Turk J Vet Anim Sci
29 (2005) 475-480

Research Article

Plasmids and Protein Patterns of *Escherichia coli* Isolated from Bovine Mastitis in Konya, Turkey

Uçkun S. UÇAN
Department of Microbiology, Faculty of Veterinary Medicine, Selçuk University, Konya - TURKEY

Leyla AÇIK*
Department of Biology, Faculty of Science and Arts, Gazi University, Ankara - TURKEY
*E-mail: leylaacik@gazi.edu.tr

Ayten ÇELEBİ
Department of Biology, Faculty of Science and Arts, Kırıkkale University, Kırıkkale - TURKEY

Osman ERGANİŞ
Department of Microbiology, Faculty of Veterinary Medicine, Selçuk University, Konya - TURKEY

Emine ARSLAN
Department of Biology, Faculty of Science and Arts, Selçuk University, Konya - TURKEY

Received: 18.12.2003

Abstract: In this study, a total of 30 *Escherichia coli* isolates obtained from milk samples of dairy cows suffering from subclinical mastitis in Konya, Turkey were typed according to plasmids and protein patterns. Agarose gel electrophoresis and sodium doedecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) methods were used to identify plasmids and whole-cell protein profiles. Of these two methods, SDS-PAGE typing proved to be more discriminate for typing the isolates.

Key Words: SDS-PAGE, plasmid, mastitis, *E. coli*, bovine.

Konya Yöresindeki Mastitisli Sığırlardan Elde Edilen *Escherichia coli* İzolatlarının Plazmit ve Protein Profilleri

Özet: Bu çalışmada Konya yöresindeki subklinik mastitisli tanısı konulan ineklerin sütlerinden izole edilen 30 *Escherichia coli* izolatının plazmit ve protein profillerine göre tiplendirilmesi amaçlanmıştır. Plazmit ve protein profillerinin saptanması amacıyla sırasıyla agaroz jel elektroforez ve sodyum dodesil sülfat poliakrilamit jel elektroforez (SDS-PAGE) metotları kullanılmıştır. SDS-PAGE metodunun izolatların tiplendirilmesinde daha ayırt edici olduğu belirlenmiştir.

Anahtar Sözcükler: SDS-PAGE, plazmit, mastitis, *E. coli*, sığır.

Introduction

Mastitis is an important problem in dairy farms and especially *Escherichia coli* mastitis is a major disease in cows (1,2). In microbiological analysis associated with the epidemiological investigation of outbreaks, it is often necessary to obtain more detailed identification and characterization of the organisms involved than can be provided by conventional methods. To investigate detailed characterization of a causative agent is also a preliminary need to start some protection studies since selection of an efficient vaccine strain(s) should begin with determining of bacterial components eliciting immune response which make them attractive as potential antigens for vaccines (3,4). Methods for typing microorganism can be divided into phenotypic or genotypic protocols. Conventional methods used for identification and characterization of bacterial strains such as morphological, biochemical and physiological tests, serotyping, phage typing, antibiogram analysis are often based on phenotypic characteristics (5). These methods can be lengthy procedures, are labor intensive, rely on specific media and multiplication of the target organism and do not use genetic information, which can be used to discriminate almost closely related organisms

(6). Most of these techniques are not sufficiently sensitive to distinguish different strains and they are affected by physiological factors. Alternative approaches such as, plasmid analysis, and sodium doedecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) have been used for typing of bacterial strains with high discriminatory potential and good reproducibility (6-8). SDS-PAGE usually combined with dendrogram derived from the numerical analysis of the whole-cell protein patterns of the strains has been used extensively to study the differences among bacterial genera, species and even strains (9,10). Plasmid analysis has also proved a useful method for differentiating isolates (11-13). The purpose of this study was to investigate the protein patterns and plasmid profiles for characterizing and differentiating *E. coli* isolates from mastitic milk samples of cow.

Materials and Methods

Sample collection and bacteriological analysis: Eight dairy farms were visited following clinical and CMT examinations; 412 samples of milk had been collected after teat ends had been disinfected and after the first streams of milk had been discarded. Strains of *E. coli* were isolated in pure culture and were identified as described before (14). A study code number was given for each isolate (Table 1).

Plasmid DNA analysis

Plasmid DNA of the isolates was prepared according to the method by Maniatis et al. (15). Plasmids were electrophoresed for 4 hours at 100 V on a 0.8% agarose gel in TAE buffer. The gel photographed on UV illumination using Polaroid film Sigma 667. The approximate molecular mass of the plasmids was determined (kb) using Lambda-pUC mix Marker 4.

