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RESEARCH ARTICLE

MOLECULAR AND BIOCHEMICAL CHARACTERIZATION OF *PSEUDOMONAS* SPECIES ISOLATED FROM SUBCLINICAL MASTITIS MILK AND ICE CREAM AND ITS SUSCEPTIBILITY TO *ALLIUM SATIVUM* AND *COMMIPHORRA MOLMOL* PLANT EXTRACTS IN EGYPT.

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Abstract

Mastitis is the most important economic illness affecting dairy cattle worldwide. Milk is nutrient and essential daily diet for growing human and young animals. Also, serve as good medium for several microbial growth. Identification of contamination milk pathogens still depends principally on culture and phenotype method. This study aimed to isolate, characterize and evaluate *Pseudomonas* spp. isolated from subclinical mastitis raw milk and ice cream by using molecular and biochemical assays manual and automated system in Egypt. Sixty (60) random milk samples were collected aseptically from dairy farms (cow and buffaloes), supermarkets and street vendors. Also, Fifty (50) Ice cream samples were collected from supermarkets and street vendors at four different Governorates in Egypt. Number of positive pathogenic bacteria were isolated from raw milk (26) and (22) from Ice cream samples. Not only *Pseudomonas SPP* were isolated from samples but also major pathogenic bacteria were isolated as *E.coli*, *Staphylococcus aureus* and *Salmonella*. Putative *Pseudomonas* spp were partial characterized by biochemical test, API 20NE, Enzymatic activity lipase, lecithinase and proteolytic activity, automated system (VETIC system). Molecular characterization by polymerase chain reaction (PCR through detection of aprA and exotoxin A genes, amplifying a 396- pb region of the exotoxin A and 1580-bp region of apr A genes. The inhibitory values of essential oil were evaluated by disc diffusion test and minimal inhibition concentration (MIC) for each oil plant extracts compared with reference antibiotics. *Commiphorra molmol* essential oil extract has a marked inhibitory effects against pseudomonas spp (26.6 ± 0.33) followed by *A. sativum* essential oil extract IZD were (21.6±0.48) respectively. *P.aeruginosa* was as the most predominant isolate followed by *P. fluoresces*.

Diagnosis of *Pseudomonas* spp with molecular methods is a rapid, sensitive and accurate method for identification, detection of virulence factors and differentiation of *Pseudomonas* spp isolated from dairy milk and ice cream. Also, essential oils from *Commiphorra molmol* and *Allium sativum* have a

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marked antibacterial effect against *Pseudomonas* spp and may be use a novel antibacterial agents and food preservative but it's still need extensive study for safety and clinical study in vivo.

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Introduction:-

Subclinical mastitis is a major problem in dairy industry. This illness affects of milk quality, yield and the infection may be transmitted to other healthy cattle in the herd. Bacteriological technique routinely used to diagnose subclinical mastitis is based on changes in milk constituents due to bacterial growth and enzymatic reactions in the infected dairy cattle [1]. Milk is a major composition of the diet; its quality is important to welfare of human and animals life. Milk is considered an excellent medium for growing of microorganisms as *Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, resultant spoilage of the milk and milk byproduct which lead to infectious or intoxications in consumers and responsible for milk borne diseases [1,2]. Ice cream and its related products have certain extrinsic factors related to storage environment. During production, transportation and storage, it may become contaminated with several microorganisms [3,4,5]. *Pseudomonas Spp* are psychotropic, gram- negative bacterium, widely distributed in nature and considered the most common dairy product spoilers [6,7]. Whenever, the *Pseudomonas spp* are not considered primary human and animals pathogens but several species of these group are associated with human and animals infections as *P. aeruginosa* which causing bovine subclinical mastitis [8,9,10]. The deficiency of ideal identification tools for these organisms lead to the misidentification of nonpathogenic *Pseudomonas* spp as pathogenic species [9]. Moreover, *P. aeruginosa* pathogenicity depends on extracellular metabolites, includes proteolytic, lipolytic enzymes, exotoxins, and pigment production (pyocyanin) [11]. *P. aeruginosa* produced several proteases which considered as virulence factors which include alkaline proteases (aeruginolysin) and two elastases, LasA (staphylolysin) and lasB (pseudolysin) [12]. which interferes with host immunity through inhibiting the host inflammatory response to infection [13]. *P. aeruginosa* produce exotoxin A is a major virulence factor similar to diphtheria toxin on its action which causes leucocytes depletion and liver necrosis [14]. Developing of accurate and rapid tools to identify pathogens causing spoilage of milk help industry meet future product quality and safety challenges[15]. Different phenotypic and molecular techniques have been used for sub typing isolates include PCR based assays [16,17]. The plant extracts are a viable option that could be decrease side effects of antibiotic treatment, limit infection and synergistically with current therapy [18]. Medicinal plant include active materials which can be used as alternative effective drugs on MRD . They would be a good source to obtain a variety of antibacterial drugs and active compound. These plants had been used for many centuries as a spices and food preservatives and uses in Modern and Folk medicine for combating of different diseases. In modern medicine they are used as antibacterial , antioxidant , antitumor, antifungal and antiviral effect [19]. *Commiphora molmol(myrrh)* is used in modern medicine as antibacterial and anti-inflammatory [20]. Several reported had proved that Garlic plant extract has antimicrobial effects [21]. In the last few years several studies have been documented to plant extracts efficacy against different microorganisms [22]. The present study aimed to isolate and characterize of *Pseudomonas spp* from Raw milk and Ice cream by using classical and molecular assays and its susceptibility to *Allium sativum* and *commiphora molmol* plant extracts in Egypt.

