Vet.Med.J.,Giza. Vol.54, No.2. (2006) :551-561.

MILK AS A VEHICLE OF MULTIDRUG RESISTANT ZOONOTIC BACTERIA TO HUMAN

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Received: 14.3.2006
Accepted: 21.3.2006

SUMMARY

This study was carried out on 72 zoonotic bacteria isolated from 115 milk samples collected from buffaloes with clinical mastitis (43 Staphylococcus aureus, 3 Streptococcus agalactiae, 14 *E. coli*, 6 *Klebsiella pneumoniae*, 4 *Salmonella typhimurium* and 2 *Serratia marescens*). Antibiotic sensitivity test was done for all the zoonotic bacterial isolates. Multiresistant isolates constituted 88.88%. Multiresistance was more among Gram -ve isolates 26/26 (100%) in comparison to Gram + ve isolates 38/46 (82.60%) ($P < 0.01$). Plasmids were extracted from 49 out of 64 multiresistant isolates (76.56%). The majority of the isolates contained more than one plasmid. Plasmids were isolated from all Gram –ve isolates (100%) and from 60.52% of Gram + ve isolates ($P < 0.01$). Twenty-one out of the 26 (80.70%) multiresistant Gram –ve isolates could transfer R-plasmids to sensitive *E. coli* K12 by conjugation. Controlled and proper use of veterinary antibiotics with continuous monitoring of milk for zoonotic multiresistant organisms are recommended to protect human from developing diseases that will not respond to treatment.

INTRODUCTION

Zoonoses and contamination of milk with bacteria or zoonotic agents leading to important and wide spread food borne diseases; are important aspect of public health. Milk borne infections are still a cause of morbidity and mortality in humans in developing countries (Wastling et al., 1999).

Raw milk is identified with increasing numbers of outbreaks of gastroenteritis and is an important vehicle for transmission of pathogenic organisms (Altekruse et al., 1998). Gillespie et al. (2003) reported 27 milk borne general outbreaks of infectious intestinal diseases in England and Wales. Unpasteurized milk (52%) was commonly report-

ed vehicle for infection.

The most frequent bacteria associated with milk contamination are Staphylococcus aureus, Streptococcus spp. (*S. agalactiae* and *S. dysgalactiae*), *E. coli*, *Salmonella*, *Klebsiella* and *Corynebacterium* (Randhawa et al., 1999; Paul et al., 2000 and Grewal et al., 2001). *Salmonella* (37%) and Verocytotoxin *E. coli* 0157 (33%) were the most commonly detected pathogens in 27 milk borne outbreaks of infectious intestinal disease (Gillespie et al., 2003).

Milk may contain antibiotic resistant bacteria posing a potential risk to consumers. Public health officials are increasingly concerned that human will be exposed to antimicrobial resistant organisms through the food they consume (Ombui et al., 2000).

Bacterial resistance to antibiotics is a long-established, widely-studied problem. Acquired resistance to antimicrobials may arise by cellular mutation or by the acquisition of genetic elements. Plasmid/transposons-mediated resistance (Russell, 1999). Plasmids are transmissible to other bacterial cells by conjugation, transformation and transduction. However, conjugative transfer of bacterial plasmid is the most efficient way for horizontal gene spread and it is considered as one of the main reasons for increase in the number of multiresistant bacteria (Grohmann et al., 2003 and Mukherjee et al., 2005).

The aim of the present study was to isolate antibiotic resistant zoonotic bacteria from buffalo's mastitic milk samples, then to investigate the origin of drug resistance by plasmid extraction and purification and transfer of the resistance plasmids to susceptible *E. coli* K12.

MATERIAL AND METHODS

For conducting this work, 115 milk samples were aseptically collected from buffaloes with clinical mastitis selected from villages at Monifia and Sharkia Governorates. The collected milk samples were examined as follows:

