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Genetic Studies on Escherichia Coli Strains Isolated From Cases of Bovine Mastttis

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دراسات وراثية على ذراري الإشريكية القولونية المعزولة من حالات التهاب الضرع في الأبقار

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أجريت الدراسة على **53** عينة حليب كانت موجبة في اختبار كاليفورنيا ، جمعت من ثمانية قطعان أبقار محموجة بالتهاب الضرع السريري في محافظة الطائف .كان متوسط تعداد الخلايا (الكريات البيضاء) **4,482,000** خلية في كل مل من الحليب .عزلت **48** ذرية (**51,06 %**) من ذراري الإشريكية القولونية من حليب الأبقار المخموجة بالتهاب الضرع ، وذلك باستخدام الطرائق الجرثومية .من بين تلك الذراري ؛ ميزت **27** ذرية إلى أنماطها المصلية وأنماطها الجينية (طرز تعدد أشكال الــ دنا) ، وذلك عن طريق تكبير أو تضخيم الــ دنا بواسطة الحروف التكرارية الطردية العكسية القراءة (**REP**) ، والحروف التكرارية التوافقية (**ERIC**) هذا وقد عني البحث عناية خاصة بالتهاب الضرع البقري المتسبب بالإشريكية القولونية المتكرر دورياً.ومن أجل دراسة وبيئات التهاب الضرع السريري الناتج عن خمج الإشريكية القولونية ؛ استخدم مايسمى بـ توافقية النمط المصلي وطراز الــ دنا للجرثومة السابقة المكتشفة في البحث. لقد أكد البحث أن تفاعل البوليميراز

السلسلي (PCR) إضافة للحروف التكرارية التوافقية(ERIC) ، يمكن أن يستعمل من أجل التعرف على ذراري الإشريكية القولونية وتحديد هويتها .وبناء على طرز تعدد أشكال الـ دنا لعزولات الإشريكية القولونية المكتشفة في الأبقار المخموجة بالتهاب الضرع ، تبين أن للعزولات أشكال مختلفة أو متغيرة في ما يتعلق بأنماطها الجينية .وخلال فترة در اللبن ذاتها ؛ اكتشفت أكثر من حالة التهاب ضرع سريري تسببت بوساطة الإشريكية القولونية؛ ولكن وقوع هذا الإلتهاب لم يكن على نحو منتظم . وأياً كان الوقوع المرضي ؛ والحالة هذه ؛ فإن ذراري الإشريكية القولونية المعزولة من الحالات العارضة لالتهاب الضرع ؛ كانت – في أغلب الأحوال – من النمط المصلي ذاته ، كما كان لها طراز تضخم الـ دنا ذاته أيضاً .

Key words: *Genetic studies, Escherichia coli, Bovine mastitis.*

ABSTRACT

This study was carried out on 53 milk samples positive for California Mastitis Test taken from eight herds infected with clinical mastitis in Taif City. Mean Somatic cell Count (SCC) was 4, 482, 000 cells / ml. Forty-eight (51.06%) *E .coli* strains were isolated from bovine mastitic milk by bacteriological methods.

From these *E .coli* strains, 27 were characterized with regard to their serotype and their DNA polymorphism pattern with REP and ERIC primers. special attention was given to recurrent *E .coli* mastitis in cows . For studying the epidemiology of clinical E . coli mastitis, the combination of serotype and DNA pattern detected , was used in this investigation .It was assured that the PCR reaction with the ERIC primers can be used in the field of *E . coli* strains identification . Based on the DNA polymorphisms patterns of *E . coli* isolates from clinical mastitic cows , it revealed that isolates have a great variations in regard to their genotypes .

During the same lactation period, it was detected more than one clinical mastitis case associated with *E . coli*, but this clinical mastitic occurrence was irregularly. Whatever the occurrence, at this rate, *E . coli* strains isolated from the separate episodes of inflammation , were for the most part, of the same serotype and had the same DNA amplification pattern . Therefore , the PCR DNA amplification method can not replace serotyping in epidemiological studies .

Introduction

Mastitis has been recognized for some time as the most costly disease in dairy herds (2) . It remains a major challenge to the worldwide dairy industry despite the

widespread implementation of mastitis control strategies (1) . The last forty years have seen a dramatic decrease in clinical mastitis incidence , but this has been accompanied by a change in the relative and absolute importance of different pathogens (1) . There are several pathogens caused clinical and subclinical bovine mastitis (16 , 28 , 36) , but the important bacterial causes of mastitis are :
Staphylococcus aureus , *Escherichia coli* , *Streptococcus* spp . *Klebsiella* spp *Pseudomonas* spp *Proteus* spp " *Candida albicans* (27) , and other microbes .

