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Norfloxacin Treatment on *E. coli* in the Urinary Tract of Mice Effect of Probiotic Lactobacilli

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Abstract: This study was performed to evaluate the effect of the treatment of norfloxacin, as an antimicrobial agent, on the uropathogenic *Escherichia coli* colonization in mice, complemented by Lactobacilli as probiotics.Norfloxacin (5.5 mg kg^{-1} dose) was administered orally before and after the challenge with *E. coli.* The animals were also inoculated intra-urethrally with agarose beads containing *L. fermentum.* The population of the microorganisms in the urogenital tract was measured various days throughout the study. Using either 1 or 3 doses of norfloxacin before or after the animals had been challenged with *E. coli* bacteria, reduced the *E. coli* populationin the urinary tract. The Norfloxacin treatment after the animals were administered 1 or 3 doses of agarose beads of lactobacilli significantly affected the viability of the lactic acid bacteria in all of the urinary tract organs and the number *of E. coli* (regardless to when the antibiotic was inoculated) was lower in the mice treated with lactobacilli than in the non-lactobacilli treated mice. Eventhough the survival of the lactobacilli was highly affected by the norfloxacin treatment, the uropathogenic *E. coli* colonization always decreased more than the lactobacilli population did and often completely disappeared in those animals which were treated with both norfloxacin and probiotic lactobacilli. Through this experimental model one can conclude that lactobacilli increases the effectiveness of the norfloxacin medication in the prevention and treatment of UTI produced by *E. coli.* Further studies are being performed to determine the mechanisms involved in this probiotic effect.

Key words: Norfloxacin, *E. coli*, probiotics, *L. fermentum*, prevention, recurrence, urinary tract infections

INTRODUCTION

Acute Urinary Tract Infections (UTI) are one of the most common ailments for which women seek medical consultation[1].They affect both pre and post-menopausal women worldwide. It has been estimated that approximately 40 to 50% of adult women have had at least one cystitis episode in their lives[2] and up to 10% of the population of premenopausal women experience recurrent symptomatic UTI[3]. *E. coli* is responsible for the 90% of the UTI´s in ambulatory patients and 30% of nosocomial infections[2]. This microorganism also causes 80 to 90% of uncomplicated UTI´s[4] and even though these infections are uncomplicated, therapy is very pricey[5].

Another problem related to the UTI is the degree of recurrence. A high percentage of at least one recurrence is produced in those women with a first UTI episode. The microorganism responsible is also *E. coli,* being the second episode produced by a genetically different *E. coli* than the first episode[6,7].

Clasically, the therapy for UTI consists of antimicrobial and wide-spectrum antibiotics. Although there are antibiotics which can be used to treat a UTI, for the many women who experience re-ocurring Urinary Tract Infections, there lacks a scientific method that effectively prevents their recurrence[8]. The most commonly used regime is a long-term, low-dose prophylaxis with thrimethoprim/ sulfamethoxazole (TMS/SMX) or nitrofurantoin that treats the disease by killing and inhibiting the growth of pathogens once they enter the bladder[1]. Such antimicrobial therapy disrupts the intestinal and urogenital flora which shows the need for a single-dose treatment[9]. Norfloxacin[10], a fluoroquinolone and TMS/SMX[11] are antibiotics which are commonly used to effectively eradicate acute cystitis.

In order to fullfill the main purpose of our research, which was to develop a protective and therapeutic device, consisting of probiotic lactobacilli, we set up an experimental model using mice[12,13]. We also searched for the role that lactobacilli and antibiotics play in mice on

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E. coli infections by studying the effects of varying doses of ampicilin which were orally administered[14], or estradiol subcutaneoslly injected[15]. The *E. coli* colonization in the urinary tract was decreased when treated with probiotic lactobacilli and three doses of ampicilin. For this work we selected the uropathogenic strain *Escherichia coli* (UPEC) and *Lactobacillus fermentum* CRL 1058 in order to examine the possible benefits of norfloxacin and lactic acid bacteria when used in treatment and prevention of UTI´s caused by *E. coli* in mice.

