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Antibiotic Residues and R-Plasmid Selection: Are in vitro Methods Good Models?

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Summary

Three clones of *E. coli*, one of which was harbouring a tetracycline resistance plasmid, were inoculated together into the stomach of axenic mice. Without antibiotic selective pressure, the R-Plasmid bearing strain became dominant in the faeces of mice, while the R-plasmid free strain was eliminated. When the R-plasmid bearing strain was given to mice 4 days after the inoculation with the R-plasmid free strain, it was repressed and remained at the stable level of $10^{4.5}$ organisms per g of faeces. But a rapid spread of the R-plasmid was observed, tetracycline resistant bacteria become dominant within one day, and replace the tetracycline sensitive *E. coli*.

The tetracycline resistance plasmid did not disadvantage the mediating strain in the gut, even in the absence of antibiotic pressure. In contrast *Lebek and Egger* (1983), studying the same strains *in vitro*, found that in a chemostat the plasmid bearing strain was overgrown by the plasmid free strain. These results strongly suggest that *in vitro* interactions between *E. coli* strains cannot be directly extrapolated to *in vivo* conditions. For the determination of the no-effect level of antibiotic residue on the selection of R-factor in the gut, studies should be made *in vivo*.

Zusammenfassung

Drei Stämme von Escherichia coli K12, von denen einer ein R-Plasmid trug, wurden zusammen in den Magen axenischer Mäuse inokuliert. Ein Stamm war plasmidfrei (Str.-R), der zweite Stamm besaß die chromosomale Nalidixinsäureresistenz und trug das Tetracyclinresistenzplasmid R 270 (Tet Suf), Inc F II rep. Der dritte Stamm (Rif-R) mit chromosomaler Resistenz gegen Rifampicin trug das Plasmid F' lac Inc F I derep. Ohne antibiotischen Selektionsdruck wurde der das R-Plasmid Nal-Tet-R tragende Stamm in-nerhalb eines Tages in den Faeces der Mäuse dominant, während der R-Plasmid-freie Stamm Str-R innerhalb von zehn Tagen eliminiert wurde (Fig. 1).

Gab man den Mäusen vier Tage nach der Einimpfung des Rif-R Stammes noch zusätzlich den das R-Plasmid tragenden Stamm Nal-Tet-R, dann wurde dieser Stamm unterdrückt, und eine konstante Zahl von 104-105 Keimen pro Gramm Faeces wurde gefunden. Jedoch wurde eine sehr schnelle Ausbreitung des R-Plasmids beobachtet; die tetracyclin-resistenten Bakterien (Rif-Tet-R) wurden dominant innerhalb eines Tages und ersetzten den tetra-cyclinsensitiven Rif-R E. coli-Stamm (Fig. 2). Diese Rif-Tet-R Transkonjuganten wurden unabhângig von der Anwesenheit von Tetracyclin im Trinkwasser der Mâuse gefunden (Fig. 2B). Diese Ausbreitung des R-Plasmids im ükologischen System des Darmes wurde auch in Mâusen beobachtet, die eine komplexe Flora besaf3en, welche durch die Beimpfung der Mäuse mit einer anaeroben Verdünnung menschlicher Faeces erhalten wurde (Fig. 3). Diese Tetracyclin-R Plasmide schienen dem Trägerstamm einen klaren Wuchsvorteil im Verdauungstrakt zu verleihen, sogar in Abwesenheit von antibiotischem Druck. Im Gegensatz dazu hatten Lebek u. Egger (1983) mit denselben Stämmen bei in vitro Versuchen im Chemostat gefunden, dag der plasmidtragende Stamm vom plasmidfreien Stamm über-wachsen wurde. Diese Resultate weisen deutlich daraufhin, dag in vitro gemessene Austausche zwischen E. coli-Stämmen nicht direkt übertragbar sind auf in vivo Bedingungen. Zur Bestimmungg der Antibiotikarückstandskonzentration, die keine Selektion von R-Fak-toren un menschlichen Darm bewirkt, sollten in vivo-Studien durchgeführt werden, entweder mit Probanden oder in einem besonders für die Fragestellung geeigneten Tierversuch-smodell.

Introduction

Antibiotics are used in food animal production for the treatment and prevention of disease. This could result in the occurrence of residues in meat or milk if witholding times are not adhered to. The question of whether or not such residues select for intestinal bacteria bearing R-plasmids therefore arises.

Lebek and Egger (7) investigated the minimum R-factor selecting tetracycline concentration in an *in vitro* model. Three clones of *Escherichia coli*, one of which was harbouring a tetracycline resistance plasmid,

were grown together in a chemostat, in the presence of various tetracycline concentrations. In this simple model, 0.25 µg tetracycline/ml of medium was enough to favour the selection of the tetracycline resistant strain. Since *in vitro* methods may not reflect conditions *in vivo*, an attempt was made to validate this model, by inoculating axenic mice *per os* with the three strains.