Material and Methods:-

Collection of samples:-

Sixty (60) random milk samples were collected aseptically from dairy cows, supermarkets, street vendors and collection milk station. Also, Fifty (50) Ice cream were collected from supermarkets and street vendors at different Governorates in Egypt. The collected samples were prepared according to [23]. Also, of collected Ice cream samples were thawed by immersing in a hot water bath at 55°C for 30 seconds or standing at room temperature for 10 minutes [24].

Plant Sources:-

The four essential oils of *Allium sativa* and *commiphora molmol* plants. were purchased from Department of Medicinal and Aromatic plants Department, National Research centre, Cairo, Egypt

Determination of microbial load in Raw milk and Ice Cream samples:-

The samples were analyzed for determination of total microbial count. Samples were serially diluted from 10 to 10⁻⁶ in ringer's solution and then culture on nutrient Agar medium, MacConky Agar medium, LED Agar medium and Mannitol salt Agar medium and then incubated at 37°C for 24-48 hrs and count the numbers of colony forming units (cfu/ml) were determined. Each assay was performed in duplicates, *Pseudomonas* isolates were subculture on to nutrient agar (NA) plates

and incubated at 30°C for 24 h. Pure cultures were inoculated into nutrient broth and incubated overnight at 30°C prior to testing according to Bergey's manual of determinative bacteriology [25]. pure bacterial culture were isolated from each Raw milk and Ice cream samples

Identification of isolates from Raw milk and Ice Cream samples:-

Classical Identification:-

The bacterial isolates were identified by cultural and physiological, morphological, Standard identification of different pathogens was carried out by a variety of phenotypic tests, as the coagulase test (Sparks, Maryland, USA), the catalase test (bioMérieux, France). For further *Pseudomonas* Spp used API and VITEK Systems (bioMérieux).

Biochemical characterization:-

Oxidation Fermentation Test. Oxidation fermentation test were applied on lactose negative colonies For *Pseudomonas* oxidative reaction, among the same samples of catalase (+), motility (+) and oxidase (+) activities, colonies were studied according to [26].

Proteolytic and Lipolytic:-

Overnight cultures were spot onto pre-poured plates of standard plate count agar with 10% skim milk. The inoculated plates were incubated at 30°C for 48-72 hrs. The inoculated plates were flooded with hydrochloric acid 1% , if clear zones around the colonies were regarded as positive. Also, cultures were streaked onto pre-poured plates of tributyrin agar and incubated at 30°C for 48-72 hrs. Hydrolysis of tributyrin resulting in clearing of the medium and formation of clear zones around lipolytic colonies were regarded as positive according to [27].

Slime production (biofilm):-

Biofilm was evaluated by the Congo red method [28]. The medium was prepared with 37g/L of brain heart infusion broth, 50 g/L sucrose, and 10 g/ L of agar and .8 g /L of Congo red stain sterile by autoclaving. Plates were inoculated and incubated at 37°C for 24 h. A positive result was indicated by black colonies on the surface.

Determination of antibacterial activity of pyocyanin pigment:-

Disc diffusion method was done according to [19], using sterile disc which impregnated with culture filtrate of pigment producing isolates (blue green pigment) against *staphylococcus aureus*.

Identification with Analytical Profile Index (API 20 System):-

API 20 NE kit (BioMérieux, Paris, France) consists of 32 cupules, 26 of which contain dehydrated biochemical agents for colorimetric. The manufacturer's recommended procedures (API System, BioMérieux) were followed. Briefly, 3-5 of bacterial colonies were prepared from overnight cultures. They were standardized with an equivalent turbidity to 0.5 McFarland standards in 6 ml of sterile distilled water. The cupped strip of inoculated API suspension medium was homogenized and 55 µL of the suspension were dispensed in each cupules of the strip. The tests URE, ADH and ODC were covered with two drops of mineral oil. After an incubation period of 18- 24 hrs at 37°C, reagents were added for the non spontaneous tests. Strain profiles were read and identified and determined of the numerical profile; the oxidase reaction constitutes the 21st test and has a value, look up the numerical profile.

