In Vitro Study of Mutagenicity and Genotoxicity of Some Bacterial Secretions associated with Bladder Tumors Tissues on Human Lymphocytes by Using Blood Tissue Culture Techniques

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Summary:

Background: Cytogenetic studies have been used to assess carcinogenic or mutagenic exposures and effects early in occupational, biological and environmental settings. Bacterial infection has not traditionally been considered asa major causes of cancer. Bacteria have been linked to cancer by two mechanisms: induction of chronic inflammation and production of carcinogenic bacterial metabolites.

Objective: Because of new concepts in relating infection with certain malignancies the present study is performed. Therefore this study was conducted to determine the effects of bacterial products associated with biopsies of bladder tumor on human lymphocytes tissue cultures in vitro. Moreover, if there is any relation between these products for induction chromosomal aberration (CA) and formations of micronucleus (MN).

Subjects and Methods: Chromosomal aberration and micronucleus assay were studied on six identified bacterial isolates of bladder tumor biopsies included 2 isolates of *Escherichia coli* , 2 isolates of *Pseudomonas aeruginosa* and 2 isolates of *Klebsiella pneumoniae*. The bacterial products activity for induction (CA) and (MN) were assayed according to the procedure mentioned in the text.

Results: The results showed that each bacterial species have significant effects for induction different types of structural chromosomal aberration (CA) and formation of (MN).

Conclusion: *K. pneumoniae* yielded high significant related predisposing factor in inducing different cytogenetic analysis followed by *P. aeruginosa* and *E. coli* .

Key words: Bladder Cancer, Bacterial Infection, Chromosomal aberration, Micronucleus.

Introduction:

Cytogenetic studies have been instrumental in the characterization of multiple cancers [1]. Blood disorders have been extensively studied and cytogenetic analysis are now considered as routine work [2]. However cytogenetic analysis have for over 30 years been used to assess carcinogenic or mutagenic exposures and effects early in occupational, biological and environmental settings. The most extensively employed method to assess the genetic effects has been the analysis of chromosomal alterations in stimulated peripheral blood lymphocytes of exposed persons [3]. Chromosomal abnormalities may be divided into two broad categories numerical and structural. Numerical abnormalities are simply abnormalities of chromosomal number. Structural abnormalities include chromosomal rearrangements, deletions and duplications.

The chromosomal aberration (CA) constitutes a significant portion of genetic damage produced by most mutagenic agents. The analysis of CA has been commonly used method of detection DNA damage in mammalian cells [4]. While micronucleus (MN) index is a test proposed for use in the mutagenicity testing and based upon the formation of MN due to chromosome structural changes or spindle dysfunction [5]. Bacterial infection has not traditionally been considered as a major cause of cancer [6]. It has long been believed that cancer arises from genetic changes and that the agents of these changes have roots in toxins or in the environment [7].

Recently, however, bacteria have been linked to cancer by two mechanisms: induction of chronic inflammation and production of carcinogenic bacterial metabolites. In general, bacteria are able to produce a wide range of carcinogens, mutagens or tumor promoters from a wide range of substances such as Tryptophan metabolites, volatile phenols, and N-nitrosamine compounds. Many bacteria present in the urine reduce diet-derived nitrate to nitrite, which under mildly acidic or neutral conditions becomes a potent nitrosating agent. The production of *N*-nitrosamines by the nitrosation of amine precursors was detected in the urine of bacterially infected rats [8]. Gram negative-bacteria were able to produce nitrosoamine compounds as a potent carcinogenic agent in the urine of patients with bladder cancer [9]. It has also been argued that bacterial infection could be linked to cancer because such infections are known to promote cell proliferation, produce toxins that directly modulate intracellular signaling pathways and even suppress

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apoptosis in host cells, many uropathogenic *Escherichia coli* species produce a toxic substance known as cytotoxic necrotizing factor, which has been shown to induce elevated expression levels of cyclooxygenase-2 in murine fibroblasts [10]. The cyclooxygenase-2 enzyme has gained recent interest because is overexpressed in many human cancers and has been linked to increase tumor invasiveness via overexpression of bladder cyclooxygenase-2 and suppression of apoptosis. Apoptotic cells have unique morphologic and biochemical characteristics that distinguish them from necrotic cells [11]. The main physiologic difference between apoptosis and necrosis is in how the cells affect the surrounding tissues: Cells dying by apoptosis are recognized and taken up by phagocytic cells before they have an opportunity to lyse and release their content into the tissue [12]. The phagocytes degrade the cells with minimal environmental disturbance and no induction of inflammation. In contrast, cell dying by necrosis lyse before being taken up by phagocytes, thereby causing an inflammatory response that can cause incidental damage to the surrounding tissue.

