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Transfer of antimicrobial resistance plasmids from *Klebsiella pneumoniae* to *Escherichia coli* in the mouse intestine

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***Objectives and methods*: *Klebsiella pneumoniae* is a nosocomial pathogen and is considered the most common Gram-negative bacterium that exhibits multiple antimicrobial resistances. In this study, the transfer of antimicrobial resistance genes from the clinical multiresistant *K. pneumoniae* MGH75875 isolate was assessed *in vitro* and *in vivo* in an intestinal colonization animal model. The ability to colonize and transfer was tested under different antimicrobial treatments. The frequency of the horizontal gene transfer was also examined *in vitro*.**

***Results*: The clinical isolate of *K. pneumoniae* colonized the intestine of mice at levels up to 10^9 cfu/g faeces in antimicrobial-treated mice. In mice without antimicrobial treatment, the strain quickly decreased to below the detection limit due to competitive exclusion by the indigenous mouse flora. Onset of antimicrobial treatment gave immediate rise to detectable levels of the strain in the faeces of up to 10^9 cfu/g faeces. The experiment clearly shows that the treatment selects resistant strains and gives advantages to colonize the gastrointestinal tract. Furthermore, high transfer frequency of different plasmids was observed during colonization of the mouse intestine. The bla_{SHV} and bla_{TEM} genotypes were transferred to both an indigenous recipient in the *in vivo* setting and to an MG1655 *Escherichia coli* recipient strain *in vitro*.**

***Conclusions*: *K. pneumoniae* is an excellent colonizer of the intestine and is extremely promiscuous with respect to the transferability of its numerous plasmids. Antimicrobial treatment enhances the selection of resistant strains and results in an increase in the resistance gene pool, which ultimately raises the risk of spreading resistance genes.**

Keywords: MGH78578, horizontal gene transfer, streptomycin-treated mice, gastrointestinal tract, indigenous flora, selective pressure

Introduction

Klebsiella pneumoniae is a common Gram-negative bacterium that exhibits multiple antimicrobial resistances.[1] *K. pneumoniae* is also an important opportunistic pathogen, and many studies observe *K. pneumoniae* as the most common source of Gram-negative nosocomial bacteraemia.[2,3] Recently, pyogenic liver abscess was linked to *K. pneumoniae* in several cases.[4,5] Furthermore, 15% to 30% of the multiresistant *K. pneumoniae* isolates are resistant to broad-spectrum cephalosporins via plasmid-encoded extended-spectrum β-lactamases (ESBLs).[6] *K. pneumoniae* is ubiquitous; the natural habitat includes soil, vegetation and surface waters and the intestinal mucosal surface of mammals.[7] In a clinical setting, the most important reservoirs for transmission are assumed to be the gastrointestinal tracts of patients and the hands of the personnel.[8] It has been shown that the majority of the infections in the urinary and/or in the respiratory tract are preceded by the colonization of the patients' gastrointestinal tract by the pathogen.[9] This observation combined with the well-known hypothesis 'that the human colon is serving

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as a reservoir for resistance genes' provides the basis of this study.[10] Transfer of resistance genes among potentially pathogenic bacteria in the gastrointestinal tract results in multiresistant bacteria that might cause infections to spread to different organs and ultimately result in treatment failure.

The goal of the present study was to investigate the transfer of antimicrobial resistance genes in the gastrointestinal tract. The gene transfer was studied *in vitro* and *in vivo* in the intestine of mice treated with antimicrobial agents from a multiresistant clinical *K. pneumoniae* isolate to a susceptible *Escherichia coli* strain.

Materials and methods

Bacterial strains and media

K. pneumoniae MGH78578 (ATCC 700721) is a clinical isolate from an intensive care unit (ICU) patient with pneumonia. The strain is resistant to numerous antimicrobial agents, including ampicillin, streptomycin, tetracycline, nalidixic acid, ticarcillin, trimethoprim/sulfamethoxazole, cefotaxime and gentamicin, and is susceptible to imipenem. The strain contains five plasmids of 176, 108, 89, 4 and 3 kb. Derivatives of the *E. coli* K12 strain MG1655 were used as recipients in the transfer experiments.[11] A spontaneous streptomycin- and rifampicin-resistant mutant (MG1655SR) and a streptomycin- and nalidixic acid-resistant mutant (MG1655SN) were used as recipients in the transfer experiments. All strains were grown in Luria–Bertani (LB) medium (Sigma, St Louis, MO, USA) at 37°C overnight.

