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Some Virulence Genes of Pathogenic Enterococci Isolated from Raw Milk and Some Milk Products

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Abstract

A total of 150 random samples of raw cow milk and some locally manufactured dairy products including yoghurt, Kareish cheese and ice cream were collected from various dairy shops, and supermarkets in Qena city, Egypt. Samples were examined for the presence of Enterococcus spp. The investigation revealed that 64, 28, 76, 72 and 16 % of the examined raw milk samples, large and small-scale yoghurt, Kareish cheese and ice cream were contaminated with Enterococcus spp., respectively. Isolates were identified as E. faecalis and E. faecium in percentages of (8 & 32), (16 & 0), (8 & 28), (8 & 36), and (4 & 0) in the examined raw milk samples, large and small-scale yoghurt, Kareish cheese and ice cream, respectively. The obtained isolates were screened for presence of some virulence genes gelE, asa1, esp and cylA using multiplex PCR. The results indicated that gelE, asa1, esp and cylA were located in 53.9, 76.9, 69.2, and 30.8% of the total E. faecalis isolates and in 46.9, 71.9, 53.1, and 34.3 % of the total E. faecium isolates, respectively. The asa1 and esp genes were the predominant virulence traits among all investigated enterococci isolates followed by gelE and cylA genes. Therefore, the results of this study showed that milk and dairy products can play an important role in the spread of Enterococci with virulence potential through the food chain to the human population.

Keywords: Enterococcus spp., milk, dairy products, PCR, virulence genes

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Introduction

The genus Enterococcus is Gram-positive, catalase and oxidase negative, non-spore-forming, facultative anaerobic bacteria that can occur both as single cocci and in chains. Enterococci belong to a group of organisms known as lactic acid bacteria (LAB) that produce bacteriocins (Thurlowet al., 2009 and VanTyne and Gilmore, 2014). Enterococci often occur in large numbers in soil, water, in the gastrointestinal tract of animals and humans, and foods especially those of animal origin such as milk and dairy products (Franz et al.,1999). The ability of Enterococci to colonize different ecological niches and spreading within the food chain is due to their resistance to the adverse environmental conditions (Giraffa, 2002). Enterococcus spp. has become one of the most common nosocomial pathogens especially in immunosuppressed patients with a high mortality rate of up to 61% (Pohet al., 2006). Enterococci have been implicated in cases of food poisoning, e.g. by production of biogenic amines. Food intoxication caused by ingestion of biogenic amines determines a number of symptoms which include headache, vomiting, increase of blood pressure and even allergic reactions of strong intensity (Giraffa, 2002). Enterococci may carry various genes such as aggregation substances (asa1), endocarditis antigen, gelatinase (gelE), Extracellular surface protein (esp), Cytolysin (cylA) and hyaluronidase or adhesion collagen protein have been described in enterococci isolated from foods (Hammad, et al.,2015). The presence of virulence genes in foods is currently a matter of concern because Enterococci may be involved in the transmission of virulence genes via the food chain (Trivediet al., 2011). Therefore, this study put a focus on isolation and identification of Enterococcus spp. from raw milk and some milk products as well as detection of some virulence genes by multiplex PCR.

Materials and Methods

products including yoghurt (25 large scale and 25 small scale samples), Kareish cheese (25 samples) and ice cream (25 samples) were collected from various dairy shops, and supermarkets in Qena city, Egypt. These samples were transferred to the laboratory without delay to be examined. Heat treated milk was detected by Storch test (Lampert, 1975).

1. Samples preparation: were done according to (A.P.H.A., 1992)

2. Enumeration and isolation of Enterococcal isolates: were done according to (Deibel and Hartman, 1982).

3. Identification of Enterococcal isolates

3.1. Microscopical examination: (A.P.H.A., 1992)

3.2. Biochemical identification: was done according to Morrison et al., (1997) and Manero and Blanch (1999).

4. Detection of some virulence genes in E. faecalis and E. faecium isolated from the examined samples by multiplex PCR (Dogru et al., 2010)