Mastitis caused by *E . coli* is a major disease in cows (35) . The reason for the importance of *E . coli* mastitis is its increasing incidence and the severe symptoms like milkdrop and fever (35) . A possible explanation for this increase is the fact that farmers for economic reasons , are forced to achieve a low somatic cell count using management practices like dry cow treatment (use of antibiotics in the non- pathogenic period) and post-milking teat disinfection (10 , 34 , 35) . Most probably, the number of nonlactating bacteria is diminshed by these methods which leads to a low concentration of polymorphic nuclear cells in the milk . This would render cows more susceptible for mastitis caused by environmental bacteria like *E . coli* (20) . Discrimination between virulent and nonvirulent strains of E .coli will allow proper diagnosis of cows infected by that bacteria and identification of carrier cows . *E .coli* strains isolated from cases of mastitis have been examined for the presence of common virulence factors sush as fimbriae (32) , but unlike in *E . coli* causing enterotoxicosis in pigs and calves no special serotyp or virulence factors (like toxins or fimbriae) were found (39, 43) . In pathogenicity studies, it could never be demonstrated that adhesion of E.coli to udder epithelium , is a first step in the mastitis infections , in analogy to adhesion of *E.coli* to bladder epithelium in uro - pathogenic infections (9) . Most urinary tract infections (UITs) among otherwise healthy women are caused primarily by *E. coli* (24) . Certain O : K : H serotypes and virulence factors occur , more frequently in urinary than faecal isolates , suggesting that uro pathogenic *E . coli* are different from normal bowel inhabitants (17) . However , they are also a diverse group :20 O : K : H serotypes that have been associated with pyelonephritic infection (17) .

Furthermore , when first – time UTI isolates were grouped by the presence or absence of nine putative UTI virulence genes , 36 groups were observed (7). Human enterohaemorrhagic *coli* (EHEC) infection most commonly arises , either directly or indirectly , from cattle , which act as a reservoir host for these bacteria (4) . Based on epidemiological studies (22) , it was hypothesized that cows are infected with E . coli strains from their environment (faeces or straw) .

However, strains originated from Finland and Israel, showed differences in the proportion of mastitis caused by *E .coli*, clincal pictures of coliform mastitis , environmental conditions and herd management(18).

Whatever the cause, the reason for that hypothesis or this establishment , it was the wide range of serotypes observed in clinical mastitis strains and the absence of long term *E .coli* udder infections that might have led to cow to

cow infections (21) . In the recent years , DNA Fingerprinting has become available for epidemiological research. A polymerase chain Reaction (PCR) with primers on repetitive sequences in the DNA can be performed to identify strains (8 , 14 , 19 , 23). This method to differentiate between *E . coli* strains is simple , fast and less expensive than traditional methods of serotyping . Chromosomal DNA (chromosomal genotypes) from Enterobacteriaceae contains several repetitive sequences e.g. Repetitive Extragenic palindromic (REP) and Enterobacterial Repetitive Intergenic consensus (ERIC) sequences (41 , 42) .These and other sequences can be used for fingerprinting strains as was shown in epidemiological studies on cases of methicillin – resistant Staphylococcuus aureus infections or S .aureus bovine mastitis (40 , 26 , 33) The band pattern of the amplified DNA obtained after electorophoresis was found to be specific for the different S.aureus strains tested as stated by several workers (22 ,13 ,19 ,8 ,23).Threfore , the presence and size of the observed PCR products can be used to genotype the bacteria (22,19,23). The present study showed for the frist time that genetically and serotypically methods for identifying, *E .coli* isolates causing clinical mastitis in cow is possible and easy . In this study it is shown that diferentiation between the genotypes of *E .coli* strains isolated from clinical mastitis of cows using a PCR amplification method with ERIC primers can also be possible (13, 22) .

Therefore , those modern methods – based on identifying genotypes and serotypes of *E . coli* isolated from clinical mastitis of cows tested , have been confirmed by many researchers (22 , 13 , 23 , 8) . The aim of this study is to screen *E . coli* isolates for serotypes and genotypes to specify its role in clinical bovine mastitis in Taif City in K . S . A. In addition, it was aimed to investigate the most accurate methods in the field of diagnosis of clinical bovine mastitis , then the bacterial strains that responsible for infection of udder in the lactation period .

Materials And Methods

Sample collection 53 samples of milk were collected from (53) cows with clinical mastitis , related to eight herds with a low Bulk milk somatic cell count as described previously (5 , 29 , 38) . Environmental samples included hay , straw , faeces and skin dirt were also taken from five farms except farms numbers 2, 7 and 8 .