Unless otherwise stated, the antimicrobial agents and chemicals used in this study were of analytical grade and were obtained from Sigma. Rifampicin was purchased from Fluka BioChemica, Buchs, Switzerland.

Transfer of antimicrobial resistance genes in vitro

Overnight cultures of donor and recipient strains were mixed in a ratio of 1:1, and the mixture was washed in saline (0.9% NaCl). An aliquot of 20 μL of the mixture was spotted on an LB plate incubated at 37°C for 24 h and plated on selective plates. Selective plates used for *K. pneumoniae* MGH75578 and *E. coli* MG1655SR contained 100 mg/L streptomycin sulphate, 200 mg/L rifampicin (for recipient + transconjugant), 50 mg/L ampicillin, 50 mg/L kanamycin (for donor + transconjugant), 100 mg/L streptomycin sulphate, 200 mg/L rifampicin, 50 mg/L ampicillin or 50 mg/L kanamycin or 20 mg/L chloramphenicol or 10 mg/L tetracycline or 15 mg/L nalidixic acid (for transconjugants).

Antimicrobial-treated mouse model of gastrointestinal tract colonization

Six- to eight-week-old, outbred albino female Ssc:CF1 mice [Statens Serum Institut (SSI), Copenhagen, Denmark] were used for colonization and antimicrobial resistance transfer studies. The mice were individually caged, and cages were changed daily. The mice had unlimited access to food and continuously received either pure drinking water or water containing antimicrobial agents, either streptomycin sulphate or ampicillin.[12] Prior to inoculation, faecal samples were tested for the presence of indigenous bacteria with similar resistance. Inoculum suspensions were prepared by 10× overnight cultures resuspended in 20% (w/v) sucrose. Each mouse was given 100 μL of suspension *per os*. Faecal samples were collected and the numbers of cfu were determined by serial dilution and spread on selective plates. The mice were euthanized, and the experimental duration time was between 3 and 6 weeks. All experiments were performed twice independently. The animal experiments were approved by The Animal Experiments Inspectorate, The Danish Ministry of Justice, and were performed by skilled personnel. Different antimicrobial concentrations were tested, and the lowest concentration that allowed *Klebsiella* MGH78578 to colonize was selected.

Effects of antimicrobial treatment on intestinal flora

Three mice per experiment were inoculated with the strain *K. pneumoniae* MGH78578 (2×10^9 cfu/mouse). The mice were provided with fresh drinking water every day. The numbers of cfu in faecal samples were determined on LB plates containing 50 mg/L ampicillin and 25 mg/L kanamycin. To mimic the treatment of infection, 0.5 g/L ampicillin was added to the drinking water 4 weeks after inoculation.

Colonization of the intestine during antimicrobial treatment

Two mice per experiment were treated with 0.5 g/L ampicillin in the drinking water prior to inoculation of the strain *K. pneumoniae* MGH78578 (1×10^9 cfu/mouse) and throughout the experiment. The numbers of cfu were determined from faecal samples homogenized and spread on LB plates containing 50 mg/L ampicillin and 25 mg/L kanamycin.

Gene transfer in the intestine of streptomycin-treated mice

Three mice per experiment were treated with 0.5 g/L streptomycin sulphate in the drinking water prior to inoculation with the recipient strain and throughout the experiment. The recipient *E. coli* MG1655SR was given *per os* at 3×10^8 cfu/mouse. The recipient strain was allowed to establish in the intestine for 1 week before inoculation of the donor *K. pneumoniae* MGH78578 (3×10^8 cfu). The numbers of cfu were determined from faecal samples on LB plates containing: 100 mg/L streptomycin and 50 mg/L rifampicin (for recipients + transconjugants), 50 mg/L ampicillin and 25 mg/L kanamycin (for donors + transconjugants) and 100 mg/L streptomycin and 50 mg/L rifampicin with 25 mg/L ampicillin or 25 mg/L kanamycin or 10 mg/L chloramphenicol (for transconjugants).

Verification of transconjugants

Biochemical assays. Bacterial species were verified on selective media and by biochemical tests: Statens Serum Institut (SSI) blue plates with lactose (SSI nr. 694, Hillerød, Denmark) were used to select Gram-negative bacilli; 4-nitro-phenyl-β-D-glukopyranosiduronic acid test (a β-glucuronidase activity test; SSI nr. 1033, Hillerød, Denmark) to differentiate *K. pneumoniae* from *E. coli*; and saccharose test (SSI nr. 3734, Hillerød, Denmark) to detect the indigenous *E. coli* transconjugants.