Identification with VITEK 2 System:-

Automated system VITEK 2 System Identification of all isolates were detected using the automated systems VITEK 2 (bioMérieux), panel Gram Negative (GN) card. The manufacturer's instructions were followed for the preparation of the inocula and incubation of the isolates. The card was automatically filled by a vacuum device, sealed and inserted into the VITEK 2 reader-incubator module. The results were interpreted by the ID-GPC database and final results were obtained automatically. All cards used were automatically discarded into a waste container.

Molecular assays:-

DNA extraction:-

DNA extraction and Genomic DNA of the strains were obtained using the genomic DNA Extraction Kit (Gene JET; Thermo scientific) following the manufacturer's instructions. PCR reaction mixture consisted of; 10 ng template DNA, 1 unit Taq DNA polymerase, 1x reaction buffer, 50 p mol of each Alkaline protease gene (aprA) primer pair:

apr A-F 5'-CCTGATCKGGCCGATAACTGCAAT-3') and
apr A-R 5'-GGAAGACASCTATCAATTGGAACAG-3').

Also, for *Exotoxin A* gene pair primers: *ETA1*: 5'-GACAACGCCCTCAGCATCACCAGC-3', *ETA2*: 5'-CGCTGGCCCATTCGCTCCAGCGCT-3'. gene, 2.5 – 3 mM Mg Cl₂, 0.2 mM dNTPs and dd H₂O to a final volume of 50 µl. The amplification conditions for detection alkaline protease gene were: denaturation at 94°C for 4 min, 25 cycles of denaturation (1 min at 94 °C), annealing (40 s at 46 °C) and extension (72 °C for 2 min), and a final extension at 72 °C for 4 min. Also, amplification for *Exotoxin A gene*, reaction mixtures were subjected to 30 cycles of the following incubations: initial denaturation at 94°C for 2 min (1 cycle), denaturation at 94°C for 2 min, annealing at 68°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 7 min. PCR products for *aprA* gene and *ETA* gene were separated by 1.5% agarose gel electrophoresis (Agarose, Sigma, USA), using Tris-boric EDTA buffer. Amplification products stained with ethidium bromide using Gene Ruler 100bp DNA Ladder (Fermentas Company, Cat.No.SM0243, USA), visualized under UV and photographed. The amplification fragment of a *aprA* gene was 1580-bp, and amplification fragment of a *ETA* gene was 396 – bp.

Antibacterial test:-

The essential oils were tested against *Pseudomonas* spp. The growth of inhibitory effect were determined by Disc Diffusion Method & Minimal Inhibition Concentration (MIC) as describe by [28].

Determination of antibacterial activity:-

Disc Diffusion Method:-

Filter paper disc 6 mm diameter were papered from filter paper 20 discs obtained by cutting filter paper circular and then put in test tube and sterilized then impregnated with 40 µl of essential oils suspension. After Mueller Hinton agar solidification, swabbed with a suspension of each bacterial species with sterile cotton swab, the pervious prepared essential oil disc was carefully placed on the surface of inoculated plate by using sterile needle. Also, standard antibiotics disc as control positive and normal saline disc as control negative and allow to diffuse at room temp for 20 minutes. The concentration of individuals oils extracts were 10 and 30 mg/ml and control positive with other antibiotic at concentration 1- 3 mg / ml. The plate was incubate at 37 C for 24-36 hrs. The plat check daily and inhibition zone diameters (IZD) formed around the disc or wells were calibration ruler (mm). All of this test done in triplicate manner and the growth inhibition activity of NEOs extracts were recorded.

Minimal Inhibitory Concentration (MIC) using micro-dilution method:-

It was done according to method describe by [29,30], guidelines for measuring of Minimal Inhibitory Concentration (MIC). The MIC was smallest concentration of oils extracts that inhibiting visible bacteria growth after incubation for 24-36 hrs at 37 C. The 96 plate was checked and the activity was measured as function of turbidity. low or absent of turbidity was further confirmed by inoculation on nutrient agar and incubate at 37 C at 24 hrs.

Statistical Analysis:-

Plate-count data were transformed into logarithms before statistical treatment. Statistical analysis was carried out using the SPSS software package for Windows 10.0 [31]. The test was performed for variation in mean microbial counts in raw milk and Ice cream samples

Results

Microbial load in different sources of raw milk and Ice cream sample. The Sixty raw milk samples were examined out of these samples, in Raw milk 71×10^3 cfu/ml and in Ice Cream 62×10^3 cfu/ml microbial were observed in Nutrient agar medium. Similarly in CLED agar in Raw milk 62.5×10^3 cfu/ml and in Ice Cream 42.2×10^3 CFU / ml microbial load were observed. While in case of MacConkey agar medium, in case of Raw milk 52.5×10^3 cfu/ml and in case Ice Cream 37.2×10^3 cfu/ml and cfu/ml microbial load were determined. But in M.S.A medium 1.2×10^3 cfu/ml microbial load were observed and in Raw milk and 0.9×10^3 cfu/ml in Ice Cream.

