ELSEVIER

Toxicology 177 (2002) 143–155

TOXICOLOGY

www.elsevier.com/locate/toxicol

Effect of the herbicide 2,4-dichlorophenoxyacetic acid on uropathogenic *Escherichia coli* virulence factors

Claudia E. Balagué [a], Clara Silva de Ruiz [b], Rosario Rey [b], Ana María Evangelista de Duffard [a,*], María Elena Nader-Macías [c]

[a] *Laboratorio de Toxicología Experimental, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, 2000 Rosario, Provinicia Santa Fe, Argentina*

[b] *Instituto de Microbiología, Universidad Nacional de Tucumán, Chacabuco 145, 4000 San Miguel de Tucumán, Argentina*

[c] *Centro de Referencia para Lactobacilos, Chacabuco 145, 4000 San Miguel de Tucumán, Argentina*

Received 21 December 2001; received in revised form 11 March 2002; accepted 23 March 2002

Abstract

The effects of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D)—widely used in the world and mainly excreted by the renal route in exposed humans—were studied on the virulence and surface characteristics of an uropathogenic *Escherichia coli* strain. When the urine was supplemented with 2,4-D in vitro, the compound significantly reduced the bacterial fimbriation assayed by hemagglutination and surface protein quantification. Protein values decreased from 0.24 mg/g dw to 0.05 or 0.12 mg/g dw by 1 or 0.1 mM 2,4-D treatment, respectively. The effects in vivo were studied in groups of mice challenged intra-urethra with *E. coli* and exposed by the oral route with three different 2,4-D doses (2.6, 25 or 70 mg/kg bw) during 22 days. Depending on the dose used, the herbicide significantly decreased or removed bacterial cells in mice bladder and kidneys; except in the group treated with the highest dose from the 9th day of treatment. The histological studies showed mononuclear cell infiltration at low doses, and toxic damage in the renal parenchyma at prolonged exposure with higher doses, up to tisular necrosis in the 70 mg/kg bw group after 9 days of treatment. Our investigations performed in an experimental model suggest that short time 2,4-D exposure at low doses could act in prevention of UTI stimulating leukocytic migration and decreasing bacterial fimbriation. On the contrary, high doses and long-term exposure enhanced renal damage resulting in infection recurrence.

Keywords: 2,4-Dichlorophenoxyacetic acid; Herbicide; Urinary tract infection; *Escherichia coli*; Virulence factors; Kidney toxicity

1. Introduction

The aromatic weak acid 2,4-dichlorophenoxyacetic (2,4-D) is a selective herbicide widely used to kill broad leaf plants. 2,4-D is used worldwide and human exposure to this chemical through agricultural or garden uses and food products was

* Corresponding author. Tel.: +54-341-480-4602; fax: +54-341-480-4598

E-mail address: aevangel@fbioyf.unr.edu.ar (A.M.E. De Duffard).

0300-483X/02/$ - see front matter
PII: S0300-483X(02)00161-0

demonstrated in several studies (Grover et al., 1986; Taskar et al., 1982). The main route of elimination is urine, so 2,4-D urine concentration is used for biological monitoring in exposed workers (Knopp and Glass, 1991). Besides, the kidney was shown as a target organ in a subchronic toxicological study of 2,4-D in rats (Gorzinski et al., 1987). Cytoplasmic alterations were present in the renal proximal tubules at dose levels from 15 to 150 mg/kg/day. Degenerative changes were also observed in the descending part of the proximal tubules. Moreover, it was shown that tubular cells express the receptor molecules for bacterial adhesins (Leffler and Svanborg Eden, 1980). One epidemiological survey entering rural citizens revealed that the frequency of infectious inflammatory lesions on the kidneys was higher in subjects exposed to pesticides (7.9–13.4%) against 1.5% in the controls (Allazov, 1994). Even though there is no evidence of increased incidence of urinary tract infections (UTIs) associated specifically with 2,4-D exposure, we do not discard an underestimated or ignored collateral effect of the herbicide.

Escherichia coli is the species responsible for most bacterial UTIs in humans (Svanborg-Eden, 1978). Although the commensal intestinal flora is the primary reservoir of infection, certain virulence factors were related to uropathogenic strains. Aerobactin and hemolysin release, as well as adhesins and fimbriae production, were clearly implicated in uropathogenic capacity (Johnson, 1991). Nonetheless, Gordon and Riley (1992) demonstrated that the ability to grow rapidly in urine may be as critical for virulence of uropathogens as adherence mediated by fimbriae.

A number of adhesins of *E. coli* uropathogenic strains were identified and studied. Type 1 fimbriae contribute primarily to bladder colonization in the mouse urinary tract (Hagberg et al., 1983); but the adhesin related to strains causing pyelonephritis, verified in both epidemiological and experimental studies, is P fimbriae (Johnson et al., 1987; O'Hanley et al., 1985; Pere et al., 1987; Svanborg-Eden et al., 1987). P fimbriae are differentiated from type 1 by their capability to agglutinate human erythrocytes in the presence of mannose (mannose-resistant). P fimbriae carry tip adhesin proteins capable of binding to the kidney and to erythrocytes globoseries glycosphingolipids receptors. The expression of these proteins changes in response to multiple environmental signals, such as temperature, media composition and salicylate (van der Woude et al., 1992; Kunin et al., 1994).

The preventive therapeutics in UTI include the use of antibiotic treatments or bacterial replacement by using lactobacilli. For the demonstration of the protective effect of probiotic micro-organisms, an experimental UTI model was set up, determining the effect produced by lactobacilli in mice and the *E. coli* T149 strain infective dose—as low as 10^3 colony forming units, CFU—(Silva de Ruiz et al., 1993; Nader-Macías et al., 1996).

Another possibility to prevent infections is to use compounds which interfere with bacteria-epithelial cell adhesion. Salicylate, an aromatic weak acid, reversibly repressed P fimbriation by pyelonephritogenic strains (Kunin et al., 1994). In addition, a group of compounds chemically related to nitrophenol were evaluated as inhibitors of the adherence of type 1 fimbriated *E. coli* to epithelial cells, but the potent inhibition observed was attributed to the interfering hydrophobic interactions between such compounds and cell surfaces (Falkowski et al., 1986). In a previous work, we described changes in fimbriation of uropathogenic *E. coli* strains after in vitro exposure to 2,4-D (Balagué et al., 2001). We observed a significant diminution of fimbriation in treated cells in fimbrial protein determinations and in electron microphotographs. These results suggested a reduced urovirulence potential, and were in contrast to the augmented frequency of inflammatory lesions observed in rural citizens.