Plasmid profile. Plasmid preparation was conducted by a modification of a previously described method.[13] Briefly, 1 mL of overnight culture was centrifuged for 5 min at 5000 **g**. The pellet was resuspended in 20 μL of TE buffer [50 mM Tris–HCl and 1 mM EDTA (pH 8.0)] and lysed by adding 100 μL of lysis buffer (3% SDS, 80 mM NaOH and 50 mM Tris–HCl). The suspension was vortexed carefully (2 s) and incubated at 56°C for 30 min, and then 100 μL of phenol mixture (phenol/chloroform/isoamyl alcohol, 25:24:1) was added. The sample was vortexed (3×3 s) and

centrifuged for 5 min at 13 000 **g** at 4°C. The top phase was transferred to a new tube and mixed with the loading buffer. The plasmid profiles were run on a 0.8% agarose gel and visualized by ethidium bromide staining. As a plasmid size marker, *E. coli* 39R861 was used, containing four plasmids of 147, 63, 36 and 7 kb.[14]

Pulsed-field gel electrophoresis. PFGE analysis was performed according to the Pulse-Net standard protocol, with restriction enzyme *Xba*I (Roche, Indianapolis, IN, USA) and *Salmonella* Branderup strain used as a molecular weight marker.[15]

Antimicrobial susceptibility testing. All strains, donors, recipients and transconjugants were tested for their antimicrobial resistance profile either by disc diffusion or by Etest as follows.

Disc diffusion was conducted on Specific agar plate (SSI nr. 22879, Hillerød, Denmark) and adding Neo-Sensitabs (Rosco Diagnostica, Taastrup, Denmark) followed by incubation at 37°C for 24 h. Diameters of the zones were measured and analysed according to Rosco manual V (Rosco Diagnostica, 2008). The antimicrobial concentrations tested were: amikacin, 40 μg; ampicillin, 33 μg; cefotaxime, 30 μg; chloramphenicol, 60 μg; ciprofloxacin, 10 μg; gentamicin, 40 μg; imipenem, 18 μg; kanamycin, 100 μg; mecillinam, 33 μg; nalidixic acid, 130 μg; nitrofurantoin, 260 μg; polymyxins, 150 μg; rifampicin, 30 μg; sulphonamide, 240 μg; tetracycline, 80 μg; tobramycin, 40 μg; and trimethoprim, 5.2 μg.

ESBL-producing phenotypes were confirmed with Etest strips (AB Biodisk, Solna, Sweden), which were applied on Mueller–Hinton plates (SSI nr. 708, Copenhagen, Denmark) with cefotaxime (CTX) and cefotaxime + clavulanic acid (CTX + CLA), and ceftazidime (CAZ) and ceftazidime + clavulanic acid (CAZ + CLA). Etest was read according to the manufacturer's protocol after overnight incubation at 37°C.

PCR detection of ESBL genes. The primers that were used to check for the presence of ESBL genes were: bla_{TEM} genes, F 5′-GTATCCGCTCATGAGACAATAACCCTG-3′, R 5′-CCAATGC TTAATCAGTGAGGCACC-3′; bla_{SHV} genes, F 5′-CGCCTGTGTA TTATCTCCCTGTTAGCC-3′, R 5′-TTGCCAGTGCTCGATCAGC G-3′; bla_{OXA} genes, F 5′-ATGAAAACATTTGCCGCATATGTA-3′, R 5′-ACACCAGGATTTGACTCAGTTCC-3′ and $bla_{\mathrm{CTX\text{-}M}}$ genes, F 5′-ATGGCGGCCGCGGCGGTGCTTAA-3′, R 5′-AGCGCGGCC GCGCTACAGTACAGC-3′.[16] The strains used as positive/negative controls were: bla_{TEM} (*K. pneumoniae* KP6T[17]/*E. coli* K12J62-2), bla_{SHV} (*K. pneumoniae* KP15[17]/*E. coli* ATCC 25922), bla_{OXA} (*E. coli* J53-2/pm202[18]/*E. coli* MG1655) and $bla_{\mathrm{CTX\text{-}M}}$ (*K. pneumoniae* KP4aC[17]/*E. coli* K12J62-2). Amplitaq polymerase (5 U/μL) and boiling lysate were used. All PCR amplifications were performed in a T3 Thermocycler (Biometra, Göttingen, Germany) using the following PCR programme: 3 min at 94°C; 29 cycles of 30 s at 94°C, 30 s at 60°C (*tem*), 62°C (*shv*), 56°C (*oxa*) and 65°C (*ctx-m*) and 60 s at 72°C; and 10 min at 72°C.[16] The PCR products were run on a 2% E-gel (Invitrogen, Carlsbad, CA, USA).