4.1 DNA Extraction and PCR amplification

DNA extraction from samples was carried out using the QIAamp DNA

Mini kit (Qiagen, Germany) according to the manufacture's recommendations. The DNA amplification was performed using the oligonucleotide primers recorded in Table 1 as described by (Dogruet al., 2010). The reaction was conducted in a thermal cycler. The cycling parameters were an initial denaturation at 95 °C for 10 min, followed by 30 cycles of denaturation (94 °C for 1 min), annealing (56 °C for 1 min), extension (72 °C for 1 min), and a final extension step at 72 °C for 10 min. The PCR reaction was performed in a mixture of 25 µl. The reaction mix consisted of 2.5 µL of bacterial lysate, 2.5 µL of Template DNA, 5 µl of 10x assay buffer for Taq polymerase containing 1.5 mM MgCl2, 2 µl of 10mM dNTP mix, 1 µl each of forward and reverse primer (10 pmol) and 2.5U of Taq DNA polymerase.

4.2. Detection of PCR products:

PCR products were analyzed by electrophoresis on 1.5% agarose gel stained with ethidium bromide and Figgraphed under UV light.

Results and Discussion

Enterococci are commonly encountered in raw milk and dairy products including even those undergo heat treatment. Their ability to withstand processing conditions renders them potentially important from the public health point of view as their presence is indicative of fecal contamination (Brooks, 1974). According to data presented in Table 2, Enterococci was counted in 64 % of the raw cow milk samples with an average count of 7.5 ×107cfu/ ml. Abd El-Hameid (2002), Abd El-Rahman (2010), Mohammad (2015) and Abd El Tawab et al., (2016) recorded higher incidences of Enterococci in dairy shops raw milk which reached 100, 83.3, 66 and 76%, respectively. While lower incidence (60%) was reported by Elmali and Hayriye (2018). The isolated Enterococci spp. recovered from the tested raw cow milk samples were biochemically identified as E. faecalis (8%), E. faecium (32%) as recorded in Table 3. Occurrence of Enterococci in milk is due to their wide distribution in nature hence it may contaminate milk through the contaminated water supply, equipment and unhygienic conditions during production and handling through the journey of milking to reach the consumer. They have been incriminated as direct or indirect cause of disease and food poisoning because of their ability to produce extracellular toxic metabolites (Roushdy et al., 1998). Regarding large scale yoghurt samples, the data summarized in Table (2) postulated that 28% of the examined samples were contaminated with Enterococci in counts ranged from < 102 to 2.4×105 with an average count of 64.8×103cfu/g. Higher incidences 40% and counts 4.7×104cfu/g was reported by Abd El-Rahman (2010). On the other hand, Abd El-Aal, (2008) demonstrated lower incidence of 20%, and lower counts of Enterococci7.3 ×10 and 1.4×103cfu/ g, respectively. Concerning small scale yoghurt samples, it was found that 76 % of the examined samples were contaminated with Enterococci with a minimum of <102, a maximum of 4. 5 ×108cfu/ g and an average value of 83.6×105cfu/ g. Lower incidences of 60 and 52% were reported by (Ahlam, et al., 2015) and Abd El Tawabet al., (2016) respectively. Higher incidence (77.5%) and lower count of (15.4×103cfu/ g)