Bacterial Examination Environmental samples and the milk samples (0.01 ml) were subjected to bacterial cultivation on Blood Agar Base No . 2 . plus 7 % sterile Blood (O xoid) or 6 % Bovine Blood Agar (Both aerobically and anaerobically) , on MacConkey Agar w/ (0.15 %) Bile salt , CV & Nacl (HiMedia L . L .) , on MacConkey Agar No.3 . (LAB . Topley House), on Brain Heart Infusion Agar (HiMedia L . L .) , and on TCT medium (Thallium Sulphate , Crystal Violet , Staphylococcus B toxin) (Merck). Mannitol Salt Agar used as a selective medium for the isolation of pathogenic Staphylococci .

Baird Parker Agar Base was used for the isolation and enumeration of coagulase positive Staphylococci from milk samples (HiMedia L . L .) . Agar plates were

incubated at 37 C and bacterial growth was evaluated after 24 and 48 hours. Gram negative microorganisms were isolated from the MacConkey Agar and determined to species level using cytochrome oxidase , triple sugar iron agar, Urea and indole tests . These tests were done according to advised laboratory methods (6 , 37) .

Isolates of *E . coli* were selected from recurrent infections (15 /27) and chosen at random (12 / 27), from a total of (94) strains isolated in this study .

Counting of Somatic Cells and California Mastitis Test (C . M . T)

Somatic cells Counting by the Coulter Counter Method , California Mastitis Test and bacteriolgical examination were performed on milk samples taken at farms as soon as after collecting by using an accurate procedure done already (38 , 12 , 5) .

Isolation of DNA

Luria broth and chromosal DNA (chromosomal genotypes) of E . coli isolates from milk samples , hay , straw , Faeces and dirt of skin) of cows with clinical mastitis were examined to find possible clonality of the isolates by standard procedures (31) .

Serotyping Methods

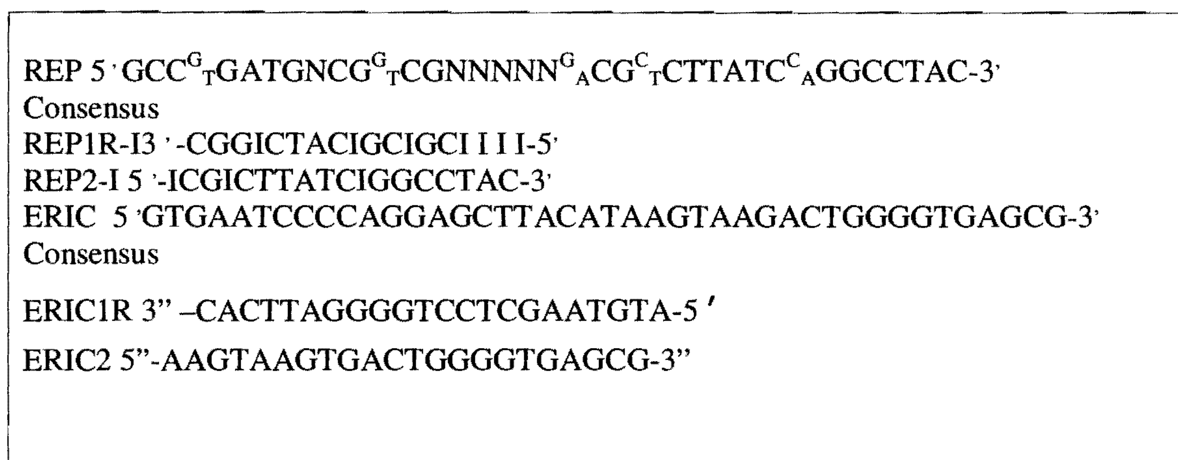
_Determination of the O-serogroups of the strains was carried out at the college of Veterinary Medicine , Al- Baath University , Homs , Syria , according to the Determinating standard methods (Lipman, 1995) .

Polymerase Chain Reaction

This was performed as described by Lipman. L . J . A .(22)Each 50 µl PCR reaction contained 50 pmol of 2 primers (Figure 1) , 100 ng of template DNA , 1.25 mM of each 4 dNTPs , 2U Taq DNA polymerase (pharmacia) in a 10 × PCR buffer containing 500 mM KCL , 100 mM Tris / HCl (pH 9.0) , 15 mM $MgCl_2$, 1 % Triton X – 100 and 0.1 % gelatine . PCR amplification was performed in an automated thermal cycler (Hybaid Omnigene) . The PCR amplification schedule was :

Denaturation (95C , 1 min) , annealing (REP 45 C 1 min, ERIC 50° C 1 min) and extension (65 C 8 min) with a final extension (65 C 16 min .) .

Electrophoresis was done in 1 % agarose gel containing 1x TBE (Tris Borate – EDTA , 0.5 µg ethidium bromide) .