The present paper aims to elucidate this apparent contradiction, determining the influence of 2,4-D on uropathogenic *E. coli* T149 virulence factors in vitro; and studying the effect of 2,4-D-administered by the oral route—on the UTI produced by T149 in mice as an experimental model.

2. Materials and methods

2.1. Chemicals

2,4-D and its sodium salt was purchased from Sigma (St. Louis, MO, USA).

2.2. Microorganisms

E. coli T149 strain was isolated from the infected urinary tract of an adult woman as described in a previous work (Silva de Ruiz et al., 1996) and identified by biochemical tests according to Orskov (1986). T149 possesses type 1 and P fimbriae, which were determined by mannose-sensitive hemagglutination of guinea pig erythrocytes and mannose-resistant hemagglutination of human type A erythrocytes, respectively. Characterization of P fimbriae was made with guinea pig red cells coated with specific globosides according to Leffler and Svanborg Eden, (1980). Additionally, T149 produced hemolysin and showed pyelonephritogenic effect in the mouse experimental model (Silva de Ruiz et al., 1996).

2.3. In vitro assays

All tests were performed in culture media (control) and in 2,4-D acid supplemented media, in concentrations from 0.1 to 10 mM. We used the acid form of the herbicide, as it is excreted in the mammalian urinary tract. In various experiments, we assayed the direct effect of the compound added to control medium after bacterial growth.

2.3.1. Growth-rate determinations

Precultures were grown statically for 8 h in Brain Heart Infusion (BHI, Britania Lab. S.A., Buenos Aires, Argentina) or in urine at 37 °C. Urine was supplied by healthy volunteers, from the first micturition of the day; it was collected under aseptic conditions and used within the hour. The absence of bacteria in urine samples was confirmed by plating 0.1 ml of the sample on a blood agar plate. In order to estimate growth, the optical density was measured every half hour at 540 nm. The following parameters were used to evaluate growth (Carlberg, 1986):

$$\text{Specific growth rate} = \ln(At_2/At_1)/T$$

$$\text{Doubling time} = \ln 2\ \text{Tm}/\ln(At_2/At_1)$$

where At_1, absorbance in time t_1, At_2, absorbance in the next time, T, time in hours and Tm = time in minutes.

Linearity at logarithmic phase was considered to estimate the maximal value.

2.3.2. Hemolytic activity determination

Alpha-hemolysin production was quantified according to the method of van den Bosch et al. (1980). Briefly, an overnight culture of bacteria grown in fresh alkaline meat extract broth containing 0.2% glucose, was diluted 1:10 in the same medium and incubated with agitation at 37 °C for 4 h. Supernatants were collected after centrifugation and adjusted to pH 7. Twofold dilutions (in 0.5 ml) of the culture supernatants in Tris–buffered saline were made and 0.5 ml of washed sheep erythrocytes (2% suspension) was added to each dilution. The mixtures were incubated at 37 °C for 2 h, the remaining erythrocytes were removed by centrifugation, and the hemoglobin release was measured at 540 nm. The values were normalized per unit of OD of bacterial growth in broth. Maximal hemoglobin release was determined. This was carried out by including in each assay a tube containing 0.5 ml of the erythrocyte suspension with 0.5 ml of distilled water, in order to adjust the erythrocyte suspensions in each independent replicate of the experiment.

2.3.3. Hemagglutination titrate

Bacteria were grown in Colonization Factor Antigen (CFA) agar (Evans et al., 1977), washed with phosphate-buffered saline (PBS) and adjusted photometrically to a concentration of 10^{10} bacteria per ml. Serial twofold dilutions were prepared in PBS using microliter plates, and an equal volume of 5% washed human type A erythrocyte suspension with 2% mannose were added to each well. Agglutinations were observed after 10 min of incubation at room temperature. The number of wells agglutinated by each culture was recorded and compared with the number of wells agglutinated by a control culture (Kunin et al., 1994).

2.3.4. Quantification of surface proteins

Bacteria were grown in CFA agar for 24 h at 37 °C and collected in 75 mM NaCl. Fimbriae were removed by the method described by Hoschützky et al. (1989). Briefly, the bacterial suspension was treated by agitation at 65 °C for 30 min. After being cooled to room temperature, the suspension was centrifuged ($15{,}000 \times g$ 30 min) to remove the defimbriated bacteria, and the supernatant was treated with EDTA 5 mM, glycine 20 mM and 10% ammonium sulfate. Lipids were dissolved in 50% ethanol and surface proteins were precipitated with 250 mM LiCl. Finally, the protein content was determined by the Bradford method (1976).

2.3.5. Determination of bacterial hydrophobicity

p-Xylene partition test was performed according to Chapman and Georgopapadakou, (1988). Cultures were incubated overnight at 37 °C in BHI broth. Samples from each culture were removed, washed in 50 mM sodium phosphate buffer (pH 7.4) and suspended to an optical density of 0.8 at 540 nm in the buffer. Then, *p*-xylene (1 ml) was added to a 2.5 ml sample, and the two phases were mixed (by vortexing, 2 min). The two phases separated, letting the solution stand at room temperature for 20 min. The lower aqueous phase was collected and the optical density was determined at 540 nm (Ax), using a sample without treatment for control (Ac). Based on these absorbance values, the partition index (PI) was calculated using the following formula:

$$PI = (Ac - Ax)/Ac$$

2.4. In vivo assays

The Ethical Committee for Animal Care of the Reference Center for Lactobacilli (CERELA-CONICET) approved the experimental protocol used. Two-month-old female BALB/c mice from the breeding colony of the Microbiology Institute of the National University of Tucumán were used throughout the investigation. The animals were housed in plastic cages, fed ad libitum keeping their environmental conditions constant. Each experiment was carried out with a group of 25–35 mice. Urine was cultured 24 h prior to challenge, and mice with bacteria present at $> 10^2$ CFU/ml were excluded. Uropathogenic *E. coli* strain was inoculated intra-urethrally in a 0.5% peptone water suspension (0.05 ml) at an infectious concentration (10^7–10^8 CFU/ml). A plastic polyethylene catheter (0.5 mm diameter) coupled to a syringe was used for this purpose.

2.4.1. Preventive assay

The 2,4-D sodium salt was administered orally in water solution (50 μl) every day, during 22 days. The suspension of *E. coli* was inoculated as a single dose 2 days after the first treatment with the herbicide. The studies were performed in the different groups of mice: (a) control mice challenged with *E.* coli; (b) reference mice treated with 2,4-D at 2.6 or 70 mg/kg bw; and (c) study group treated with three different doses of 2,4-D (2,6; 25 or 70 mg/kg bw) and challenged with *E. coli*.

