INVESTIGATION OF PHYLOGENIC RELATIONSHIP AMONG *ESCHERICHIA COLI* ISOLATED FROM CLINICAL AND SUBCLINICAL MASTITIS IN DIFFERENT ANIMALS IN BASRAH PROVINCE

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(Received 1 October 2017 ,Accepted 11November 2017)

Keywords:*Escherichia coli*, Phylogenetic groups, PCR

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ABSTRACT

*E.coli*can be assigned to one of the four main phylogenetic groups A, B1, B2 and D, which can be divided into seven and then into subgroups: A_0, A_1, B1, $B2_2$, $B2_3$, D_1, and D_2, in addition group B1can be divided into subgroups $B1_1$ and $B1_2$, using multiplex PCR according to the presence/absence or combinationof the three phylogeny genetic markers *chuA*, *yjaA* and DNA fragment TspE4.C2.In the currentstudy atotal of 30*E. coli* isolates were obtained from clinical and subclinical samples from mastitis in cows, sheeps and goats by standard bacteriological methods. Results found that the most isolates of *E. coli* belong to the phylogeny groups A and B1. Group Aincluded (14 isolates, 46.7%) belonged to subgroup A_0 about (6 isolates, 20.0%), and (8 isolates, 26.7%) to A_1 subgroup. On the other hand results showed group B1 composed (14 isolates, 46.7%). Group B1 can be also classified into subgroups $B1_1$ included (8 isolates, 26.7%) and $B1_2$ about (6 isolates, 20.0%). In addition our results showed (1 isolate, 3.3%), assigned to B2 belonged to subgroup $B2_3$ and (1 isolate, 3.3%), fitted in D belonged to subgroup D_1. No isolates were found to belong to subgroups $B2_2$ and D_2. Phylogeny pedigree was done according to the data recovered previously. This study explains that the distributions of *E. coli* isolates in phylogenetic groups (A, B1, B2 and D) varied depending on the climatic zone and environmental factors such as dietary, climatic conditions and geographic.

INTRODUCTION

Escherichia coli, a bacterium widely spread among warm blooded animals,has been used as an indicator of water fecal contamination (1).Phylogeny is the study of evolutionary relatedness among various groups of organism. *E. coli* strains can be assigned to one of the main phylogenetic groups (2). Recently, developed a multiplex PCR based method to characterize the phylogroups to assign of *E.coli* into four main phylogenetic groups: A, B1, B2 and D using the genetic markers *chuA*, *yjaA*and the DNA fragment TspE4.C2 (3). To increase the discrimination power of *E. coli*population analyses, *E. coli* can be categorized into subgroups: A_0, A_1, B1, $B2_2$, $B2_3$, D_1, and D_2. The groups and subgroups were determined based on the presence/absence and combination of genetic markers(4,5).The genetic markers include*chuA* had been established to be involved with heme transport *E. coli* O157:H7 (6), while *yjaA* was involved in cellular response to hydrogen peroxide and acid stress (7), and DNA fragment TSP4.C2 that has been recently known as part of a putative lipase esterase gene (8). According to (9) groups A and B1are sister groups whereas group B2 is included in an ancestral branch. Differences of phenotypic characteristics of these groups including the ability to use certain sugars, antibiotic resistance profiles and growth rate temperature relationships (10). These phylogroups apparently differ in their ecological niches, life history (11).For example, groups B2 and D strains are less frequently isolated from environment (12), than A and B1 strains (11). Furthermore, genome size differs among these phylogroups, with A and B1 strains having smaller genomes than B2 or D strains. The commensal strains of *E. coli* had been placed into the phylogenetic groups; A and B1, while the extraintestinal pathogenic *E.coli* (ExPEC) strains into group B2 (14), and the (ExPEC) strains into group B2 and, to a lesser extent, group D (15-16). The intestinal pathogenic *E.coli* (InPEC) strains are usually assigned to groups A, B1 and D (17).Some authors analyzed the distribution of the main phylogenetic groups among *E. coli*strains isolated from mastitis in cows and goats. (11) observed that the relative abundance of phylogeneticgroups among mammals is dependent on the host diet, body mass and climate. (18) analyzing mastitic strains isolated from bovine mastitis, observed the prevalence of A and B1..(19) analyzed mastitic milk in goats and found a prevalence of group B1.

The particular study was amid to analyze the distribution of phylogenetic groups and subgroups in mastitic milk from different animals and assess the potential application of this analysis in identifying the major source of mastitis infection.

MATERIALS AND METHODS:

Samples and Bacterial Culture

A total of 30 *E. coli* isolates were isolated from various milk samples of clinical and subclinical mastitis from cows, sheeps and goats by standard bacteriological methods, 6 isolates were isolated from clinical and 9 isolated from subclinical mastitis in cows, 7 clinical isolates and 5 isolates were isolated from the subclinical mastitis in sheeps, while 3 isolates isolated from subclinical mastitis in goats. All samples were obtained from mastitic animals who were admitted to Basrah Veterinary Hospital and from different fields and regions of Basrah Province in the period from October 2016 to January 2017 in two phases. Phase 1 was from animals with clinical mastitis according to clinical signs and phase 2 was from animals with subclinical mastitis according to California Mastitis Test (20). The samples were processed on MacConkey sorbitol agar, Eosin methylene blue agar, Endo agar and on KIA agar and were incubated at 37°C overnight. The identification of Gram negative bacteria, purple color was confirmed by API 20 E system (BioMérieux, Inc., France).

DNA extraction for *E.coli*

Bacterial DNA was obtained by suspending colonies of bacteria growth in 500 µl of sterile distilled water and boiling at 100 °C for 10 min to lyse the organisms. After heating, the DNA harvested by centrifugation at 12,000 rpm for 15 min(21).