Materials and Methods

The strains, provided by Professor *Lebek*, were three *E. coli* K12 921 lac-, chromosomally resistant to streptomycin or nalidixic acid or rifampicin. The streptomycin resistant strain was plasmid free, the nalidixic acid resistant strain harboured a tetracycline resistance plasmid R 270 (Tet, Suf) IncFII rep, and the rifampicin resistant strain a plasmid R- F' lac+ IncFI derep (7). Each strain was kept at -25°C in a broth containing 45% glycerol, and for the tetracycline resistant strain 10 µg/ml of tetracycline. It was then cultured overnight at 37°C in a nutrient broth, and diluted in phosphate buffer (KH_2PO_4 0.75 mg/ml, K_2HPO_4 3 mg/ml) on the basis of its optical density (400 nm, Spectrophotometer Uvikon 820, Kontron), to a concentration of 2×10^4 *E. coli* per ml of inoculum. The inoculum was buffered in order to avoid destruction of viable bacteria by the stomachal acidity (5). Number of viable *E. coli* and resistance pattern were checked *a posteriori* in the inoculum. The transfer frequency of plasmid R 270 was measured *in vitro* to confirm the repression of fertility. In standardized conditions of transfer (10) a transfer frequency of 3×10^{-4} was found, which is normal for a repressed plasmid.

The axenic mice, adult female OF1 (Iffa Credo, F 69210 St Germain) were maintained in plastic isolators(La Calhene, F 95870 Bezons). A commercial diet sterilized by a 4 Mrad irradiation (Diet C04, UAR, F 91360 Villemoisson), and autoclaved acidified water were both available *ad libitum*. The water was brought to pH 2.5 with HCI in order to avoid bacterial multiplication and tetracycline degradation in the drinking bottle. Each experiment was conducted in three mice, which were maintained in individual cages in one isolator. After confirmation of their axenic status, the mice were given 0.5 ml of the inoculum, which was administered by gavage directly into the stomach.

Freshly passed faeces were collected directly at the anus, and after decimal dilution in sterile saline, were plated within 30 minutes onto desoxycholate agar (Difco) which contained either no antibiotic or the following: streptomycin (1 mg/ml), nalidixic acid (50 µg/ ml), rifampicin (100 µg/ml), tetracycline (20 µg/ml) or a combination of tetracycline with streptomycin, nalidixic acid or rifampicin (all antibiotics came from Sigma). A preliminary experiment had shown that there was no toxic synergy against the resistant strains between this selective medium and these antibiotic concentrations.

Results

Axenic mice were inoculated with the three strains simultaneously, which became established and were non-pathogenic. On two sequential experiments, the nalidixic acid-tetracycline resistant strain became dominant and the streptomycin resistant strain was eliminated (Fig. 1). The *in vivo* interactions between the three strains were very different from the results obtained *in vitro*, where the plasmid-bearing strains were repressed by the plasmid-free strain (7).

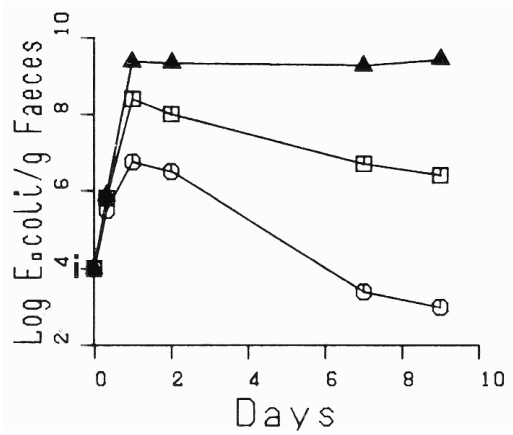

Fig. 1. Competition between three isogenic strains of *E. coli* in gnotobiotic mice. Each point represents the mean log number of CFU/g faeces from three mice.
Black triangles: nalidixic acid-tetracycline-R, Empty squares: rifampicin-R, Empty circles: streptomycin-R, **i**: inoculation.

When the nalidixic acid-tetracycline resistant strain was given to mice four days after inoculation with the rifampicin resistant strain and the streptomycin resistant strain, it was repressed. This antagonistic effect was not observed when mice colonised by the streptomycin resistant strain alone were inoculated with the nalidixic acid-tetracycline resistant strain. The latter became dominant five days after introduction into the monoxenic mice. In contrast, the rifampicin resistant strain, already established alone in the mice, was able to antagonize subsequent infection with the nalidixic acid-tetracycline resistant strain. On each of four experiments, the same equilibrium was reached of $10^{9.5}$ rifampicin resistant and $10^{4.5}$ nalidixic acid-tetracycline resistant organisms per g of faeces. This system was stable and reproducible and seemed to be a suitable model in which to determine the minimum tetracycline concentration in the drinking water which would select the tetracycline resistance plasmid.

