Molecular and Virulence Characterization of *Escherichia.coli* strains Isolated from Persistent Bovine Mastitis.

[1]Salwa, M. Helmy; [2]Ammar, M. A.; [3]Aisha R. Ali; [4]Mona, A. El-Shabrawy; [4*]Hakim.A.S.; [4] Bakry, M.A.; [4]Azza, S.M. Abuelnaga and[4]Eraqi, M. M.

[1]Bacteriology, Mycology and Immunology Department Faculty of Veterinary Medicine Kafrelsheikh University,
[2] Microbiology, Faculty of Veterinary Medicine, Zagazig University, Zagazig , Egypt
[3] Serology Unit Animal Health Research Institute Dokki, Giza, Egypt
[4]Microbiology and Immunology National Research Center, Dokki, Giza, Egypt
migris410@yahoo.com *

Abstract: Four hundred and fifty lactating cows were examined according to the clinical observation and the California mastitis test, 181 were clinical mastitis with the percentages of 40.2%, and revealed 57 *E.coli* isolates, the incidence of clinical mastitis is higher in hind quarters (63.97%) than the fore quarters (36.02%). Serotyping of *E.coli* revealed 8 different serovars of *E.coli* according to somatic antigen O55 (19.2%), O111 (15.8%), O124 (12.3%), O119 (12.3%), O114 (10.5%), O26 (7%), O157 (7%) and O44 (3.5%), in addition, (12.2%) of isolated *E.coli* strains could not be serologically identified by the available antisera. The incidence of recurrent *E.coli* mastitis, 26.3% (15 of 57) occurred in 5 of 56 quarters 8.9% of 5 cows, the most *E.coli* serogroups recovered from recurrent *E.coli* mastitis from 5 quarters of 5 cows were O55, O119, O111, and O157. The adherent and invasive property were the most common factors in *E.coli* serogroups (O55, O119, O111 and O157) which were isolated from recurrent mastitis and give positive results with (*eae*A) gene but it is less in *E.coli* serogroups(O124, O114, O26 and O44) which give negative results with (*eae*A) gene**.**

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1. Introduction:

Mastitis is considered the most costly disease in dairy herds due to discarded milk and lowered milk production for approximately 80% of costs associated with mastitis, treatment costs, veterinary fees, labor costs early culling and death (Miller *et al*., 1993). Lowered milk quality due to increased somatic cell count (SCC) in the milk decreases shelf life of milk and cheese making quality (Klei *et al*., 1998 and Ma *et al*., 2000).

The importance of mastitis in public perception should not be over looked. The general public is more and more concerned with animal welfare, possible antibiotic residues in the milk and a disease such as mastitis that can cause severe distress to the cow shouldn't be ignored(Bradely, 2002). Mastitis is considered of vital importance due to its association with many zoonotic diseases in which milk acts as a source of infection (APHA., 1993).

E.coli is one of the most frequently isolated pathogens from both clinical and chronic infection, it was more severe than the other bacterial causes and it tended to be more severe in early lactation and during the housing period, resulting in inflammation that ranges from sub-acute to per-acute. Necrosis of the mammary epithelium occurs during severe, naturally occurring clinical *E.coli* mastitis, as well as during severe experimental *E. coli* mastitis. In moderate cases of *E. coli* mastitis, there is minimal alveolar tissue damage (Bradley and Green, 2001).

Recurrent clinical mastitis caused by *E. coli* in a cow that express persistent intra-mammary infection (IMI) is known to exist in the same quarter can be caused by a persistent IMI or may be associated with recurrent IMI. (Lipman *et al*., 1995).

Adherence of microorganisms to the host cells is the first step in colonization on the host surface (Finlay and Falkow, 1997). Invasion and adhesion are important virulence mechanisms in the bacterial infection, Therefore persistent bacterial infection generally involves adhesion, invasion and intracellular survival (Finaly and Cossart, 1997). Pathogenic *E.coli* which can cause persistent intramammary infection have several fimbrial and afimbrial adhesions that mediate adhesion to host epithelial cell through the cell surface compound

like proteins, glycolipids and carbohydrates (Le Bouguenec, 2005).

The present study was directed to detection and characterization of virulent E.coli pathogen recovered from mastitic milk with special reference to recurrent mastitis.