2.4.2. Bacterial counts in tissue homogenate

The animals were killed by cervical dislocation. Prior to killing, urine was collected in a microtube by pressing the mouse bladder. The quantitative determination of micro-organisms was carried out by plating urine onto Cysteine-Lactose-Electrolite-Deficient agar (CLDE) following the standard technique of Barry et al. (1975). The kidneys and empty bladders were removed aseptically, placed in 0.5% peptone–water and homogenized with a Teflon pestle. Dilutions were made and the samples were plated onto MacConkey agar plates. The plates were incubated for 48 h and counts of viable cells were made for each organ. Results were shown as the mean value of the data obtained from four to six animals.

2.4.3. Bleeding procedure

Before killing the animals, they were bled at the retroorbital venous plexus. Serum was obtained to make the determination of antibody levels. The anti-*E. coli* T149 antibodies were quantitatively determined by an agglutination test using suspensions with 10^9 CFU/ml of dead micro-organisms. These suspensions and the serum dilutions were incubated for an hour at 37 °C and refrigerated overnight, before titer specification. The presence

of agglutinating antibodies in animals prior to infection was also examined (basal values).

2.5. Histological procedures

The organs of the urinary tract were aseptically removed. Kidneys and bladder from control, reference and treated mice were fixed in 10% formaldehyde for 24 h. The organs were processed and sectioned according to routine procedures. Paraffin sections (5 μm each) were stained with hematoxylin–eosin techniques. The microscopic observation was performed by using a light microscope. Pyelitis was defined as uroepithelial damage, infiltration of neutrophils into the epithelium and purulent exudade observed only in the pelvic cavity. Pyelonephritis was defined as tubular damage and infiltration of inflammatory cells into the medulla and cortex. The medullar and cortical lesions were classified as following:

- mild: leukocytic perivascular infiltration;
- moderate: predominant perivascular and glomerular infiltrate of mononuclear cells (MNs), tubular cellular desquamation;
- severe: in addition to moderate lesions, detection of tubular acidosis and necrosis. Tubular acidosis was related to colorless tubular cells without nucleus or with protruding nucleus.

2.6. Statistical analysis

All data was presented as mean ± SD, statistical differences were assessed by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparisons test. Levels of significance are shown in figures and tables, in all the cases, the differences were considered significant at $P < 0.05$.

3. Results

3.1. In vitro assays

3.1.1. Growth curves in different culture media

T149 *E. coli* strain with pyelonephritogenic effect was previously selected by the in vitro characteristics: α-hemolysin production and agglutinating pattern of different erythrocytes (guinea pig + + +, guinea pig plus mannose +; human + + +, human plus mannose + + +; guinea pig coated with globoside + +, guinea pig coated with globoside plus mannose + +) indicating type 1 and P fimbriae. Since it is now suggested that the growth rate in urine may influence the uropathogenic capability of the strains, as had been postulated by Gordon and Riley (1992), we considered analyzing this parameter. The strain was cultured in BHI and urine in order to evaluate its specific growth rate and doubling time. Significant differences between both media were observed. However, T149 could reach, in urine, 42% of the OD in stationary phase and 166% of the doubling time as compared with the values in BHI (Table 1). This data supported the contention of Gordon and Riley, demonstrating that the uropathogenic strain was capable of growing in urine while fecal strains tested did not (data not shown).

3.1.2. Effect of 2,4-D on strain T149 growing parameters

The effects of the herbicide 2,4-D acid on growth rate, doubling time, OD at stationary phase and duration of lag phase, were represented in Table 1. 2,4-D influenced the growth curves of the uropathogenic *E. coli* in urine, revealing a growth inhibition at 5 and 10 mM concentrations. However, when the strain was grown in BHI plus the herbicide, significant differences (in growth rate and doubling time) with control values were detected only at the highest concentration (10 mM).

3.2. Influence of 2,4-D on virulence factors

It is known that hydrophobicity was considered a substrate unspecific adherence factor, as opposed to hemagglutination which revealed specific adhesin–receptor interactions (Fletcher and Marshall, 1982). However, several studies demonstrated a relationship between both factors, as type 1 fimbriae conferred hydrophobicity to bacterial surfaces (Lachica, 1990).

Herbicide concentrations chosen for our studies (0.1 and 1 mM) did not significantly modify

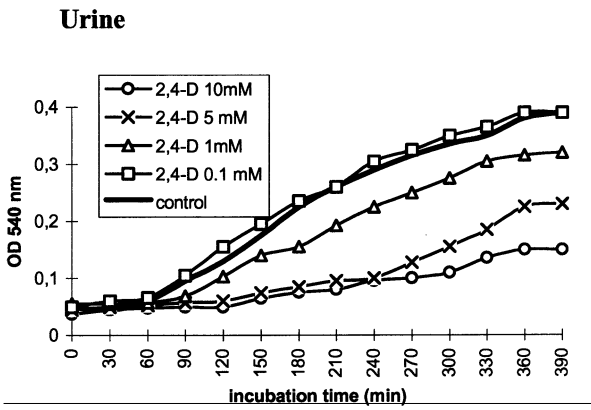
growth curves and represented, approximately, the range of values described to be excreted in urine or accumulated in the kidneys of the mice treated with 5 or 90 mg/kg body weight (Eiseman, 1984). In this condition, after having grown exposed to 2,4-D acid, the isolate T149 showed no statistically significant changes in hydrophobicity index values or hemolysin activity, but hemagglutination titer and surface protein content were further diminished (Table 2). We also observed that the inhibition of hemagglutination required growth of the bacteria in the presence of 2,4-D.

