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The Sensitivity of PCR to the Number of *Lactococcus garvieae* in Different Concentrations of NaCl: FTA Technique

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Abstract

The major objective of this study is to investigate the sensitivity of PCR to the number of *Lactococcus garvieae* (*L. garvieae*) in sterilized milk containing different concentrations of NaCl using FTA technique for genomic DNA extraction. This technique is a simple, rapid, precise and highly sensitive even to low count of bacterial cells in samples. The study used M17 medium to determine the number of bacterial cells, thereafter, FTA method was used for genomic DNA extraction. The results using M17 medium, indicated that the *L. garvieae* increased in all concentrations of NaCl during an incubation period of 24 hours, on the other hand, pH value decreased. However, the increase in bacterial count was statistically significant only on 0, 2% and 4% of NaCl concentrations. In conjunction with this, the PCR showed sensitivity to both low and high numbers of bacterial cells in the samples. Increasing bacterial numbers led to an increase in the amount of DNA extracted via FTA cards and therefore, the intensity and clarity of bands of PCR reaction was also increased. This is the first study that uses FTA technique for DNA extraction in dairy products. Using FTA cards and subjecting to PCR in quantifying the bacterial growth is a very important issue because of its direct application in the field of milk, agricultural and animal products research and development.

Keyword: *Lactococus garvieae,* FTA, specific PCR, molecular method

1. Introduction

L. garvieae is a major pathogen of fish that causes fatal hemorrhagic septicemia such as yellow tail and trout (Vendrell et al., 2006). It is isolated from buffalos with mastitis (Teixeira et al., 1996) and clinical specimens of human blood, urine, and skin (Vinh et al., 2006; Wang, 2007; Villani et al., 2001). Moreover, *L. garvieae* is isolated from various kinds of food products including cow's milk (Villani et al., 2001), cheeses (Alegr´ıa et al., 2009; Fortina et al., 2007; El-Baradei et al., 2007; Florez & Mayo, 2006), meat products (Aquilanti et al., 2007; Barakat et al., 2000; Rantsiou et al., 2005) and sprout (Kawanishi et al., 2007). *L. garvieae* is detected in most of Egyptian cheeses (El-Baradei et al., 2005) and also found in Jordanian cheese (Alrabadi, 2009). It is considered as one of microorganism causing mastitis (Collines et al., 1984; Teixeira et al., 1996). *L. garvieae* is a salt tolerant bacterium (6.5% salt) (Eleder et al., 1999).

Researchers have used many techniques for *L. garvieae* identification. The methods that were based on biochemical and antigenic characteristics and other semi-automated bacteriological systems such as API Rapid ID 32 Strep (bioMe´rieux) could not precisely differentiate between *L. garvieae* and *L. lactis subsp*. *Lactis* (Elliott et al., 1991; Holt et al., 1994). Utilizing phenotypic characterization with other characterization techniques; determination of clindamycin susceptibility, sodium dodecyl sulphate–polyacrylamide gel electrophoresis (Elliott et al., 1991), DNA, RNA and restriction fragment length polymorphism (Eldar et al., 1999; Eyngor et al., 2004) has resulted in a better identification and discrimination between *L. garvieae*, *L. lactis* and related bacterial species. However, Zlotkin et al. (1998) have developed a new polymerase chain reaction (PCR) assay based on primers deduced from the regions carrying the 16S rRNA genes of *L. garvieae*. They argued that PCR assay is the only practical test besides the clindamycin test which can specifically identify the *L. garvieae* and can differentiate it from *L. lactis*. Thereafter, PCR assays have became the most widely used technique in identifying *L. garvieae* in various sources (Aoki et al., 2000; Goh et al., 2000; Pu et al., 2002; Mata et al., 2004; El-Baradei et al., 2008; Fadaeifard et al., 2012). It is a quick, inexpensive and simple technique

(Erlich, 1989). Jung et al. (2010) developed a real-time quantitative PCR (qPCR) assay with primers for CAU12F and CAU12R based on the 16S rRNA gene of *L. garvieae*.

This study proposes FTA cards for DNA extraction. To the best of author's knowledge, this is the first study that uses FTA technique in identification of *L. garvieae* in dairy products. FTA is a method of collecting, transporting, storing, and purifying DNA safely and securely at room temperature. It is a cotton-based cellulose paper, impregnated with anionic detergent and buffer that provide chelating and free radical trapping properties. Moreover, it contains lyophilized chemicals that lyses many types of bacteria and viruses (Bayoumi et al., 2007). The FTA cards were originally used as blood storage media. Stored at room temperature, genomic DNA on FTA is documented by its manufacturer's to be stable for at least 17 years. Mullen et al. (2009) reported that these cards provided viable DNA extractions after 8 years of storage. They were mostly used to extract DNA from blood samples and generally fresh human fluids. Then it has been used for a variety of zoological materials (Crabbe, 2003; Smith & Burgoyne, 2004; Harvey, 2005; Borisenko et al., 2008), plant (Ndunguru et al., 2005; Owor et al., 2007) and fungi sampling (Barlocher et al., 2010). Recently, FTA cards were introduced to detect bacterial DNA or viral RNA from different biological samples. Specifically, they have been used in template preparation for PCR detection of pathogenic microorganisms. For example, Lampel et al. (2000), Orlandi and Lampel (2000), Lampel and Orlandi (2002), Hide et al. (2003), Warren (2003), Lampel et al. (2004) and Warren et al. (2005) used PCR to detect pathogenic microorganisms from different foods using FTA cards. FTA technique is a simple, rapid, precise and highly sensitive even to low count of bacterial cells in samples.