2. Materials and methods

Samples:

A total of 450 milk samples were collected from cows from 4 farms in Kafrelsheikh and Dakahlia Governorates. All samples were examined for mastitis according to clinical observation and California mastitis test as shown in Table (1).

Bacteriological examination of milk samples (Quinn et al., 2002):

The collected milk samples were incubated aerobically at 37°C for 18-24 hours, then centerifugated at 3000 rpm for 20 minutes. The cream and supernatant fluid were discarded. The sediment was streaked onto blood agar, MacConkey agar and EMB agar. The inoculated plates were incubated aerobically at 37ºC for 24-48 hours and examined for bacterial growth.

Identification of isolates:

Pure cultures were prepared from all suspected colonies. The shape, size and type of colonies either lactose or non-lactose fermenting colonies onto MacConkey's agar and blood agar were recorded. Gram's stained films from the purified isolates were made on clean slides films to be examined microscopically for detection their stain reaction and morphological characters. Gram negative rood shape bacilli or coccobacilli, with parallel sides and round ends. Such colonies were picked on slope agar for preservation of isolates and for further studies (Koneman et al., 1995).

Biochemical identification:

Members of this group were initially identified by their catalase positive, oxidase negative and gram negative bacilli in Gram stained smears. Further identification was done according to Quinn et al. (1994).

Serological identification of *E.coli*

Serotyping of *E.coli* isolates was performed according to (Edwards and Ewing, 1972).

Fifty seven isolates of *E.coli* from mastitic cows were sub-typed by using 8 polyvalent and 43 monovalent "O"antisera. All isolates were taken from mastitic cows showing sever manifestation. Each isolate was first tested for its agglutinability of the diagnostic polyvalent "O" antisera, which are intended for use by slideagglutination technique. Once the pathogenic type has been indicated by the use of polyvalent sera, further serogrouping was made with the appropriate "O" monovalent antisera.

Identification of K99 pilus in *E.coli* strains:

From *E.coli* suspected colonies from each culture plate were selected and used for K99 pilus detection.Proposed positive *E.coli* samples were collected and cultured on Minica Iso Vitalex medium (Guinee *et al*., 1977). Colonies from each cultured plate were selected and subculture on Minica Iso Vitalex agar plate for purification .Plates were inoculated for 16-18 hr. at 37ºC. All selected colonies were smooth translucent circumscribed, completely separate colonies. These colonies were subjected for K99 pilus antigen detection by slide agglutination test using ready made international trading diagnostic serum.

Extraction of DNA from the bacterial isolates according to Sambrook *et al.* (1989):

Bacterial culture was grown in 5 ml Tryptic Soy broth (TSB) and then 1.5 ml of the culture were microfuged at 6000 rpm for 2 minutes. Pellets were resuspended in 567µl Tris-EDTA by repeated pipetting. 30µl of 10% SDS and 3µl of 20 mg/ml proteinase K were added, mixed and incubated for 1 hour at 37°C. 100µl of 5M NaCl were added and mixed thoroughly. 80µl CTAB/NaCl solution were added, mixed and incubated for 10 minutes at 65°C. Equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added, mixed and microfuged for 5 minutes. The supernatant was transferred to a fresh tube then 0.6 volume of isopropanol was added and mixed gently. After storage at 20°C overnight, the DNA was pelleted at 14,000 xg/min for 20 minutes, followed by washing with 500µl 70% ethanol and recentrifuged for 10 minutes at 14,000 xg/min. The supernatant was discarded carefully and the precipitate was dried briefly in laminar air flow, and resuspended in 20µl sterile distilled water.

PCR amplification of the extracted DNA from the bacterial isolates using species-specific primers according to Riffon *et al.* (2001):

All reactions were carried out in a final volume of 50µl in micro amplification tubes (PCR tubes). The reaction mixture consisted of 1µl (200ng) of the extracted DNA template from the bacterial cultures 5µl 10X PCR buffer (75 mM Tris-HCl, pH 9.0, 2 mM $MgCl_2$, 50 mM KC1, 20 mM $(NH_4)_2SO_4$), 1µl dNTPs (40 uM), 1µl (1 U AmpliTaq DNA polymerase), 1µl (50 pmol) from the forward and reverse primers (Eco223-Eco455) primer pairs, each

primer pair used separately and the volume of the reaction mixture was completed to 50µl using DDW. 40µl paraffin oil was added and the thermal cycler was adjusted as follows:

Initial denaturation at 94°C for 4 min followed by 30 cycles of denaturation at 94°C for 45 seconds, annealing step at 65°C for 1 min and extension at72°C for 2 min. A final extension step was done at 72°C for 10 min. The PCR products were stored in the thermal cycler at 4°C until they were collected.