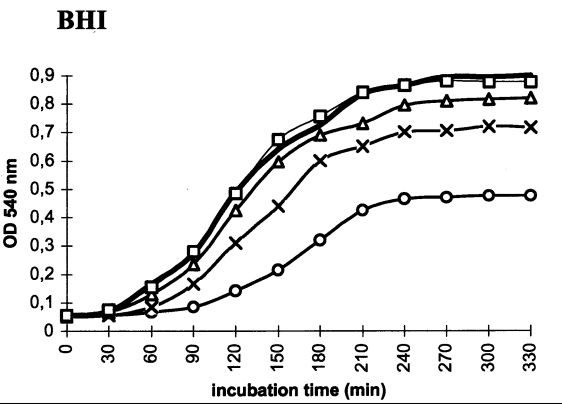
3.3. In vivo assay

3.3.1. Pyelonephritogenic capacity

To explore the possibility of a protective effect of the herbicide on UTI, we treated different groups of mice daily with 2,4-D sodium salt by the oral route (refer to Section 2). We challenged the animals intraurethra with the uropathogenic strain T149. Groups of animals were killed on day 2, 5, 9, 15 and 20 after infection. Urine, bladder and kidneys were homogenized and viable bacterial counts were performed. *E. coli* was recovered from the experimental infection in the

Table 1
Influence of 2,4-D on *E. coli* growth parameters





Medium supplement	Growth rate	Doubling time (min)	OD stationary phase	Lag phase (min)
Urine				
Control	0.69 ± 0.10	62.53 ± 6.32	0.38 ± 0.02	60
2,4-D 0.1 mM	0.70 ± 0.06	59.55 ± 5.16	0.39 ± 0.01	60
2,4-D 1 mM	0.58 ± 0.01	71.70 ± 1.27	0.32 ± 0.04	60
2,4-D 5 mM	0.38 ± 0.07***	110.15 ± 21.85*	0.23 ± 0.01***	120
2,4-D 10 mM	0.10 ± 0.02***	404.10 ± 89.37***	0.15 ± 0.01***	120
BHI				
Control	1.11 ± 0.07[a]	37.60 ± 2.4[b]	0.90 ± 0.01[b]	30
2,4-D 0.1 mM	1.06 ± 0.07	39.10 ± 0.28	0.89 ± 0.02	30
2,4-D 1 mM	1.12 ± 0.08	37.05 ± 2.61	0.85 ± 0.02	30
2,4-D 5 mM	1.09 ± 0.14	38.55 ± 5.16	0.73 ± 0.09**	30
2,4-D 10 mM	0.67 ± 0.08**	62.55 ± 7.28***	0.48 ± 0.03***	60

Data are means ± standard deviations of three independent assays.
[a] $P<0.01$.
[b] $P<0.001$ comparing each parameter in both control medium.
* $P<0.05$
** $P<0.01$.
*** $P<0.001$ comparing each parameter related to differences from the respective control medium.

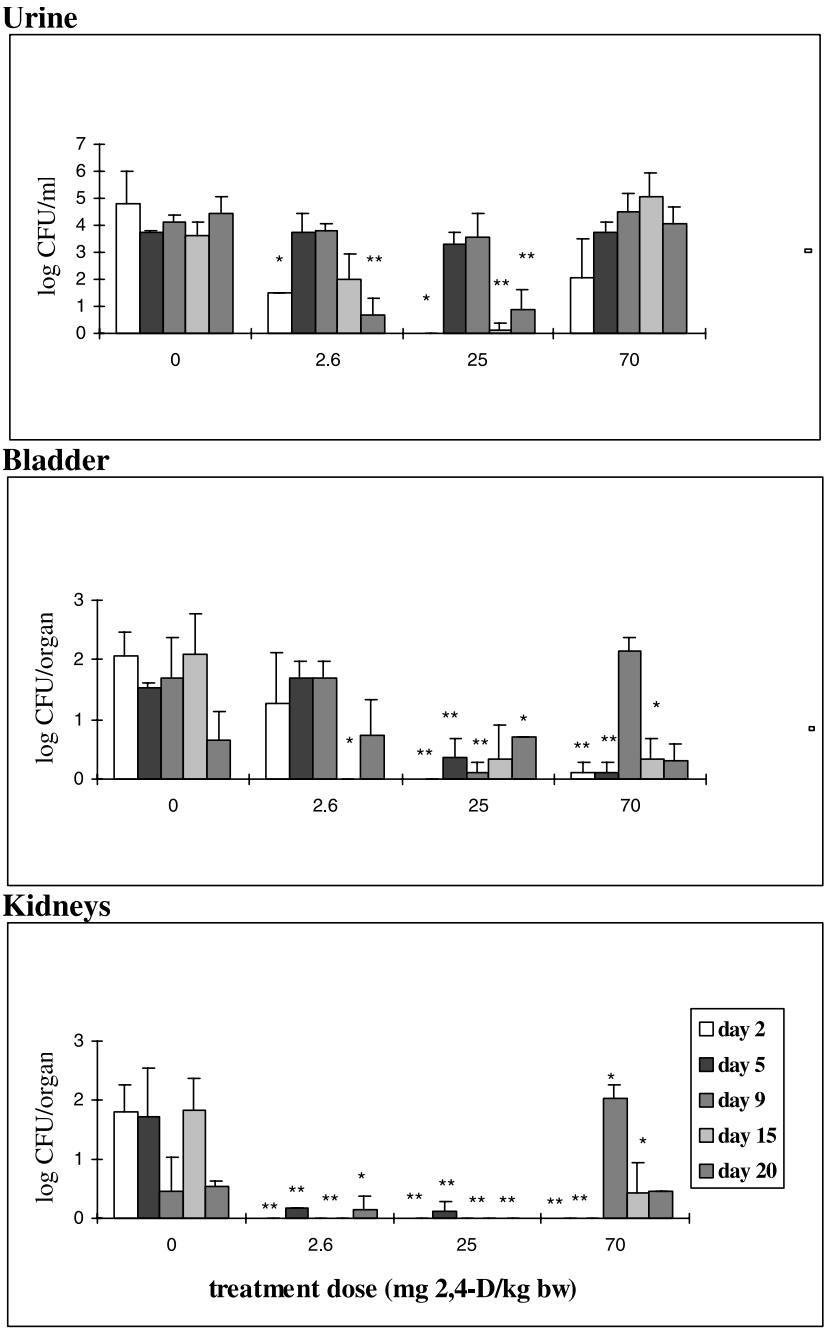


Fig. 1. *E. coli* CFU recovered from the urine, bladder and kidneys. The day numbers indicate time after bacterial challenge; daily oral treatments with the herbicide sodium salt (2.6, 25 or 70 mg 2,4-D/kg bw) during 22 days, start 2 days before intra-urethral bacterial inoculation. Each bar represents the mean + SD, $*P < 0.05$, $**P < 0.01$ with reference to control mice (only challenged with *E. coli*) values, $n = 4–6$.