2. Materials and Methods

2.1 Bacterial Source

The L. garvieae that was used in this study was isolated and identified by PCR technique from four dairy products including, raw milk, Nabulsi cheese, Jordanian Jameed (Dried yoghurt) and Labaneh (fermented milk).

2.2 Enumeration of Lactococcus garvieae

In this test, 1 ml of an overnight culture (M17) was inoculated to 100 ml sterilized milk bottles containing different concentrations of NaCl and incubated for 24 hours .During this period, serial dilutions in sterile 1% (w/v) peptone water from the bottles were prepared at different times of incubation, plated on M17 agar medium and incubated at 30˚C.

2.3 PH Value

The pH of inoculation milk was measured using a glass electrode pH meter Accumel PH meter model 810 (fisher scientific) according to the British Standard Institution (B.S.L) bulletin no. 770 (1952).

2.4 Genomic DNA Extraction

FTA paper Technology was used to extract DNA as follow: First, 5 µl of milk that contain *L. garvieae* was taken and directly spotted onto FTA matrix cards. Second, samples were air dried at room temperature and stored at plastic sealed bags with desiccant. Third, 1.2mm and 2mm diameter FTA punches from the area of the dried spot were removed using micro punch. Fourth, the disc was transferred into a micro-tube and washed twice with 200 µl of Whatman FTA purification reagent. Finally, rinsed once with 200µl of TE buffer and dried at 55 C for 15 minutes. The washed and dried disc was directly used for molecular detection.

2.5 PCR Amplification

Specific PCR test was carried out on DNA extracted from *L. garvieae* using species-specific primers 1RL (TTTGAGAGTTTGATCCTGG) and LgR (AAGTAATTTTCCACTCTACTT) (Pu et al., 2002) in order to confirm identity of *L. garvieae*.

Species-specific primers based on previously published data were synthesized by MWG Biotech AG (Ebersberg, Germany). Primers were prepared at a final concentration of 60 µM in deionized autoclaved water. PCR amplification was performed in a Primus 25 advanced thermocycler (Peqlab, Biotechnology GmbH, Germany). The reaction mixture contained 1 µl of each primer, 50 µl of master mix and 25 µl of DNA solution extracted from FTA. Sizes of PCR products were determined by 1% agarose gel electrophoresis (Seakem CTG agarose, TEBU, France).

3. Result and Discussion

Table 1 shows the mean values of *L. garvieae* count which was isolated from four different diary sources; raw milk, Nabulsi cheese, Jordanian Jameed (Dried yoghurt) and Labaneh (fermented milk). The results indicate that the *L. garvieae* increased in all concentrations of NaCl during an incubation period that extended to 24 hours.

However, the increase in bacterial count was statistically significant (according to Least Significant Difference test (LSD) only on 0%, 2% and 4% of NaCl concentrations and specifically most significant at 0% concentration after 24 of incubation. The average log cfu/ml increased from 5.21 to 9.98 at 0 concentration, from 5.20 to 9.80 at 2% concentration and from 5.14 to 8.58 at 4% concentration. Moreover, Table 1 shows that the significance level of *L. garvieae* where the count was increased as the incubation period lengthened. Additionally, the control sample (sterilized milk free of *L. garvieae*) was stable with zero bacteria count. Table 2 reports changes in average pH values during the incubation periods. The pH value decreases after 24 hours of incubation from 6.80 to 5.01 at 0 concentration, from 6.66 to 5.31 at 2 % concentration and from 6.80 to 5.71 at 4% concentration. This refers to an increase in *L. garvieae* numbers. These results are in agreement with Eleder et al. (1999) who reported that *L. garvieae* could grow at 6.5% NaCl concentration. In addition to it, *L. garvieae* can grow in different level of pH values (El-Baradei et al., 2005).

As shown in Figure (1), FTA method has proven to be an excellent method for identifying the existence of *Lc.garvieae* in different concentration of DNA extraction. Thus, the bands appear in all samples that we used in PCR reaction. The bands became more clear and intense when the numbers of bacterial cells increased. They were very clear in band numbers (4 & 5). While the control sample which contained no bacterial cells did not show any band. These results are consistent with Respire (1997) who has shown that PCR's sensitivity is related to the number of organisms present in the sample.

