spoilage in milk products (Rajmohan *et al.*, 2002; March and *et al.*, 2009). The metalloprotease oprL is one of the enzymes produced by *Pseudomonas* spp., and it is of particular interest to the dairy industry due to its specific spoilage activity in casein, which results in significant modifications to the physico-chemical and organoleptic properties of raw milk (Dufour*et al.*, 2008).So this study aimed to isolate *Pseudomonas* spp from different milk product then identify virulence genes of *Pseudomonas* using molecular technique.

2. MATERIAL AND METHODS

2.1. Samples:

A total of 100 random samples of milk and milk products asraw milk, kareish cheese, yoghurt and ice cream (25 of each) were collected from different supermarkets at Shebin el-kom city El-menoufia Governorate. Each sample was kept in a separate sterile plastic bag and preserved in an insulated icebox then transferred directly to the laboratory under complete aseptic conditions without delay and examined as quickly as possible.

Accurately 25 grams of the examined yoghurt, kareish cheese and ice cream and 25 ml from raw milk and 225 ml of 0.1% sterile buffered peptone water were aseptically

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added to sterile containers. One ml from the original dilution was transferred with sterile pipette to another sterile test tube containing 9 ml of sterile buffered peptone water and mixed well to make the next dilution, from which further decimal serial dilutions were prepared.

2.2. Counting of Pseudomonas species:

Accurately, 0.1 ml of each sample homogenate were separately inoculated into duplicate petri-dishes of *Pseudomonas* selective agar medium supplemented with glycerol and evenly spread. The inoculated plates were incubated at 25 °C for 48 hours after which all developed colonies (greenish yellow colonies) were enumerated. The average count was calculated and recorded.

Then isolation had been done then the average count was calculated and recorded. The suspected colonies were purified and sub-cultured onto nutrient agar slopes and incubated at 37°C for 24 hrs. The purified colonies were subjected for further identification either morphologically or biochemically according to ISO, (2004). In motility test, all isolated bacterial strains showed positive. In methyl red test and indole test, they give negative results on all strains.

In catalase test, they were observed to give positive results. In voges-proskauer (VP) test, all isolated bacterial strains showed negative. All strains gave negative results on the urease test. In starch hydrolysis test could hydrolyze the starch.

2.3. Pseudomonas species molecular identification and detection of their virulent genes:

Isolates that were preliminary identified as *Pseudomonas auroginosa* were subjected to molecular analysis to confirm their identification and genetic profiles. Polymerase chain reaction was conducted to confirm the isolates as belonging to the genus *Pseudomonas* auroginosa and to identify presence of oprl, txoA and opSl virulence gene. Single colonies from each isolate were transferred to trypticase soy broth (Oxoid) and incubated at 25°C for 24 h. The aliquots were subjected to DNA extraction using the Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI). The concentration of DNA was estimated by spectrophotometry (Sambrook*et al*., 1989).

3. RESULTS

After isolation and identification of isolated *Pseudomonas* species to found that incidence percent of *Pseudomonas spp* in examined samples of raw milk, karish cheese, yogurt and ice cream 80%, 56 %, 24% and 20% respectively as shown in fig "1".

Also, the count of *Pseudomonas* in the examined samples were the count of *Pseudomonas* (log10 CFU/ml) were from range of Min count of 3.5, 6.6, 2.7 and 2.5 to Max count of 5.9, 3.1, 5.8 and 3.4 with mean± SD count of 1.9± 2.1, 6.3±1.1, 1.8 ± 2.2and 1.0±1.6 in Raw milk, kareish cheese, Yogurt and Ice cream respectively as shown in fig "2".