MATERIALS AND METHODS

Microorganisms: The *Escherichia coli* strain was isolated from the infected urinary tract of adult women and identified by biochemical tests according to Orskov[16]. It was also demonstrated that it infects to 2-month-old BALB/c mice. The culture media and the protocol used has been described previously[17]. *Lactobacillus fermentum* CRL 1058 agarose beads[13] were used in this experiments. The isolation, identification, maintenance and characterization procedures of the microorganisms have also been previously described[13,14].

Mice: 2 months old adult inbred BALB/c female mice from the stock breeding of our Institute, fed *ad libitum*, were used for the experiments. The protocol used for animals was approved by the CERELA ethics committee. 12-15 mice were used for each experiment.

Bacterial inoculation of animals: The *E. coli* was inoculated in a 0.5% peptone water suspension at an infectious concentration (higher than 10^3 CFU mL^{-1}), as aforementioned[18]. Bacterial doses are indicated in each experiment. *L.fermentum* was administered intraurethrally in agarose beads in doses higher than 10^7 CFU, enough to produce colonization.

***In vitro* antibiotic sensitivity of the strains:** was previously reported[17]. The *E. coli* strain was sensitive to Norfloxacin (0.1 ug mL^{-1}) and the sensitivity of *L. fermentum* was 1 ug mL^{-1}

Antibiotic treatment in mice: Animals were orally administered 1 or 3 doses (5.5 mg kg^{-1} per dose) of norfloxacin with a 12 h duration between intakes.

Norfloxacin as a prevention treatment: (Norfloxacin-*E. coli*). The antibiotic was orally administered (type and doses are indicated in each experiment) and a suspension of *E. coli* was inoculated as a single dose; and each inoculation has a 12 h. respite in between. One group of animals was infected with *E. coli* and not treated with the antibiotics.

Another group of mice was treated with a threefold intra-urethral administration of *L. fermentum* administered as agarose beads; afterwards norfloxacin was given as described and the *E. coli* was intraurethrally inoculated.

Norfloxacin as therapeutic treatment: (*E. coli*-norfloxacin) A group of mice was infected with *E. coli*. After 12 h, they were orally administered either with 1 or 3 doses of norfloxacin. Another group of mice was treated intraurethrally with *E. coli*.

A third group of mice was treated with three doses of *L. fermentum*, which was administered intraurethrally to the mice and 12 h later they were challenged with *E. coli*. After 12 h, mice were treated orally with either 1 or 3 doses of norfloxacin

Quantification of the microorganisms in the organs: These figures were determined as previously described[17].

Statistical analysis: Experimental values (mean and standard deviation) obtained from 3 to 4 animals were analysed according to the Student's t test.

RESULTS

Preventive assay using one dose or three doses of norfloxacin. (Norfloxacin-*E. coli*): Mice which were preventively treated with norfloxacin, before the *E. coli* challenge showed lower counts of the pathogenic microorganisms in all urinary tract organs from the day 3 and on Fig. 1b than mice without norfloxacin treatment Fig. 1a. *E-coli* infected mice presented values between 10^4 and 10^6 CFU in all organs during all the days assayed, up to the 7^{th} post-challenge Fig. 1a, while norfloxacin-treated mice showed values around 10^3 CFU Fig. 1b. Results show the data obtained in experiments with three doses of norfloxacin, being similar to those obtained with one dose of antibiotic.

Probiotic *L. fermentum* treatment (*L. fermentum*-norfloxacin-*E. coli*): The mice received a threefold dose of *L. fermentum* (6.4×10^8 CFU each), three doses of norfloxacin (5.5 mg kg^{-1}/dose) and one dose of *E. coli* (8.00×10^7 CFU) Fig. 1c. This experiment was not successful, from the lactobacilli permanence point of view, because the antimicrobial agent completely killed the probiotic *L. fermentum*, (data non shown). But the *E. coli* numbers recovered was lower in these lactobacilli-treated animals Fig. 1c than in norfloxacin or no-norfloxacin