Multiplex PCR assay for the simultaneous detection of intimin gene (encoded by *eae*A) in the extracted DNA of *E.coli* according to Paton and Paton (1998):

The amplified reactions were performed in 50µl volumes in PCR tubes. The reaction mixture consisted of: 1µl (200µl) of the extracted DNA template from the *E.coli* isolates, 5µl 10X PCR buffer [75 mM Tris-HCl, pH 9.0, 2 mM $MgCl_2$, 50 mM KCl, 20 mM $(NH_4)_2SO_4$], 1µl dNTPs (40µM), 1µl (50 pmol) *eae*A primer, 1µl (50 pmol) *eae*A primer, 1µl (1.5 U) Ampli Taq DNA polymerase and 38µl double distilled water.

Then the reaction mixture was overlaid with 40µl paraffin oil and subjected to 35 PCR cycles, each consisting of 1 min. of denaturation at 95°C; 2 min of annealing at 65°C for the first 10 cycles, decrementing to 60°C by cycle 15; and 1.5 min. of elongation at 72°C, incrementing to 2 min from cycles 25 to 35. After the final cycle, the preparation was kept at 72°C for 10 min to complete the reaction. The PCR products were stored in the thermal cycler at 4°C until they were collected.

Agarose gel electrophoresis according to Sambrook *et al*. (1989):

Electrophoresis grade agarose was prepared of IX electrophoresis buffer (TAE) to reach the required concentration 2% and volume (to make 4 mm thick layer). The mixture was heated in a microwave with periodical agitation to check melting degree in between bursts. It was allowed to cool to 70°C, and then the ethidium bromide (0.5µg/ml) was added and mixed thoroughly.

The PCR product and the suitable molecular weight marker were mixed with the loading buffer (15µl PCR product + 3µl loading buffer) followed by loading of the samples into the gel, then the tank gel was closed and attached with the power supply. The running parameters were 1-5 Volts / cm of the tank length and for many gels, 5-20 Volts / cm. Bromophenol blue was allowed to run 2/3 of the gel length before termination of the run, and after the run was stopped, the gel was transferred to the trans-illuminator to observe the amplified DNA on the gel in comparison to the molecular weight marker.

Table (1): Oligonucleotide primers used for amplification of *eae*A gene from the DNA of *E.coli* isolates and primers for detection of mastitic *E.coli* isolates

Primers	Sequence(5 -3)	Specificity	Amplicon size	Annealing temp.
Eco 223-f	ATC AAC CGA GAT TCC CCC AGT	*E.coli*	231 bp	64°C
Eco 445-R	TCA CTA TCG GTC AGT CAG GAG			
***eae*A-F**	GAC CCG GCA CAA GCA TAA GC	Intimin gene (encoded by *eae*A)	384 bp	65°C
***eae*A-R**	CCA CCT GCA GCA ACA AGA GG			

Congo red (CR) binding activity (Berkhoff and Vinal, 1986):

E.coli strains were cultured onto Congo red medium. The reaction is best seen after 24 hr. of incubation at $36C^o$ and then left at room temperature for additional 3 days (not to exceed 4 days).

Adherence assays (Donnenberg and Nataro, 1995):

Ten ml of overnight bacterial cultures in peptone water (containing 1% D-mannose) was inoculated into cover slip – containing 24 – well plates which had been seeded with 5×10^5 HEp-2 cells 48h before. Cultures were incubated at 37°C for 3h. The cells were washed. Fresh RPMI 1640 was added and then the cells were incubated for other 3hours, the cells were fixed with 3% formalin, and cultures were stained with Giemsa solution. The adhesion was determined by light microscopy covering the whole slide. Bacteria were recorded as adhered if a cluster of at least 10 bacteria adhered per HEp-2 cell.