REP 5'GCC$^{G}_{T}$GATGNCG$^{G}_{T}$CGNNNNN$^{G}_{A}$CG$^{C}_{T}$CTTATC$^{C}_{A}$GGCCTAC-3'
Consensus
REP1R-I3'-CGGICTACIGCIGCI I I I-5'
REP2-I 5'-ICGICTTATCIGGCCTAC-3'
ERIC 5'GTGAATCCCCAGGAGCTTACATAAGTAAGACTGGGGTGAGCG-3'
Consensus
ERIC1R 3"–CACTTAGGGGTCCTCGAATGTA-5'
ERIC2 5"-AAGTAAGTGACTGGGGTGAGCG-3"

Figure 1: Oligonucleotide design

Results

It is evident from the results given in table(1), that the mean Somatic Cell Count (SCC) per/ ml for all samples was (4,482,000).

Table (1): Somatic cells (leucocytes) count (SCC) (cells/ml) of milk samples

Herd number	SCC (cells / ml)		Mean of the total Minimum & Maximum SSC (cells / ml)	Mean of SCC in eight herds
	Minimum	Maximum		
1	2,500,000	4,160,000		
2	3,220,000	5,630,000		
3	3,470,000	5,972,000		
4	2,992,000	3,889,000		
5	4,763,000	5,924,000		
6	3,551,000	4,672,000		
7	5,111,000	5,844,000		
8	4,775,000	5,238,000		
Total	30,382,000	41,329,000	35,856,000	4,482,000

Moreover, the isolation percentages of *E . coli* and staphylococcus *aureus* were 51 .06 % and 48 . 94 % , respectively (Table 2). Neither other bacterial strains nor fungi could be detected .

Table (2): Bacterial strains isolated from mastitic milk samples.

Hard and farm number	No. of examined %	No. of strains isolated	Types of microorganisms isolated and their number	
			E. coli **spp No.**	***Staphylococcus aureus*** **No.**
1	7	12	5	7
2	5	10	6	4
3	6	13	8	5
4	6	8	4	4
5	5	9	4	5
6	8	17	9	8
7	9	11	5	6
8	7	14	7	7
Total	53	94	48	46
%			51.06 %	48.94 %

The results indicated that 27 *E . coli* strains were isolated from milk samples of 15 cows from eight herds (Table 3) .

In 3 cows, repeated episodes of *E . coli* clinical mastitis cases occurred during one lactation in the same quarter .

Table (3) : Serotype and genotype code of *E . coli* strains isolated form cows infected with clinical mastitis occurred during a period of 1-6-2002 to 11-10-2002 .

Herd and Farm number	Strain number	Serotype	Genotype
1	1	O4K-	A
	2	O4K-	A
	3	O150K-	B
	4	O150K-	B
	5	O150K-	B
	6	O150K-	B
	7	O150K-	B
	8	O150K-	B
	9	O150K-	B
2	10	O8K-	C
	11	O8K-	C
	12	O8K-	C
3	13	Untyp.	D
	14	Untyp.	D

4	15	O104K-	E
	16	Untyp.	E
5	17	Untyp.	F
	18	Untyp.	G
	19	Untyp.	G
	20	Untyp.	G
	21	Untyp.	G
6	22	Untyp.	H
	23	O150K-	I
	24	O5K-	J
7	25	O105K-	J
	26	O1K-	K
8	27	O74K-	L

Serotyping and genotyping of *E . coli* strains isolated from cows infected with clinical mastitis , could be differntiated from each other on the basis of DNA isolation , determination of the O – serogroups , and polymerase chain reaction (PCR), i . e . serotype and genotype classification .

Seven different serotypes and seven untypable strains were determined (Table 3) . Differentiation of isolated strains could be achieved by the presence and size of their PCR products (genotype) . The sizes of the PCR products ranged from 1.5 kb to 250 bp (Figure 2) .

Amplification of DNA with ERIC primers was found to be more reproducible , compared to DNA amplification with REP primers . For example strains 5 and 6 , did not react at all with the primers or yielded non reproducible patterns (results not shown) . A total of 12 different genotypes was detected, out of the 27 isolated strains, with the ERIC primers (Table 3 , Figure 2) .

Nine strains that were untypable with reference to their serotype could be typed in different genotypes (e .g strain 13 genotype D and strain 22 genotype H) and strains with the same serotype could be differentiated in different genotypes (e . g . strain 3 serotype O150k- , genotype B , and strain 23 serotype O150k- , genotype I) .

On the other hand , out of 15 *E . coli* strains isolated from the environment of cows infected with clinical mastitis . 10 different genotypes of these isolates could be differentiated With regard to *E . coli* serotyping and genotyping , and by comparing the results of this study , it is clear that there is no special pathogenic *E . coli* serotype / genotype , that infects cows with mastitis .

Also , the present study reveals no prevalent serotype / genotype of *E . coli* is predomianted within farms investigated . It is also evident that, a total of 15 recurrent mastitis cases with *E. Coli* in one lactation period occurs infrequently (15 cases from 53 cases) .