1. Isolation of antibiotic resistant bacteria:

Milk sediment of each milk sample (obtained by centrifugation of 10 ml of the sample for 20 min at 3000 r.p.m.) was seeded onto plates of nutrient agar, MacConkey agar and blood agar. Inoculated plates were incubated at 37°C for 48 hr. Suspected colonies appearing on the different media were examined microscopically and isolated in pure culture for further identification according to Holt et al. (1994). The recovered *Salmonella* isolates were identified serologically using the diagnostic polyvalent and monovalent antisera according to Kauffmann (1972). All zoonotic bacterial isolates (*Citrobacter* isolates were excluded) were tested for their antibiotic sensitivity by the routine disc diffusion method (National Committee for Clinical Laboratory Standards, 1997) using the following 12 commonly used antibiotics (Oxoid); ampicillin 10 µg, chloramphenicol 30 µg, amikacine 30µg, gentamicin 10 mg, cephalothin 30µg, ceftri-

axone 30μg, tetracycline 30μg, enrofloxacin 5 μg, Levofloxacin 5μg, vancomycin 30μg, sulphamethoxazole-trimethoprime 25μg and amoxyclavulanic acid 30μg. *E. coli* ATCC (25922) lactose fermenter and sensitive to all antibiotics was used as control to the antibiotic sensitivity test. Bacterial isolates found to be resistant to three or more antibiotics were considered multiresistant.

II) Plasmid extraction and detection :

A single colony from each multiresistant isolate was grown on 5 ml of LB (Luria – Bertani, Difco) broth containing ampicillin 100 μg/ml and chloramphenicol 170 μ g/ml to obtain the highest plasmid yields. Plasmids were extracted and purified according to QIA prep. Miniprep. Kit protocol (QIAGEN, 2005). Purified plasmid DNA was separated by horizontal electrophoresis on 0.7% agarose gel according to Sambrook et al. (1989). DNA Hind III Digested (7 fragments ranging in size from 23130-564 bp) served as marker in electrophoresis.

III) Conjugation experiments :

Conjugation experiments were done on 26 resistant Gram-ve isolates proved to contain plasmids according to Chowdhurg et al. (1994). Nalidixic acid-resistant, plasmid free recipient (*E. coli* K12 ATCC 12435) was used in all matings. Donor and recipient strains were grown separately in Muller-Hinton broth overnight at 37°C. The conjugation mixtures were prepared by adding 1 ml of the donor culture to 9 ml of the recipient *E. coli* K12 culture. The mixture was then vortexed and incubated at 37°C for 2 hr.

Resistance to ampicillin and chloramphenicol were used as selective markers to select transconjugates from recipients. 0.01 ml of the mixed culture was seeded onto MacConkey agar plates in duplicate. One plate was supplemented with ampicillin (50 μg/ml) and nalidixic acid (50 μg/ml) and the other plate was supplemented with chloramphenicol (25 μg/ml) and nalidixic acid (50 μg/ml). The plates were then incubated overnight at 37°C. Transfer of resistance plasmid was indicated by appearance of lactose fermenting *E.coli* K12 colonies onto the plates containing antibiotics. Rose pink colonies of resistant transconjugates were tested for sensitivity to other antimicrobials to determine pattern of resistant trait transferred.

VI) Statistical analysis :

Standard normal deviation (S.N.D.) test was used for analysis of the results.

RESULTS

Microbiological examination of the 115 collected buffalo's milk samples revealed that 74 milk samples (64.34%) were positive for bacterial isolation. The isolates were identified as 46 (62.16%) Gram + ve strains and 28 (37.84%) Gram –ve isolates (Table 1).

Table (1): Results of bacteriological examination of 115 buffalo's milk samples.

Bacterial isolates	No. of isolates	%
Gram + ve isolates:	46	62.16
S.aureus	43	58.11
Streptococcus agalactiae	3	4.05
Gram -ve isolates:	28	37.84
E.coli	14	18.92
Klebsiella pneumoniae	6	8.11
Salmonella typhimurium	4	5.41
Serratia marescens	2	2.70
Citrobacter	2	2.70
Total	74	100

Antimicrobial susceptibility testing revealed that 64 out of the 72 (88.88%) zoonotic bacterial isolates (as 2 *Citrobacter* isolates were excluded) were multiresistant. Multiresistance was detected in 100% and 82.60% of Gram –ve and Gram +ve zoonotic bacterial isolates, respectively ($P < 0.01$) (Table 2).

Table (2): Relation between the incidence of multiresistance to antibiotics and the type of isolates

Type of isolated strains	No.	Multiresistance to antibiotics		
		No.	%.	P.value.
Gram (-) ve strains	26	26	100.00	
Gram (+) ve strains	46	38	82.60	*P<0.01
Total No.	72	64	88.88	

* There is highly statistically significant difference.