Invasion assays (Tang *et al.*,1993 and Janda and Abbott, 1998):

Ten ml of bacterial culture in peptone water was incubated with HEp-2 cells usually for 2-3h to allow attachment and penetration of the epithelial cell. Gentamicin, (which is unable to penetrate mammalian cells) was added to eliminate extracellular bacteria, and the tissues cell sheet was again incubated 24 hr. to allow multiplication of the bacteria that had invaded. The cells sheet was washed, fixed and Giemsa stained for visual examination of internalized bacteria. The bacteria can be seen in Giemsa– stained preparations as dark blue forms within, and usually filling the cytoplasm, or within cytoplasmic vacuoles.

Antibiogram assay for the local isolates recovered from the examined cows:

The test diffusion technique was applied according to Finegold and Martin (1982).

3. Results and Discussion:

From the results presented in Table (2) Out of 450 lactating cows examined according to clinical observation and California mastitis test, 181 were clinical mastitis with the percentages of 40.2%, these results nearly similar to that reported by Bartlett *et al*. (2001) with an incidence of 38.7%.

Out of 56 quarters affected, the prevalence was higher in hind quarter (63.97%) (116 of 181) than the fore quarters (36.02%) (65 of 181), this result nearly similar incidence was recorded by Wadhwa *et al*. (1996) who reported that out of 93 quarters affected by clinical mastitis, the prevalence was higher in hind quarters (57%) than the fore quarters (43%) and related these result to higher contamination of hind quarters with urine and feces. *E.coli* was the most common cause of clinical mastitis, accounting for 57 isolates in percentages 31.4% of all isolates. Fifty seven cases of clinical *E.coli* mastitis occurred in 56 quarters of 49 cows, Similar results were observed by Aziz (2002) who found that *E.coli* was predominant isolate (37.7%) and Bradley and Green (2001) who demonstrated that the most common cause of clinical mastitis was *E.coli* (34.7%).

Table (2): Incidence of clinical *E.coli* mastitis among lactating cows:

Farm Locality	No. of cows in farm	Clinical mastitis cases		Quarters affected				Clinical *E.coli* cases	
				Hind		Fore			
		No.	%	*No.*	%	*No.*	%	*No.*	%
1	115	56	48.60%	36	64.28%	20	35.71%	15	26.70%
2	80	32	40%	20	62.50%	12	37.50%	12	37.50%
3	135	50	37%	32	64%	18	36%	14	28%
4	120	43	35.80%	28	65.11%	15	34.88%	16	37.20%
Total	**450**	**181**	**40.20%**	**116**	**63.97%**	**65**	**36.02%**	**57**	**31.40%**

The results of the serogrouping of 57 *E.coli* isolates from clinical mastitis is indicated as shown in Table (3), in which distribution of *E.coli* serogroups according to somatic "O" antigen and capsular (K99) antigen. The most prevalent serogroups recovered from mastitic cases were O55 (19.5%), O111 (15.8%), O124 (12.3%), O119 (12.3%), O114 (10.5%), O26 (7%), O157 (7%) and O44 (3.5%).

While mean, 7 (12.2%) isolates were untypable with the available antisera, this observation is in agreement with Aziz (2002) who cited that *E.coli* recovered from mastitic cases belong to different serogroups and nearly in agreement with Correa and Marin (2002) who determined (12) O-serogroups, belong to the classical enteropathogenic serogroups (O26, O55, O11, O114, O119, O125, O126, O127, O128, O142, O158) represented 77.4% of the isolates. However, 12.5% of the obtained *E.coli* isolates were found to be untyapable. Similar observation was recovered by Lipman *et al*. (1996) who found that 7 *E.coli* isolates out of 30 (23.33%) were untypable. This could be attributed to the presence of other serogroups against with no diagnostic antisera were available.

Also it's clear from Table (3) *E.coli* strains O55, O111, O119 and O157 were given positive results with slide agglutination test for detection K99 pilus, while O124, O114, O26 and O44 give negative results,this result agree with Galone and Le-Roux (2001) which found the strains O111 and O119 isolated from mastitic cow contain K99 antigen.

Table (3): Serotypes of *E.coli* isolated from clinical mastitis:

Total *No.* of *clinical E.coli* isolates	**Serogroup**	***No.***	***%**	**K99**
57	O55	11	19.2%	+
	O111	9	15.8%	+
	O124	7	12.3%	_
	O119	7	12.3%	+
	O114	6	10.5%	_
	O26	4	7%	_
	O157	4	7%	+
	O44	2	3.5%	_
	Total	**50**	**87.6%**	
	Untyable	**7**	**12.3%**	

*The percentage was calculated according to the number of *E.coli* serogroups and total number of clinical *E.coli* isolates (57).