was reported by Al-Hawary (2000). In contrary with the obtained results, lower incidences and counts were recorded by El-Malt et al., (2013b), El-Ansary (2014) and Gorgy et al., (2016), where they could isolate Enterococci from small scale yoghurt in percentages of 58, 28 and 32%, with counts of 1.7×104, 5.8 x 104 ± 5.43 x 103 and 5.5x103± 0.64 x103cfu/g, respectively. The existence of Enterococci in yoghurt is indicator of neglected sanitary measures during production and distribution. Moreover, Enterococcus able to survive the unfavorable microenvironment as the low pH value of yoghurt and other types of fermented milk (El-Ansary, 2014). The results obtained in Tables 2 revealed that (72%) 18 out of the 25 examined Kareish cheese samples were contaminated with Enterococci at levels varied from <102 to 1.8×108 with an average count of 3.94×107cfu/ g. Enterococci could be isolated in higher incidences and lower counts by Abd El-Rahman (2010) and Mohammad (2015).They isolated Enterococci in percentages of 83.3 and 86.7 % with counts of 1.5×106 and 3.4×106cfu/g. respectively. Likewise, Ahlam et al., (2015) and Abdeen (2016) recovered higher incidence 86.6 and 90%, respectively. On contrary, Hussien et al., (2013) and Gorgy et al., (2016) recorded lower incidence 65.7 and 36% and count 2.4×106 and 5.7x103±1.6x103cfu/ g. respectively. High contamination levels of Enterococci are considered to cause the deterioration of organoleptic properties in some cheese (Lopez-Diaz et al., 1995). The obtained high levels of Enterococci in Kareish cheese samples may be contributed to the lack of pasteurization of milk. Also, the production of Kareish cheese in Egypt is generally a house-hold process which takes place under poor sanitary practices during manufacturing, handling, storage and distribution of cheese that may constitute a public hazard and induce food poisoning. According to the data presented in Tables 2, it was found that 16% of the examined ice cream samples were contaminated with Enterococci with an average value of 1×104cfu/ g. Higher incidences (54%) and lower count (6.9x 103cfu/ ml) were recorded by El-Malt et al., (2013a). The presence of Enterococci in ice cream samples seems to be illegal, because no acceptable level of these microorganisms could be present. Their occurrence may be attributed to post- manufacture contamination, heat resistance character of the organism and contamination during packaging or improper methods of distribution. Furthermore, at below freezing temperature, Enterococci remained viable for long periods and able to multiply at temperature below 4.5 and 10^{o}C (Angelottiet al., 1963). Several studies have recently shown that Enterococci spp. possess putative virulence factors that play important role in its pathogenesis such as gelatinase (gelE), aggregation substance (asa1), extracellular surface protein (esp) and cytolysin (cycl A) (Barbosa et al., 2010). E. faecalis and E. faecium remain the species of greatest importance amongst the different Enterococci spp. that found in milk and dairy products, so the present study focused on detection of some virulence genes in these two species because virulence genes may be transmitted via the food chain (Trivediet al., 2011). Data presented in Table (3), and Figs (1 & 2) showed that the gelE gene was

found in 50 and 62.5 % of E. faecalis and E. faecium isolates from raw milk submitted to PCR. Similar result was reported by Hussein (2013) while, higher incidence (86%) obtained by Inhoque et al., (2017). Lower incidences of gelE (33.3 and 17.24 %) in E. faecium were reported by Abd El Tawab et al., (2016) and Inhoque et al., (2017). In the present study the gelE gene was detected in a total of 50 &42.9% of E. faecalis and E. faecium strains isolated from yoghurt samples, respectively (Table 3 and Figs 1&2). The same incidence of gelE gene in E. faecalis isolates was reported by Abd El Tawab et al., (2016). From data illustrated in Table (3), and Figs (1& 2), the gelE gene was found in 50 and 22.2 % of E. faecalis and E. faecium isolates from Kareish cheese samples, respectively. Also, Gomes et al., 2008 recorded higher incidences (95.1%) of gelE of E. feacalis. The gelE gene was detected in all investigated E. faecalis obtained from large scale ice cream samples, while none of E. faecium harbored gelE virulence gene Table (3), and Figs (1&2). Aggregation substance (asa1) is a surface protein adhesion encoded by asa1 and is exclusively found in E. faecalis strains however, its incidence among food isolates seems to be high (Franz et al., 2001). The present study showed that all E. faecalis isolates obtained from raw cow milk carried asa1 gene, while 75 % of E. faecium strains isolated from raw milk were positive for presence of asa1 gene (Table 3 and Figs 1&2). Lower incidence was recorded by Hussein (2013), Hosseini et al., (2016) and Inhoque et al., (2017) as they detected asa1 gene in 75, 84.6 and 26 %of E. faecalis, respectively. As well, the asa1 gene was found in a total of 66.7 & 57.1 %of E. faecalis and E. faecium strains isolated from yoghurt samples, respectively. Abd El Tawab et al., (2016) found that none of E. faecalis obtained from yoghurt samples harbored asa1 gene. Regarding Kareish cheese samples asa1gene was found in 50 &77.8 % of E. faecalis and E. faecium, respectively. Hosseini et al., (2016) detectedasa1 gene in all E. faecalis strains obtained from dairy cheese, While Gomes et al., (2008) recorded lower incidence of E. faecalis asa1 gene 26.8. Higher incidence80% of asa1 genes in E. faecium was reported by Hosseini et al., (2016). The asa1 gene was detected in all investigated E. faecalis isolates from large scale ice cream samples, while

The present study showed that esp gene was found in 66.7 &71.4%of E. faecalis and E. faecium strains obtained from yoghurt samples, respectively Table (3). Higher results obtained by Abd El Tawab et al., (2016) as he found that all E. faecalis isolates obtained from yoghurt samples harbored esp gene. None of virulence genes was recorded for E. faecium in large scale yoghurt samples.