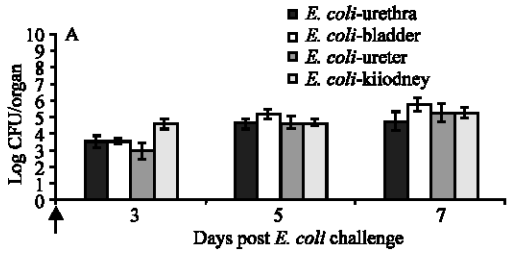

Fig. 1a: Control mice inoculated with *E. coli*. Mice were challenged with uropathogenic *E.coli* (one dose of 8.00×10^7 CFU/mice)(black arrow). Results are expressed as the mean±S.D. of the log CFU *E. coli*/organ from 3 to 4 mice killed each day. (Bars labels are showed in Fig. basis)

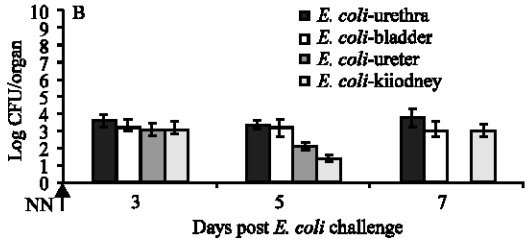

Fig. 1b: Mice treated preventively with Norfloxacin. Mice received orally 3 doses of norfloxacin (N)(5.5 mg kg^{-1} per dose). Each dose were administered 12 h in between. After the antibiotic treatment, mice were challenged with uropathogenic *E.coli* (one dose of 8.00×10^7 CFU/mice)(black arrow). Results are expressed as the mean±S.D. of the log CFU *E.coli*/organ. (Bars labels are showed in Fig. basis)

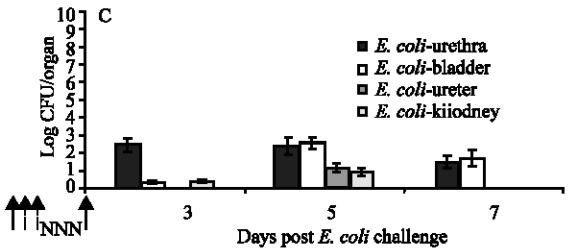

Fig. 1c: Mice treated with *L. fermentum*-norfloxacin-*E.coli*. Intra-urethral administration of a three-fold dose of *L. fermentum* in agarose beads (6.40×10^8 CFU per dose) (dotted arrows). Later, mice received orally 3 doses of norfloxacin (N)(5.5 mg kg^{-1} per dose). Each dose were administered 12 h in between. After the antibiotic treatment, mice were challenged with uropathogenic *E.coli* (one dose of 8.00×10^7 CFU/mice)(black arrow). Results are expressed as the mean±S.D. of the log CFU *E.coli*/organ. (Bars labels are showed in Fig. basis)

Fig. 1: Preventive treatment with norfloxacin

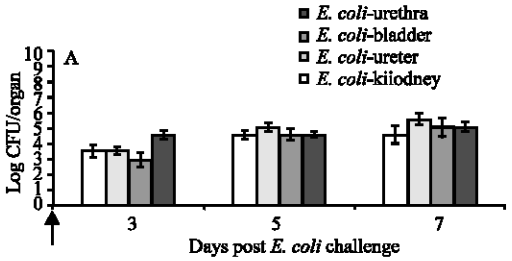

Fig. 2a: Control mice inoculated with *E. coli*. Mice were challenged with uropathogenic *E.coli* (one dose of 8.00×10^7 CFU/mice)(black arrow). Results are expressed as the mean±S.D. of the log CFU *E. coli*/organ from 3 to 4 mice. (Bars labels are showed in Fig. basis)

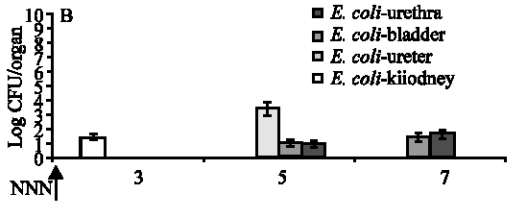

Fig. 2b: Mice treated therapeutically with norfloxacin. Mice were challenged with *E.coli* (8.00×10^7 CFU/mice)(Black arrow). 12 h later, mice received three doses of norfloxacin( )(5.5 mg kg^{-1} per dose) by the oral way. (Dotted arrows) Results are expressed as the mean±S.D. of the log of CFU *E.coli*/organ.(Bars labels are showed in Fig. basis)