Recurrent *E.coli* mastitis results are outlined in Table (4). Five quarters of five cows experienced more than cases of clinical mastitis, recurrent *E.coli* mastitis occurred in 8.9% (5 of 56) of all affected quarters (56).One quarters experienced four cases of clinical *E.coli* mastitis of each one , two quarters experienced two cases for each one and one quarters experienced three cases for each one .The recurrence mastitis is high in hind quarter 80% (4 of 5) than forward one 20 (1 of 5).The most *E.coli* serogroups recovered from recurrent *E.coli* mastitis were O55, O119, O111, and O157 as shown in Table (4). The same serogroups of *E.coli* recovered from one quarter in more cases of episodes. Of all cases of clinical *E.coli* mastitis, 26.3% (15 of 57) occurred in quarters that experienced two or more cases of clinical *E.coli* mastitis in persistently infected quarters as measured by recurrence of clinical mastitis is much higher than those in previous reports (20.2%) (Bradley and Green, 2001). This apparent shift in behavior could be indicative of either a change in the susceptibility of the bovine population to persistent infection or a change in the behavior of *E.coli*.

Table (4): Incidence of recurrence *E.coli* mastitis

Quarters affected	No.of times affected	*No. of *E.coli* isolates from quarters	*E.coli*serogroup isolates
Left hind	4	4	O55
Right hind	3	3	O157
Left forward	2	2	O111
Left hind	2	2	O157
Left hind	4	4	O119
Total	**15**	**15**	

*No of *E.coli* isolates from quarter= (No. of quarters affected × No. of times affected inquarters).

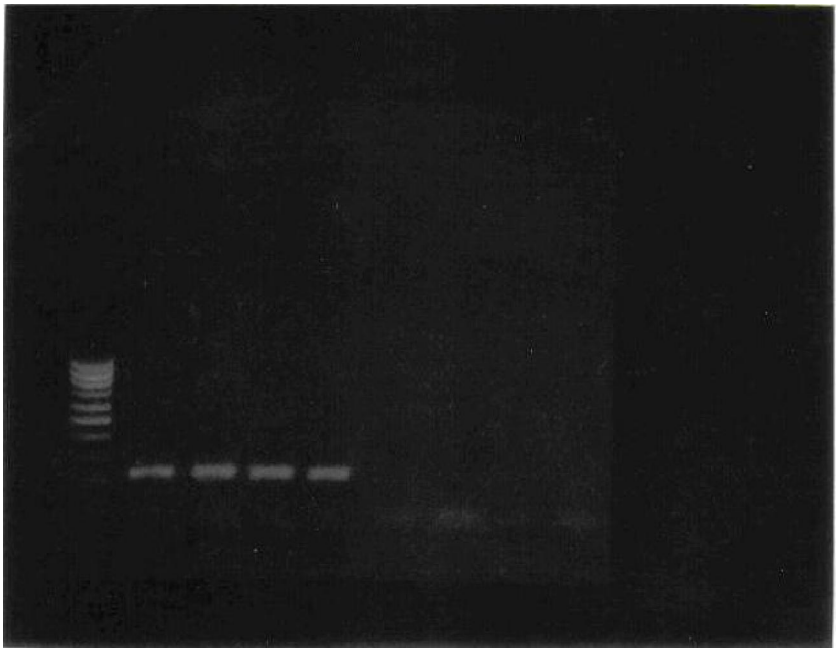

Photo (1): Agarose gel electrophorasis showing the specificity Eco 223 and Eco 455 primers. Amplification of 232 bp fragment was observed with extracted DNA of *E.coli* (Lanes 1, 2, 3, and 4), lane M showing marker.

The extracted DNA of 8 *E.coli* serogroups isolated from clinical *E.coli* mastitis were tested with multiplex PCR using primers for intimin gens (*eaeA*). Results observed in Table (5) and Photo (2) revealed positive amplification of 384 bp fragment of intimin gene from the extracted DNA of 4 *E.coli*serogroups isolates belong serogroups (O55, O119, O111 and O157) in lane (3,4,7 and 8 respectively). This observation is similar to those reported by Kobori *et al*., (2004) who study 31 *E.coli* strains isolated from mastitic cows. He found that *E.coli* serogroups (O111, O119, and O55) were positive *eae*A gene but serogroups (O125, O86 and O142) were negative *eae*A gene.