And incidence of different species of *Pseudomonas* in examined raw milk, kareish cheese yogurt and ice cream , were *Ps.* auroginosa was present in 20, 16, 8 and 8 % respectively in examined samples while ,*Ps.* Fluorescence present in 28, 20, 12 and 8% respectively in examined samples in the meanwhile *Ps.* Putida was present in 8 and 4% that not indicated yogurt and ice cream , *Ps.* diminuta and *Ps.* Fragi 12 and 8 % respectively in examined samples of raw milk and karish cheese while not indicated in yogurt and ice cream as shown in table "1".

Then isolated strains of *Pseudomonas auroginosa* were sent to molecular identification and detection of virulent genes of oprl, txoa and oprI. to give positive results for three genes as indicated in table "2".

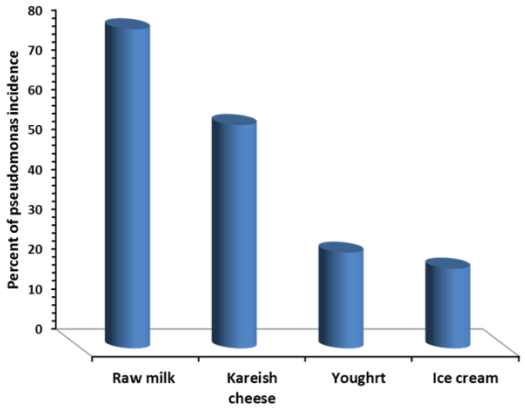

Figure 1 Incidence of *Pseudomonas* in different samples of raw milk and milk products.

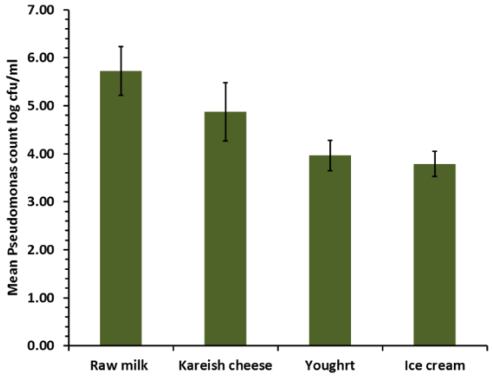

Figure 2 *Pseudomonas* counts (log10 CFU/ml) in the examined samples of raw milk and some milk products (n=100).

Table 1 Incidence of different strains of isolated *Pseudomonas* species from examined samples

Strains	Raw Milk		Kareish cheese		Yoghurt		Ice cream	
	NO	%	NO	%	No	%	NO	%
Ps. Aeurginosa	5	20	4	16	2	8	2	8
Ps. flourescence	7	28	5	20	3	12	2	8
Ps. putida	2	8	1	4	-	-	1	-
Ps. diminuta	3	12	2	8	-	-	-	-
Ps. fragi	3	12	2	8	1	-	-	-

Table 2 Molecular identification of virulence genes of *Pseudomonas* auroginosa

Sample	OprL	toxA	pslA
1	+	+	+
2	+	+	+
3	+	+	+
4	+	+	+

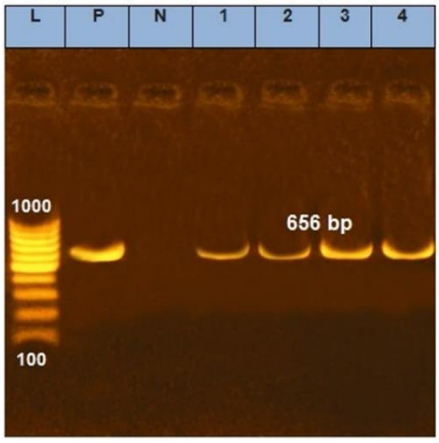

Figure 3 Agarose gel electrophoresis of PSLA gene amplified at 656 bp for characterization of *Pseudomonas*
Lane L: 100 bp ladder as molecular size DNA marker.
Lane P: Control positive for PSLA gene (656) bp
Lane N: Control negative.
Lanes 1 and 4: Positive *Pseudomonas* strains for PSLA gene isolated from raw milk and milk products.