Table (3) shows that the predominant resistance pattern was Amp.C.TE.KF.G.CRO.AMC.Lev.in Gram -ve isolates and Amp.C.TE. KF.AMC in Gram +ve strains.

Table (3): Patterns of antibiotic resistance in Gram –negative and Gram - positive bacteria isolated from milk

Bacterial isolates	No. of organism	Resistance Pattern
Gram -ve	14	Amp.C. TE. KF. CRO. AMC. Lev
	6	Amp. C. TE. KF. AK. CRO. AMC. SXT
	4	Amp. C. KF. G. CRO. AMC. SXT. EnR
	2	Amp. C. TE. KF. AK. CRO. AMC. SXT. Lev.
Gram +ve	15	Amp. C. TE. KF. AMC.
	13	Amp. TE. KF. CRO. AMC.
	8	Amp. C. KF. G. CRO.
	2	Amp. C. KF. CRO. SXT

Amp: Ampicillin, C: Chloramphenicol, TE: Tetracycline, KF: Cephalothin, CRO: Ceftriaxone, AMC: Amoxy-Clavulanic acid, G: Gentamicin, SXT: Sulphamethoxazole Trimethoprim, Lev: Levofloxacin, EnR: Enrofloxacin, AK: Amikacine.

Multiresistance plasmids were detected in 76.56% (49/64) of the resistant bacteria. Resistance plasmids were isolated from all multiresistant Gram -ve isolates (100%) and 60.52% of resistant Gram + ve bacteria, with a highly significant difference ($P < 0.01$) (Table 4).

Table (4): Relation between the incidence of plasmid detection and the type of the isolates

Type of isolates	No.	Isolates with plasmid detection		
		No.	%	Pvalue
Gram (-) ve strains	26	26	100	
Gram (+) ve strains	38	23	60.52	*P<0.01
Total No.	64	49	76.56	

* There is highly statistically significant difference.

Agarose gel electrophoresis of the plasmids extracted from the resistant isolates revealed the presence of more than one and up to six plasmids within each strain except *S.agalactiae* isolate (Fig. 1). Molecular sizes of the plasmids varied widely from 2.3 to 18 Kbp. The multiresistant Gram -ve strains contained large plasmid with molecular weight (mol. wt.) of 18 and 12 Kbp (large plasmid > 10 Kb; QIAGEN, 2005). Similar plasmid patterns are shown in lanes 2 & 3 (*E. coli*), 4 & 6 (*S. aureus*) and 7 & 8 (*Salmonella typhimurium*) (Fig. 1). Plasmids of similar molecular size were present in the different strains.

Conjugation experiments showed that among 26 multiresistant Gram -ve strains having plasmids, 21 (80.70%) strains could transfer resistance plasmids (R-plasmids) to the sensitive *E. coli* K12.

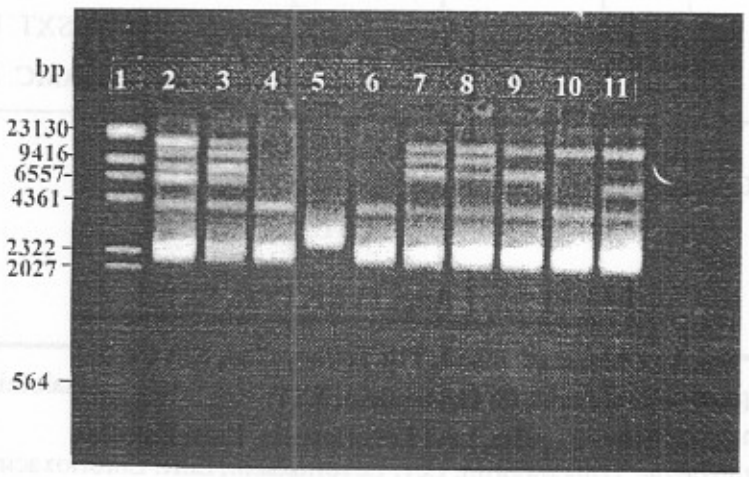

Fig. (1): Agarose gel electrophoresis of plasmids extracted from the multiresistant Gram -ve and Gram +ve strains