No amplification could be observed with extracted DNA of other serogroups (O124, O114, O26 and O44) in lane (1, 2, 5 and 6 respectively).

Table (5): Characterization of 8 *E. coli*serogroup isolates recovered from milk samples of mastitic cow by multiplex PCR assays for intimin (eaeA) gene:

***E.coli*serogroup**	**Multiplex PCR for *eae*A gene**
O55	+
O111	+
O124	–
O119	+
O114	–
O26	–
O157	+
O44	–

Table (6): Congo red binding activity of *E.coli*serogroups isolated from mastitic cow:

Mastitic*E.coli*serogroup	**CR binding activity**
O55	+
O111	+
O124	-
O119	+
O114	-
O26	-
O157	+
O44	-
Total positive %	**50%**

Photo (2): Ageros gel electrophoresis showing the amplification of 384 bp fragment of intimin (eaeA) gene from the extracted DNA of *E. coli*serogroup (O55, O119, O111 and O157) in lane (3,4,7 and 8 respectively) while other serogroup (O124, O114 and O44) give negative results with intimin (eaeA) gene in lan (1,2,5 and 6 respectively), lane M showing marker.

Table (6) demonstrated the Congo red activity of 8 *E.coli*serogroups isolates from mastitic cows.The (CR+) was observed in *E.coli*serogroups(O157,O111,O55 and O119) but other serogroups give (CR-).

As shown in Photos. (2,3) , out of 4 *E.coli*serogroups (O157,O111,O55 and O119) isolated from recurrent clinical *E.coli* mastitis which give positive results with (*eae*A) gene could adhere to Hep-2 cells..From this results it clear that *E.coli*serogroups which contain contains intmine (*eae*A) gene could be adhere to Hep-2 cells. All isolates gave 100%. These results were nearly similar to those reported by Wieler *et al.* (1998) who found that the isolates possessed adhesion at percentage 88.1%.

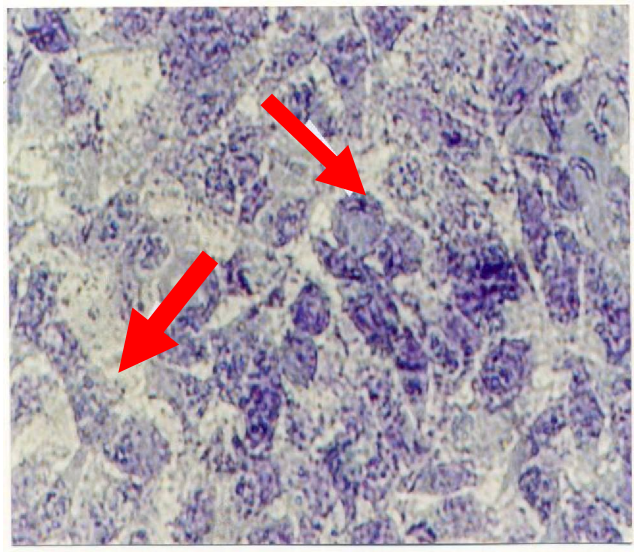

Photo (1): Normal rounded Hep2-cell complete sheet (control) (Geimsa – X:100)

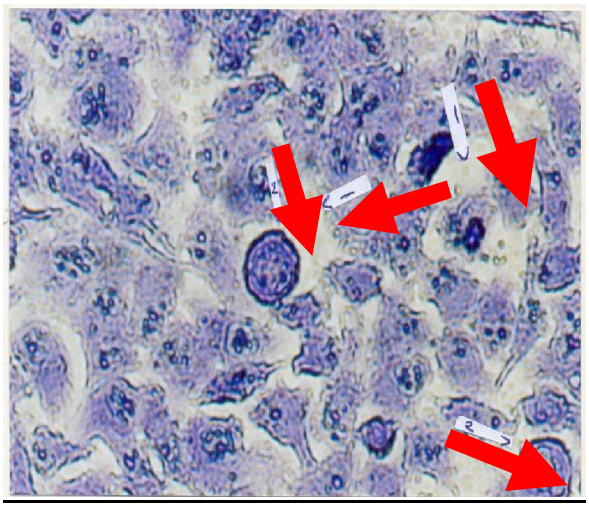

Photo (2): Vaculation of the Hep2- cell sheet & incomplete adhesion of *E.coli* cells after 3 hours (Geimsa– X: 100)