Table 1: - Microbial load in Raw milk and Ice Cream sample growing on different type of medium

Samples	Average bacteria count (10^3 cfu/ml)			
	Nutrient Agar	CLED Agar	MacConky Agar	M.S. Agar
Raw milk	51	47.5	41.5	1.2
Ice Cream	52	41.2	37.5	0.9

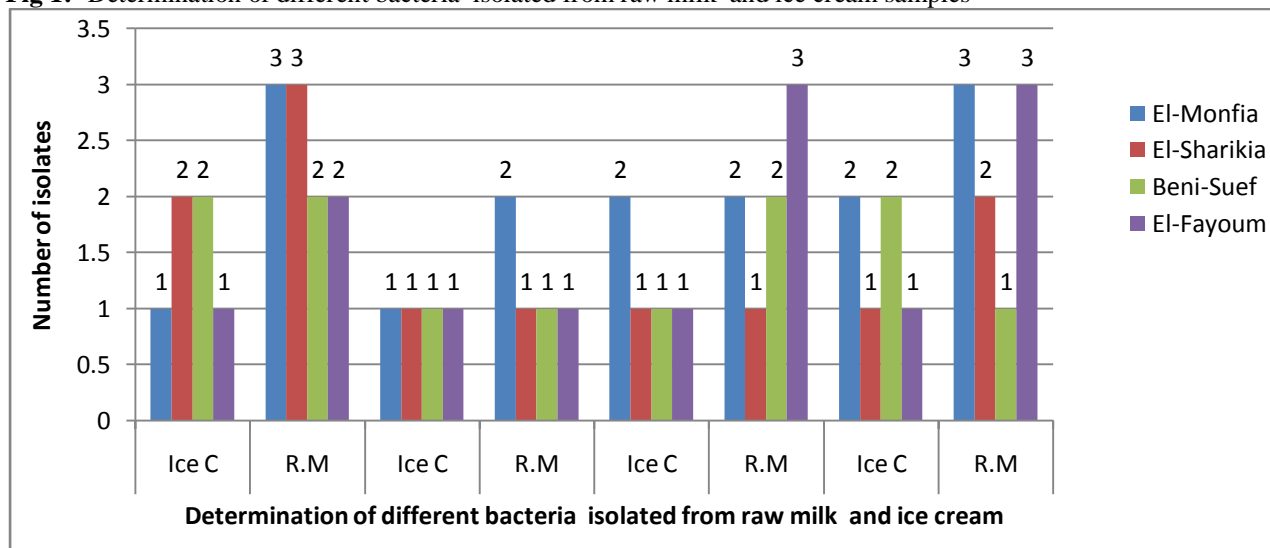
Incidence of bacterial isolates from raw milk out of 60 samples 26 samples were positive and out from 50 samples 22 samples were positive from Ice Cream.

Morphological, physiological and biochemical identification. The results of the phenotypic characterization, based on physiological, morphological and biochemical tests, allowed all the strains to be assigned different bacteria pathogen isolated from Raw milk and Ice Cream were performed as Bergey’s Manual. Results of laboratory examination of Raw milk and Ice Cream samples indicated that Raw milk samples found that microbes *E.coli* (9), *S. aureus* (8), *S. typhi* (4) and *Pseudomonas spp* (10). A total of Ice Cream samples were examine for the isolation of bacteria, (18) isolates were isolated among which (6) were *E.coli*, (5) were positive for *S.aureus*, (4) for *S.typhi* and (6) for *Pseudomonas spp* were isolated

Table 2:- Incidence of bacterial isolates from Raw milk and Ice Cream samples

Isolated organisms	Samples		<i>E coli</i>		<i>S. aureus</i>		<i>Salmonella</i>		<i>Pseudomonas SPP</i>		Total	
	R.M	Ice C	R.M	Ice C	R.M	Ice C	R.M	Ice C	R.M	Ice C	R.M	Ice C
El-Monfia	14	13	3	2	2	2	2	1	3	4	9	9
El-Sharikia	16	12	2	1	1	1	1	1	3	2	8	5
Beni-Suef	18	11	1	2	2	1	1	1	2	2	4	5
El-Fayoum	12	14	3	1	3	1	1	1	2	1	5	3
Total	60	50	9	6	8	5	5	4	10	9	26	22

Fig 1:- Determination of different bacteria isolated from raw milk and ice cream samples



Incidence of *Pseudomonas spp* among isolated bacteria from Raw milk and Ice cream samples. The results showed that 19 samples were positive for occurrence of *Pseudomonas spp.*, with a percentage of (17.27 %) Identity of the isolates was confirmed by using API 20 NE for detection and isolation and differentiate of *Pseudomonas spp*, *Pseudomonas aeruginosa* (12 isolates) isolates of *Pseudomonas fluorescens* (4 isolates), *Pseudomonas alkaligene* (2 isolates) and *Pseudomonas putida* (1 isolate).