Detection of Phylogenetic Groups

PCR was conducted to determine the phylogenetic grouping of the isolates by targeting two genes, *chuA*, *yjaA* and anonymous DNA fragment TspE4.C2 (3). Each 25 µl of PCR reaction mixture for PCR contained 2.5 µl of upstream primer, 2.5 µl of downstream primer, 2.5 µl of free nuclease water, 5 µl of DNA extraction and 12.5 µl of master mix. Thermal cycler conditions were as follows: 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 59°C for 30 s and 72°C for 30 s. A final extension of 72°C for 7 min was performed at the end of PCR. The primers used were *chuA*, *yjaA* and TspE4.C2 which

generated 279, 211 and 152bp fragment respectively. The data of the three amplification resulted in assignment of the isolates to phylogenetic groups (Fig. 1).

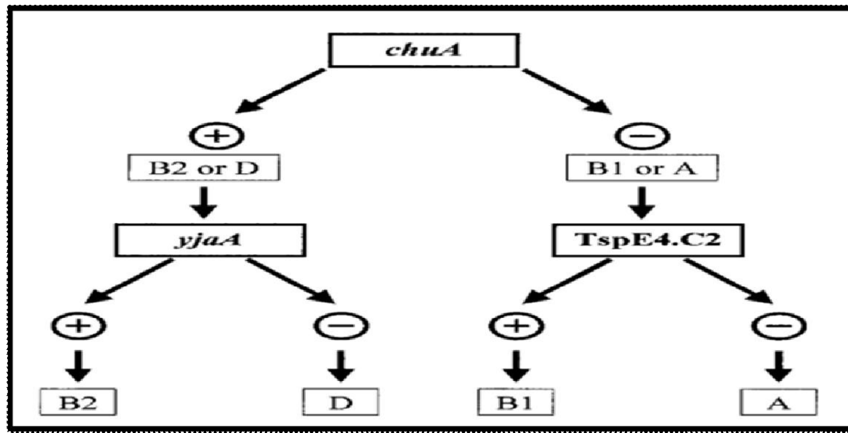

Figure 1: Dichotomous decision tree to determine the phylogenetic group of *E. coli* isolates by using the results of PCR amplification of the *chuA* and *yjaA* genes and DNA fragment TSPE4.C2

Statistical Analysis:

Chi-square test (P) was used to determine the phylogenetic groupings that revealed significant differences (P≥0.05) between distributions of four main phylogenetic groups and subgroups among isolates derived from different origins.

RESULTS

The suspected lactose fermenter colonies *E. coli* on MacConkey sorbitol agar were bright pink colonies with red halo, appeared as fluorescent blue black color reflecting greenish metallic sheen when exposed to light and a dark or black center in transmitted light on EMB, deep red colonies with a permanent metallic sheen on Endo agar and yellow colonies on KIA due to fermentation of lactose and glucose.(Fig.2,3,4,5). Additionally the results on (Tab.1) revealed the prevalence of *E.coli* isolation from milk samples from different infected animals based on culturing identification.

Figure 2: *E.coli* isolates on MacConkey Sorbitol Agar

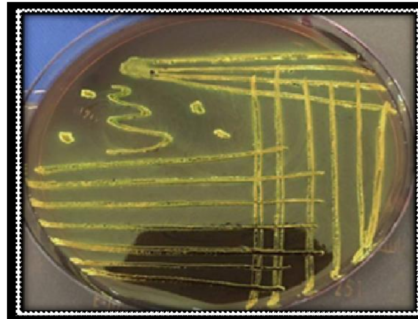

Figure 3: *E.coli* isolates on EMB agar

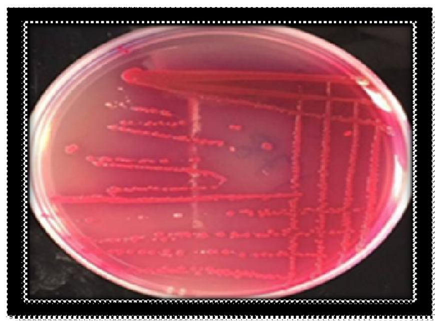

Figure 4: *E.coli* isolateson Endo agar

Figure 5: *E.coli*isolates on KIA agar

Table 1: Prevalence of *E.coli* Isolation from Milk Samples from Different Infected Hosts

Source of Host	Disease	Examined Samples (No= 180)	Positive Samples (No= 30)	Isolation %
Cows	Clinical Mastitis	30	6	10.0
	Subclinical Mastitis	30	9	15.0
Sheeps	Clinical Mastitis	30	7	11.7
	Subclinical Mastitis	30	5	8.3
Goats	Clinical Mastitis	30	0	0
	Subclinical Mastitis	30	3	5.0
$P \geq 0.05$				

On the other hand the results from the API 20 E test showed that 30 isolates earlier identified as *E. coli* on EMB and Endo agars. Using of API 20 E system revealed that only 9 (30 %) isolates were identified as *E. coli* (Tab. 2).