Table 1. Primer sequences used for PCR

Target gene	Virulence factor	Oligonucleotide sequence (5′ → 3′)	Primer name	Product size (bp)	References

***gelE* (F)**	Gelatinase	5′ TATGACAATGCTTTTTGGGAT ′3	GEL 11	213	Vankerckhoven *et al*., (2004)
***gelE* (R)**		5′ AGATGCACCCGAAATAATATA ′3	GEL 12		
***asa1* (F)**	Aggregation substance	5′ GCACGCTATTACGAACTATGA ′3	ASA 11	375	
***asa1* (R)**		5′ TAAGAAAGAACATCACCACGA′3	ASA 12		
***esp* (F)**	Enterococcal surface protein	5′ AGATTTCATCTTTGATTCTTGG′3	ESP 14F	510	Willems *et al*. (2001)
***esp* (R)**		5′ AATTGATTCTTTAGCATCTGG ′3	ESP 12R		
***cylA* (F)**	Cytolysin	5′ ACTCGGGGATTGATAGGC′3	CYT I	688	Coque *et al*., (1995)
***cylA* (R)**		5′ GCTGCTAAAGCTGCGCTT′3	CYT IIb		

Table 2. Statistical analytical results of *Enterococcus spp*. in the examined raw milk, yoghurt, Kareish cheese and ice cream samples

Type of samples	No. of examined samples	Positive samples		Count/g or ml		
		No.	%	Minimum	Maximum	Average
Raw milk	50	32	64	*$<10^2$	4.3×10^8	7.5×10^7
Large scale yoghurt	25	7	28	*$<10^2$	2.4×10^5	64.8×10^3
Small scale yoghurt	25	19	76	*$<10^2$	4.5×10^8	83.6×10^5
Kareish cheese	25	18	72	*$<10^2$	1.8×10^8	3.9×10^7
Large scale Ice cream	25	4	16	*$<10^2$	3.9×10^4	1×10^4

*No colonies could be detected on the plates.

Table 3. Incidence of *gelE*, *asa1*, *esp* and cylA *genes could be detected in E. faecalis and E. faecium* isolated from examined sample

Type of samples	***Enterococci spp.***	**No. of isolated strains**		**Virulence genes**							
				gelE		***asa1***		***esp***		***cylA***	
		No.	%	No.	%	No.	%	No.	%	No.	%
Raw milk	*E. faecalis*	4	8	2	50	4	100	2	50	2	50
	E. faecium	16	32	10	62.5	12	75	9	56.3	3	18.8
Large scale yoghurt	*E. faecalis*	4	16	2	50	3	75	3	75	N	N
	E. faecium	N	N	N	N	N	N	N	N	N	N
Small scale yoghurt	*E. faecalis*	2	8	1	50	1	50	1	50	1	50
	E. faecium	7	28	3	42.9	4	57.1	5	71.4	4	57.1
Kareish cheese	*E. faecalis*	2	8	1	50	1	50	2	100	N	N
	E. faecium	9	36	2	22.2	7	77.8	3	33.3	4	44.4
Large scale Ice cream	*E. faecalis*	1	4	1	100	1	100	1	100	1	100
	E. faecium	N	N	N	N	N	N	N	N	N	N

On the contrary, esp gene was detected in all investigated E. faecalis isolates obtained from Kareish cheese samples Table (3) and Figs (1& 2). Lower incidence (75%) obtained by Abdeen et al., (2016). Moreover, esp gene was detected in 33.3% of E. faecium isolates from Kareish cheese samples. Hosseini et al., (2016) could not detect esp in E. faecalis strains obtained from cheese but detect esp genes in 40 %of E. faecium. Although the esp gene was detected in all investigated E. faecalis strains, it couldn't be located in E. faecium isolates obtained from large scale ice cream samples Table (3) and Figs (1& 2). Cytolysin (cylA) is one of the best characterized enterococci virulence factors. It has β-haemolytic properties which considered undesirable in foods and their use as starters in food fermentation is not recommended (Fifadaraet al., 2003). The results achieved in Table (3) and Figs (1&2) revealed that cyclA gene was found in 50 and 18.8 % of E. faecalis and E. faecium strains isolated from raw milk, respectively. Higher incidence of cyclA gene in E. faecalis isolates obtained by Gomes et al., (2008) as they found cylA in 88% of the obtained isolates, while Hussein (2013) detect lower incidence of cylA gene (25%). In yoghurt samples it was found that cyclA gene was detected in 16.7 and 57.1 %of E. faecalis and E. faecium, respectively. Abd El Tawab et al., (2016) couldn’t detect cyclA gene in E. faecalis obtained from yoghurt samples.