Total protein analysis

The total protein samples were extracted as described by Kishore et al. (16). Total protein analysis was carried out by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method described by Laemmli (17). Each run included marker proteins of

Table 1. Code, plasmid numbers and sizes of the *E. coli* isolates

Code numbers of the isolates with plasmid	Plasmid number	Plasmid profile (mDa) (~)
1	-	-
2	3	>13, 13, 0.9
3	5	>13, 13, 5.1, 1.7, 0.7
4	3	>13, 13, 0.9
5	2	>13, 13
6	2	>13, 13,
7	2	>13, 13, 5.1, 1.7, 0.7
8	-	-
9	4	>13, 13, 5.1, 1.7
10	3	>13, 13, 2.8
11	6	>13, 13, 5.1, 3.6, 2.8, 2
12	4	>13, 13, 2.1, 1.7
13	7	>13, >13, 13, 3.6, 2.8, 2.1, 1.7
14	7	>13, >13, 13, 3.6, 2.8, 2.1, 1.7
15	1	13
16	1	13
17	1	13
18	1	13
19	1	13
20	1	13
21	1	13
22	2	13, 1.5
23	2	13, 2.6
24	1	13
25	1	13
26	3	>13, 13, 3.6
27	4	>13, >13, 13, 3.6
28	1	13
29	2	13, 5.1
30	1	13

which molecular weights were known (Bio-Rad). The gels were stained overnight with Coomassie Brilliant Blue G-250 according to Bushuk et al. (18) and Demiralp et al. (19).

Cluster analysis

Different fragments on the gel were numbered sequentially and presence and absence of fragments in each sample were scored (present 1, absent 0) and compared with each other. Cluster analysis of whole cell proteins was performed according to the genetic distance method of Nei (20).

Results

Plasmid profiles of the strains isolated from the 412 mastitic milk samples are detailed in Table 1. Plasmid profiling demonstrated that 28 of 30 isolates contained plasmid. 11 different molecular weights were identified. Most of the isolates showed multiple plasmid bands with sizes ranging from >13 to 0.9 mDa. The most common plasmid of 13 mDa was detected almost in all strains isolated. The isolates 1 and 8 were plasmid-free. The plasmid profiles were examined by naked eye and compared each other by POPGENE computer program. Table 2 shows genetic distances based on plasmid profiles of isolates. Cluster dendrogram produced by numerical analysis of plasmid profiles is shown in Figure 1. Dendrogram is divided into five clusters. First cluster included only isolate number 11. The second cluster included 27, 29, 23, 22, 26, 1, 8, 30, 28, 25, 24, 21, 20, 19, 18, 17, 15, and 16 isolates. The third cluster included 10, 2, 4, 5 and 6. The fourth cluster included 9, 3 and 7. The last cluster included 12, 13 and 14. Genetic similarity among isolates changed from 0% to 61%.

Figure 2 illustrates a typical SDS-PAGE gel in which different protein patterns are shown for *E. coli* isolates. SDS-PAGE of whole-cell protein extracts of *E. coli* strains produced patterns containing 26 to 35 discrete bands with molecular weights of 6500-200,000 Da. The whole-cell protein patterns of the *E. coli* isolates are fairly homogeneous with some variability primarily localized in the low molecular weight region estimated molecular weight, 116-45 kDa. In order to estimate relationship among *E. coli* isolates, a similarity matrix using Nei's genetic distance (20) was constructed (Table 3). Dendrogram produced by numerical analysis of whole–cell protein profiles by POPGEN program is shown in Figure 3. The *E. coli* isolates divided into six clusters according to whole-cell protein patterns. The first group contained isolates 9, 1, 6, 4, 5, 10, 12, and 13. The second cluster included isolates numbered 27, 26, 28,

Table 2. Genetic distances based on plasmid profiles of E. coli isolates (%). Iso: isolate no