Table 2
Effect of 2,4-D on *E. coli* hydrophobicity, adherence to erythrocytes and hemolysin activity

Medium supplement	Partition index (%) (xylene/water)	Surface protein content (mg/g dw)	Hemagglutination titer[a]	Hemoglobin release[b]
Control	15.03 ± 3.09	0.24 ± 0.06	1.81 ± 0.17	0.66 ± 0.06
2,4-D 1 mM[c]	12.55 ± 3.05	0.20 ± 0.04	1.81 ± 0.17	0.66 ± 0.10
2,4-D 0.1 mM	13.25 ± 2.20	0.12 ± 0.04*	1.20 ± 0.00*	0.68 ± 0.04
2,4-D 1 mM	11.43 ± 4.58	0.05 ± 0.01**	0.90 ± 0.17**	0.64 ± 0.02

dw, dry weight of the bacterial suspension. Data are means ± standard deviations of three independent assays.
[a] Log inverse of the maximal dilution with visible agglutinates.
[b] OD_{540} supernatants/OD_{540} bacterial growths in broth, as described in Section 2.
[c] The herbicide was added after bacterial growth in a control medium.
* $P<0.05$.
** $P<0.01$.

renal tissue of mice after 2 days of inoculation, demonstrating its rapid pyelonephritogenic capacity.

The values of colony forming units (CFU) recovered from urine, bladder and kidneys of control and 2,4-D exposed animals were summarized in Fig. 1. Although no difference was detected in urine CFU/ml at 70 mg/kg bw exposure, the effect of 2,4-D on recuperated CFU/organ was significant at all herbicide concentrations in mice bladders and kidneys. It is important to point out that even though the lowest dose (2.6 mg/kg bw) had little influence on bladders and urines, it had a significant effect on kidneys homogenates decreasing colonization (Fig. 1C). When 25 mg/kg bw doses were used, *E. coli* was isolated from urines and bladders to a lesser extent than in control mice, but no microorganisms were recovered in the kidneys by our assay after the 5th day. The dose of 70 mg/kg bw showed a particular effect. While a very small number or no microorganisms at all were recovered from bladders and kidneys on the 2nd and 5th days respectively, on the 9th day after the challenge the amount of *E. coli* increased. This result revealed an induction of the infection after the initial protection.

Fecal isolates tested were not infectious in the urinary tract of mice (data not shown).

3.4. Antibodies titrate

Little specific serum response occurred prior to 5 days after bacterial challenge. Antibody levels increased during the period comprised from 5th to 9th day in treated mice groups; but control group levels persisted up to the 20th day. The main decreases in antibody levels of treated groups, compared with control group, were detected on day 15 after *E. coli* inoculation. Interestingly, antibodies increased to the same level than control group on day 20 when the treatment dose was 70 mg/kg bw (Table 3), again suggesting a recurrence phenomenon.

3.5. Histological modifications

In light of the renal alterations by the herbicide described by other authors (Gorzinski et al., 1987), we decided to investigate tissue damage. The most severe histological modifications produced by 2,4-D in each group were summarized in Table 4. In the animals only treated with 2.6 mg 2,4-D/kg bw (reference group) and in those later challenged with *E. coli* (study group), the significant histological modifications related to control group were observed on the 20th day after bacterial inoculation (which means after 22 days of exposure to the toxic compound). There was MNs interstitial infiltration around the vessels and cellular desquamation into the tubules related to a moderate pyelonephritis in three out of five mice and four out of five mice, respectively. On the contrary, histological examination of the control group showed pyelitis (four out of four mice).

Kidneys were also modified with 25 mg 2,4-D/kg bw treatment, but to a greater extent than with

the lowest dose. Leukocytic infiltration and cellular desquamation into the tubules started on the 9th day and was enhanced by the 20th day when cortical and medullar structures were altered. The pyelonephritis was mild or moderate in most animals.

In the mice exposed to 70 mg 2,4-D/kg bw and challenged with *E. coli*, high MNs infiltration was observed from the 5th day, and tubular necrosis from the 15th day, when four out of six mice showed severe pyelonephritis. On the 20th day, tubular acidosis and necrosis in the proximal tubules were detected in all animals examined. The modifications produced by the administration of 70 mg 2,4-D/kg bw (reference animals) were as follows: from the 9th day (11th day of treatment) the compound produced small areas of necrosis in the cortex. These areas were formed by cellular debris and increased cytoplasmic and nuclear alterations in tubules (Fig. 2A). In addition, on the 15th day a perivascular infiltrate of leukocytes was observed (Fig. 2B).

Control mice showed pyelitis and a substantial PMNs intraepithelial and subepithelial infiltrate.

Interestingly, it was observed that all animal groups did not show histological modifications in the bladder (in comparison with control mice) according to the methodology applied to perform the studies.

4. Discussion

One of the purposes of our investigation was to determine if virulence factors detected in vitro in an *E. coli* strain isolated from a human UTI, could be reflected in a mouse ascending infection model. Our results showed that the studied strain had a pyelonephritogenic capability since it was able to produce a kidney infection when inoculated intra-urethrally to mice. Besides, it persisted in the bladder, kidney and urine up to the 20th day, according to Johnson et al. (1998), who described that persistence over the 7th day after challenge could distinguish pyelonephritogenic from cystitogenic strains.

In this work, we also analyzed the effect of the aromatic weak acid 2,4-D on the mouse UTI. There were reports describing the influence of several aromatic compounds—not structurally related to each other—on the adherence inhibition of uropathogenic strains, all of them assayed exclusively in vitro (Vosbeck et al., 1982; Falkowski et al., 1986; Kunin et al., 1994). We performed comparisons of the in vitro and in vivo effects of the herbicide.

Knopp and Schiller (1992) determined that a treatment of 2.6 mg/kg bw 2,4-D applied to rats was representative of occupational exposure. This compound, essentially unaltered, is mainly ex-

Table 3
Antibody anti-*E. coli* levels in mice serum

Days [a]	Antibody level (log inv. dil.)[b]				
	Basal	Control	2,4-D 2.6 mg[c]	2,4-D 25 mg	2,4-D 70 mg
2	0.70 ± 0.17	0.90 ± 0.30	0.70 ± 0.17	0.60 ± 0.30	1.10 ± 0.17
5	0.50 ± 0.30	1.91 ± 0.17***	1.81 ± 0.17	1.71 ± 0.17	1.50 ± 0.00
9	nd	2.41 ± 0.30	1.81 ± 0.30	1.81 ± 0.17	1.71 ± 0.17*
15	nd	2.61 ± 0.17	1.61 ± 0.17**	1.81 ± 0.30*	1.71 ± 0.17**
20	0.50 ± 0.17	2.11 ± 0.17***	1.61 ± 0.17	1.41 ± 0.17*	2.41 ± 0.17

Data are means $\pm$ standard deviations of values from four or six mice in each group. nd refers to not determined.

[a] Days after *E. coli* intra-urethral inoculation.