Table 2: Prevalenceof *E. coli* isolates by API 20 E Test

Isolate No. N= 30	Identification by EMB	Identification by API 20 E Test
9 (30 %)	Identified as *E. coli*	*E. coli*
16 (53.33 %)	Identified as *E. coli*	Out of specification
5 (16. 66 %)	Identified as *E. coli*	-

The study was conducted to show the phylogeny of *E. coli* isolated from clinical and subclinical mastitic samples in different animals. However, the phylogenetic groups of *E. coli* isolates were detected by identifying the presence/absence of specific multiplex PCR amplified fragments. The results showed the *chuA* gene was found only insubclinical mastitis sample incow and one sample in sheep. The *chuA* represents the phylogenetic marker for extraintestinal *E. coli* isolates. Additionally the *yjaA* marker found in *E. coli* isolated from clinical mastitis which occur in five samples in sheeps and one sample in cow and subclinical mastitis in four , two and three samples in cows, sheeps and goats respectively,*yjaA* represents the phylogenetic marker for intestinal *E. coli* isolates. While TspE4.C2 fragment found in clinical mastitis in four samples in cows and five samples in sheeps and in subclinical mastitis was occurred in four samples in cows and two samples in sheeps using PCR amplification. (Fig. 6,7,8,9). TspE4.C2 represents a phylogenetic marker for intestinal *E. coli* isolates.

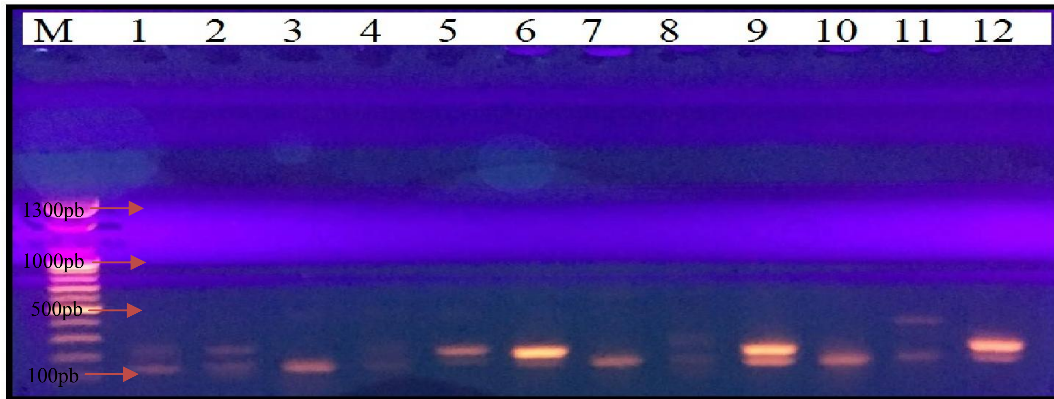

Figure 6: Multiplex PCR Amplification of *chuA, yjaA* genes andTspE4.C2 fragmentfor *E. coli* isolates. Lane M: molecular size marker (100bp); lane 11, positive result for *chuA* gene (279bp), lanes 5, 6, 9, 12, positive result for *yjaA*gene (211pb) and lanes 3, 7, 9, 10, 12, positive result for TspE4.C2 fragment (152pb).

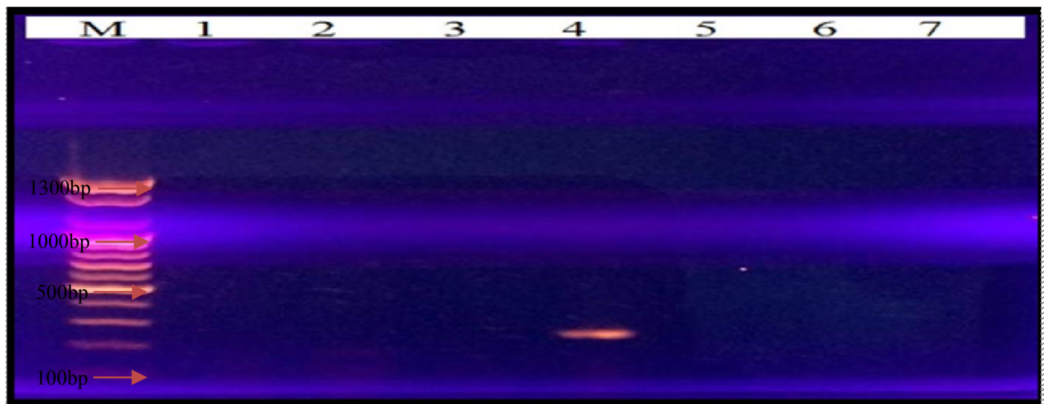

Figure 7: Conventional PCR amplification of *chuA* gene for *E. coli* isolates. Lane M: molecular size marker (100bp); lane 4, positive result for *chuA* gene (279bp).

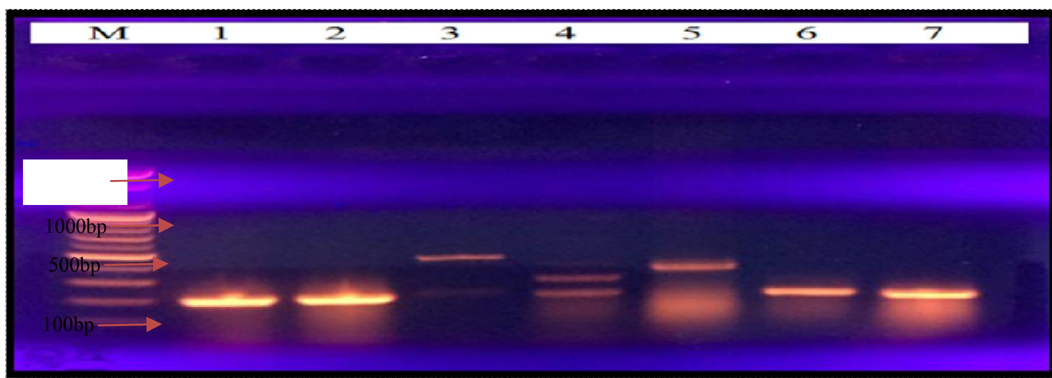

Figure 8: Conventional PCR amplification of *yjaA* gene for *E. coli* isolates. Lane M: molecular size marker (100bp), lanes 1,2,4,6-7, positive result for *yjaA* gene(211pb).