Iso	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	**																													
2	26	**																												
3	48	36	**																											
4	26	00	36	**																										
5	16	08	26	08	**																									
6	16	08	26	08	00	**																								
7	48	36	00	36	26	26	**																							
8	00	26	48	26	16	16	48	**																						
9	36	26	08	26	16	16	08	36	**																					
10	26	16	36	16	08	08	36	26	26	**																				
11	61	48	48	48	36	36	48	61	36	26	**																			
12	36	26	26	26	16	16	26	36	16	26	61	**																		
13	61	48	48	48	36	36	48	61	36	26	61	16	**																	
14	61	48	48	48	36	36	48	61	36	26	61	16	00	**																
15	08	16	36	16	08	08	36	08	26	16	48	26	48	48	**															
16	08	16	36	16	08	08	36	08	26	16	48	26	48	48	00	**														
17	08	16	36	16	08	08	36	08	26	16	48	26	48	48	00	00	**													
18	08	16	36	16	08	08	36	08	26	16	48	26	48	48	00	00	00	**												
19	08	16	36	16	08	08	36	08	26	16	48	26	48	48	00	00	00	00	**											
20	08	16	36	16	08	08	36	08	26	16	48	26	48	48	00	00	00	00	00	**										
21	08	16	36	16	08	08	36	08	26	16	48	26	48	48	00	00	00	00	00	00	**									
22	16	26	48	26	16	16	48	16	36	26	61	36	61	61	08	08	08	08	08	08	08	**								
23	16	26	48	26	16	16	48	16	36	26	61	36	61	61	08	08	08	08	08	08	08	16	**							
24	08	16	36	16	08	08	36	08	26	16	48	26	48	48	00	00	00	00	00	00	00	08	08	**						
25	08	16	36	16	08	08	36	08	26	16	48	26	48	48	00	00	00	00	00	00	00	08	08	00	**					
26	00	26	48	26	16	16	48	00	36	26	61	36	61	61	08	08	08	08	08	08	08	16	16	08	08	**				
27	36	26	48	26	16	16	48	36	36	26	36	36	36	36	26	26	26	26	26	26	26	36	36	26	26	36	**			
28	08	16	36	16	08	08	36	08	26	16	48	26	48	48	00	00	00	00	00	00	00	08	08	00	00	08	26	**		
29	16	26	26	26	16	16	26	16	16	26	36	36	61	61	08	08	08	08	08	08	08	16	16	08	08	16	36	08	**	
30	08	16	36	16	08	08	36	08	26	16	48	26	48	48	00	00	00	00	00	00	00	08	08	00	00	08	26	00	08	**

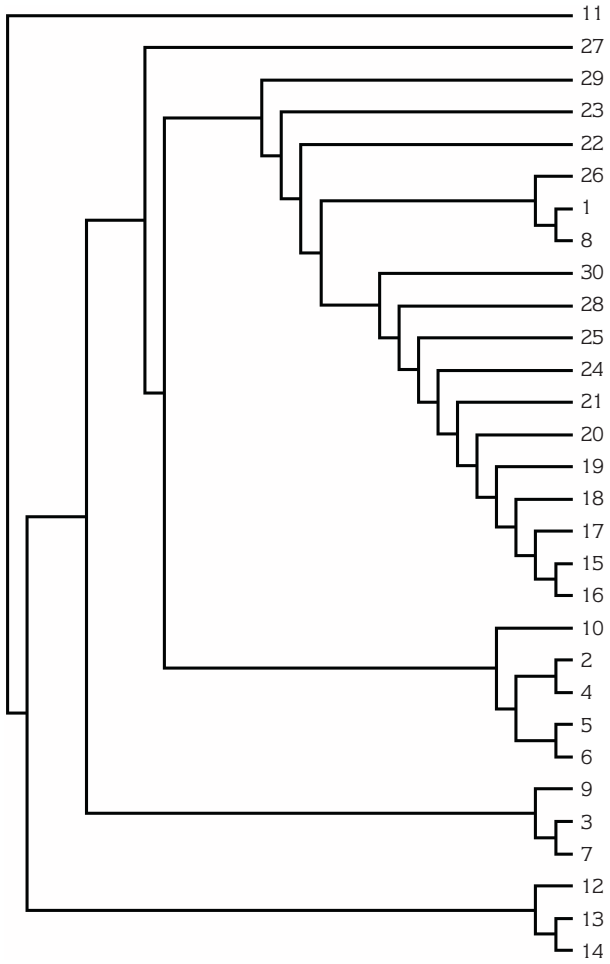

Figure 1. The dendrogram based on plasmid profiles of *E. coli* isolates.

30, 11, 2, 8, 3, 7, 24, and 25. The third cluster included isolates numbered 23 and 29. The fourth cluster included isolates 16, 15 and 17. The fifth cluster included isolates 19, 20, 21, and 22. The last cluster contained isolates 14 and 18. Genetic similarity among isolates changed from 0% to 55%.