[b] Log inverse dilution of the last tube with visible agglutinates.

[c] Milligram per kg body weight daily supplied by oral administration.

* $P < 0.05$.

** $P < 0.01$ related to differences from the controls.

*** $P < 0.05$ related to differences between control and basal values.

Table 4
Histological modifications in kidneys of 2,4-D treated mice related to differences from the mice control group only inoculated with *E. coli*

2,4-D doses[a] (mg/kg bw)[b]	*E. coli* inoculation (CFU/ml)	Kidney histological modifications at the following time			
		5th day	9th day	15th day	20th day
0	1×10^8	Pyelitis (3/5)[c]	Pyelitis (3/5)	Pyelitis (4/5)	Pyelitis (4/4)
2.6[d]	0	Normal	Pyelitis (1/5)	Pyelitis (4/5)	Moderate pyelonephritis (3/5)
2.6[e]	1×10^8	Pyelitis (3/5)	Pyelitis (3/6)	Pyelitis (5/6)	Moderate pyelonephritis (4/5)
25[e]	1×10^8	Pyelitis (3/5)	Moderate pyelonephritis (4/5)	Moderate pyelonephritis (2/4)	Moderate pyelonephritis (5/5)
70[d]	0	Pyelitis (3/6)	Severe pyelonephritis (2/6)	Severe pyelonephritis (4/6)	Severe pyelonephritis (6/6)
70[e]	1×10^8	Moderate pyelonephritis (3/6)	Moderate pyelonephritis (3/5)	Severe pyelonephritis (4/6)	Severe pyelonephritis (6/6)

The 2,4-D sodium salt was administered orally every day. The bacterial suspension was inoculated as a single dose.
[a] 2,4-D oral administration was started 2 days before *E. coli* intra-urethral inoculation.
[b] Milligram of 2,4-D daily supplied per kg body weight.
[c] Number of mice with renal pathology/number of animals examined.
[d] Groups correspond to reference mice 2,4-D treated and non-inoculated with *E. coli*.
[e] Study groups.

creted by the renal route in treated mammalian species (Sauerhoff et al., 1977; Erne, 1966). Besides, in a toxicokinetic study performed in mice dosed with single 2,4-D oral dosage, the urinary clearence appeared to be a saturable process at doses over 45 mg/kg bw (Eiseman, 1984). Subchronic dietary studies in rats, given 60 mg/kg bw/day and higher of 2,4-D, revealed treatment-related cytoplasmic alterations in the renal proximal tubules (Gorzinski et al., 1987). Considering these references, the doses 2.6, 25 and 70 mg/kg/day were chosen as a wide range of exposure.

Interestingly, the largely different pattern of *E. coli* CFU recovered in the organs of exposed mice demonstrated the effectiveness of the aromatic weak acid 2,4-D in the prevention of pyelonephritis in vivo, except with 70 mg/kg bw, when the renal tissue injury reached necrosis and a reinfection was observed (Fig. 1). Different results were obtained with the lower doses, although urine samples showed pathogens during the whole exposure, 25 mg/kg bw 2,4-D was enough to eliminate and significantly decrease *E. coli* from the kidney and bladder, respectively, provoking minor tisular damage. Besides, 2.6 mg/kg bw largely reduced renal tissue colonization. We suggest that the increased CFU number detected in kidney after the first 5 days of 70 mg/kg bw exposure could be related to 2,4-D saturated renal clearance and tissue damage corresponding to severe pyelonephritis. Accordingly, the levels of anti-*E. coli* antibodies obtained from the different groups of animals were lower on the 15th day of treatment, but increased to the same level than control group on the 20th day, when the exposure was 70 mg/kg bw. Antibody titers were considered significant over 1/250. These values were only detected in the group control of infection and in the last days of treatment with the highest dose.

The mechanism involved in the reduction of the CFU number recovered from the kidneys of mice treated with 2,4-D, could be a difficulty for the microorganisms to adhere to the kidney, because studies in vitro showed a decreased fimbriation in uropathogenic strains (Balagué et al., 2001). On the other side, the comparison of the results obtained in vitro in our work revealed an important decrease in the adherence to erythrocytes and in

the surface proteins, while direct hemagglutination inhibition when 2,4-D was added to the assay was not observed, suggesting any immediate interference with hydrophobic or specific lectin-like interactions. These results agree with our previous experiments and Kunin et al. (1994) studies with salicylate, referred to P-fimbriation inhibition mediated by these compounds. We suggest that the diminished bacterial ability to adhere to the erythrocyte specific receptor, also produced a significant decrease on renal tissue colonization, mediated by adherence to the same receptor (Gal 1–4 Gal) in the globoseries glycosphingolipids.

The herbicide concentrations assayed in vitro represent an expected available range in the urinary tract of exposed mice (Eiseman, 1984).

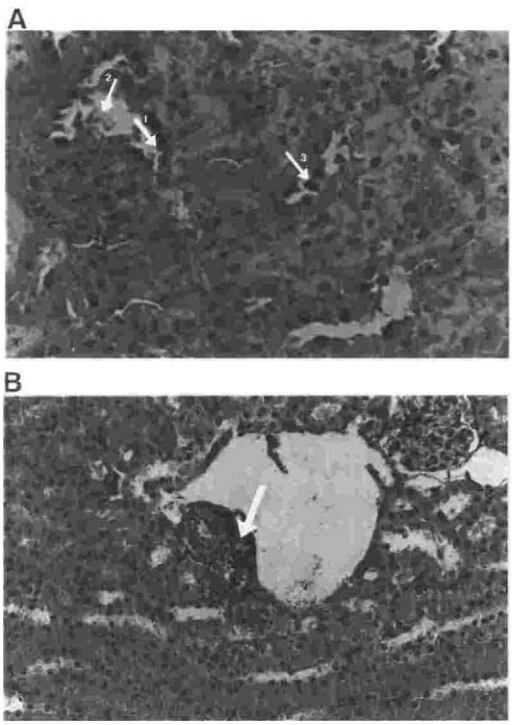


Fig. 2. Paraffin-embedded sections stained with hematoxylin and eosin demonstrate histological modifications observed in kidneys of the mice treated with 70 mg 2,4-D/kg bw after 11 (A) and 17 (B) days of treatment. A-1, 2 show cytoplasmic alterations and cellular detritus in tubules; A-3 shows cellular desquamation and tubular cells with protruding nucleus or without them; and in B perivascular infiltration of leukocytes is observed (A; magnification, $\times 40$ and B; magnification, $\times 20$).