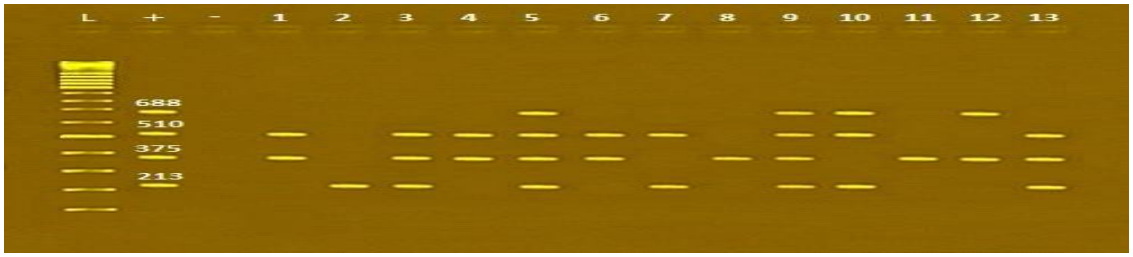

Fig. 1. Detection of *gelE*(213 bp), *asa1* (375 bp), *esp* (510 bp) and *cylA* (688 bp) encoded virulence genes of *E. faecalis* strains isolated from the examined samples by multiplex PCR*Lane (L) (DNA ladder 1000 bp),*Lane (+) (positive control)*Lane (-) (negative control)*Lanes 8, 9, 12 and13: DNA of *E. faecalis* strains isolated from raw milk samples,*Lanes 1, 2, 3, 4, 10 and 11: DNA of *E. faecalis* strains isolated from yoghurt samples,*Lanes 6 and 7: DNA of *E. faecalis* strains isolated from Kareish cheese samples,*Lane 5: DNA of *E. faecalis* strains isolated from large scale ice cream samples.

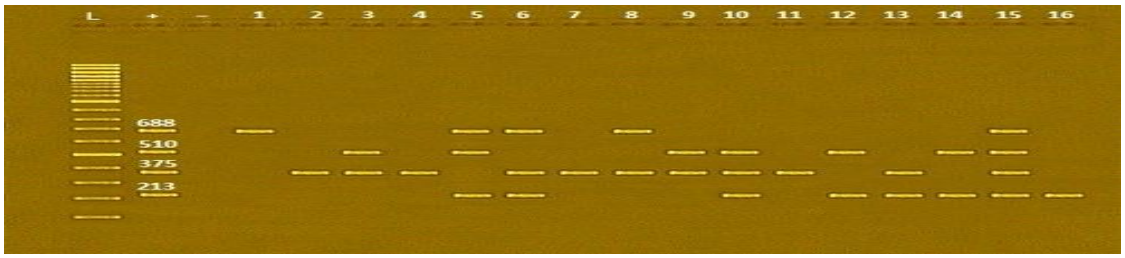

Fig. 2. Detection of *gelE* (213 bp), *asa1* (375 bp), *esp* (510 bp) and *cylA* (688 bp) encoded virulence genes of *E. faecium* strains isolated from the examined samples by multiplex PCR*Lane (L) (DNA ladder 1000 bp), *Lane (+) (positive control) *Lane (-) (negative control) *Lanes 10- 16: DNA of *E. faecium* strains isolated from raw milk samples*Lanes 1- 9: DNA of *E. faecium* strains isolated from Kareish cheese samples.

Just like the finding in the present study and presented in Table (3) and Figs (1 & 2), none of E. faecalis isolated from Kareish cheese samples and obtained by and Abdeen (2016) submitted to PCR harbored cycl Agene. All the investigated E. faecalis isolates obtained from large scale ice cream was found to carry cylA gene, while none of E. faecium strains had cyclA gene Table (3) and Figs (1 & 2).

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