Discussion

Although immunization has often been attempted in efforts to control mastitis, the diversity of potential *E. coli* has vaccine development difficulties (4). To overcome this problem, use of one or likely several strains of *E. coli* that are antigenically representative of the majority of the causative strains in a herd or even in herds located in the same geographical region is recommended. Decision on representative strains can be made based on whole cell proteins since such proteins of strain *E. coli* 0111:B4 has already been shown (21) to elicit a high level of immune response to the strain in question. The isolates could be characterized by their plasmid profiles. However, plasmid profiles do not reveal stable genetic differences of the strains. In fact, plasmid profiles of the isolates are generally a useful tool for obtaining knowledge about resistance of the isolates to the antimicrobial substances and transfer of a plasmid among closely related isolates from different sources. Plasmid profile is one of several useful methods for determining the relatedness or unrelatedness of bacterial strains that contain plasmid DNA. However some of the strains do not harbor any

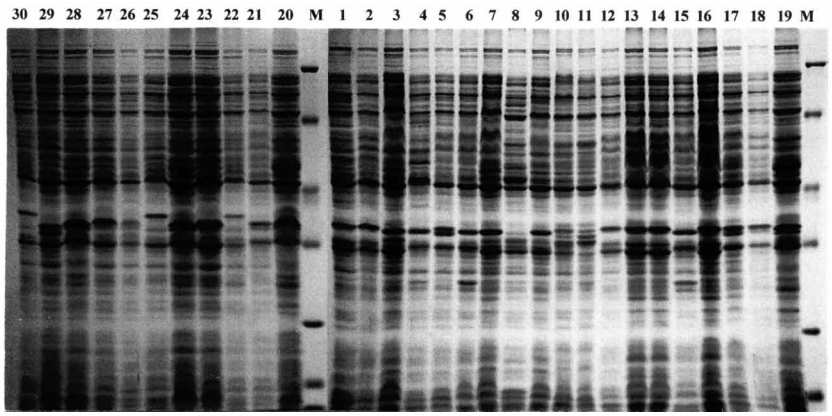

Figure 2. SDS-PAGE protein profiles of *E. coli* isolates. M: molecular weight standards (kDa), 116 (β-galactosidase), 66 (bovine serum albumin), 45 (ovalbumin), 35 (lactate dehydrogenase), 25 (restriction endonuclease *Bsp*981) 18 (β-lactoglobulin) 14 (lysozym).

Table 3. Genetic distance below diagonal based on whole cell protein analysis (%). Iso: isolate no.

Iso	30	29	28	27	26	25	24	23	22	21	20	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
30	**																													
29	22	**																												
28	18	02	**																											
27	12	08	05	**																										
26	05	15	12	05	**																									
25	00	22	18	12	05	**																								
24	18	02	00	05	12	18	**																							
23	18	02	00	05	12	18	00	**																						
22	02	18	15	08	08	02	15	15	**																					
21	18	29	25	18	12	18	25	25	22	**																				
20	22	05	02	08	15	22	02	02	18	29	**																			
1	22	25	22	15	15	22	22	22	25	15	25	**																		
2	18	22	18	12	12	18	18	18	22	12	22	02	**																	
3	55	25	29	37	46	55	29	29	51	46	25	41	37	**																
4	41	22	25	33	41	41	25	25	37	51	22	46	41	37	**															
5	41	29	33	33	41	41	33	33	37	51	29	46	41	46	12	**														
6	37	18	22	29	37	37	22	22	33	46	18	41	37	33	02	08	**													
7	51	29	33	41	51	51	33	33	46	51	29	46	41	08	33	41	29	**												
8	51	37	41	51	51	51	41	41	46	41	46	37	41	29	41	51	37	33	**											
9	41	22	25	33	41	41	25	25	37	41	29	37	33	29	25	33	22	33	12	**										
10	46	41	46	46	37	46	46	46	51	37	51	25	29	51	55	55	51	55	22	29	**									
11	46	33	37	46	37	46	37	37	51	46	41	41	37	51	46	46	41	55	29	22	12	**								
12	25	37	41	33	25	25	41	41	29	25	37	29	25	46	41	41	37	51	33	25	29	29	**							
13	29	18	22	29	29	29	22	22	33	37	25	25	22	41	29	29	25	37	37	22	41	33	29	**						
14	29	18	22	29	29	29	22	22	33	37	25	25	22	41	29	29	25	37	37	22	41	33	29	00	**					
15	29	12	15	22	29	29	15	15	25	37	18	33	29	41	15	22	12	37	37	22	51	41	37	18	18	**				
16	41	15	18	25	33	41	18	18	37	41	22	29	25	29	41	51	37	33	33	25	37	37	51	22	22	22	**			
17	29	12	15	15	22	29	15	15	25	29	18	18	15	33	29	22	25	37	37	22	33	25	29	18	18	18	22	**		
18	22	18	22	22	22	22	22	22	18	22	25	33	29	51	29	29	25	37	37	29	41	33	22	25	25	18	37	18	**	
19	29	05	08	15	22	29	08	08	25	37	05	33	29	25	22	22	18	29	46	29	41	33	37	25	25	18	22	12	25	**