Results

Susceptibility testing of K. pneumoniae MGH78578

The MGH78578 strain was characterized in our laboratory for the presence of ESBL and was found to be resistant towards cefpodoxime. Etest confirmed an ESBL-producing phenotype (CTX/CTX + CLA = phantom inhibition of zone/0.064 and CAZ/CAZ + CLA = >32/>4 = out of range). The *K. pneumoniae* strain was further tested and found to possess two β-lactamase genes, *tem* and *shv*, revealed by PCR (data not shown).

Transfer of antimicrobial resistance genes in vitro

The ability of the clinical strain *K. pneumoniae* MGH78578 to transfer its antimicrobial resistance genes was studied *in vitro*. *K. pneumoniae* MGH78578 was used as a donor and *E. coli* MG1655SR was used as a recipient. Transconjugants were detected on plates containing streptomycin, rifampicin and either ampicillin or kanamycin after both solid and broth mating. The transfer frequency obtained was $1.3–1.9 \times 10^{-5}$ transconjugants/recipient from mating on plates and $1.1–1.8 \times 10^{-4}$ from mating in liquid media.

All transconjugants were tested against a number of antibiotics and were found to be resistant to ampicillin, kanamycin and cefpodoxime, and intermediately resistant to amikacin, cefotaxime and tobramycin (Table 1, TC MG *vitro* 2). The plasmid profiles showed that the transconjugants had obtained the second largest plasmid from the donor (Figure 1a, lane 5). ESBL Etest confirmed ESBL phenotype (CTX/CTX + CLA = deformation of CTX zone/0.016 and CAZ/CAZ + CLA = deformation of CAZ zone/0.064) and PCR showed the presence of two ESBL genes, *shv* and *tem* (data not shown). A few of the transconjugants were seen to harbour the largest plasmid from the donor strain and exhibited resistance towards ampicillin, kanamycin, chloramphenicol, tobramycin, trimethoprim and sulphonamide, and intermediate resistance towards gentamicin and amikacin (Figure 1a, lane 4 and Table 1, TC MG *vitro* 1). The PFGE profiles verified that the transconjugants were MG1655SR (Figure 1b, lanes 2, 4 and 5).

Effects of antimicrobial treatment on colonization of the intestine by K. pneumoniae

The colonization ability of the clinical isolate of *K. pneumoniae* and persistence in the gut was investigated using mice with normal flora. In this experiment, three mice were provided with drinking water without antimicrobial agents, and on day 0, the mice were inoculated with *K. pneumoniae* MGH78578 (2×10^9 cfu/mouse). Only 1 day after inoculation, the level of the strain was reduced to $10^4–10^5$ cfu/g faeces (Figure 2), and from day 6, *K. pneumoniae* was only detectable in two mice. After day 16, the level of the strain was below the detection limit (DL = 50 cfu/g faeces, corresponding to the minimum number of detectable colonies when 200 μL is plated) in all three mice and remained undetected. To investigate the effects of antimicrobial treatment and to mimic a clinical setting, 0.5 g/L ampicillin was added to the drinking water at day 28 and throughout the rest of the experiment. The cfu levels of the strain quickly rose above the detection limit and colonized the intestine at a high level of $\sim 10^9$ cfu/g faeces for the rest of the experiment (Figure 2).

Colonization of the intestine during continuous antimicrobial treatment

The mouse model was used to investigate the colonization ability of *K. pneumoniae* during the antimicrobial treatment with ampicillin. Prior to inoculation with *K. pneumoniae*, faeces from mice were tested for the presence of indigenous bacteria resistant