In any case , results showed that cows developed symptoms of recurrent *E . coli* mastitis (Two cows of herd (1) and one cow of herd 3) , each time were re-infected , or more likely , remained infected with strains of the same serotype / genotype (one cow of herd 1 on farm 1 is considered as one case of clinical mastitis with 3 samples) .

Two recurrent infections were detected to be caused by *E . coli* of different serotypes /genotypes , such as one cow with serotype O4k- , genotype A of the herd 1 , and one cow with serotype O105k- , genotype J of the herd 7.

These results could be established again , the possibility of recurrent infections occurrence with bovine mastitis on the same farm screened .

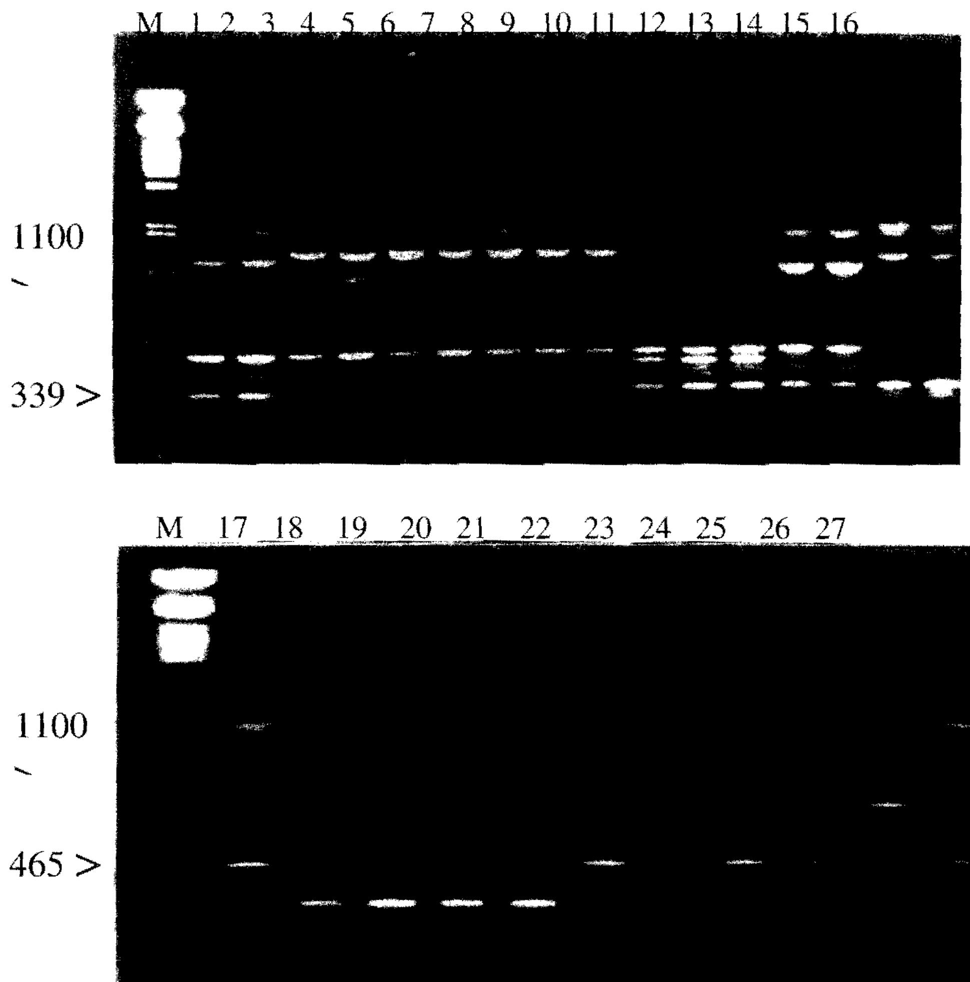


Figure (2): ERIC DNA amplification pattern of 27 *E . coli* clinical mastitis strains. (M: DNA size marker bp.)

Discussion

Mastitis is an inflammation of the udder and as such is a disease complex resulting from any condition or combination of factors leading to injury to the internal structure of one or more quarters . There is no standard definition of the various types of mastitis , generally it could be considered as clinical or subclinical , and symptoms may be acute or chronic . Latent infection with mastitic organisms also occurs (12) .