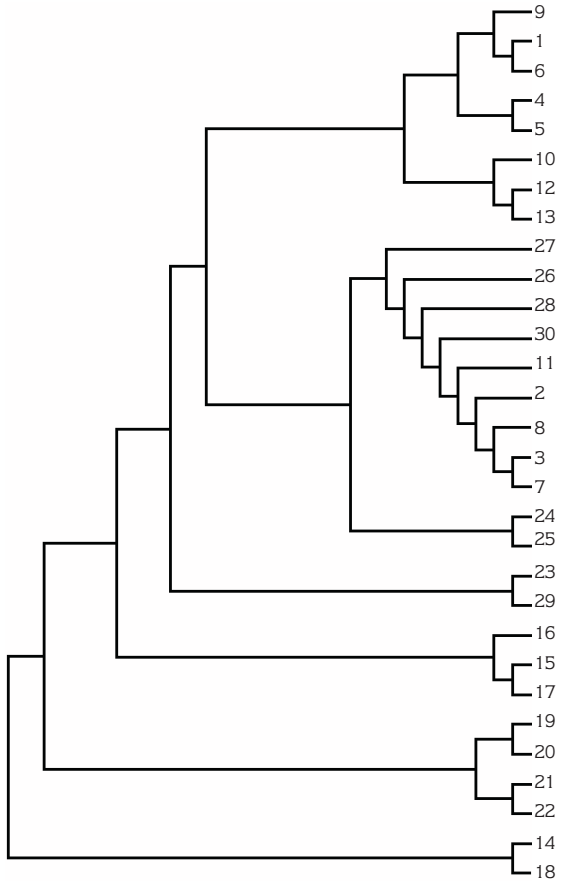

Figure 3. The dendrogram based on whole cell protein profiles of *E. coli* isolates.

plasmid such as isolates 1 and 8. Calculation of genetic distance based on plasmid profiles reveals that isolate 1 is 100% similar to the isolate 8. However, calculation based on protein profiles reveals that in fact these two isolates are 63% similar. Thus, it should be used in combination with other epidemiological and molecular methods.

It was hypothesized that mastitic cows are infected with *E. coli* strains from their environment (feces or straw) (22). In this study, strains that showed protein similarities in some degree were collected from very different farms. On the other hand, those isolated from the same herd showed little similarities in terms of protein patterns (data not shown). The diversity observed in protein patterns supports the dogma that cows are infected by strains from their environment and not through cow to cow transmission of a single udder-pathogenic strain.

All the results of this study clearly show high discriminatory potential of the protein profile analysis in typing the *E. coli* strains isolated from cow. The differentiation of the isolates from each other by using protein and plasmid profiles can provide a reliable additional method to aid in the characterization of the bacteria.