Lane 1: Molecular weight marker Lambda DNA Hind III Digested (23130-564 bp)
Lane 2: Plasmids extracted from *E. coli* (mol. wt. 18, 12, 8, 6.5, 4 & 2.3 Kbp)
Lane 3: Plasmids extracted from *E. coli* (mol. wt. 18, 12, 8, 6.5, 4 & 2.3 Kbp)
Lane 4: Plasmids extracted from *S. aureus* (mol. wt. 4 & 2.3 Kbp)
Lane 5: Plasmids extracted from *S. agalactiae* (mol. wt. 2.8 Kbp)
Lane 6: Plasmids extracted from *S. aureus* (mol. wt. 4 & 2.3 Kbp)
Lane 7: Plasmids extracted from *S. typhimurium* (mol. wt. 18, 12, 8, 4 & 2.3 Kbp)
Lane 8: Plasmids extracted from *S. typhimurium* (mol. wt. 18, 12, 8, 4 & 2.3 Kbp)
Lane 9: Plasmids extracted from *K. pneumoniae* (mol. wt. 18, 8, 4 & 2.3 kbp)
Lane 10: Plasmids extracted from *K. pneumoniae* (mol. wt. 18, 4 & 2.3 Kbp)
Lane 11: Plasmids extracted from *Serratia marescens* (mol. wt. 18, 6.5, 4 & 2.3 Kbp)

DISCUSSION

Transmission of milk borne pathogens often involve complex interactions among the pathogen, the environment, and one or multiple host species. Some bacteria are naturally resistant to many antibiotics and most can become multiresistant. Antibiotics may alter its environment, creating selective pressure and advantage for resistance organisms (Seppala et al., 1992). Resistance genes located on conjugative plasmids can be found in species living in habitats (e.g. human and animal intestines) regularly challenged with antibiotics (Teuber et al., 1999).

In the present study, high percentage (64.34) of the collected buffalo's milk samples was positive for bacterial isolation. Similar result was recorded by Nazem et al. (1998). However, Thirunavukkarasu and Prabahran (1999) and Paul et al. (2000) reported lower infection rate. All the isolated strains (except 2 *Citrobacter* isolates) were zoonotic including, *S.aureus* (58.11%), *E.coli* (18.92%), *K.pneumoniae* (8.11%), *S. typhimurium* (5.41%), *S. agalactiae* (4.05%) and *Serratia marescens* (2.70%). Mahmoud (1990) and Nazem et al. (1998) recorded higher isolation rate of *S.agalactiae* and lower isolation rate of *S.aureus* than those in the present study.

Antimicrobial susceptibility testing of the 72 zoonotic bacterial milk isolates showed high percentage (88.88) of multiresistant strains. Annibu et al. (1986) and Martel et al. (1995) stated that use of veterinary drugs in animal feed for purpose of growth promotion and animal husbandry has resulted in pollution of farms with resistant strains.

Both Gram -ve (100%) and Gram +ve (82.60%) isolates showed high percentage of multiresistance with highly significant difference ($P < 0.01$). In Gram -ve bacteria, the strong barrier to permeability, in addition to the greater diversity of plasmids and chromosomal enzymes, probably have greater potential for them to become multiresistant (Gould, 1994).

In the current study, the isolated multiresistant zoonotic bacteria showed resistance to 5-9 of the 12 tested antibiotics. Sherley et al. (2004) stated that plasmids allow the movement of genetic material, including antimicrobial resistance genes, between bacterial species and genera. They frequently mediate the resistance to multiple antimicrobials and can result in the acquisition by a pathogen of resistance to all or most clinically relevant antimicrobials.

Muliresistant bacterial isolates whether Gram -ve or Gram +ve exhibited high resistance to ampicillin, chloramphenicol, tetracycline, cephalothin, ceftriaxone and amoxy-clavulanic acid. Similar results were recorded by Senerwa et al. (1991), Thomson and Amyes (1992) and Wan et al. (2003) for E. coli, Vahabouglu et al. (1995) and

Baggesen et al. (2000) for *S.typhimurium*, Schmid and Kayser (1976) and Shaokat et al. (1985) for *K.pneumonia* and *Serratia marescens* and Nawal et al. (1999) for *S. aureus*.