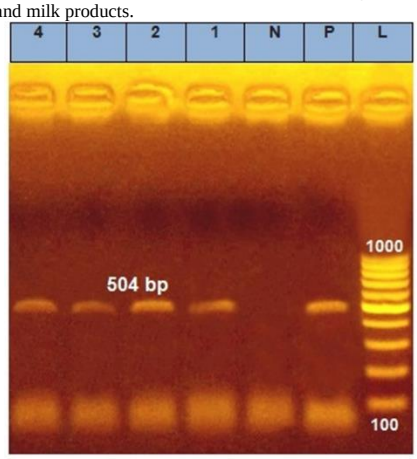

Figure 4 Agarose gel electrophoresis of OPRL GENE amplified at 504 bp for characterization of *Pseudomonas*
Lane L: 100 bp ladder as molecular size DNA marker.
Lane P: Control positive for OPRL gene (504) bp
Lane N: Control negative.
Lanes 1 and 4: Positive *Pseudomonas* strains for OPRL gene isolated from raw milk and milk products.

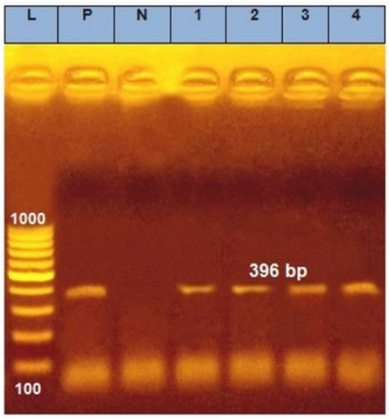

Figure 5 Agarose gel electrophoresis of ToxA GENE amplified at 396 bp for characterization of *Pseudomonas*
Lane L: 100 bp ladder as molecular size DNA marker.
Lane P: Control positive for ToxA gene (396) bp
Lane N: Control negative.
Lanes 1 and 4: Positive *Pseudomonas* strains for ToxA gene isolated from raw milk and milk products.

4. DISCUSSION

Pseudomonas species represent the awfully common group of psychrotrophic bacteria related to milk spoilage. On the other side, *Pseudomonas* species are able to grow at refrigeration temperatures (7 °C or less), causing putrefaction, fermentation, fruity- and bitter flavors as they metabolize fat and protein.

Fig "1" illustrated the incidence of *Pseudomonas* spp in examined samples of raw milk, kariesh cheese, yoghurt and ice cream80%, 56 %, 24% and 20% respectively.

These results were higher than those recorded by Arslan (2011) and these results were lower than results recorded by Ibrahim *et al* (2008).

Fig "2" illustrated that count of *Pseudomonas* (CFU /ml) were from range of Min count of 3.5, 6.6, 2.7 and 2.5to Max count of 5.9 ,3.1, 5.8 and 3.4

With mean ±sd of 1.9±2.1, 6.3±1.1,1.8 ±2.2 and 1± 1.6 in raw milk, kareish cheese, yoghurt and ice cream respectively. These results came in agreement to results of Olfa *et al.* (2013) while it were lower than those recorded by Ahlam A. El-Leboudy *et al.* (2015) and Esmat (2005) and also lower than that recorded by sadek *et al*. (2006) and ELSayed *et al*.(2011) and these results were higher than that recorded by Ibrahim *et al* (2008) that isolated *Pseudomonas* from following products; raw milk ,yoghurt and White soft cheese and also higher than results recorded by Kelly Molin de Almeida (2017).

In addition to growing at low temperatures, a variety of psychrotrophic bacterial species (primarily represented by pseudomonads) found in raw milk produce heat-stable proteases and lipases, generally during the late log or early stationary growth phases when the cell density is high (Sadek *et al.,* 2006).

psychrotrophic bacteria produce soluble casein that gives negative effects on cheese production as reduction in cheese yield and tainting that lead to degradation of products(Bintsis *et al.*, 2000) .