Table 3:- Isolation of *Pseudomonas spp* from examined Raw milk and Ice Cream samples.

Isolated organisms	No. of RM Samples	Isolates from Raw Milk		No. of IC Samples	Isolates from Ice Cream	
		No.	%		No.	%
<i>Pseudomonas aeruginosa</i>	60	7	11.66	50	5	10
<i>Pseudomonas fluorescens</i>		2	3.33		2	4
<i>Pseudomonas alkaligene</i>		1	1.6		1	2
<i>Pseudomonas putida</i>		0	0		1	2
Total			10		16.66	

RM: Raw Milk samples IC: Ice Cream samples

The *Proteolytic* and *Lipolytic* activities of *Pseudomonas* spp. The high spoilage potential of *Pseudomonas* spp virulence were produced thermostable proteases and lipases. *Pseudomonas* spp. isolates did not show proteolytic and lipolytic activities at 4°C, while at 20°C the *P. aeruginosa* and *P. fluorescens* isolates showed these activities as shown in Table (3) isolates were positive for proteolytic;(9 isolates), lipolytic (6 isolates) and both proteolytic and lipolytic;(2 isolates) .

Table 4: - Evaluation of Lipolytic and proteolytic activities of different isolated strains

Isolated strains	No. isolates	<i>Lipolytic</i>		<i>Proteolytic</i>	
		No.	%	No.	%
<i>Pseudomonas aeruginosa</i>	12	3	25	7	58.33
<i>Pseudomonas fluorescens</i>	4	2	50	2	50
<i>Pseudomonas alkaligene</i>	2	1	50	0	0
<i>Pseudomonas putida</i>	1	0	0	1	100
Total	19	6	31.57	9	47.36

Gram-negative, oxidase and catalase positive rods, identification by commercial biochemical reaction Analytical Profile Index (API 20 NE) test kits and some biochemical tests. Also, enzyme activities of the strains were determined by proteolytic and lipolytic and lecithinase. The isolated strains included (12 of *P. aeruginosa*, 4 of *P. fluorescens*, 2 *P. alkaligene*, and 1 *P. putida*).

Fig 2:- The result of PCR for detection of *aprA* and *exotoxin A* genes:

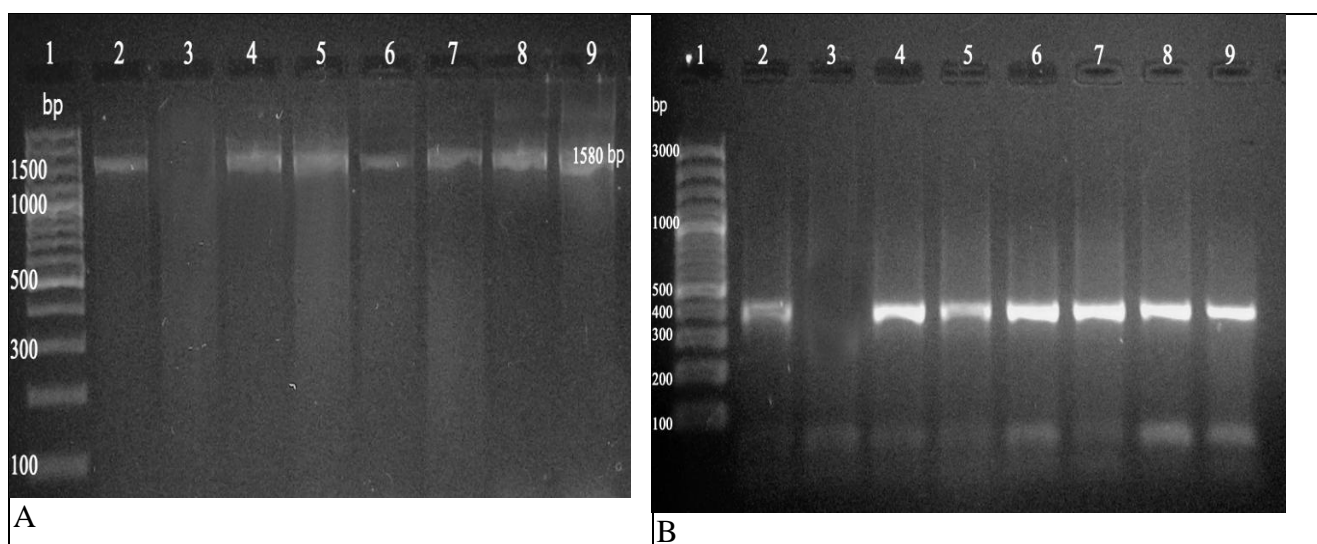
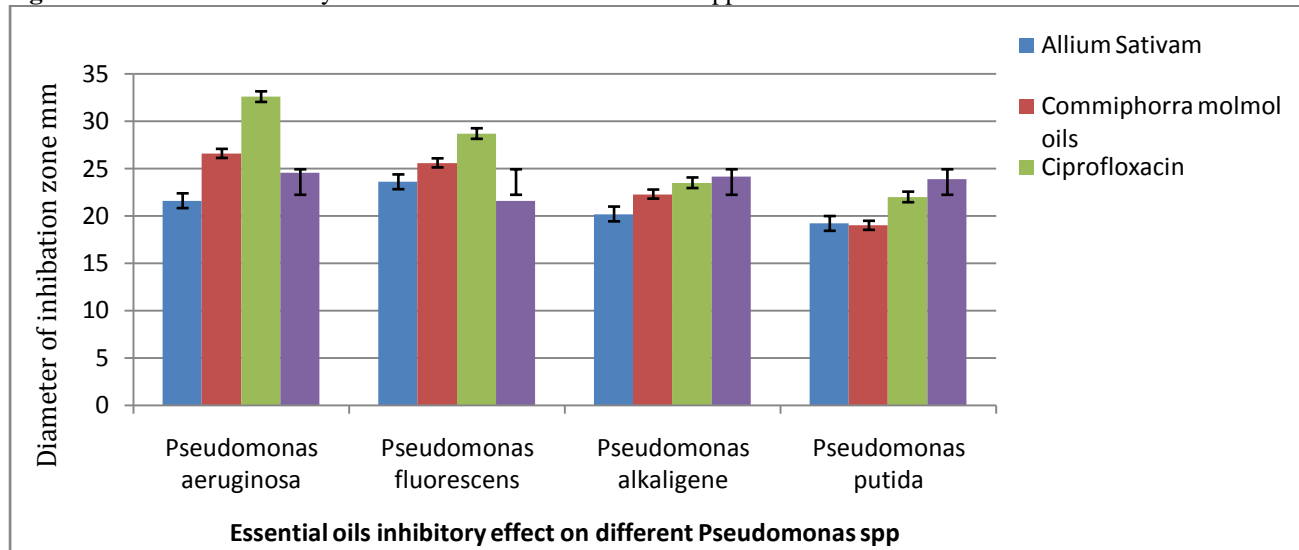