Table 1. Resistance and plasmid profiles of recipient, donor and transconjugants

	Recipient MG	Donor MGH	Transconjugants				
			TC MG *vitro* 1 (MGH + MG *vitro*)	TC MG *vitro* 2 (MGH + MG *vitro*)	TC IG *E. coli vivo* (MGH + flora *vivo*)	TC MG *vitro* 3 (TC IG *E. coli* + MG *vitro*)	TC MG *vivo* (MGH + MG *vivo*)
Plasmids	0	5	1 (176 kb)	1 (108 kb)	1 (108 kb)	1 (108 kb)	1 (89 kb)
Antibiotic							
amikacin	S	**I**	**I**	**I**	**I**	**I**	**I**
chloramphenicol	S	**R**	**R**	S	S	S	**R**
gentamicin	S	**I**	**I**	S	S	S	S
kanamycin	S	**R**	**R**	**R**	**R**	**R**	**R**
streptomycin	R	R	R	R	I	R	R
tetracycline	S	R	S	S	S	S	S
tobramycin	S	**R**	**R**	**I**	**I**	**I**	S
ampicillin	S	**R**	**R**	**R**	**R**	**R**	**R**
cefotaxime	S	**R**	S	**I**	**I**	**I**	S
cefpodoxime	S	**R**	S	**R**	**R**	**R**	S
imipenem	S	S	S	S	S	S	S
mecillinam	S	R	S	S	S	S	S
ciprofloxacin	S	I	S	S	S	S	S
nalidixic acid	S	R	S	S	S	S	S
rifampicin	R	I	R	R	I	R	R
sulphonamide	S	**R**	**R**	S	S	S	S
trimethoprim	S	**R**	**R**	S	S	S	S
polymyxins	S	I	S	S	S	S	S
nitrofurantoin	S	I	S	S	S	S	S

Donor: *K. pneumoniae*, MGH; recipient: *E. coli*, MG; flora *vivo*: natural flora of mice in colonizing experiment; IG, indigenous; TC, transconjugant; R, resistance; I, intermediate resistance; S, susceptible; bold indicates resistance transferred from the donor.

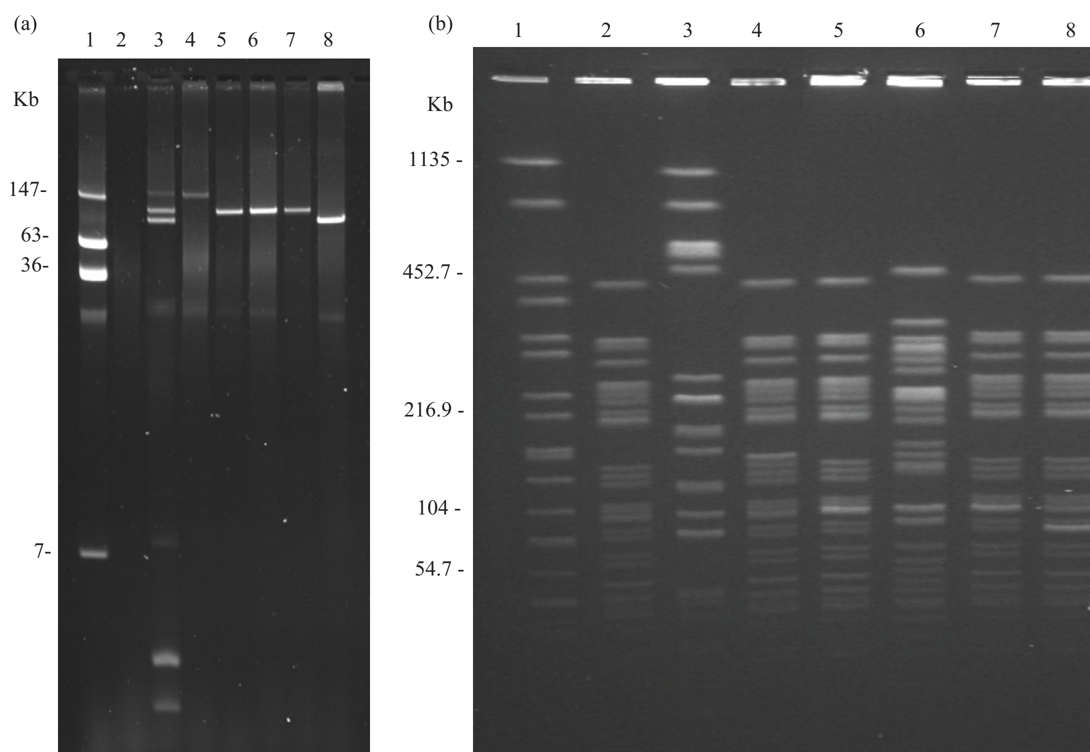

Figure 1. Plasmid profiles and PFGE profiles of donor, recipient and transconjugants. (a) Plasmid profiles where the marker is *E. coli* 39R861 (147, 63, 37 and 7 kb).[12] (b) PFGE profiles using the restriction enzyme *Xba*I and *Salmonella* Branderup strain as a molecular weight marker. Lane 1, marker; lane; 2, recipient *E. coli* MG1655SR; lane 3, donor *K. pneumoniae* MGH75875; lanes 4–8, transconjugants (TCs) from different experiments (lane 4, TC MG *vitro* 1; lane 5, TC MG *vitro* 2; lane 6, TC indigenous *E. coli vivo*; lane 7, TC MG *vitro* 3; and lane 8, TC MG *vivo*).