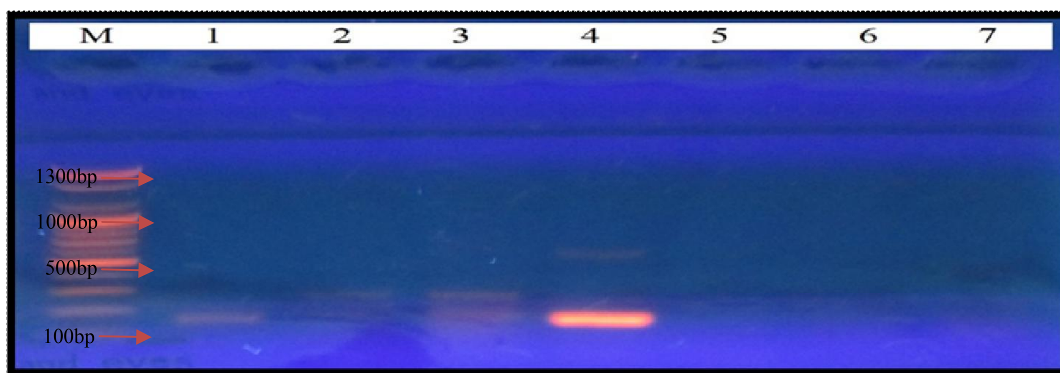

Figure 9: Conventional PCR amplification of TspE4.C2 fragmentfor *E. coli* isolates. Lane M: molecular size marker (100bp); lane 4, positive result for TspE4.C2 fragment (152bp).

Consequently, the result showed that most strains of group A (14 isolates, 46.7%) belonged to subgroup A_0 about (6 isolates, 20.0%), and (8 isolates, 26.7%) to A_1 subgroup. On the other hand results showed group B1 included (14 isolates, 46.7%). Group B1 can be classified into subgroups $B1_1$ included (8 isolates, 26.7%) and $B1_2$ about (6 isolates, 20.0%). In addition our results showed (1 isolate, 3.3%), assigned to B2 belonged to subgroup $B2_3$ and (1 isolate, 3.3%), fitted in D belonged to subgroup D_1.No isolates were found to belong to subgroups $B2_2$ and D_2. (Tab. 3) and (Fig. 10)

Table 3: Distribution of the *E.* coli isolates into main four phylogenetic groups and subgroups

Phylogenetic Groups	Phylogenetic Subgroups	*ChuA* Gene	*YjaA* Gene	TspE4.C2 Fragment	Phylogenetic Subgroups% N= 30	Phylogenetic Groups% N= 30
Group A	A_0	-	-	-	6 (20.0%)	14 (46.7)
	A_1	-	+	-	8 (26.7%)	
Group B1	$B1_1$	-	-	+	8 (26.7%)	14 (46.7)
	$B1_2$	-	+	+	6 (20.0%)	
Group B2	$B2_2$	+	+	-	0 (.0%)	1 (3.3%)
	$B2_3$	+	+	+	1 (3.3%)	
Group D	D_1	+	-	-	1 (3.3%)	1 (3.3%).
	D_2	+	-	+	0 (.0%)	