Materials and Methods:

Six identified bacterial isolates were used include (2 isolates of *E. coli*, 2 isolates from *Pseudomonas aeruginosa* and 2 isolates of *Klebsiella pneumoniae*) for testing the ability and activity to induce some cytogenetic analysis (CA and MN index) as a marker for genotoxicity and mutagenicity. These bacterial isolates were sub-cultured separately in 5ml of RPMI 1640 (Flow Laboratories, U.K.) tissue culture broth (without penicillin and streptomycin) were grown overnight at 37°C. Three tubes were used for each isolate. Each one of the tubes centrifuged for 10 minutes, 15 and 30 minutes at (4000 rpm/minute). The supernatant was used to study chromosomal and some cytogenetic analysis.

Cell culture technique and chromosomal preparations were carried out as follow:

1 1ml of fetal calf serum was added to each 5 ml of RPMI 1640 prepared as mentioned previously. 0.3 ml of lymphocyte-rich plasma was added to each culture tubes and 0.4 ml of phytohaemagglutinine was added then mixed well. The cultures were incubated for 24 hours at 37°C.

2 - 0.3 ml of external bacterial products was added to each culture tube and the contents were mixed gently by inverting them few times.

3 - Cultures were incubated for 2 days at 37°C in a slanting position (this position creates more surface area between the liquid and gaseous phase and allows the cells to settle over a large area of culture tubes, which provides optimal culture conditions for cell growth and proliferation).

4 - The cultures were harvested after 46-48 hours of incubation.

Harvesting and slide preparation**:**

1 - 0.1 ml of colcemid was added to each culture tube containing 5ml of medium (to arrest the cells in metaphase), then mixed by gently shaking the tube and incubate the culture for an additional 60 minutes at 37°C.

2 - After colcemid treatment, the culture tubes were centrifuged at 2000 rpm for 10 minutes. (If there is any tough cell aggregate that cannot be dissociated, they should be removed before centrifugation).

3 - The supernatant was discarded by pipetting off media, leaving small amount of medium as possible over the cell button.

4 - The pellet was shaked well by using vortex, then resuspend the cell button in 5-10 ml of hypotonic solution (0.075 M KCl) and incubate for 15 minutes in water bath at 37°C. (Hypotonic solution was prewarmed to 37°C before use).

5 - Centrifugation was done at 2000 rpm in cooling centrifuge for 10 minutes.

6 - The supernatant was discarded. The pellet was disturbed thoroughly by taping at the bottom of the tube. The pellet was suspended in 5 ml of fresh fixative (pipetting was avoided).

7 - Centrifugation of the tubes was done again, the supernatant was discarded and the cells were suspended in fresh fixative. This step was repeated for three times.

8 - The cells were suspended in a small volume of fixative (approximately 0.5 to 1 ml, depending on the size of cell button to give a slightly opaque suspension).

Slides preparation: Grease-free slides were used in this work and were dried on hot plate at 50°C for 30 minutes. The slides were kept in refrigerator to become cool. Cells suspension were dropped by using Pasteur pipette, on to chilled slides.

Staining: Giemsa stain added on slides for 2-2.5 minutes, and then the slides were washed by Sorenson's buffer (pH 6.8) and left to dry.

Screening:The slide is screened by light microscope (100X), using oil immersion, to account CA.

Chromosomal aberration (CA): It was counted by enumerating the structural chromosomal changes randomly in 50 cells throughout metaphase from cell cycle progression [13].

Preparation of Micronucleus:Following the same steps in harvesting of cells and slide preparation but with some alteration:

1 - The speed, which was used in centrifugation must be (1000-rpm for 10 minutes).

2 - Concentration of KCl used must be 0.1 M.

Preparation of slides: After cells suspended by using fixative solution KCl (0.1 M), the tubes were centrifuged at 1000 rpm for 5 minutes. The supernatants were discarded by Pasteur pipette. The pellet was shaked for mixing and suspend the cells with remaining fluid. A drop from prepared mixture was put onto labeled clean slides after mixing well, the drop pulled out (smeared) by using cover slide, which was put at 45 degree angle to be pulled at the

same angle, then the slides are dried and stained according to the same procedure mentioned above.

Screening:The slide is screened by light microscope (100X), using oil immersion. The MN was determined by counting the number of micronucleus in 1000 cells in metaphase and calculated according to the following equation [14]:

$$MN = \frac{\text{No. of MN in each cell}}{1000}$$

Statistical Analysis: Were employed or performed according to the ready statistical program statistical package for social sciences (SPSS) and then Duncan test were made to know the significant differences between the means.