Growth rate, doubling time, OD in the stationary phase, as well as hemolysin and hydrophobicity were unaltered, even at the highest concentration expected in the kidney (1 mM). On the contrary, 0.1 and 1 mM 2,4-D drastically reduced mannose-resistant hemagglutination capacity and surface protein content. From the results previously described, it can be concluded that the reduced fimbriation is directly responsible for the decreased pyelonephritogenic capability of *E. coli*, at least until the 15th day after challenge, which is the 17th day of 2.6 mg/kg bw 2,4-D exposure. Later, the observed cortical mononuclear cell (MN) infiltration might contribute to eliminate the pathogen from the renal tissue. As this infiltrate had a larger extent in high dose-treated and infected animals than in infected animals, and as the 2,4-D treatment alone induced MNs migration, these results suggest an additional induced defense to the pathogen. While cellular inflammatory response was enhanced by the herbicide, serum antibody levels were correlated with bacterial renal colonization, resulting in a diminished serum immune response.

The phenomenon of uroepithelial shedding was originally described in the bladder by Orikasa and Hinman (1977), as involving dying and dead cells. These authors suggested that epithelial desquamation serves as a defense mechanism, since the adhering bacteria are washed out together with the cells. Later, other authors (Dalal et al., 1994) observed epithelial shedding and rapid mobilization of PMN cells into the bladder in mice treated with hydrocortisone. This compound and moderate stress protect female animals against *E. coli* infection. Accordingly, renal tubular cellular desquamation might also contribute to avoid the kidney colonization, by decreasing the density of fimbrial receptors in renal tissue. The histological alterations observed were rather different from those detected previously in Fischer rats by Gorzinski et al. (1987). We detected similar epithelial cell degeneration of the renal proximal tubules, but we also detected tubular necrosis and acidosis instead of interstitial fibrosis. Tubular injury was expected as the result of selective 2,4-D accumulation in this segment due to its transport by the organic anionic carriers (Orberg, 1980).

Several studies have shown that kidney cells can produce local inflammatory mediators in response to invading pathogens (Rugo et al., 1992; Wada et al., 1994); our results showed that herbicide treatment increased the inflammatory response, probably augmenting mediators release by tubular cellular injury. Renal microscopic modifications were enhanced with the highest 2,4-D dose used and the treatment time, suggesting a dose–response close relation.

The use of aromatic weak acids as therapeutic agents to prevent infections of the urinary tract must be obviously related to the balance between benefits and adverse toxic effects. One important conclusion of the present study is that accidental exposure to 2,4-D in rural workers may have a protective effect when they are exposed to very low levels. These levels are highly-correlated with i.e. the amount sprayed, the duration of the spray operation, the hands washing after spraying, the use of protective clothes and the days of exposure. In addition, it must be considered that high doses and several days of exposure had adverse effects in renal tissue as severe as necrosis in this mouse model. Probably the epidemiological studies that associate herbicides exposure with renal infectious inflammatory damage were due to the last kind of exposure described.

Acknowledgements

This work was supported by CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas de Argentina) PID 0385(1998/2000) and CIUNT (Consejo de Investigaciones de la Universidad Nacional de Tucumán) Project D128 grants. FOMEC SPU for Biochemistry and Pharmaceutical Faculties, law 24938, art. 21 incise B.

References

Allazov, S., 1994. The characteristics of acute infectious-inflammatory kidney diseases in pesticide exposure. Urol. Nefrol. 2, 14–16.

Balagué, C., Sturtz, N., Duffard, R., Evangelista de Duffard, A.M., 2001. Effect of 2,4-dichlorophenoxyacetic acid herbicide on *Escherichia coli* growth, chemical composition and cellular envelope. Environ. Toxicol. 16, 43–53.

Barry, A.L., Smith, P.B., Turk, M., 1975. Laboratory diagnosis of urinary tract infections. Cumitech 2. American Society for Microbiology, Washington DC.

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Carlberg, D.M., 1986. Determining the effects of antibiotics on bacterial growth by optical and electrical methods. In: Antibiotics in Laboratory Medicine. Williams & Wilkins, Baltimore, pp. 64–92.

Chapman, J.S., Georgopapadakou, N.H., 1988. Routes of quinolone permeation in *Escherichia coli*. Antimicrob. Agents Chemother. 32, 438–442.

Dalal, E., Medalia, O., Harari, O., Aronson, M., 1994. Moderate stress protects female mice against bacterial infection of the bladder by eliciting uroepithelial shedding. Infect. Immun. 62, 5505–5510.

Eiseman, J., 1984. The pharmacokinetics evaluation of 14C-labelled 2,4-D in the mouse. Unpublished report No. 2184-104 from Hazleton Laboratories America, Inc., Vienna, VA, USA. Submitted to WHO by Industry Task Force II on 2,4-D Research Data, Indianapolis, Indiana, USA.

Erne, E., 1966. Distribution and elimination of chlorinated phenoxyacetic herbicides. Acta Vet. Scand. 7, 240–256.

Evans, D.G., Evans, D.J. Jr., Tjoa, W., 1977. Hemagglutination of human group A erythrocytes by enterotoxigenic *Escherichia coli* isolated from adults with diarrhea: correlation with colonization factor. Infect. Immun. 18, 330–337.

Falkowski, W., Edwards, M., Schaeffer, A.J., 1986. Inhibitory effect of substituted aromatic hydrocarbons on adherence of *Escherichia coli* to human epithelial cells. Infect. Immun. 52, 863–866.

Fletcher, M., Marshall, K.C., 1982. Bubble contact angle method for evaluating substratum interfacial characteristics and its relevance to bacterial attachment. Appl. Environ. Microbio1. 44, 184–192.

Gordon, D.M., Riley, M.A., 1992. A theoretical and experimental analysis of bacterial growth in the bladder. Mol. Microbiol. 6, 555–562.

Gorzinski, S.J., Kociba, R.J., Campbell, R.A., Smith, F.A., Nolan, R.J., Eisenbrandt, D.L., 1987. Acute, pharmacokinetic, and subchronic toxicological studies of 2,4-dichlorophenoxiacetic acid. Fundam. Appl. Toxicol. 9, 423–435.

Grover, R., Franklin, C.A., Miur, N.I., Cessna, A.J., Riedel, D., 1986. Dermal exposure and urinary metabolite excretion in farmers repeatedly exposed to 2,4-D amine. Toxicol. Lett. 33, 73–83.

Hagberg, L., Enberg, I., Freter, R., Lam, J., Olling, S., Svanborg-Eden, C., 1983. Contribution of adhesion to bacterial persistence in the mouse urinary tract. Infect. Immun. 40, 265–272.