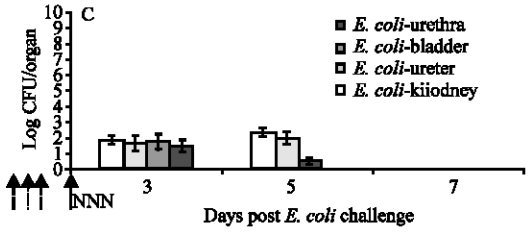

Fig. 2c: Mice treated with *L.fermentum-E.coli*-norfloxacin. Mice were inoculated by intra-urethral administration of a three-fold dose of *L. fermentum* in agarose beads (6.40×10^8 CFU per dose) each dose with 12 h in between (dotted arrows). With the same interval, the mice were challenged with *E.coli* (8.00×10^7CFU/mice)(Black arrow). 12 h later, mice received three doses of norfloxacin (N)(5.5 mg kg^{-1} per dose) by the oral way. Results are expressed as the mean±S.D. of the log of CFU *E.coli*/organ. (Bars labels are showed in Fig. basis)

Fig. 2: Therapeutic assay with norfloxacin

treated mice Fig. 1a and 1b. Becauser there wasn´t any recovered lactobacilli from the organ homogenates or urine, the figures include only *E. coli* data.

Therapeutic assay using three doses of norfloxacin (*E. coli*-norfloxacin): During the entire study, the mice treated with either 1 or 3 doses of norfloxacin, showed a dramatic decrease in their *E. coli* levels Fig. 2b, in comparison to the mice that were not treated with norfloxacin Fig. 2a. However, there were traces of pathogens in the organs until the seventh day.

Mice treated with *L. Fermentum* as probiotic (*L. fermentum*-*E. coli*-norfloxacin): The mice received a three-fold dose of *L. fermentum* (6.40×10^8 CFU each), one dose of *E. coli* (8.00×10^7 CFU) and three doses of norfloxacin. There was a lower *E.coli* colonization in the mice which were treated with lactobacilli and norfloxacin Fig. 2c than in the mice which were treated with antibiotics or those mice not treated with antibiotic Fig. 2a, 2b. No lactobacilli were recovered from the organ homogenates. *L. fermentum* demonstrates high sensitivity to norfloxacin in this *in vivo* model, for the antibiotic treatment completely eliminated this bacteria from the urogenital tract. On the 7th day, *E. coli* had been completely eradicated in all the homogenates of the urogenital tract organs.

DISCUSSION

UTI´s are a serious health problem, especially to women. Although a first infection can generally treated with wide varieties of antibiotics, UTI´s are reoccurring[18,19]. One of the main challenges for the UTI therapy is the degree of recurrence in adult women, because, even though one episode can be rapidly eliminated, is followed by other produced generally by the same (genetically different strains)[6] or sometimes by a completely different microorganism. The majority of these females are not unhealthy women; they do not generally experience recurrences by anatomic failures. Rather, it has been proposed that they have a biological predisposition or behaviours which promotes their recurrences or sensibility to them[2]. There are, of course, some treatments adapted for the UTI recurrences: local hygiene with disinfectants, prophylaxis with orally administered antibiotics during long or short periods with low doses, or estrogens therapies. Despite these possible treatments, there are no available description of a method which can actively prevents the recurrency of UTI´s[8].