Coliform mastitis is one of the most difficult diseases to treat in the modern dairy industry (3) . This study was conducted to determine the prevalence of bovine mastitis including , isolation , classification , serotyping and genotyping of *E . coli* causing the disease. (53) California Mastitis Test positive milk samples were taken from eight herds infected with clinical mastitis . Mean Somatic Cell Count (SCC) was 4,482,000 cells / ml . Previous studies carried out for SCC measurments have shown that mean SCC was 418000 cells / ml , and mean SCC for duplicates ranged from 9000 to 3 966 000 cells / ml , (5) . It is generally concluded that reaction represents 2,000,000 leucocytes (grade 2) or more and reaction indicates 4,000,000 (grade 3) or more leucocytes per ml of milk suggests a serious mastitis of the herd (12) . The grade of the reaction depends upon the amount of gel formation in the sample .

Forty- eight (51.06%) *E . coli* strains isolated from bovine mastitis milk on 8 dairy farms in Taif City were examined for prevalence of mastitis . Previously , many studies have indicated that the predominant organism among the microbes caused bovine mastitis was *E . coli* with a percentage of 16.7% (15) , whereas the isolation percentage of this bacteria was 2% by some workers 30 .

However, recent studies have shown different results . Out of (160) Finnish and 113 Israeli *E . coli* isolates , altogether 49% of Finnish and 42% of Israeli isolates had at least one virulence gene (18) .

In this study, *E . coli* mastitis strains recovered from eight herds were characterised with respect to serotype and DNA amplification pattern using PCR with ERIC and REP primers . By using this method, it was possible to differentiate strains of the same serotype in different genotypes (strain 5 , O150k- , genotype B and strain 23 O150k- , genotype I , Table 3 and Figure 2) , and to differentiate strains that are untypable with regard to their serotype in different genotypes (e .g strain 13 genotype D and strain 16 genotype E , Table 3 and Figure 2) . These results were supported by the previous studies (13 , 19) , and established with recent observations and researches (2 , 18) . The poor reproducibility , mentioned above , of the PCR amplification with the REP primers , can be explained by the fact that the primers are shorter than the ERIC primers and , compared to the ERIC primers , contain a high proportion of deoxyinosines (25) . The REP PCR DNA amplification pattern confirms the results of the ERIC DNA amplification pattern but was less reliable (22) . Few researchers have concluded that O – serogroups , eae gene and EAF plasmid in *E . coli* isolates from cases of bovine mastitis were the most important in determination of *E . coli* isolates effectually (2) .

Results demonstrated that PCR amplification with ERIC primers is a fast and reliable method for differntiation and identification of *E . coli* isolates. Also , this method is a valuable compared to serotyping , since the different genotypes can be determined even in strains with the same serotype or in untypable isolates . The PCR DNA amplification method can not replace serotyping in epidemiological studies , so strains with a different serotype can have the same genotype e.g. strains 15 and 16 (Table 3 and Figure 2).

Identification of *E .coli* mastitis isolates or other *E . coli* isolates was carried out a accurately by a combination of serotyping and genotyping method , compared with other ways in this field .

Serotypes and genotypes diversity detected , assures the dogma that cows are infected by their environmental strains and not through cow to cow transmission of a single udder pathogenic isolate .

A group of cows were infected again with the same serotype and genotype of *E .coli* isolate , on the same farm , hence , recurrent infections are at this rate very unlikely, in respect that the large variety of strains distributed in the environment .

Depending upon the PCR results , concerning the recurrent infections of clinical *E .coli* mastitis the present study is mainly demonstrating that the invased bacteria survive in the udder tissues . Experimentally , this conclusion has been confirmed by few researches (11) .

Such observations and valuable serotyping and genotyping methods provide an evidence of the importance of the udder examinations either clinically or microbiologically , which have a good indications upon the comfort of herd health and production .

The obtained results revealed clearly that *E. coli* strains isolated from repeated episodes of clinical mastitis in the same cow, have identical genotypes . Testing for the bovine mastitis *E. coli* strains in which identified by serotyping and genotyping according to the mentioned methods , can replace the traditional tests.

REFERENCES

[1] Bradley, A. (2002) . Bovine mastitis : an evolving disease, *Vet. J.* **164** **(2)** : 116-128.

[2] Correa, M. G. and Marin, J. M. (2002) . O – serogroups, eae gene and EAF plasmid in *Escherichia coli* isolates from cases of bovine mastitis in Brazil ,*Vet. Microbiol.* **85** **(2)** : 125 – 132.

[3] Dosogne, H., Vangroenweghe, F. and Burvenich, C. (2002) . Potential mechanism of action of J5 vaccine in protection against severe bovine coliform mastitis , *Vet. Res* . **33(1)** : 1-12.

[4] Elaine Hoey, D. E., Currie, C. Else, R. W., Nutikka, A., Lingwood, C.A., Gally, D.L. and Smith, D. G. E. (2002). Expression of receptors for verotoxin 1 from *Escherichia coli* O157 on bovine intestinal epithelium , *J. Med. Microbiol* . **51:** 143 – 149.