References

1. Schukken, Y.H., Kremer, W.D.J., Lohuis, J.A.C.: *E. coli* mastitis. bij het rund. Tijdschr. Diergenskd., 1989; 114: 829-838.
2. Döpfer, D., Barkem, H.W., Lam, T.J.G.M., Schukken, Y.H., Gaastra, W.: Recurrent clinical mastitis caused by *E. coli* in dairy cows. Dairy Cows. J. Dairy Sci., 1999; 82: 80-85.
3. Tyler, J.W., Spears, H., Erskine, R.J., Martin, J., Nelson, R.: Antigenic homology among bacteria isolated from clinical Gram-negative mastitis. Internat. Symp. on Bovine Mastitis, Indianapolis. 1990; 196-198.
4. Hadimli, H.H., Erganiş, O.: Mastitis ve Bağışıklık. Mastitis Sempozyumu, Akdeniz Üniversitesi, Burdur Veteriner Fakültesi. Burdur. 2001; 27-28.
5. Fantasia, M., Ricci, N., Manuppella, A., Martini, A., Filetici, E., Laurelli, T.: Phage type and DNA plasmid of *Salmonella typhimurium* isolates in the area of Isernia, Italy. Epidemiol. Infect., 1990; 105: 317-323.
6. McClure, P.J.: Microbiological hazard identification in the meat industry. HACCP in the meat industry. CRC Press Woodhead Publishing Limited, Cambridge. 2000; 157-176.
7. Parisi, J.T., Hecht, D.W.: Plasmid profiles in epidemiologic studies of infections by *S. epidermidis*. J. Infect. Dis., 1980; 141: 637-643.
8. Shi, Z.H., Liu, P.Y.F., Lau, Y.J., Hu, B.S., Lin, Y.H.: Comparison of polymerase chain reaction and pulsed-field gel electrophoresis for the epidemiological typing of *Campylobacter jejuni*. Diag. Microbiol. Infect. Dis., 1996; 26: 103-108.
9. Tanner, A.C.R., Listgarten, M.A., Ebersole, J.L.: *Bacteroides forsythus* sp. nov., a slow-growing, fusiform *Bacteroides* sp. from the human oral cavity. Int. J. Syst. Bacteriol., 1986; 36: 213-221.
10. Walia, T.M., Williamson, T., Kaiser A., Tewari, R.: Usefulness of protein patterns, serotyping and plasmid DNA profiles in the epidemiologic finger printing of *Pseudomonas aeruginosa*. Eur. J. Clin. Microbiol. Infect. Dis., 1988; 7: 248-255.
11. Dorn, C., Silapanuntakul, R., Angrick, E., Shipman, L.: Plasmid analysis and epidemiology of *Salmonella enteritidis* infection in three commercial layer flocks. Avian Dis., 1992; 38: 844-851.
12. Neuwirth, C., Siebor, E., Lopez, J., Pechinot, A., Kazmierczak, A.: Outbreak of TEM-24-producing *Enterobacter aerogenes* in an intensive care unit and dissemination of the extended-spectrum beta-lactamase to other members of the family enterobacteriaceae. J. Clin. Microbiol., 1996; 34: 76-79.
13. Wachsmuth, I., Kiehlbauch, J., Bopp, C., Cameron, D., Strockbine, N., Wells, J.A.B.P.: The use of plasmid profiles and nucleic acid probes in epidemiologic investigations of foodborne, diarrheal diseases. Int. J. Food Microbiol., 1991; 12: 77-90.
14. Lassen, J.: Rapid identification of Gram-negative rods using three tube methods combined with a dichotomic key. Acta. Path. Microbiol. Scand. sect B., 1975; 83: 525-533.
15. Maniatis, R., Fritsh, E.F., Sambrook, J.: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, New York. 1989.
16. Kishore, L., Natarajan, K., Babu, L.R.: Total soluble protein and membrane lipopolysaccharide profiles in differentiating Rhizobium isolates. Microbios., 1996; 86: 143-156.
17. Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature., 1970; 227: 680-684.
18. Bushuk, W., Hay, R.L., Larsen, N.G., Sara, R.G., Simmons, L.D., Sutton, K.H.: Effect of mechanical dough development on the extractability of wheat storage proteins from bread dough. Cereal Chem., 1997; 74: 389-395.
19. Demiralp, H., Çelik, S., Köksel, H.: Effects of oxidizing agents and defatting on the electrophoretic patterns of flour proteins during dough mixing. Eur. Food Res. Technol., 2000; 211: 322-325.
20. Nei, M.: Genetic distance between population. Am. Natural., 1972; 949; 283-292.
21. Tomita, G.M., Ray, C.H., Nickerson, S.C., Owens, W.E., Gallo, G.F.: A comparison of two commercially available *E. coli* J5 vaccines against *E. coli* intramammary challenge. J. Dairy Sci., 2000; 83; 2276-2281.
22. Lipman, J.A., Nijs, A., Lam, T.J.G.M., Gaastra, W.: Identification of *Escherichia coli* strains from cows with clinical mastitis by serotyping and DNA polymorphism patterns with REP and ERIC primers. Vet. Microbiol., 1995; 43: 13-19.