Using antibiotics to cure recurrent UTI´s is a very common practice. This therapy is sometimes given as a prophylactic measure[11] and in pregnant women, trimethoprim[10], cephalexin, or furantoin are used[20]. Antibiotics are also frequently used to eliminate symptomatic UTI[1-3,8,9,19,20]. Antibiotic administration is often accompanied by gastrointestinal disturbances, including nausea, vomiting, abdominal pain and diarrhoea. Gotz stated that these side effects probably related to qualitative and quantitative changes in the intestinal flora[22]. There have been numerous intents to overcome this effect produced by antibiotics, through the use of lactobacilli to restore the ecological balance of the microflora. This theory has been studied this subject in monkeys[23], rats[24] and man, administering lactobacilli with different antibiotics such as ampicillin[22], enoxacin, or enoxacin and clindamycin.

All the described situations have shown the importance of the indigenous microflora of humans and animals because it provides protection against infections[25,26]. Therefore, the use of probiotics to improve the individual´s health has a great value. In recent years, the use of probiotics, as a way to help and improve the animal and human health, was increased. As defined by Havennar[27] probiotics are viable cultures of one or several microorganisms that produce a beneficial effect on the host through its influence on the indigenous flora. We began our study with the purpose of examine the probiotic influence in the urogenital tract.

Lactobacilli are the predominant microorganisms which were isolated from the urogenital tract[28]. They play a very significant role in the maintenance of low pH in this area, mainly by their lactic acid production. We found that lactobacilli are also the predominant specie isolated from the vagina of BALB/c mice at levels of 10^5-10^6 CFU mL^{-1} vaginal fluid (non published results). In previous works, we isolated 40 lactobacillus strains[12] from the vagina of 2-month-old BALB/c mice. We then selected one particular *Lactobacillus fermentum* strain (CRL 1058) based on its adhesion properties. This strain was used for the preparation of agarose beads, which were inoculated in the urethra of mice after having determined that three doses of lactobacilli, each one higher than 10^5 CFU mL^{-1}, was the optimal concentration for these microorganisms to remain in the urinary tracts of the animals until the 7th day after the inoculations[13].

This study was focused on the possible *L. fermentum* CRL 1058 contributions and on understanding its capability for colonization when used in treatments with norfloxacin to prevent and cure *E. coli* UTI´s in mice. *L. fermentum* was administered in agarose beads in order to increase lactobacilli permanence in the urinary tract and to avoid their clearance by urine. We used a lactobacilli strain from mouse origin, based on the host-specificity of

the normal flora and lactobacilli[29]. This specificity is not the same in the case of *E. coli*, for it produces infections in different types of hosts[30]. Furthermore, the *E. coli* strain used had a pyelonephrytogenic capability because it was able to produce a kidney infection when inoculated intra-urethrally. Optimal concentrations for both microorganisms have previously been determined[13-16].

For this study, the antibiotic norfloxacin was selected because it is frequently used in patients suffering from UTI´s and because *E.coli* is highly sensitive to this drug. The in vitro sensitivity of *E. coli* to norfloxacin was tested previously, showing a MIC of 0,1 ug mL^{-1}[14]. There is not a reference lactobacilli strain to state the *L. fermentum* degree of sensitivity, since the MIC of the lactobacilli is 10 times higher (1 ug mL^{-1}) than the MIC of *E. coli.* Although it is not know if this value represents sensitivity to or resistance of *L. fermentum,* we decided to test this antibiotic in mice, based on the high frequency of the norfloxacin employment in the UTI therapy.

Eventhough lactobacilli disappeared from the urogenital tract after either one or three doses of antibiotics were administered to the mice, the lactobacilli treatment was able to decrease the number of *E. coli* in the urogenital tract in the preventive assays. In the therapeutical assays, *E. coli* disappeared completely from all of the organs of the urinary tract. Our results are similar to those obtained by Reid *et al.*[8] who treated 41 adult women with acute lower UTI for three days with either norfloxacin or TMS to eradicate the infection. The recurrence rate of UTI was reduced from 47% to 21% with the post-therapy administration of lactobacilli vaginal suppositories.

Although lactobacilli were not recovered from the urogenital tract when mice were treated with norfloxacin, they obviously produce some type of protective effect when inoculated, because a lower number of *E. coli* is present in the urogenital tract of the treated mice. More studies are being performed to further determine the mechanisms which are responsible for the protective effect of lactobacilli.

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