Results:

Table (1) illustrates the bacterial products induced different types of structural CA (Fig. 1, 2,3) and number of abnormal metaphase and MN formation compared with control (Fig. 4,5). These products significantly increase CA and MN formation (P<0.01). Also it is clear that there is a correlation between the effects of bacterial products upon induction of CA and formation of MN which was also a positive proportional correlation.

Table (1): Effects of external products of different bacterial isolates for inductions of chromosomal aberration and abnormal metaphase on human lymphocytes *in vitro*.

Treatment	Total no. of examined metaphase	No. of abnormal metaphase	No. of different types of chromosomal aberration			
			Gap chromatid	Gap chromosome	Break chromatid	Break chromosome
Control	50	6 (a)	5	3	1	
E. coli	50	16 (b)	10	8	7	3
E. coli	50	13 (b)	8	6	6	4
P. aeruginosa	50	21 (c)	16	13	11	5
P. aeruginosa	50	24 (c)	18	13	10	7
K. pneumoniae	50	30 (d)	18	20	19	12
K. pneumoniae	50	30 (d)	18	18	21	15

Table (1) : Continue

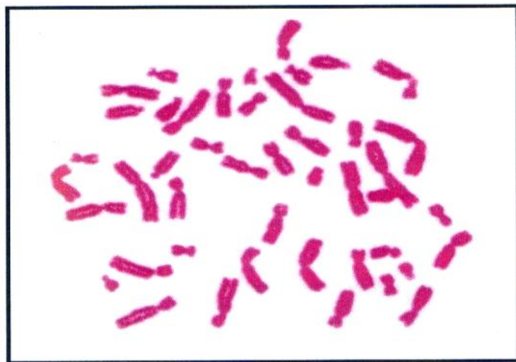
Treatment	No. of different types of chromosomal aberration			
	Deletion	Acentric chromosome	Dicentric chromosome	Total of chromosomal aberration
Control				9 (a)
E. coli				28 (b)
E. coli				24 (b)
P. aeruginosa				45 (c)
P. aeruginosa	2			50 (c)
K. pneumoniae	2			71 (d)
K. pneumoniae	2	1	1	76 (d)

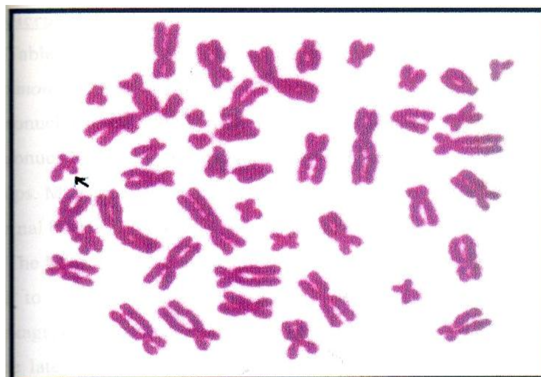
The mentioned letters (a,b,c,d) in the statistical field in above tables (1,2) confirm with this study are related to statistical analysis by (Duncan) test to indicate presence of significant differences or no significant differences between the mean values observed. Meanwhile, the presence of the same letters indicate that no significant differences are present between study group and control, while different letters indicate presence of significant differences between study groups.

The effects of *E. coli*, *P. aeruginosa* and *K. pneumoniae* products on human lymphocytes through application of MN test were shown in Table (2). The results showed that the number of MN in study groups are more than the number of MN in control groups. Micronucleus significantly induced when treated with different external bacterial products (P<0.01).

Table (2): Effect of external products of different bacterial isolates on formation and number of Micronucleus *in vitro* on human lymphocytes.

Treatment	Distribution of MN to each cell				No. of cell containing MN	Total no. of MN	No. of MN in 1000 cells	biostatistics
	0	1	2	3				
Control	996	4	0	0	4	4	0.004	a
E. coli	990	2	6	2	10	20	0.020	b
E. coli	989	2	5	4	11	24	0.024	b
P. aeruginosa	986	4	4	6	14	30	0.030	c
P. Aeruginosa	984	5	4	7	16	34	0.034	c
K. pneumoniae	982	4	6	8	18	40	0.040	d
K. pneumoniae	980	5	6	9	20	44	0.044	d





ure (2) illustrate structural chromosomal aberration from Deletion ,, observed in human lymphocytes exposed to *K. pneumoniae* external ducts (1000X). (Block stain method)

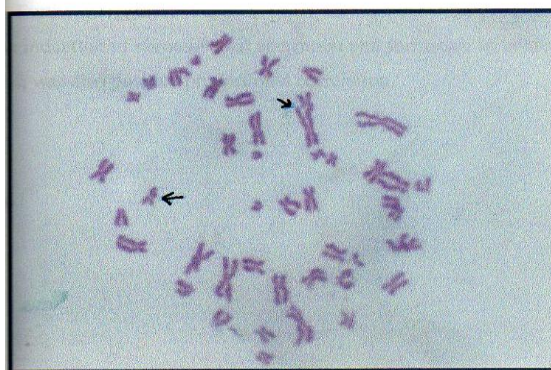


Figure (3): Illustrate structural chromosomal aberration from Deletion and Dicentric type, observed in human lymphocytes exposed to *K. pneumoniae* external products (1000X), (Block stain method).