Resistance plasmids were detected in 49/64 (76.56%) of multiresistant strains. Higher results were obtained by Malkawi and Youssef (1998), Ombui et al. (2000) and Wernicki et al. (2002). On the other hand, low incidence of resistance plasmid was reported by Murray et al. (1985) and Wan et al. (2003).

Plasmid profiles of the multiresistant Gram -ve strains showed large plasmids with molecular weight of 18 and 12 Kbp. Large plasmids are likely to contain virulence related sequences. These data are consistent with those cited by Singh et al. (1992).

Twenty-one out of 26 (80.70%) resistance plasmids were transferred to sensitive *E.coli* K12. Similar results were recorded by Malkawi and Youssef (1998) and Wan et al. (2003). While Oyofo et al. (1996) and Moustaoui et al. (2005) reported that all their multiresistant strains could transfer resistance plasmids to *E.coli* K12. This difference may be attributed to; *E.coli* K12 strains were incompatible with donor multiresistant isolates and or the resistance plasmids of some donor strain might have defective or non transfer factor gene.

We can conclude that the occurrence of multiresistant zoonotic bacteria in raw milk constitutes a reservoir of antibiotic resistance genes which may spread to other bacterial species in human intestinal tract through conjugative transfer. This can cause diseases in human difficult to treat. Therefore, pasteurization of the raw substrate milk, continuous monitoring of milk for zoonotic multiresistant bacteria together with controlled and proper use of veterinary antibiotics should be recommended.

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اللبن كحامل للبكتريا المشتركة المتعددة المقاومة للمضادات الحيوية للإنسان

نوال عبدالحفيظ حسنين

قسم الأمراض المشتركة، المركز القومى للبحوث ، الدقى ، الجيزة

أجريت هذه الدراسة على ٧٢ سلالة من البكتريا المشتركة التي تم عزلها من ١١٥ عينة لبن مجمعة من الجاموس المصاب بالتهاب الضرع ، وقد تم عزل وتصنيف ٤٦ سلالة من البكتريا المشتركة إيجابية الجرام وهى ٤٣بكاريا عنقودية ذهبية و٣ ستربتوكوكس أجالاكتى وكذلك ٢٦ سلالة من البكتريا المشتركة سالبة الجرام وهى ١٤ ايشيريشياكولاى ، ٦ كلبسيلا نيمونى، ٤ سالمونيلا تيفى ميوريم و٢ سيراتيا مارسينيس، ولقد تم عمل اختبار لحساسية هذه البكتريا للمضادات الحيوية فوجد أن هناك ٧٢/٦٤ بنسبة ٨٨.٨٨٪ من هذه السلالات متعددة المقاومة للمضادات الحيوية وأن نسبة هذه البكتريا أكثر فى السلالات سالبة الجرام ٢٦/٢٦ (١٠٠٪) بالمقارنة بالسلالات إيجابية الجرام ٤٦/٣٨ (٨٢.٦٪)، ولقد تم استخلاص البلازميد الحامل لعامل مقاومة المضادات الحيوية من ٦٤/٤٩ (٧٦.٥٦٪) من السلالات متعددة المقاومة، وقد وجد أن هناك أكثر من بلازميد فى معظم السلالات وأن جميع سلالات البكتريا المشتركة سالبة الجرام تحتوى على بلازميد (١٠٠٪) فى حين أن هناك فقط ٥٢ .٦٠٪ من سلالات البكتريا المشتركة إيجابية الجرام تحتوى على بلازميد. أما بالنسبة الى انتقال البلازما بين السلالات سالبة الجرام فقد وجد أن ٢٦/٢١ (٧٠ .٨٠٪) من البكتريا قد تم نقل البلازميد الحامل لعامل مقاومة المضادات الحيوية الذى بها الى عصيات القولون المرجعية ك١٢ بالتزواج وأ كل أنواع المقاومة التى كانت موجودة فى هذه السلالات قد نقلت الى عصيات القولون المرجعية ك١٢، ومن هذا البحث نوصى بأن الاستخدام الأمثل للمضادات الحيوية فى الحقل البيطرى والتحكم فى هذا الاستخدام مع المتابعة المستمرة لوجود الميكروبات المشتركة المتعددة المقاومة للمضادات الحيوية فى اللبن يحمى الانسان من الاصابة بالأمراض التى من الممكن أن لاتستجيب للعلاج.