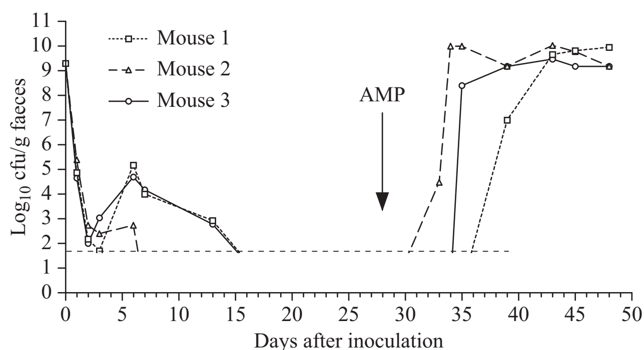

Figure 2. cfu counts of *K. pneumoniae* from faecal samples of mice. Arrow indicates the shift in drinking water from 0 to 0.5 g/L ampicillin (AMP). The detection limit is illustrated by the dashed line. cfu of the inoculation suspension is shown at day 0 (2×10^9 cfu/mouse).

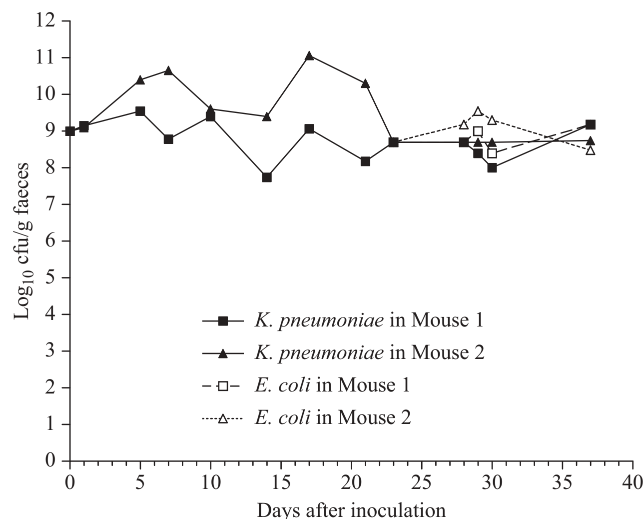

Figure 3. cfu counts of *K. pneumoniae* from faecal samples of mice treated with 0.5 g/L ampicillin. The level of *K. pneumoniae* (filled squares and filled triangles) and indigenous *E. coli* transconjugants in Mouse 1 and 2 (open squares and open triangles). cfu of the inoculation suspension is shown at day 0 (1×10^9 cfu/mouse).

towards ampicillin and kanamycin—no bacteria were detected on the selective plates. On day 0, the mice were inoculated with MGH78578 (1×10^9 cfu/mouse), which colonized the intestine in high numbers 10^7–10^{10} cfu/g faeces (Figure 3). At day 23, non-mucoid colonies were noticed on the selective plates (containing ampicillin and kanamycin) at levels of 3×10^8–4×10^9 cfu/g faeces. Biochemistry assays identified the unknown colony to be an *E. coli* that is able to grow on the selective plates. The indigenous *E. coli* and the *Klebsiella* strains co-colonized the intestine throughout the experiment (Figure 3). The indigenous *E. coli* was resistant to ampicillin, kanamycin and cefpodoxime, and intermediately resistant to amikacin, cefotaxime and tobramycin, which is an antimicrobial profile similar to that of *K. pneumoniae* MGH78578 (Table 1, TC IG *E. coli vivo*). The indigenous *E. coli* strain was also confirmed ESBL-positive with Etest ESBL (CTX/CTX + CLA = deformation of CTX zone/0.032 and CAZ/CAZ + CLA =