Table one showed that incidence of *Pseudomonas* spp in collected samples of raw milk, kareish cheese, yoghurt and ice cream. *Ps.* auroginosa was present in 20 ,16 ,8 and 8 % respectively in the examined samples while, *Ps. Fluorescence* was found in 28, 20,12 and 8%, respectively in the examined samples. Meanwhile, *Ps. Putida* was present in milk and kareishcheese8 and 4 %that not indicated in yoghurt and ice cream, *Ps. diminuta* and *Ps. fragi* were found in 12 and 8 % respectively in examined samples of raw milk and karish cheese while not indicated in yogurt and ice cream. Abdel hameed- Abeer (2016) isolated *Ps. auroginosa, Ps. flouresence* and *Ps. Alkali genes* in higher incidence from raw milk by 11.6 %, 3.3% and 1.6 %, respectively while, *Ps. aeuroginosa, Ps.* fouresence, *Ps.* Alkali genes and *Ps.* Putida could be isolated from Ice cream by 10 %, 4 %, 2 % and 2 %, respectively

These results were lower than those recorded by Amin - Ahlam et al (2015) that isolated *Ps. aeuroginosa, Ps. flouresence* and *Ps. Putida* by 15.2%, 35.4 % and 18.2 % respectively from raw milk and results recorded by S. Banerjee (2017) that found that *Ps. aeuroginosa, Ps. flouresence* and *Ps. Fragi* by 42.4, 24.4 and 12%, respectively.

Among the pathogenicity caused by virulence factors, can be cited Lipopolysaccharide, Flagellum, Secretion System, Exotoxin A, Proteases, Alginate, Quorum Sensing, Biofilm

Formation, These are major virulence factors acting in different manners in the immune system (Bintsis *et al.*, 2000).

Toxic gene like toxA, oprl and opra were presented in all examined samples. These results were to that recorded by Lnotte *et al.* (2010) also reported by Badr *et al (*2017) and Nikbin *et al.* (2006) and Sharma *et al.* (2017) one for the human beings.

PCR results also nearly to that indicated by Mo'men M. Al-Adl (2016) indicated the presence of each virulence gene among studied isolates. *Exo* SHabibi and Honarm and (2015) while these results were lower than those recorded by Dadmanesh *et al.* (2014) Mitov, *et al*. (2010), but Jabalameli, *et al*. (2012) *exo*U, and 26.3% carried *exo*S, total prevalence of *exo*S, *exo*U and *las*B virulence factors in the pediatric patients were 92.95%, 56.33% and 91.54%, respectively. Heidary *et al*. (2016) and Mitchell, *et al*. (2017) stated that all tested isolates (100%) were positive for *las* Bwhile 9 out of 17 strains (53%) were positive for *exo*S gene, Fazeli and Momtaz (2014) and In Nikbin *et al*., (2012) reported that all isolates examined harbored *las*Bgene. Investigation of virulence factors associated genes revealed that 81.25%, 69.23% and 69.7 % of the isolates harboring *tox*A, *las*B and *exo*S genes, respectively Ameen (2014).

The exotoxin A is produced by most of *Ps. aeruginosa* strains with greatly similar to diphtheria toxin. It can inhibit eukaryotic protein biosynthesis at the level of polypeptide chain elongation factor. In this study, 95% of tested isolates carried *tox*A gene. This was similar to Mitchell, *et al*., (2017) and Elsayed *et al*. (2016) who found that all tested isolates (100%) were positive for *tox*A, but it decreased to (69.56%) and (35.29%) as mentioned by Dadmanesh *et al.*, (2014) and Fazeli and Momtaz (2014), respectively.

5. CONCLUSION

Pseudomonas spp. found mainly in raw milk producing proteolytic enzymes that will next effect on its products quality as bitter taste, bad odor and also affect texture so that production of milk should be in hygienic conditions to decrease their bacterial load and avoid their bad effects.

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