Hoschützky, H., Lottspeich, F., Jann, K., 1989. Isolation and characterisation of the α-Galactosyl-1,4-β-Galactosyl-Specific adhesin (P Adhesin) from fimbriated *Escherichia coli*. Infect. Immun. 57, 76–81.

Johnson, J.R., 1991. Virulence factors in *Escherichia coli* urinary tract infections. Clin. Microbiol. Rev. 4, 80–128.

Johnson, D.E., Lockatell, C.V., Russell, R.G., Hebel, J.R., Island, M.D., Stapleton, A., Stamm, W.E., Warren, J.W., 1998. Comparison of *Escherichia coli* strains recovered from human cystitis and pyelonephritis infection in transurethrally challenged mice. Infect. Immun. 66, 3059–3065.

Johnson, J.R., Roberts, P.L., Stamm, W.E., 1987. P fimbriae and other virulence factors in *Escherichia coli* urosepsis: association with patients characteristics. J. Infect. Dis. 156, 225–229.

Knopp, D., Glass, S., 1991. Biological monitoring of 2,4-dichlorophenoxiacetic acid-exposed workers in agriculture and forestry. Int. Arch. Occup. Environ. Health 63, 329–333.

Knopp, D., Schiller, F., 1992. Oral and dermal application of 2,4-dichlorophenoxiacetic acid sodium and dimethylamine salts to male rats: investigations on absorption and excretion as well as induction of hepatic mixed-function oxidase activities. Arch. Toxicol. 66, 170–174.

Kunin, C.M., Hua Hua, T., Guerrant, R.L., Bakaletz, L.O., 1994. Effect of salicylate, bismuth, osmolytes and tetracycline resistance on expression of fimbriae by *Escherichia coli*. Infect. Immun. 62, 2178–2186.

Lachica, V.R., 1990. Significance of hydrophobicity in the adhesiveness of pathogenic Gram-negative bacteria. In: Doyle, R.J., Rosenberg, M. (Eds.), Microbial Cell Surface Hydrophobicity. American Society for Microbiology, Washington DC, pp. 297–313.

Leffler, H., Svanborg Eden, C., 1980. Chemical identification of a glycosphingolipid receptor for *Escherichia coli* attaching to human urinary tract epithelial cells and agglutinating human erythrocytes. FEMS Microbiol. Lett. 8, 127–134.

Nader-Macías, M.E., Silva de Ruiz, C., Lopez de Bocanera, M.E., Pesce de Ruiz Holgado, A., 1996. Behavior of lactobacilli on prevention and therapeutic effects on urinary tract infections (UTI) in mice. Anaerobe 2, 85–93.

O'Hanley, P., Lark, D., Falkow, S., Schoolnik, G., 1985. Molecular basis of *Escherichia coli* colonization of the upper urinary tract in BALB/c mice: Gal–Gal pili immunization prevents *Escherichia coli* pyelonephritis in the BALB/c mouse model of human pyelonephritis. J. Clin. Invest. 75, 347–360.

Orberg, J., 1980. Effects of low protein consumption on the renal clearance of 2,4-dichlorophenoxyacetic acid (2,4-D) in goats. Acta Pharmacol. Toxicol. 46, 138–140.

Orikasa, S., Hinman, F. Jr., 1977. Reaction of the vesical wall to bacterial penetration: resistance to attachment, desquamation, and leukocytic activity. Invest. Urol. 15, 185–195.

Orskov, F., 1986. Genus I *Escherichia*. In: Bergey's Manual of Systematic Bacteriology, 9th ed. pp. 175–289. The Williams & Wilkins Co, Baltimore, MD.

Pere, A., Nowicki, B., Saxen, H., Siitonen, A., Korhonen, T.K., 1987. Expression of *Escherichia coli* P, type 1 and type 1C fimbriae in the urine of patients with acute urinary tract infection. J. Infect. Dis. 156, 567–574.

Rugo, H.S., O'Hanley, P., Bishop, A.G., 1992. Local cytokine production in a murine model of *Escherichia coli* pyelonephritis. J. Clin. Invest. 89, 1032–1039.

Sauerhoff, M.N., Braun, W.H., Blau, G.E., Gehring, P.J., 1977. The fate of 2,4-dichlorophenoxyacetic acid (2,4-D) following oral administration to man. Toxicology 8, 3–11.

Silva de Ruiz, C., Lopez de Bocanera, M.E., Nader-Macias, M.E., Pesce de Ruiz Holgado, A., 1996. Effect of lactobacilli and antibiotics on *E. coli* urinary infections in mice. Biol. Pharm. Bull. 19, 88–93.

Silva de Ruiz, C., Nader-Macias, M.E., Lopez de Bocanera, M.E., Pesce de Ruiz Holgado, A., 1993. *Lactobacillus fermentum* administered in suspensions and in agarosa beads to mice: a comparative study. Microbiol. Alim. Nutr. 11, 391–397.

Svanborg-Eden, C., 1978. Attachment of *Escherichia coli* to human urinary tract epithelial cells. Scand. J. Inf. Dis. Suppl. 15, 1–69.

Svanborg-Eden, C., Hagberg, L., Hull, R., Hull, S., Magnusson, K.E., Ohman, L., 1987. Bacterial virulence versus host resistance in the urinary tracts of mice. Infect. Immun. 55, 1224–1232.

Taskar, P.K., Das, I.T., Trout, J.R., Chattopadhyay, S.K., Brown, H.D., 1982. Measurement of 2,4-dichlorophenoxiacetic acid (2,4-D) after occupational exposure. Bull. Environ. Contam. Toxicol. 29, 586–591.

van den Bosch, J.F., Postma, P., de Graaff, J., MacLaren, D.M., 1980. Detemunation of the α-haemolytic urinary *Escherichia coli* strains. FEMS Microbiol. Lett. 8, 75–77.

van der Woude, M.W., Braaten, B.A., Low, D.A., 1992. Evidence for global regulatory control of pilus expression in *Escherichia coli* by Lrp and DNA methylation: model building based on analysis of *pap*. Mol. Microbiol. 6, 2429–2435.

Vosbeck, K., Mett, H., Huber, U., Bohn, J., Petignat, M., 1982. Effects of low concentrations of antibiotics on *Escherichia coli* adhesion. Antimicrob. Agents Chemother. 21, 864–869.

Wada, T., Yokohama, H., Tomogushi, N., 1994. Detection of urinary interleukin-8 in glomerular diseases. Kidney Int. 46, 455–460.