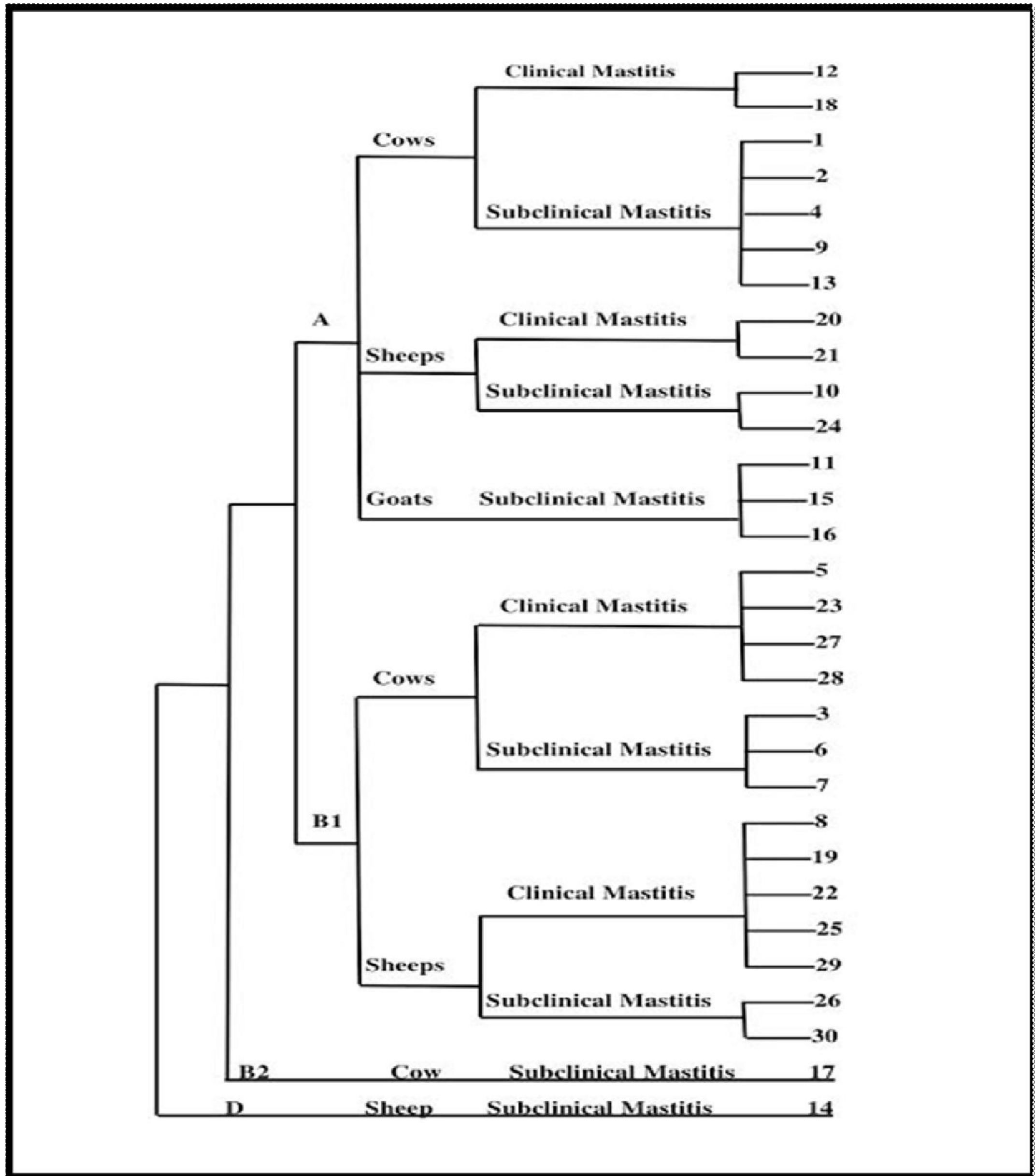

Figure 10: Dendrogram shows the similarity relationships between the phylogenic groups of *E. coli* isolated from clinical and subclinical mastitis samples in cows, sheeps and goats.

DISCUSSION

All suspected *E.coli* strains showed lactose fermentation (pink colonies) on MacConkey sorbitol agar, green metallic sheen colonies on EMB, red colonies on Endo agar and yellow colonies on KIA. In previous studies reported that all *E.coli* isolates showed lactose fermentation (pink colonies) on MacConkey agar and green metallic

sheen colonies on EMB (22). In contrast, (23,24), demonstrated that *E.coli* isolates showed deep red colonies on Endo agar and yellow colonies on KIA.

Previous study suggested that MacConkey sorbitol and EMB agars are used to identify and differentiate Gram negative mastitis pathogens. MacConkey sorbitol agar is used to differentiate *E. coli* from other Gram negative and like EMB agar, inhibits the growth of most Gram positive organisms. EMB agar provides a rapid and accurate method of distinguishing *E. coli* from other Gram negative mastitis pathogens. Direct inoculation of mastitic milk on EMB agar does not allow differentiation of *E.coli* and does not produce a green metallic sheen. Lack of sheen production could be due to the alkalinity of mastitic milk interfering with the acidic requirement of EMB agar for production of the green metallic sheen (25).

The results from the API 20 E test showed that 9 (30%), isolates out of the 30 isolates, earlier identified as *E. coli* using EMB and Endo agar gave a positive result as *E. coli* and 16 (53.33 %), were out of specification (did not recognized in the index). Only 5 isolates (16. 66 %), were negative and not confirmed as *E. coli.* Taken together, the results from the present study, the 8 isolates out of 16 isolates were determined out of specification give positive result as *E.coli* after applied multiplex PCR targeting of *E.coli* for the same16 isolates.

(26) reported that used the API 20 E system was accurately identified 96% of the veterinary isolates and misidentified 3%. Previous investigation also demonstrated that suggested confidence of *E. coli* identification using API20 E were excellent confidence where 88% isolates were identified as *E. coli* (27). A recently report revealed *E.coli* isolated from mastitic milk samples were identification by the API 20 E showed about 12.4% as *E.coli* (28). In addition a study showed that overall 95% and 100% of the clinical and environmental isolates respectively were identified with various degrees of accuracy as *E. coli* by API 20 E (29).

The variations in the biochemical behavior of the *E.coli*, may be attributed to genetic variations of different stains resulting in different phenotypic characteristics. These genetic variations may be of chromosomal or plasmid origin (30).This is probably because most of *E. coli* isolated from clinical samples are tend to be biochemically typical (31). In contrast, environmental *E. coli* isolates may exhibit atypical biochemical

characteristic due to physiological changes required for better survival in environments (32). Based on previous data (28), suggested that API 20E may produce more accurate identification with *E.coli* of clinical origin but not environmental *E. coli* isolates.

The results also showed that the *chuA* gene was found in two of *E.coli* isolated from subclinical mastitis incows and sheeps that belonging groups B2 and D, while *yjaA*gene more frequent common in *E. coli* isolated fromclinical mastitis (5 samples) in sheeps, (1 sample) in cow and subclinical mastitis (4 samples) in cows, (2 samples) in sheeps and (3 samples) in goats. Those findings disagreed with those in previous research by (5), which reported that the *chuA* and *yjaA*genes were rarely found in *E. coli* strains isolated from cows, sheep's and goats. The *yjaA* gene allowed perfect discrimination between group B2 and group D and it was find in all *E.coli* isolates belonging to group A. While, the TspE4.C2 is found in group B1 isolates and absent from all group A isolates (3).

chuA was less detected. We hypothesized that the variations in detection of *chuA* compared with other genetic markers is due to the source of mastitis infection which is intestinal *E.coli* andin our study most *E.coli* isolates were intestinal *E.coli* because of *chuA* represents the phylogenetic marker for extraintestinal *E.coli* and *yjaA* and DNA fragment represents the phylogenetic markers for intestinal *E. coli* isolates. Few previous studies suggested little information is available on *yjaA* and DNA fragment to speculate on their evolutionary history.