[5] Faust, M. A. and Timms. L. L. (1995). Estimates of variability for somatic cell count measurements in the Iowa dairy industry , *Journal of Dairy Science.* **78:** 546-551.

[6] Finegold, Sydney. M. and Baron, E. J. (1986). *Bailey and Scott's Diagnostic Microbiology.* 7th Edition, The C. V. Mosby Company, St. Louis, Toronto.

[7] Foxman, B., Zhang, L., Palin, K., Tallman, P. and Marrs, C.F. (1995). Bacterial virulence characteristics of *Escherichia coli* isolates from first-time urinary tract infection , *J. Infect. Dis.* **171:**1514 – 1521.

[8] Foxman, B. Zhang, L. Tallman, P. et. al. (1997) . Transmission of uropathogen between sex partners , *J. infect. Dis.* **175:** 989 – 992.

[9] Garcia Gonzalez, E. (1992) . Adhesion factors of extraintestinal *Escherichia coli* strains. Thesis, University Utrecht, the Netherlands 1992.

[10] Hill, A. W. (1981). Factors influencing the outcome of *Escherichia coli* mastitis in the dairy , cow. *Res. Vet. Sc.* **31:** 107 – 112.

[11] Hill, A. W. and Shears, A. L. (1979) . Recurrent Coliform mastitis in dairy cow , *Vet. Rec.* **105**: 299 – 301.

[12] Hungerford, T. G. (1975) . Diseases of livestock , 8th revised Edition, McGraw – Hill, Australia.

[13] Jantunen, M. E. Saxen, H., Lukinmaa, S. Ala – Houhala, M. and Siitonen, A. (2001) . Genomic identity of Pyelonephritogenic *Escherichia coli* isolated from, urine and faeces of children with urosepsis , *J. Med. Microbiol.* **50:** 650 – 652.

[14] Jayarao, B. M. Keane, K. A., Gillespie, B. E., Luther, D. A. and Oliver, S. P. (1993) . DNA fingerprinting : a new technique for identifying mastitis pathogens , National Mastitis Council Annual Meeting Proceedings, Kansas City, Missouri, USA. 167 – 176.

[15] Jha, V. C., Thakur, R. P. and Yadav, J. V. (1994) . Bacterial species isolated from clinical bovine mastitis and their antibiotic sensitivity patterns , *Veterinary Review* (Kathmandu). **9:** 21 – 23.

[16] Jha, V. C., Thakur, R. P. , Yadav, J. N. and Rai, L. B. (1993) . Epidemiological investigation of subclinical bovine mastitis in the eastern hills of Nepal , *Veterinary Review* (Kathmandu). **8:** 35 – 39.

[17] Johnson, J. R. (1991) . Virulence factors in *Escherichia coli* urinary tract infection. *Clin. Microbiol. Rev.* **4:** 80 – 128.

[18] Kaipainen, T., Pohjanvirta, T. Shpigel, N. Y., Shwimmer, A., Pyorala, S. and Pelkonen, S. (2002). Virulence factors of *Escherichia coli* isolated from bovine clinical mastitis , *Vet. Microbiol.* **85:** (1), 37 – 46.

[19] Karkkainen, U – M., Kauppinen, J., Ikaheimo, R., Katila, M – L. and Siitonen, A. (1998). Rapid and specific detection of three different G adhesin classes of P-fimbriae in uropathogenic *Escherichia coli* by polymerase chain reaction , *J. Microbiol. Methods.* **34:** 23 – 30.

[20] Lam, T. J. G. M., Barkema, H. W., van Vliet, J. A. H., Schukken, Y. H., Grommers, F. J. and Brand A. (1993) . Intervention study on post milking teat