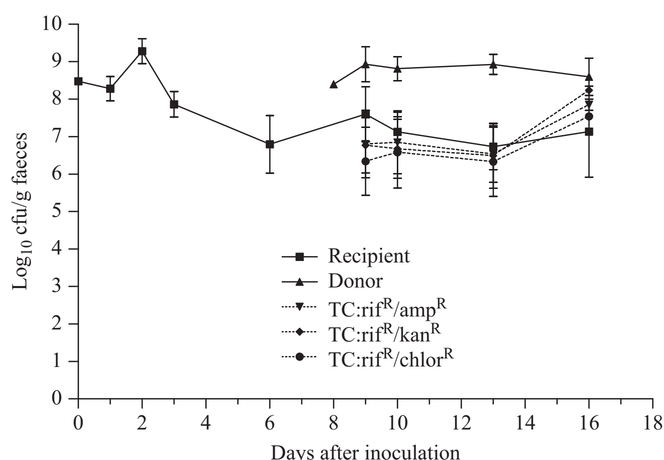

Figure 4. cfu counts of donor and recipient inoculated in three mice treated with 0.5 g/L streptomycin. Recipient *E. coli* MG1655SR (filled circles) was inoculated first, and at day 7, the clinical *K. pneumoniae* strain (filled triangles) was introduced. Transconjugants (TCs) selected on different media: rifampicin and ampicillin (filled upside-down triangles), rifampicin and kanamycin (filled diamonds), and rifampicin and chloramphenicol (filled circles). cfu of the inoculation suspension of *E. coli* MG1655SR shown at day 0 (3×10^8 cfu/mouse) and inoculation suspension of *K. pneumoniae* at day 7 (3×10^8 cfu/mouse) (not depicted).

deformation of CAZ zone/0.064). ESBL PCR revealed the presence of *shv* and *tem* genes, which were also present in the *K. pneumoniae* strain used for colonization (data not shown). A plasmid profile revealed that the indigenous *E. coli* contained a plasmid of ~108 kb (Figure 1a, lane 6), which corresponds to the second largest plasmid in the MGH78578 strain—the indigenous *E. coli* was in fact a transconjugant.

The indigenous *E. coli* transconjugant was used as a donor in order to assess the transferability of resistance genes. The indigenous *E. coli* transconjugant showed good ability of secondary transfer to recipient *E. coli* MG1655SR. The frequency of transfer in the liquid mating experiment was 1.26×10^{-3} transconjugants/recipient on average; however, no transconjugants were detected from mating on solid media. Plasmid and resistance profiles were as expected—plasmid of 108 kb (Figure 1a, lane 7) and resistance to ampicillin, kanamycin and cefpodoxime, and intermediate resistance towards amikacin, cefotaxime and tobramycin (Table 1, TC MG *vitro* 3). ESBL was confirmed with Etest (CTX/CTX + CLA = deformation of CTX zone/0.016 and CAZ/CAZ + CLA = deformation of CAZ zone/0.064) and ESBL PCR gave *shv* and *tem* genes (data not shown). The PFGE profile of the indigenous *E. coli* transconjugants verified the difference from MG1655 (Figure 1b, comparing lanes 2 and 6). The transconjugants from the secondary transfer study showed a profile identical to the transconjugants obtained from the conjugation between *Klebsiella* MGH78578 and MG1655SR *in vitro*—containing the ESBL genes (Figure 1b, lanes 5 and 7). Comparing all three transconjugant PFGE profiles (Figure 1b, lanes 5–7), there are similarities around the marker size 104 kb, which is consistent with the size of the plasmid containing the ESBL genes.

Gene transfer in the intestine of streptomycin-treated mice

To explore the transferability of the clinical *K. pneumoniae* strain to a specific *E. coli*, the streptomycin-treated mouse model was used. The mice were tested prior to inoculation and no resistant bacteria were present in faeces either before or after 24 h of streptomycin treatment. The recipient *E. coli* MG1655SR (3×10^8 cfu/mouse) was inoculated 1 week prior to the donor MGH78578 (3×10^8 cfu/mouse) to allow the strain to colonize the intestine. The recipient *E. coli* strain colonized the gut at a level of 10^7 cfu/g faeces throughout the experiment (Figure 4). The donor was inoculated at day 8 and colonized the intestine at 10^8–10^9 cfu/g faeces. Transconjugants in all three mice were detected the day after introduction of the donor and colonized throughout the experiment. The level of transconjugants was equal to that of the recipients, suggesting that all recipients become transconjugants (Figure 4).

Transconjugants were verified by plasmid and resistance profiles. The isolated transconjugants showed identical resistance profiles and all contained a large plasmid of ~89 kb, which corresponds to the third largest plasmid in the donor strain (Figure 1a, lane 8). Resistance patterns showed resistance towards ampicillin, kanamycin and chloramphenicol, and intermediate resistance towards amikacin as the donor strain (Table 1, TC MG *vivo*). The transconjugants from the *in vivo* conjugation experiments were able to re-transfer the plasmid to a nalidixic acid-resistant *E. coli* MG1655 derivate at a frequency of 1.94×10^{-2} transconjugants/recipients from mating in liquid media. All tested transconjugants showed resistance profiles and plasmid profiles as expected—resistance towards ampicillin, kanamycin and chloramphenicol, and intermediate resistance towards amikacin (data not shown).