In contrast, the study by. (33), they suggested that *chuA* was acquired by sister groups B2 and D (9), soon after their emergence rather than being present in common ancestor and subsequently being lost by groups B1 and D. The distribution of phylogenetic groups differs considerably between intestinal and extraintestinal *E. coli* isolates (34).

The results showed that most strains of group A (14 isolates, 46.7%) belonged to subgroup A_0 about (6 isolates, 20.0%), and (8 isolates, 26.7%) to A_1 subgroup. On the other hand results showed group A an equal B1, where group B1 (14 isolates, 46.7%) belonged to subgroup $B1_1$ (8 isolates, 26.7%) and $B1_2$ about (6 isolates, 20.0%). Group B1 can be classified into subgroups; $B1_1$ included (8 isolates, 26.7%) and $B1_2$ about (6 isolates, 20.0%).

Based on previous studies of. (3,4,5), *E.coli* assigned into four main groups A, B1, B2 and D, and classified into seven subgroups: A_0, A_1, B1, $B2_2$, $B2_3$, D_1, and D_2, we can classify group B1 into two subgroups $B1_1$ and $B1_2$ and this classification accordance and compatible with previous classification (3-5). In this field study, (1 isolate, 3.3%), assigned to B2 belonged to subgroup $B2_3$ and (1 isolate, 3.3%), fitted in D belonged to subgroup D_1. No isolates were found to belong subgroups $B2_2$ and D_2. This findings go hand to hand with the previous studies (35-39), which reported that the *E.coli* isolates isolated from bovine mastitis have been generally belonged to A and B1 groups.

In similar study (40), reported *E.coli* isolates from bovine mastitis were belonged to A (44.88%), B1(38.58%) and D (16.53%) groups. About (61.41%) of isolates fell into four phylogenetic subgroups:(18.11%) into A_0, (26.77%) into A_1, (6.29%) into D_1 and (10.23%) into D_2. None ofthe isolates belonged to B2group or its subgroups. Previous investigation of Dubravka *et al.* (41), suggested results of phylogenetic typing confirmed that *E. coli* strains isolated from milk of cows with mastitis are typical commensals mainly belongingto phylogenetic groups A and B1.

In addition for this, report conducted (42), suggested that *E.coli* isolated from bovine mastitis belonging to *E. coli* phylogroup A are most frequently. (43), also reported that*E. coli* bovine mastitis isolates, (76%), were assigned to group A1 and (46%) were assigned to group B1. In contrast,. (19), showed *E.coli* isolated from goats was the most prevalent and belong phylogroup B1 (57.6%), this disagreed with our results that showed all *E.coli* isolated from goat belonged group A.

Finally we concluded that *E. coli* isolates from mastitis cases were mainly of commensal phylogeny types A and B1 groups which are play important role in infection of mastitis.Our data further support recent findings demonstrating that the A and B1 commonly isolated from mastitis.

التحري عن العلاقة التطورية بين *E.coli* المعزولة من التهاب الضرع السريري والتحت السريري في الحيوانات المختلفة في محافظة البصرة

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يمكن تقسيم*E.coli*الى واحدة من اربعة مجاميع رئيسة:A،B1،B2 وDالتي يمكن تقسيمها الى سبعة مجاميع فرعية:A_0،A_1،B1،$B2_1$،$B2_2$،D_1وD2 بالإضافة الى ذلك مجموعة B1 يمكن تقسيمها الى مجموعتين فرعية $B1_1$ و$B1_2$ باستخدام اختبار تفاعل البلمرة المتعدد وفقاً لوجود/عدم وجود أو مزج مجموعة مكونة من ثلاث بادئات تطورية: جين *chuA*، جين *yjaA* وقطعة TspE4.C2.في الدراسة الحالية تم الحصول على ٣٠ عزلة*E.coli*من العينات السريرية والتحت السريرية من التهاب الضرع في الأبقار،الأغنام والماعزبواسطة الطرق البكتريولوجية القياسية.اظهرت النتائج أن معظم العزلاتتخص المجموعة Aحيث ضمت ١٤ عزلة (٤٦.٧%) تنتمي إلى المجموعة الفرعية A_0حوالي ٦ عزلة (٢٠%) و٨ عزلة (26.7%) انضمت الى المجموعة الفرعية A_1. من الناحية الاخرى المجموعة B1 ضمت ١٤ عزلة (٤٦.٧%) موزعة الى مجموعتين فرعية حيث كانت المجموعة الفرعية $B1_1$ ٨ عزلة (٢٦.٨%) في حين ٦ عزلة (٢٠.٠%) ادرجت في المجموعة الفرعية $B1_2$.بالإضافة إلى ذلك أظهرت نتائج ١عزلة(٣.٣%)مخصصة المجموعة B2تنتمي إلى المجموعة الفرعية $B2_3$ و١عزلة(٣.٣%) في المجموعةDتنتمي إلى المجموعة الفرعية D_1. لم يتم العثور على أي عزلات تنتمي إلى المجموعات الفرعية $B2_2$ وD_2.تم تكوين الاصول التطورية وفقا للبيانات المستردة سابقا. توضح هذه الدراسة أن توزيع عزلات*E.coli* الى مجموعات تطورية (A، B1، B2 و D) تختلف تبعا للمنطقة المناخية والعوامل البيئية مثل الظروف الغذائية والظروف المناخية والجغرافية.