disinfection on 7 dairy herds with a low somatic cell count and a high clinical mastitis incidence , *Proceeding of the 6th Symposium of the Dutch Society for Veterinary Epidemiology and Economics,* Boxtel, The Netherlands. 113 – 123.
[21] Linton, A. H., Howe, K., Sojka, W. J. and Wray, C. (1979) . A note on the range of *Escherichia coli* O-serotype causing clinical bovine mastitis and their antibiotic resistance spectra. *J. Appl. Bacteriol.* **46**: 590 – 595.
[22] Lipman, L. J. A. (1995) . *Escherichia coli* and *Staphylococcus aureus* mastitis : epidemiology and pathogenesis, Thesis, University Utrecht, The Netherlands 1995.
[23] Manning, S. D., Zhang, L., Foxman, B., Spindler, A., Tallman, P. and Marss, C. F. (2001) . Prevalence of known P-fimbrial G-alleles in Escherichia coli, and identification of a new adhesin class , *Clin. Diagn. Lab. Immunol.* **8:** 637 – 640.
[24] Marrs, C.F., Zhang, L. Tallman, P., Manning, S . D., Comsel, P., Raz, P., Colodner, R., Jantunen, M. E., Siitonen, A., Saxen, H. and Foxman, B. (2002) . Variations in 10 putative uropathogen virulence genes among urinary, Faecal and Peri-urethral *Escherichia coli* , *J. Med. Microbiol.* ..**51:** 138 – 142.
[25] Martin, F. H., Castro, M. M., Aboul – ela, F. and Tinco, I. jr. (1985) . Base pairing involving deoxyinosine: implications for probe design , *Nucleic Acids Res.* **13:** 473–478.
[26] Matthews, K. R. O'Conner, S. A., Kumar, S. J. and Oliver, S. P. (1993) . Differentiation of *Staphylococcus aureus* species of bovine origin by DNA amplification fingerprinting National Mastitis Council Annual Meeting Proceedings, Kansas City, Missouri, USA, 214.
[27] Misra, P. R., Roy, P. K. and Das, K. L. (1993) . Bovine mastitis in Orissa : predominant microflora and antibiotic sensitivity test pattern. *Indian Journal of Dairy Science.,* **46**, 543.
[28] Myllys, V. and Rautala, H. (1995) . Characterization of clinical mastitis in primiparous heifers. *Journal of Dairy Science.* **78**: 538 – 545.
[29] Philipsson, J., Ral, G. and Berglund, B. (1995) . Somatic cell count as a selection criterion for mastitis resistance in diary cattle , *Livestock Production Science.* **41:**195 – 200.
[30] Rule, R., Franco, M., Pracca, G., Grande, F. and Veneroni, R. (1992) . Bacterial diagnosis of acute mastitis in dairy cows and its sensitivity to chemotherapeutic drugs , *Veterinaria Argentina.* **9:** 604 – 608.
[31] Sambrook, J., Fritsch, E. F. and Maniates, T. (1989) . *Molecular Cloning.* A Laboratory Manual (second edition), Cold Spring Harbor Laboratory Press.
[32] Sanchez – Carlo, V., McDonald, J. S. and Packer, R. A. (1984) . Virulence Factors of *Escherichia coli* isolated from cows with acute mastitis, Am. *J. Vet. Res.* **45:** 1775 – 1777.
[33] Saulnier, P., Bourneix, C., Prevost, G. and Andremont, A. (1993) . Random amplified polymorfic DNA assay is less discriminant than pulse – field gel electrophoresis for typing strains of methicillin-resistant staphylococcus aureus, *J. clin. Microbiol.* **31:** 982 – 985.
[34] Schukken, Y. H., Buurman, J., Brand, A., van der Geer. D. and Grommers, F. J. (1990). Population dynamics of bulk milk somatic cell counts, *J. Dairy Science .* **73:** 1343 – 1350.

[35] Schukken, Y. H., Kremer, W. D. J. and Lohuis , J. A. C. (1989) . *Escherichia coli* – mastitis bij het rund , *Tijdschr. Diergenskd.* **114:** 829 – 838.
[36] Schukken, Y. H., Lam, T. J. G. M., Nielen, M., Hogeveen, H. and Barkema, H. W. (1995) . Subclinical and clinical mastitis in Dutch dairy herds : epidemiological developments, *Tijdschrift voor Diergeneeskunde.* **120:** 208 – 213.
[37] Skinner, F. A. and Lovelock, D. W. (1979) . *Identification Methods for microbiologists,* Second Edition. Academic Press, London.
[38] Stefanakis, A.; Boscos, C., Alexopoulos, C. and Samartzi, F. (1995) . Frequency of subclinical mastitis and observations on Somatic Cell Counts in ewes' milk in northern Greece, *Animal Science.***61:** 69 – 76.
[39] Valente, C., Cardaras, P., Ciorba, A. and Tesi, B. (1988) . Studies on virulence factors of *Escherichia coli* isolated from cows with acute mastitis, *Arch. Vet. Ital.* **39:** 254 – 260.
[40] Van Belkem, A., Bax, R., Peerbooms, P., Goessen, W. H. F., van Leeuwen, N. and Quint, W. G. V. (1993) . Comparison of phage typing and DNA fingerprinting by polymerase chain reaction for discrimination of methicillin – resistant *Staphylococcus aureus* strains, *J. Clin. Microbiol.* **31**: 798 – 803.
[41] Versalovic, J., Koeuth, T. and Lupski, J. R. (1991) . Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res,***19**: 6823 – 6831.
[42] Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafslski, J. A. and Tingey, S. V. (1991) . DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res,* **18:** 6531 – 6535.
[43] Wray, C. Callow, R. J. and Sojka, W. J. (1983) . An examination of *Escherichia coli* strains isolated from cases of bovine mastitis for possible virulence determinants. *Vet. Microbiol,* **8:**141 – 145.