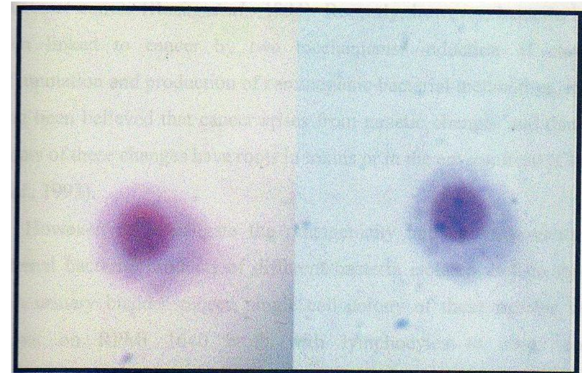


Figure (4): Show normal human lymphocytes (1000X) . (Control).

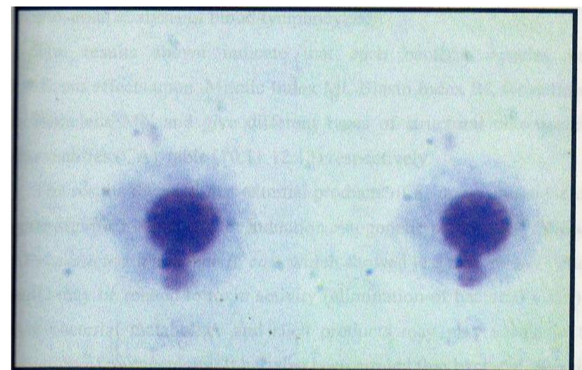


Figure (5): Micronucleus formation in human lymphocytes exposed to *K. pneumoniae* external products (1000 X), *in vitro*.

Discussion:

Urinary tract infection and its complication is a common health problem in patients attending hospitals in Baghdad. To explore the possibility that urinary tract infections enhance the urinary bladder cancer, this study was performed. Hence, the mutagenicity and carcinogenicity of such isolated organisms from biopsies of bladder cancer patients were examined *in vitro* by using cells that are actively dividing. Peripheral blood leukocytes, were used for routine chromosomal disorder, induction of cell division and formation of micronucleus. Association of few *in vivo* studies to test the possibility of bacterial infection as a cause of cancer have been reported [15,16]. *In vitro*, it has been shown that bacterial species within the colon can deconjugate the 7α - dehydroxylating bile acids

(deoxycholate and lithocholate). These compounds are reported to promote cell proliferation and growth of adenomas [17]. Bacteria are also thought to activate exogenous mutagen precursors. Examples observed *in vitro* and *in vivo* are hydrolysis of rutin to quercetin (amutagenic aromatic amine), hydrolysis of cycasin tomethylaz-oxymetnol, and hydrolysis of glucuronide-conjugated polycyclic hydrocarbons to their unconjugated mutagenic forms [18]. The present results showed that bacterial products induced different types of structural chromosomal abnormalities and number of abnormal metaphase compared with control. These products significantly increase chromosomal aberration (P<0.01). Effects include formation of different structural abnormalities. Break chromosome,Gap chromosome, Gap chromatid and Deletion. These results may be relating with bacterial products effects upon either protein nature present in chromosomes or DNA molecules [19]. The MN may be produced as an exposure to bacterial products, lead to form chromosome fragments as a result of chromosomes breakage, chromosomes deletion or loss of chromosomes centromers late in anaphase and telophase of cell division, therefore normal chromosomes forming normal nuclei while chromosomes fragmented convoluted around themselves then converted to micronuclei [5]. The results revealed that external products of *K. pneumoniae* yielded higher significant analysis for induction of cytogenetic parameters, followed by *P. aeruginosa* and then *E. coli*, which showed less than others did. These results may be related to toxin activity (elimination of bacterial toxin) or other bacterial metabolites and such products may play role in the induction of mutagenicity. Thomas *et al.* (2001) [10] reported that infection could be linked to cancer because such infections are known to promote cell proliferation, produce toxins that directly modulate intracellular signaling pathways and even suppress apoptosis in host cells.

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