REFERENCES

1-Field, K.G. and Samadpour, M. (2007). Fecal source tracking, the indicator paradigm, and managing water quality. Water Research.41:3517-3538

2-Herzer, P.J.; Inouye, S.; Inouye, M. and Whittam, T.S. (1990). Phylogenetic distribution of branched RNA-linked multi-copy single-stranded DNA among natural isolates of *Escherichia coli*. J. Bacteriol. 172:6157-6181

3- Clermont, O.; Bonacorsi, S. and Bingen, E. (2000). Rapid and simple determination of the *Escherichia coli* phylogenetic group. Appl. Environ. Microbiol. 66: 4555-4558

4- Escobar-Páramo, P.; Menac'h, A. L.; Gall, T. L.; Amorin, C.; Gouriou, S.; Picard, B.; Skurnik, D. and Denamur, E. (2006). Identification of forces shaping the commensal *Escherichia coli* genetic structure by comparing animal and human isolates. Environmental Microbiology. 8 (11): 1975 -1984

5- Carlos, C.; Pires, M.M.; Stoppe, N.C.; Hachich, E.M.; Sato, M.I.; Gomes, T.A. Amaral, L.A. and Ottoboni, L.M. (2010).*Escherichia coli* phylogenetic group determination andits application in the identification of the major animal source of fecal contamination.BMC Microbiology. 10:161

6-Bonacorsi, S.P.; Clermont, O. and Tinsley, C. (2000). Identification of the *Escherichia coli* chromosome specific for neonatal meningitis associated strains. Infect. Immun. 68: 2096-2101

7- Lee, C. C. Y. (2011). *Escherichia coli* Isolates from Duck, Goose, and Gull Fecal Samples with Phylogenetic Markers using Multiplex Polymerase Chain Reaction for Application in Microbial Source Tracking. Journal of Experimental Microbiology and Immunology. 130 -135

8- Gordon, D. M.; Clermont, O.; Tolley, H. and Denamur, E. (2008). Assigning *Escherichia coli* strains to phylogenetic groups: multi-locus sequence typing versus the PCR triplex method. Environmental Microbiology.10: 2484-2496

9-Lecointre, G.; Rachdi, L., Darlu, P. and Denamur, E. (1998).*Escherichia coli* molecular phylogeny using the incongruence length difference test. *Mol. Biol. Evol.* 15(12):1685–1695

10- Touchon, M.; Hoede, C.; Tenaillon, O.; Barbe, V.; Baeriswyl, S.; Bidet, P.;Bingen, E.; Bonacorsi*et al.,* (2009).Organised Genome Dynamics in the *Escherichia coli* Species Results in Highly Diverse Adaptive Paths. PLoS Genet, 5(1), e1000344

11- Gordon, D. M. and Cowling, A. (2003). The distribution and genetic structure of *Escherichia coli* in Australian vertebrates: host and geographic effects. Microbiology. 149: 3575 - 3586

12- Walk, S. T.; Alm, E. W.; Calhoun, L. M.; Mladonicky, J. M and Whittam, T. S. (2007). Genetic diversity and population structure of *Escherichia coli* isolated from freshwater beaches. Environmental Microbiology. 9: 2274 - 2288

13- Bergthorsson, U. and Ochman, H. (1998). Distribution of Chromosome Length Variation in Natural Isolates of *Escherichia coli*. *Mol. Biol. Evol.* 15 (1):6 -16

١4-Johnson, J. R.; Delavari, P.; Kuskowski, M. and Stell, A. L. (2001). Phylogenetic Distribution of Extraintestinal Virulence-Associated Traits in *Escherichia coli.* Infect. Immun. 183:78 - 88

15- Picard, B.; Garcia, J.S.; Gouriou, S.; Duriez, P.; Brahimi, N.; Bingen, E.; Elion, J. and Denamur, E. (1999). The link between phylogeny and virulence in *Escherichia coli* extraintestinal infections. Infect. Immun. 546 -553

16- Johnson, J. R. and Stell, A. L. (2000). Extended Virulence Genotypes of *Escherichia coli* Strains from Patients with Urosepsis in Relation to Phylogeny and Host Compromise. The Journal of Infectious Diseases.181:261-72

17- Pupo, G. M.; Karaolis, D. K.; Lan, R. and Reeves, P. R. (1997). Evolutionary relationships among pathogenic and nonpathogenic *Escherichia coli* strains inferred from multilocus enzyme electrophoresis and *mdh* sequence studies. Infect. Immun. 65: 2685-2692

18- Blum, S. E. and Leitner, G. (2013). Genotyping and virulence factors assessment of bovine mastitis *Escherichia coli.* Veterinary Microbiology. 163: 305-312

19- Jajarmi, M.; Ghanbarpour, R.; Sharifi, H. and Golchin, M. (2015). Distribution Pattern of ECOR Phylogenetic Groups among Shiga Toxin-Producing and Enteropathogenic *Escherichia coli* Isolated From Healthy Goats.