Figure (2 A) . PCR amplification of *aprA* gene in genome Lane 1: 200bp DNA ladder. Lane 2: Control Positive Lane 3: Control Negative Lane 4-9: samples positive for *aprA* gene at 1580-bp : (Fig 2 B)] . PCR amplification of *ETA* gene in genome Lane 1: 100bp DNA ladder. Lane 2: Control Positive Lane3: Control Negative Lane 4-9: samples positive for *ETA* gene at 396bp

The result revealed that the detected of *P. aeruginosa* were positive for *aprA* and *ETA* genes at 1580-bp and *ETA* gene at 396 – bp. as shown in Fig. 2 (A and B).

Table 5:- Determination of Antibacterial activity of essential oils against *Pseudomonas* spp tested by disc diffusion method and Minimal inhibition concentration

<i>Pseudomonas</i> Spp	Antibacterial IZD \pm SD * EO				Antibacterial MIC ** EO			
	<i>A. Sativam</i>	<i>C. mol oils</i>	Cipx	Ery	<i>A. Sativam</i>	<i>C. mol</i>	Cipx	Ery
<i>P.aeruginosa</i>	21.6 \pm 0.33	26.6 \pm 0.33	32.6 \pm 0.33	24.6 \pm 0.33	6.25	3.25	1.625	3.25
<i>P. fluorescens</i>	23.6 \pm 0.33	25.6 \pm 0.33	28.7 \pm 0.33	21.6 \pm 0.33	3.25	6.25	1.625	3.25
<i>P. alkaligene</i>	20.2 \pm 0.59	22.3 \pm 0.96	23.5 \pm 0.48	24.2 \pm 0.25	6.25	3.25	0.125	1.625
<i>P. putida</i>	19.2 \pm 0.58	19. \pm 0.85	22. \pm 0.65	23.9 \pm 0.56	6.25	3.25	0.125	1.625

Fig3:- Essential oils inhibitory effect on different *Pseudomonas* spp**Discussion:-**