Table 1. Viability of *L. garvieae* on different concentrations of NaCl

NaCl concentration%	Time (hour)					
	0	2	4	8	12	24
0	5.21 ^{NS}	7.22 ^{NS}	7.38 ^{NS}	8.39*	8.46*	9.98**
2	5.20 ^{NS}	6.76 ^{NS}	7.80*	8.33*	8.45*	9.80**
4	5.14 ^{NS}	5.34 ^{NS}	5.50 ^{NS}	6.99 ^{NS}	7.50 ^{NS}	8.58*
6	5.16 ^{NS}	5.19 ^{NS}	5.39 ^{NS}	5.71^{NS}	5.83 ^{NS}	6.00 ^{NS}
8	5.13 ^{NS}	5.22 ^{NS}	5.30 ^{NS}	5.38 ^{NS}	5.43 ^{NS}	5.63 ^{NS}
Control	0.00 ^{NS}	0.00 ^{NS}	0.00 ^{NS}	0.00 ^{NS}	0.00 ^{NS}	0.00 ^{NS}

** indicates significant LSD value at p value ≤ 1%;

* indicates significant LSD value at p value ≤ 5%;

^{NS} denotes not significant.

Table 2. Change in pH value during incubation period

NaCl concentration%	PH value					
	0	2	4	8	12	24
0	6.80	6.71	6.58	6.32	5.58	5.01
2	6.66	6.59	6.43	6.28	6.09	5.31
4	6.80	6.71	6.60	6.42	6.24	5.71
6	6.39	6.39	6.34	6.29	6.22	6.11
8	6.40	6.40	6.38	6.36	6.30	6.24
Control	6.88	6.88	6.88	6.88	6.88	6.88

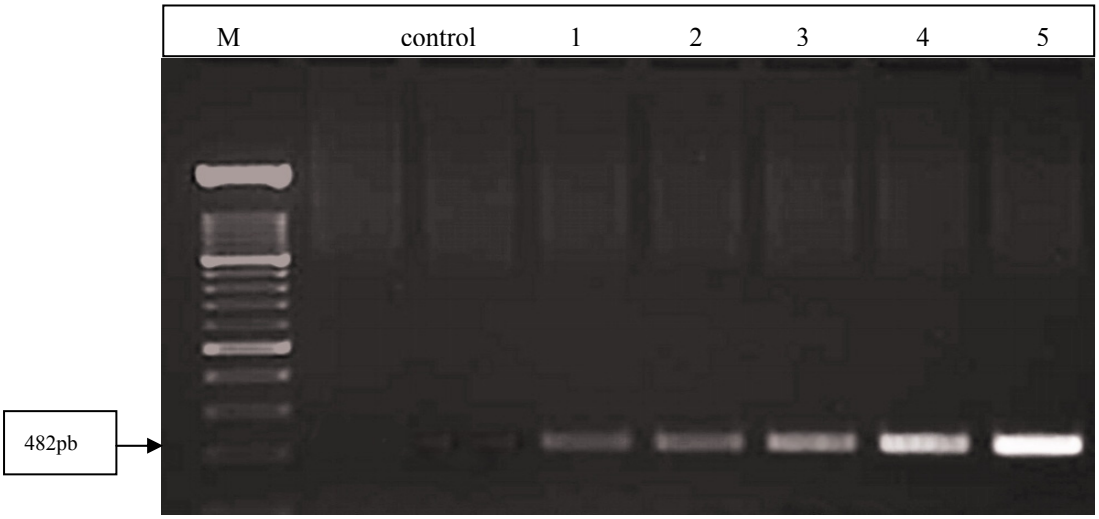

Figure 1. Presence of *L. garvieae* using species-specific PCR test

The figure shows Agarose gel of the amplification products of DNA extracted from FTA cards with species-specific primers (bands1,2,3,4,5 were from samples that showed these numbers 5.13, 6.00, 7.22, 8.33 and 9.98 log cfu/ml, respectively, M: Smart Ladder Marker).

4. Conclusion

The FTA technique for genomic DNA extraction is a simple, rapid, precise and highly sensitive method that enables to detect even low count of bacterial cells in samples. This study investigated the sensitivity of PCR to the number of *L. garvieae* in sterilized milk containing different concentrations of NaCl. To our knowledge, this is the first study that used FTA technique in dairy products. The count of *L. garvieae* increased significantly in concentrations of 0%, 2% and 4% NaCl. The PCR showed sensitivity to both low and high numbers of bacterial cells in the samples. Increasing bacterial numbers led to an increase in the amount of DNA extracted via FTA cards and therefore, the intensity and clarity of bands in the PCR reaction also increased. Our findings will play a major role at both the academic and practical levels in the field of dairy products.

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