Int. J. Enteric. Pathog. 3(3): e27971

20- Abera, M.; Megersa, B. and Asmare, K (2016). Bovine mastitis: prevalence, risk factors and isolation of Staphylococcus aureus in dairy herds at Hawassa milk shed, South Ethiopia. BMC Veterinary Research. 12:270

21- Barzan, M.; Rad, M.; Tabar, G. R. H. and Azizzadeh, M. (2016). Phylogenetic analysis of *Escherichia coli* isolates from healthy and diarrhoeic calvesin Mashhad, Iran.B. J. V. M. 1- 8

22- Abd El Tawab, A. A.; Ammar, A. M.; Nasef, S. A. and Reda, R. M. (2015). Prevalence of *E.coli* in diseased chickens with its antibiogram pattern. B. V. M. J. 28 (2): 224 - 230

23- Arshad, R.; Farooq, S. and Ali, S. S. (2006). Manipulation of different media and methods for cost effective characterization of *Escherichia coli* strains collected from different habitats. *Pak. J. Bot*. 38(3): 779 -789

24- Ahmad, K.D. and Baker, K. I. (2007). Isolation, Identification and Partial Characterization of Plasmid DNA of Different Bacterial Species Isolated from

Clinical Specimens of Patients Suffering from Diarrhea.Raf. Jour. Sci. 18 (12): 6 - 12

25- Leininger, D.J. Roberson, J. R. and Elvinger, F. (2001). Use of eosin methylene blue agar to differentiate Escherichia coli from other gram-negative mastitis pathogens. J. Vet. Diagn. Invest. 13:273 - 275 4-Abebe, R.; Hatiya, H.;

26- Swanson, C. E. and Collins, M.T. (1980).Use of the API 20E System to Identify Veterinary Enterobacteriaceae. J. Clin. Microbiol.10-14

27- Julian, T. R.; Islam, M. A.; Pickering, A. J.; Roy, S.; Fuhrmeister, E. R.; Ercumen, A.; Harris,A.; Bishai, J. and Schwab, K. J. (2014). Genotypic and Phenotypic Characterization of *Escherichia coli* Isolates from Feces, Hands, and Soils in Rural Bangladesh via the ColilertQuanti-Tray System. Appl. Environ. Microbiol.1735- 1743

28- Amani B. A, Osman, I. A. M.; El Ayis, A. A. and Almofti, Y. A. (2016). Studies on Coliform Mastitis in River Nile State, Sudan. Int. j. sci. footpr. 4 (2): 82 - 96

29- Abulreesh, H.H. (2014). Efficacy of Two Commercial Systems for Identification of Clinical and Environmental *Escherichia coli*. International Journal of Biology. 6 (2): 31- 41

30- Oliver, S.P. and Bushe, T. (1987). Growth inhibition of *Escherichia coli* and *Klebsiella pneumoniae* during involution of the mammary gland: relation to secretion composition. American Journal of Veterinary Research. 48: 1669 - 1673

31- Janda, J. M. and Abbott, S. L. (2006). The Enterobacteria. Washington D. C., USA: ASM Press. (2nded.)

32- Jordan, K. N.; Oxford, L. and O'Byrne, C. P. (1999). Survival of low-pH stress by *Escherichia coli* O157:H7: correlation between alterations in the cell envelope and increase acid tolerance. Appl. Environ. Microbiol. 65:3048 - 3055

33- Wyckoff, E. E.; Duncan, D.; Torres, A. G.; Mills, M.; Maase, K. and Payne, S. M. (1998). Structure of the *Shigella dysenteriae* haem transport locus and its phylogenetic distribution in enteric bacteria. Molecular Microbiology. 28 (6): 1139 -1152

34- Duriez, P.; Clermont, O.; Bonacorsi, S.; Bingen, E.; Chaventre, A. ; Elion, J.; Picard, B. and Denamur, E. (2001). Commensal *Escherichia coli* isolates are

phylogenetically distributed among geographically distinct human populations.Microbiology.147:1671-1676

35- Dosogne, H.; Meyer, E.; Sturk, A.; Loon, J. V.; Massart-Leën, A. M. and Burvenich, C. (2002). Effect of enrofloxacin treatment on plasma endotoxin during bovine *Escherichia coli* mastitis. Inflamm. Res. 51: 201-205

36- Dogan, B.; Klaessig, S.; Rishniw, M.; Almeida, R.A.; Oliver, S.P.; Simpson, K. and Schukken, Y.H. (2006). Adherent and invasive *Escherichia coli* are associated with persistent bovine mastitis. Veterinary Microbiology. 116: 270 - 282

37- Fernandes, J. B. C.; Zanardo, L. G.; Galvão, N. N.; Carvalho, I. A.; Nero, L. A. and Moreira, M. A. S. (2011).*Escherichia coli* from clinical mastitis: serotypes and virulence factors. Journal of Veterinary Diagnostic Investigation.23(6) 1146 - 1152

38- Blum, S. E. and Leitner, G. (2013). Genotyping and virulence factors assessment of bovine mastitis *Escherichia coli.* Veterinary Microbiology. 163: 305-312

39- Leimbach, A.; Poehlein, A.; Vollmers, J.; Görlich, D.; Daniel, R. and Dobrindt, U. (2017).No evidence for a bovine mastitis *Escherichia coli* pathotype.BMC Genomics.18:359

40- Ghanbarpour, R. and Oswald, E. (2010). Phylogenetic distribution of virulence genes in *Escherichia coli* isolated from bovine mastitis in Iran. Research in Veterinary Science. 88: 6 -10

41- Dubravka, M.; Bojana, P.; Maja, V.; Dalibor, T. and Vladimir, P (2015). Investigation of Biofilm Formation and Phylogenetic Typing of *Escherichia Coli* Strains Isolated From Milk of Cows with Mastitis. ActaVeterinaria-Beograd. 65 (2): 202-216

42- Goldstone, R. J. and Harris, S. and Smith, D. G. E. (2016). Genomic content typifying a prevalent clade of bovine mastitis associated *Escherichia coli*. Scientific Reports. 6: 30115

43- Mustak, H. K.; Gunaydin, E.; Kaya, I. B.; Salar, M. O.; Babacana, M.; Onat, K.; Ata, Z. and Diker, K. S. (2015). Phylotyping of clinical *Escherichia coli* isolates originating from bovine mastitis and canine pyometra and urinary tract infection by means of quadruplex PCR. Veterinary Quarterly.1 - 6