Mastitis is one of the most important problems in dairy production from the socioeconomic, , public health and diagnostic point of view. About several species of pathogens, mostly bacteria is able to infect mammary gland. The pathogens are divided into contagious and environmental pathogens which causing mastitis in dairy cattle worldwide.[32] These pathogens have adapted to survive within the mammary gland and distribute among cattle nearly at the time of milking and early diagnosis of the initial cases of mastitis is necessary for prevention and control of infection spreading in the herds. Although the milk culture is considered as a gold standard test for diagnosis of mastitis, there are several disadvantages associated with bacterial culture of milk is time consuming and species identification by standard biochemical methods requires more than 72 hrs to be completed. Polymerase Chain Reaction (PCR) has been developed to identify different mastitis microbes due to the above-mentioned limitations of cultural methods. The progress of PCR based technique is rapid and accurate detection of different microorganisms. Results in table (1) classical microbiological methods applied to detect bacterial load in raw milk and ice cream samples. The Sixty raw milk samples examined were 71×10^3 cfu/ml and in ice cream 62×10^3 cfu/ml microbial were observed in nutrient agar medium, CLED agar raw milk samples were 62.5×10^3 cfu/ml and in ice cream samples were 42.2×10^3 cfu / ml microbial load were observed. While in case of MacConkey agar medium, in raw milk samples were 52.5×10^3 cfu/ml and in ice cream samples were 37.2×10^3 cfu/ml microbial load. But in MSA medium 1.2×10^3 cfu/ml microbial load were observed in Raw milk samples and 0.9×10^3 cfu/ml in ice cream samples. These method is still employ as classical approaches based on culture, colony growth, morphological and biochemical identification of microorganism. Nature of milk and dairy products are considered a good media for the growth of microorganisms due to abundant water, nutrients, nearly neutral pH and the proteins and lipids must be broken down by enzymes to allow sustained microbial growth. [32, 33, 34]. The results of the phenotypic characterization as physiological, morphological and biochemical tests for pathogens isolated from raw milk and ice cream were performed according to Bergey's Manual of laboratory examination. Raw milk and ice cream samples indicated that raw milk samples found that microbes *E.coli* (9), *S. aureus* (8), *S. typhi* (4) and *Pseudomonas* spp (10). A total of ice cream samples were examine for the isolation of bacteria, (18) isolates which *E. coli* (6), *S.aureus* (5), *S. typhi* (4) and *Pseudomonas* spp (6) as in Table (2) and Fig (1) and this result is agree with [33,34]. Some pathogens may also be contaminants and if temperature abuse occurs then they proliferate as was shown in the results by growth of contaminated bacteria species at stimulated temperature abuse conditions. This can then result in spoilage or food poisoning. The results showed that ice cream can be an ideal medium for growth of these organisms . This was more evident when the samples were incubated at 25°C which is equivalent to ambient temperatures in Egypt specially in winter, spring and autumn. This is due to the fact that even small doses of pathogenic organisms in foods, such as ice cream, constitutes a potential risk particularly to children, pregnant women, immuno compromised adults and the elderly. This shows that at refrigeration temperatures the organisms are capable of growing if the commodity is kept for prolonged time. Though ice cream is not kept at refrigeration temperatures, temperature abuse and inadequate freezing during storage of ice cream may result in the organisms proliferating. *Pseudomonas* spp are important pathogenic microorganisms which associated with food spoilage which is a serious problem in developing countries, because of inadequate processing and refrigeration facilities [35]. In Table (3). Incidence of *Pseudomonas* spp among isolated bacteria from raw milk and ice cream samples. The results represented that 19 samples were positive for *Pseudomonas*

spp., with (17.27 %). Identity of the isolates were confirmed by API 20 NE for detection of differentiate *Pseudomonas* spp. *P. aeruginosa* (12 isolates) isolates of *Pseudomonas fluorescens* (4 isolates), *Pseudomonas alkaligene* (2 isolates) and *P. putida* (1 isolate) this result is agree with [36, 37]. While the prevalence rat in mastitis milk was (12%). the lower prevalence rat (3.85%) in mastitis cases, were achieved by [37]. *P. aeruginosa* depend on their pathogenesis on lipolytic and proteolytic enzymes which play as important factors in infection and in milk industries through degradation of milk casein and lipolysis of milk fat which affect on quality and self life of product. [38]. In the present study, most of *p. aeruginosa* isolates were produced for extracellular enzymes: protease, lipase similar result obtained by [39,40]. The result shown that most isolates of *P. aeruginosa* was positive for slime production which has important role in identification and enhancement virulence of microorganism through act as a protective adherence surface against phagocytosis, and play role in rapines of milk products [5]. Pigment production was detected in most isolates and the result confirmed that *P. aeruginosa* was well producer for pyocyanin pigment which is considered a virulence factor through, increase the resistance of the microbe to antibiotics, also has antibacterial effect on some microorganisms as shown in the present study against staph. aureus and similar result achieved by [41]. Molecular diagnosis of virulence genes is new, rapid, sensitive and accurate method for detection of *aprA* gene encoded for alkaline protease, which considers one of virulence factors of *P. aeruginosa* through interfere with host immunity [42] who revealed that alkaline proteases is responsible for reduce phagocytosis of *P. aeruginosa* by macrophages.