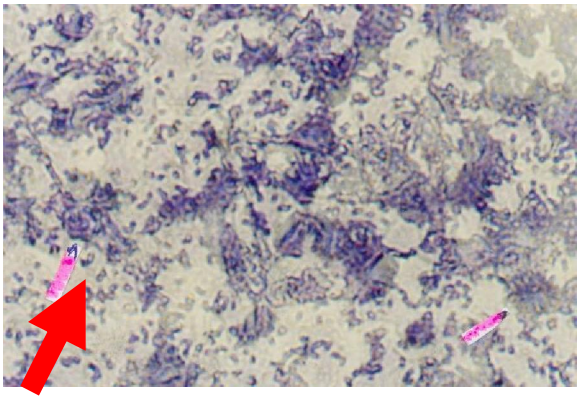

Photo (3): Adhesion of *E.coli*cells to Hep2- cells after 7 hours (Geimsa– X:100)

As shown in Photo (4&5) *E.coli* which isolated from recurrent clinical *E.coli* mastitis from cows

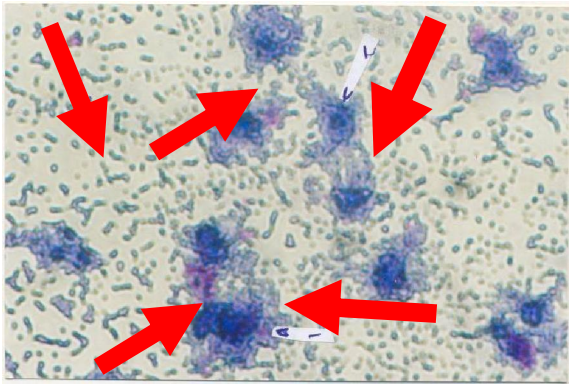

Photo (6): The separated cell sheet and high invasion of *E.coli* cells for Hep2- cells after 24 hours (Geimsa– X:100)

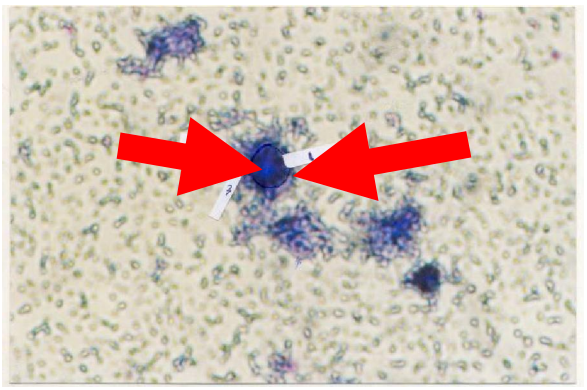

Photo (7): Shows complete invasion of *E.coli*to Hep2- cells &the changes of Hepto cell morphology. (Geimsa– X:100)

As showed in Table (7) . *E.coli* is highly sensitive to nalidixic acid, pefloxacin, gentamycin, sulbactam+ampicillin , cefoperazon flucloxacillin , ampicillin , amoxicillin + clavulonic acid andofloxacin, these results simulate what was reported by Jha *et al* (1994) who found that *E.coli* isolates recovered from clinical mastitis were highly sensitive to genyamycin, kanamycin and ampicillin.

Sympol	O26	O119	O124	O157	O55	O44	O114	O111
TOB	Int	S	R	S	R	S	Int.	Int.
AMC	R	R	SS	S	Int.	S	SS	R
CEP	R	R	Int.	R	R	R	SS	R
CAZ	R	Int.	S	R	R	R	R	R
NA	R	SS	SS	Int.	R	Int.	Int.	R
FL	S	S	S.	R	S	S	SS	S
PEF	Int.	SS	Int.	SS	R	R	SS	R
SAM	R	R	S	SS	R	Int.	S	R
OFX	R	R	Int.	R	Int.	Int.	Int.	R
AM	R	Int.	S	R	R	SS	SS	R
CN	R	SS	R	R	S	R	R	S

Corresponding author
Hakim .A. S
Microbiology and Immunology National Research Center, Dokki, Giza, Egypt
migris410@yahoo.com

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