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MULTIPLE ANTIBIOTIC RESISTANCE AMONG GRAM NEGATIVE BACTERIA ISOLATED FROM MILK IN KARACHI

AZEEM AHMED, NUSRAT JAMEEL, FASIHUDDIN AHMED ANSARI AND HAJRA KHATOON
Department of Microbiology, University of Karachi
Karachi-75270, Pakistan

ABSTRACT:
A total of 100 Gram negative bacteria isolated from milk in Karachi were screened for their resistance to eleven commonly used antibiotics. The concentration of antibiotics used was 100 μg/ml. About 46% were found to resist antibiotics at the concentration of 100 μg/ml giving different patterns. The resistant bacteria were tested for the presence of R plasmids, both transferable and non-transferable, by conjugation, spontaneous segregation and curing with acridine orange and ethidium bromide. Among the resistant bacteria tested, eight were found to carry non-transferable R plasmids.

INTRODUCTION

Milk is an excellent bacteriological medium for a large number of microorganisms. At the time it is drawn from the udder of a healthy animal, milk contains organisms that have entered the teat canal through the teat opening. They are mechanically flushed out during milking. The number present at the time of milking has been reported to range between several hundred and several thousand per milliliter. The source of contamination includes the air in the environment, the milking equipment, and the personnel. A variety of diseases are potentially transmissible through milk. The source of pathogenic agent occurring in milk may be either a cow, or a human, and it may be transmitted by both.

Antibiotics are used to treat diseases of cattle and as preservatives in milk as well. The indiscriminate use of antibiotics in milk has led to the development of multiple antibiotic resistant populations of bacteria thereby rendering antibiotic treatment ineffective (Johnston *et al.,* 1983). Multiple drug resistant bacteria have been reported from all over the world.

Multiple antibiotic resistance bacteria can transfer the resistances to a sensitive bacterium by conjugation. Such type of resistance is known as R plasmid mediated antibiotic resistance and is quite dangerous from view point of chemotherapy. R plasmids have also been detected in isolates from milk. Most studies have been made on R plasmids isolated from Gram negative bacteria. Some R plasmids are non-transferable and can be detected by performing curing or spontaneous segregation (Hirota 1960, Mitsuhashi *et al.,* 1961, Watanabe & Iyang, 1962). However the extent of spontaneous loss or segregation is a property of a particular plasmid. Susceptibility to curing agents also varies among plasmids. Some plasmids are not curable by acridine orange. Such R plasmids are quite dangerous from public health point of view (Amir Ali & Khatoon 1976; Khatoon & Jehan 1995 and Platt *et al.,* 1986).

The present work deals with the antibiotic resistance of Gram negative bacteria isolated from milk in Karachi. Multiple antibiotic resistance in some strains were associated with R plasmdis.

MATERIALS AND METHODS

Materials:

1) Bacterial Strains

The bacterial strains screened for their antibiotic resistance were obtained from raw milk samples collected from different localities of Karachi city, coming from two main sources (i) Cattle Colony, Landhi and (ii) Cattle Colony, Ghadab.

Genetically marked bacterial strain used in these studies was *E. coli* BU 40. It was obtained from A.I. Bukhari of USA.

2) Media

(a) Nutrient Agar (N.A.)
(b) Mac Conkey's (M.A.): It was from E. Merck, Germany.
(c) Nutrient Broth (N.B.): Composition similar to Nutrient Agar except that agar agar was not added.
(d) Tryptone agar: It had the similar composition as described by Khursheed and Khatoon (1984).
(e) TSI Medium: It was from Oxoide, U.K.
(f) Simmon citrate medium: It was from Difco, U.S.A.
(g) Urea broth: It was from Difco, U.S.A.
(h) Peptone water: It was from Oxoide, U.K.
(i) Sugars: Sugars used were lactose, sucrose, maltose and mannitol.
(j) Kovac's reagent.

3) Drugs/Antibiotics

Ampicillin trihydrate, tetracycline and oxytetracycline were from Opal Laboratories Limited, Karachi. Furazolidone was from Risma Laboratories, Karachi. Nalidixic acid was from Winthrop Sterling Products, Pakistan, Limited. Neomycin sulphate, Gentamicin sulphate, Chloramphenicol levo, Streptomycin sulphate were from Glaxo Laboratories, Karachi. Kanamycin sulphate was from Continental Pharma, Belgium. Erythromycin was obtained from Department of Microbiology, University of Karachi.