PCR technique has rapid, sensitive, specific and can improve the level of detection. Both biochemical and genetic analyses are accurate, but cost comparatively high. Overall, novel rapid identification of microorganisms are still important step toward a appropriate treatment of infectious diseases in veterinary and medical diagnosis today [43]. Identification of different microorganisms with mass spectrometry has accurate and rapid diagnosis of bacteria. The result of PCR, showed that Figure (2 A). PCR amplification of *aprA* gene in genome Lane 1: 200bp DNA ladder. Lane 2: Control Positive Lane3: Control Negative Lane 4-9: samples positive for *aprA* gene at 1580-bp : (Fig 2 B)] . PCR amplification of *ETA* gene in genome Lane 1: 100bp DNA ladder. Lane 2: Control Positive Lane3: Control Negative Lane 4-9: samples positive for *ETA* gene at 396bp, similar result obtained by [43]. Exotoxin A is a lethal virulent factors of *P. aeruginosa* which play important role in pathogenesis through inhibit macrophage progenitor cell proliferation [45]. Molecular detection of exotoxin A gene is rapid, specific, sensitive method for identification of *P. aeruginosa* In our research, [46].

Table (5) and Fig (3) showed clearly that *Commiphora molmol* oils have the highest inhibitory effect were (26.66± followed by *Allium sativum* IZD were (21.6± and 23.6) against *Pseudomonas* spp comparing to ciprofloxacin and Erythromycin respectively. Sensitivity of bacteria needed a largest concentration of essential oils to induce bactericidal activity as in Table (5) and this finding is agreement with [47].

The unexpected results was no variation between inhibition zone of susceptible strain and resistant strain this explanation may be due to essential oils caused disrupted bacterial cells and this finding is in agreement with [48]. They found that natural essential oils caused damaged and lysis of bacterial cells. The mechanism of spreading of antibacterial resistance from animals to humans. The explanation of antibacterial activities of natural essential oils on bacterial cells depend on the action of active principle as terpenes fraction that involve their action by causing membrane perforating and leakage of cytoplasmic materials lead to irreversible destruction of cytoplasmic membrane but still fully action unknown and this explanation is agreement with [49].

Minimal Inhibition Concentration inhibitory values were determined for each oil plant extract showed inhibitory effect, compared with antibiotics. The obtained results from Table (5) showed clearly that the *Commiphora molmol* oils has the highest inhibitory effect determined by MIC ranged from (3.25 and 6.25mg/ml) followed by *Allium sativum* ranged from (3.25 to 6.25 mg/ml) comparing to ciprofloxacin and Erythromycinranged from (3.25 to 6.25 mg/ml) respectively. Our results showed that the inhibitory effects of E.O. on bacteria may be due to reduce or modification resistance bacteria but real mechanism still unknown. Whenever, essential oil is explained by structural change in bacterial cell especially in MDR Firstly, essential volatile oil fraction can be connect with perforation of bacterial cell membrane. Secondly, the essential oil action may be inhibited protein binding protein production (PBP) by inhabited some related gene lead to suppression of cell wall synthesis(Transpeptidation mechanism) which take place outside the cell membrane and this opinion is confirmed by [23, 50,51]. Finally *P. aeruginosa* was the most predominant isolate followed by *P. fluoresces*. Diagnosis of *Pseudomonas* spp with molecular methods is a rapid, sensitive and accurate method for identification, detection of virulence factors and differentiation of *Pseudomonas SPP* isolated from dairy milk and ice cream .Also, essential oils from *Commiphora molmol* and *Allium sativum* have a marked antibacterial effect against *Pseudomonas* spp and may be use a novel antibacterial agents but it's still need extensive study for safety and clinical study in vivo.

Conclusion:-

Successful strategy for detection of clinical and subclinical mastitis can be established with an effective monitoring system of all dairy cattle and accurate rapid diagnosis with automated VITEK 2 system and molecular methods consider Novel technique for routinely diagnosis in microbiology diagnosis for animals as using in human laboratory diagnosis would

enable a change in work practices. but still need complete integration into laboratory workflow and several steps need develop such as detection sensitivity and specificity before use as diagnostic tools directly from clinical samples. *P. aeruginosa* was the most predominant isolate from raw milk and ice cream. Diagnosis of microorganisms with molecular methods is a rapid, sensitive and accurate method for identification isolates from dairy milk and ice cream. Also, essential oils from *Commiphora molmol* and *Allium sativum* have a marked antibacterial effect against different microbial species and may be use a novel antibacterial agents but it's still need extensive study for safety and clinical study in vivo

Authors Contributions:-

Dr. Abeer M. Abdalhamed Dr. Gamil S.G. Zeedan authors, design study plan, data and samples collection, part of laboratory work and interpret the data; drafting and reviewed the manuscript. Dr. Heba Hussein author, study design, data and samples collection and part of laboratory work. Dr. Eman Abdeen author, study design; samples collection and part of laboratory work and sharing in drafting manuscript. All authors have read and approved the final revised manuscript

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The authors don't have any conflict of interests regarding the content of the manuscript

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