Mice containing only the rifampicin resistant strain were given tetracycline continuously in the drinking water at concentrations of 1, 4, and 16 µg/ml and then challenged orally with the nalidixic acid-tetracycline

resistant strain. The administration of tetracycline did not influence the number of nalidixic acid-tetracycline resistant bacteria in faeces (Fig. 2A). When faecal dilutions were plated on agar containing tetracycline and rifampicin, it was found that a rifampicin resistant strain that carried the tetracycline resistance plasmid had evolved after the introduction of the nalidixic acid-tetracycline resistant strain into the ecosystem. This strain became dominant, and replaced the tetracycline-sensitive rifampicin resistant strain. This rapid spread of the R-plasmid occurred independently of the presence of tetracycline in the drinking water (Fig. 2B). These transconjugants had not been observed in the *in vitro* model (7).

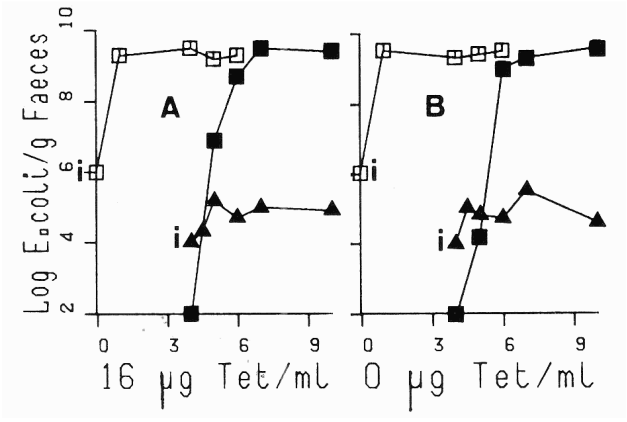

Fig. 2. Effect of tetracycline on the interaction between two *E. coli* strains in gnotobiotic mice.
2A: tetracycline (16 µg/ml) was added into the drinking water at day 3.
2B: No tetracycline in the water.
See Fig. 1 for symbols, except
Solid black squares: rifampicin-tetracycline-R.

In the presence of a more complex flora, obtained from the anaerobic 10^{-6} dilution of human faeces (chosen because it harboured less than 10^6 coliforms per g), given together with the rifampicin resistant strain to axenic mice on two consecutive days, the number of rifampicin resistant *E. coli* decreased from $10^{9.5}$ to 10^7 as shown in Fig.3. Again, the tetracycline resistance plasmid spread to the rifampicin resistant strain after the introduction of the nalidixic acid-tetracycline resistant strain, in the absence of tetracycline selective pressure. This occurred less quickly, however, than in the previous experiment (see Fig. 3).

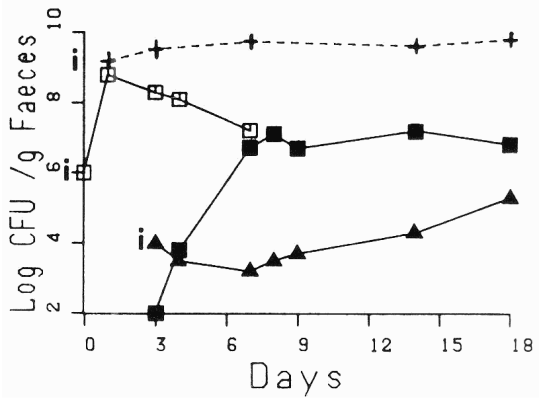

Fig. 3. Competition between two *E. coli* strains in gnotobiotic mice, in the presence of a human faecal flora. See. Fig. 2 for symbols +---+: total counts (anaerobic).

Control experiments were realized, in which the plasmid bearing tetracycline resistant strain was replaced by a cured strain, obtained from the action of acridine orange on the nalidixic acid-tetracycline resistant strain. The inoculation of the three strains together led to the domination of the nalidixic acid resistant strain (tetracycline susceptible) over the other strains, the kinetics of the three populations being exactly the same as in Fig. 1. The experiment which is shown in Fig. 2B, i.e. inoculation of the nalidixic acid resistant strain three days after the rifampicin resistant strain, was repeated with the cured strain. Obviously, no tetracycline resistant transconjugants appeared, and an equilibrium was reached of $10^{9.5}$ rifampicin resistant and $10^{7.5}$ nalidixic acid resistant organisms per g of faeces.