4) Chemicals

Acridine orange, Ethyl alcohol and NaCl were from E. Merck, Germany. Ethidium bromide and Crystal violet were from Department of Microbiology, University of Karachi.

Methods:

I) Cultivation of bacteria

Stock cultures as well as cultures used routinely were maintained in Tryptone agar stabs. The cultures were kept refrigerated at $4^{o}C$.

II) Screening for antibiotic resistance

Cultures were identified on the basis of colonial morphology, straining reactions, pigment production, IMViC tests, reaction on TSI, urease test, sugar formentation, motility test, oxidase and catalase tests.

Screening for antibiotic resistance was performed by replica plate technique. Approximately 20 individual colonies were picked onto a master plate (M.A.), grown overnight and replicated on

M.A. plates containing desired concentration of the test antibiotics. Sterile velveteen replica was used for replication. Growth of replicated colonies on M.A. plates containing test antibiotics was considered as resistance for that particular antibiotic.

III) Bacterial conjugation, Curing and Spontaneous segregation

Bacterial conjugation:

The cultures of donor and recipient were grown overnight in Nutrient broth. Next morning, the overnight culture was diluted 50-fold and grown with shaking at 37^{o}C for ½ hour or till slight turbidity was visible. Donor were then mixed with recipients in the proportion of 1:10 and incubated at 37^{o}C for two hours. Controls, which consisted of the donor or recipient cells, were treated similarly.

After proper incubation the conjugation mixture was plated on media containing appropriate antibiotic to contraselect recipient and in some cases, also donors. Control platings of unmated donor and recipient cells were always made. Transconjugants appeared after an incubation of 24 hours at 37^{o}C. These were purified twice on the selective medium used and replicated to check for the donated markers.

Curing and Spontaneous segregation:

Extrachromosomal elements, including R plasmids may be lost spontaneously from the host cell because of some errors in replication or segregation. These losses (elimination or curing), which may be partial or complete, can be increased by treating the host cells which certain chemical agents such as acridine orange or ethidium bromide (Ansari & Khatoon 2001, Jahan & Khatoon 1999, Khatoon & Jahan 1995).

The R plasmids were studied for segregation and curability in *E. coli* host. For spontaneous segregation, the bacterium bearing R plasmid was grown in antibiotic free nutrient broth, through 30-45 consecutive transfers. Each time the culture was grown for 24 hours and transferred to the fresh broth to give a 20 fold dilution. At the end of 30-45 transfer, the culture was diluted and plated on M.A., to obtain isolated colonies. Some 10-20 colonies were gridded on to master plates which were then replicated on the antibiotic containing M.A. plates to check for losses of resistance determinants.

For studying curing by ethidium bromide (EBr), nutrient broth (5 ml) tubes containing graded concentrations of EBr were incubated with log phase cultures of *E. coli* host bearing R plasmid to give a 20 fold dilution. A control tube lacking EBr was always included. All the tubes were incubated overnight at 37^{o}C. The contents of the control tube and of the EBr-containing tubes were plated on M.A. to obtain isolated colonies. Some 10-20 colonies from each plating were grided onto M.A. plates. After overnight incubation at 37^{o}C, these were replicated on antibiotic containing plates to check for the loss (or its absence) of antibiotic resistance determinants. Same procedure was adopted for studying curing by acridine orange.

RESULTS

1. Gram negative bacteria isolated from Milk:

These included Escherichia (52), Enterobacter (6), Klebsiella (27), Salmonmella (2), Pseudomonas (2), Providencia (2), Moraxella (7), Enterobacter (6) and Citrobacter (2).

2. Screening for antibiotic resistance:

Some 46 of the total 100 cultures screened were found to resist one or more drugs/antibiotics (Table 1). Different patterns of drugs/antibiotics resistance were observed which are given in Table 2.

3. Bacterial conjugations

Among the 46 antibiotic resistant bacteria tested none transferred the resistance by conjugation.

4. Curing with Acridine orange, Ethidium bromide and Spontaneous segregation

Out of 46 resistant strains, 15 carrying multiple antibiotic/drug resistance were treated with 100 μg/ml of acridine orange and ethidium bromide to check for the loss of antibiotic/drug resistance markers. Of the 15 multiple drug/antibiotic resistant strains tested, 8 were found to lose all or part of their antibiotic/drug resistance by treatment with acridine orange (Table 3) and only one, Enterobacter 6, lost the Sm resistance by treatment with ethidium bromide. Only two strains were found to lose resistance by spontaneous segregation (Table 4).