Discussion

K. pneumoniae is a nosocomial pathogen often isolated from ICU patients. Outbreak of *Klebsiella* in ICUs has been described relatively frequently;[19–22] a recent cohort study observed that 52% of the *K. pneumoniae* infection was caused by patient-to-patient transmission.[23] *K. pneumoniae* MGH78578, a clinical isolate, was used to investigate the colonization ability of an opportunistic pathogen to colonize the gastrointestinal tract with and without antimicrobial treatment and to investigate whether the antimicrobial resistance genes are transferred in the gastrointestinal tract.

In this study, it was experimentally shown that antimicrobial treatment provides a major advantage to bacteria harbouring antimicrobial resistance genes. Thus, it was shown that *K. pneumoniae* was able to proliferate quickly from undetectable numbers and colonize the gut in high numbers within a few days, during the antimicrobial treatment (Figure 2). This experimental model mimicking a patient treated with antimicrobial agents revealed a time line that indeed explains how patients, admitted to hospitals, within days of antimicrobial treatment get colonized with resistant strains—resistant to the antimicrobial agents given as therapy. Graffunder *et al.*[24] also concluded that giving third-generation cephalosporins or aminoglycosides is a risk factor that enhances the acquisition of ESBL bacteria by adding selective pressure.

Besides the selection of antimicrobial-resistant strains during therapy, *K. pneumoniae* transferred plasmids containing resistance genes to an indigenous *E. coli*. The transferability of these plasmids was assessed in the *in vivo* model using *E. coli* MG1655SR as a specific recipient. Transconjugants were

detected 24 h after inoculation of the donor in all three mice at a level of 10^6–10^8 cfu/g faeces. All the transconjugants contained a common large plasmid (89 kb) from *K. pneumoniae* strain resistant to ampicillin, kanamycin and chloramphenicol and intermediately resistant to amikacin.

Furthermore, we found that which plasmids are transferred is influenced by environmental conditions (*in vitro* versus *in vivo*). *In vitro* experiments showed transfer of the 108 or 157 kb plasmid, while the *in vivo* conjugation experiment showed transfer of the 89 kb plasmid. Nevertheless, transfer of the 108 kb plasmid was also observed in the first *in vivo* colonizing experiment where the plasmid was transferred to the indigenous *E. coli*.

Plasmids from the clinical *K. pneumoniae* strain were seen to be highly conjugative. Thus, both plasmids (89 and 108 kb) were transferred at high frequency to another recipient *E. coli*. This also shows that the plasmids are conjugative on their own.

Transfer of antimicrobial resistance genes has been investigated by different methods, both *in vitro* (filter, plate and liquid mating)[25,26] and in animals—'worst-case' models such as gnotobiotic rats or mice,[27–30] and conventional colonization models such as the streptomycin-treated mice used in this study.[31,32] Using the animal model containing normal bacteria, flora gives more realistic results than any *in vitro* or gnotobiotic study. The normal bacterial flora barrier and the present immune system give the used animal model advantages in mimicking the human gastrointestinal tract. Transfer *in vivo* cannot be calculated from the extrapolation of *in vitro* experiments.

The indigenous flora can act as a reservoir and transfer resistance genes to pathogenic bacteria that might lead to infections with limited treatment possibilities. Transfer of any antimicrobial resistance genes is a threat, but transfer of ESBL resistance genes is in a category of its own, which might result in the limitation of treatment and in worst cases treatment failure.[33,34] Transfer of ESBL *shv* and *tem* genes from *K. pneumonia* to *E. coli* was obtained both in *in vitro* and *in vivo* studies—an illustration of how quickly ESBL genes can be spread.

The fact that transfer of antimicrobial resistance genes happens in patients has been previously reported by Karami *et al*.[35] where transfer of ampicillin resistance genes between two *E. coli* strains was observed in the gastrointestinal tract of infants. Also, Bidet *et al*.[36] described plasmid pACC-1 harbouring a β-lactamase resistance gene being transferred from *K. pneumoniae* to an *E. coli* during the antimicrobial treatment of infants. Our studies clearly show that transfer can occur at a relatively high level in the gastrointestinal tract of mice.

This study also clearly shows that resistant strains can be present in the gastrointestinal tract at a low level, and after the antimicrobial treatment, they can be selected to colonize the intestine. The ecological effect of antimicrobial treatment on the commensal microflora warrants further study in the future. The rational use of antimicrobial agents together with infection control will aid in controlling further spread of these multiresistant bacteria.

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Transparency declarations

None to declare.

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