Discussion

There is a general agreement that *in vitro* results cannot be simply extrapolated to the *in vivo* situation. The results presented in this paper confirm this in a well control-led system: (i) the chromosomal resistance markers seemed to have no effect on the *in vitro* behaviour of the three *E. coli* strains (7) but played a major role in the *in vivo* interactions between the same strains (the nalidixic acid resistant strain overcame by far the streptomycin resistant strain), and (ii) the R-plasmid appeared to confer a disadvantage to the mediating strain *in vitro*, but gave no clear ecological disadvantage in the digestive tract (the nalidixic acid resistant strain reached the same level if it was plasmid free or harbouring a R-plasmid, in the presence of the two other strains, cf. Fig. 1).

An explanation for this discrepancy between *in vitro* and *in vivo* results comes from the differences in the ecological conditions: In the rich oxygenated broth of the chemostat, the shorter generation time (32 min) of the plasmid free strain allows it to overgrow the plasmid bearing strains (generation time of 42 min) (7). In contrast, in the distal part of digestive tract, where little oxygen and nutrients are available, generation times are usually estimated to range between 4 and 12 h, and other factors may have greater importance, such as attachment to the gut wall or metabolic efficiency in a limiting substrate (5). Although plasmids are important

determinants of the bacterial ecology, little is known about the colonisation factors they encode. According to Richmond (9), most plasmid bearing strains are disadvantaged against their plasmid free counterparts, likely because of a reduction in the *in vivo* growth rate. The factor which may explain the lower *in vivo* growth rate of plasmid bearing bacteria, is a higher saturation constant for limiting substrate (6), itself likely due to the cost of expressing the plasmidic genes in proteins, which is much more expensive than the energetic cost of duplicating the plasmidic DNA. The presence of a plasmid may sometimes have the opposite effect as well, i.e. to increase the *in vivo* fitness of a bacterium, likely because of adhesion to the gut wall, the proteic adhesins being often encoded by plasmid. For example, Duval-Iflah and others (3, 4) showed that in gnotobiotic mice, gnotobiotic piglets, and in human neonates, plasmid free strains generally inhibited the establishment of plasmid bearing strains. They found on one occasion, however, a tetracycline resistance plasmid which gave an ecological advantage to the strains bearing it. Therefore, whilst it has already been reported, the type of interaction presented here seems to be a rare occurrence.

We are now going to discuss the design of the experimental model: Numerous authors (e.g. 6, 10) have studied R-factor transfer in gnotobiotic animals. Their results have also shown that plasmid transfer can take place *in vivo* without antibiotic pressure. It is important to consider this phenomenon, which was not observed *in vitro* by Lebek and Egger (7) at their detection level (10^5 CFU/ml), in the development of any model designed to assess antimicrobial safety in vivo. A possible explanation for the lack of R-plasmid transfer *in vitro* is that the coculture was made for seven hours in the chemostat (7), whereas experiments in mice were conducted for several days. It was not possible in our experimental model to study the early evolution of faecal flora, since the first positive sample arrived at the anus of the mice about eight hours after inoculation in the stomach. To our opinion, long term experiments simulate much better the normal human gut bacterial interactions than short term ones. Another explanation for the difference in the R-plasmid transfer between the continuous flow culture and the gut is that the total number of *E. coli* in the gut is much higher ($10^{9.5}$) than in the chemostat (10^7). When the experimental data from *in vitro* and *in vivo* models were gathered together in a mathematical model of the plasmid transfer (2, 6), and simulated numerically on a computer, it was found that this difference in the number of potential recipient bacteria was alone sufficient to explain the differences observed in the transfer rates.

The competition between isogenic *E. coli* strains *in vivo*, differing in their chromosomal pattern of resistance, had also been studied. Onderdonk and others (8) showed that chromosomal resistance are detrimental to *E. coli*. It was clear in the results obtained here from the use of the cured nalidixic acid resistant strain, that the chromosomal resistance to streptomycin was much more detrimental *in vivo* to the bacteria than the resistance to nalidixic acid. The presence of both types of resistance (namely plasmidic and chromosomal) together in the same experiment was found to be rather confusing, and, in the author's opinion, this kind of marker should be avoided in further studies.

The faecal human microflora given to the mice, has an antagonistic effect against the *E. coli* population (see Fig. 3), and seems to simulate the bacterial interactions of the human intestine. This kind of ecological situation must be a better model of the digestive tract than the mice harbouring only *E. coli* strains. It should nevertheless be explored more deeply, particularly in order to obtain a well defined and reproducible anaerobic flora, as Freter and others (5, 6) did with the mouse microflora.

From the point of view of the determination of the no-effect level of an antibiotic residue on the selection of R-factor in the gut, studies should be made *in vivo*. Axenic mice, inoculated with bacteria of human origin may be a suitable test system (1). Experiments in human volunteers may also to be useful, though the natural variation in the components of the gut flora, due to contaminations or diet modifications, may hide the effect of low levels of antibiotics.

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