Table 1
Gram negative bacteria isolated from milk resistant
to different drugs/antibiotics

Drugs/Antibiotics	No. of resistant strains
Ampicillin	35
Chloramphenicol	-
Erythromycin	24
Furazolidone	-
Gentamycin	-
Kanamycin	-
Nalidixic acid	-
Neomycin	-
Oxytetracycline	21
Streptomycin	13
Tetracycline	3

Note:

a) All the drugs/antibiotics were tested at the level of 100 μg/ml.
b) Total No. of cultures tested were 98 out of 100 isolates. Two isolates died.

Table 2
Antibiotic resistance patterns of Gram negative bacteria isolated from milk samples

S. No.	Resistance patterns	No. of cultures with a pattern
1.	ApOtSmTc	3
2.	ApOtSm	5
3.	ApOt	2
4.	OtSm	5
5.	Ap	25
6.	Ot	6

Ap = Ampicillin, Ot = Oxytetracycline, Sm = Streptomycin, Tc = Tetracycline.

Table 3
Curing with acridine orange

Strain	Pattern	No. of colonies with lost marker	Marker(s) lost	% loss	R plasmid designation
Klebsiella 2	ApOtSmTc	10	Tc	100	pAK1
Escherichia 9	ApOt	-	-	-	
Escherichia 10	OtSm	1	Sm	10	pAK2
Escherichia 13	ApOtSm	-	-	-	
Escherichia 14	ApOt	-	-	-	
Escherichia 17	OtSm	-	-	-	
Escherichia 22	ApOtSmTc	10	Tc	100	pAK3
Klebsiella 22	ApOtSm		-	-	
Enterobacter 6	ApOtSm	10	OtSm	100	pAK4
Escherichia 23	ApOtSm	1	Ap	10	pAK5
Klebsiella 27	OtSm	-	-	-	
Escherichia 25	OtSm	1	Sm	10	pAK6
Escherichia 43	ApOtSmTc	10	Tc	100	pAK7
Escherichia 44	ApOtSm	-	-	-	
Escherichia 48	OtSm	1	Sm	10	pAK8

No. of colonies tested were 10. Concentration of acridine orange was 100 μg/ml.

Table 4
Spontaneous segregation of R plasmids

R plasmid	Strain	Pattern	No. of colonies with lost marker	Markers lost	% loss
pAK1	Klebsiella 2	ApOtSmTc	-	-	-
pAK2	E. coli 10	OtSm	-	-	-
pAK3	E. coli 22	ApOtSmTc	-	-	-
pAK4	Enterobacter 6	ApOtSm	20	OtSm	100
pAK5	E. coli 23	ApOtSm	-	-	-
pAK6	E. coli 25	OtSm	-	-	-
pAK7	E. coli 43	ApOtSmTc	20	Ap	100
pAK8	E. coli 48	OtSm	-	-	-

No. of colonies tested were 20.
Concentration of antibiotics tested was 100 μg/ml.

DISCUSSION

Out of 100 Gram negative bacteria screened, 46 were found to resist one or more antibiotics, which indicates a 46% incidence of antibiotic resistance and may be considered as quite high. The fact that 15 of the 46 resistant bacteria are multiply resistant also indicates that multiple antibiotic resistance among Gram negative bacteria isolated from raw milk may not be uncommon.

Most effective antibiotics to treat infections which spread through contaminated milk were chloramphenicol, gentamycin, furazolidone, kanamycin, neomycin and nalidixic acid, as all the strains tested were sensitive to these antibiotics at the level of 100 μg/ml (Table 1).

The resistance to ampicillin, tetracycline, oxytetracycline, erythromycin and streptomycin is very high, one of the reason is the frequent use of these antibiotics in treating the infections of cows, given to increase the yield of milk and to prevent contamination by adding them in bulk quantities of raw milk. The presence of R plasmids in Gram negative bacteria isolated from raw milk is significant from the view point of genetics as they can be used as vehicles in the process of "genetic engineering" (Ansari & Khatoon 2001 and Jehan & Khatoon 1999).

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