Natural transfer of sulphonamide and ampicillin resistance between *Escherichia coli* residing in the human intestine

Margarita Trobos¹,², Camilla H. Lester¹, John E. Olsen², Niels Frimodt-Møller¹ and Anette M. Hammerum¹*

¹National Center for Antimicrobials and Infection Control, Statens Serum Institut, Artillerivej 5, 2300 Copenhagen S, Denmark; ²Department of Disease Biology, Faculty of Life Sciences, University of Copenhagen, Stigbøljen 4, 1870 Frederiksberg C, Denmark

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**Objectives:** The aim of this study was to investigate whether the sulphonamide resistance gene sul2 could be transferred between *Escherichia coli* in the human gut.

**Methods:** Nine volunteers ingested a $10^9$ cfu suspension of sulphonamide-susceptible, rifampicin-resistant *E. coli* recipients of human origin. Three hours later, they ingested a $10^7$ cfu suspension of a sulphonamide-resistant (MIC > 1024 mg/L) *E. coli* donor of pig origin. Stool samples were collected 24 h prior to ingestion, daily for 7 days and at days 14 and 35. Samples were plated on selective plates and monitored for the acquisition of sulphonamide-resistance by the recipient from the indigenous or administrated donor *E. coli*. Possible transconjugants were typed by PFGE and tested for the presence of plasmids containing the sul2 gene, which was also sequenced.

**Results:** Concentrations of the human and animal *E. coli* reached a maximum of $7.5 \times 10^6$ cfu/g faeces and colonized for more than 7 days, and $2 \times 10^8$ cfu/g for more than 14 days, respectively. On day 2, a transconjugant was detected in one volunteer. This volunteer was colonized with sulphonamide-resistant *E. coli* at day 0. The transconjugant was sul2-positive, had an MIC > 1024 mg/L for sulfamethoxazole and the same PFGE profile as the recipient. The resident *E. coli* transferred a plasmid (>63 kb), containing the sul2 gene, to the recipient. The sul2 sequence of the transconjugant was identical to that of the volunteer’s own *E. coli* from day 0, but differed from the animal strain. Co-transfer of ampicillin resistance was also demonstrated.

**Conclusions:** Transfer of sul2 was observed between *E. coli* bacteria in the human intestine. The transconjugant’s sul2 gene came from the volunteer’s own flora. The origin of the *E. coli* donor is unknown.

Keywords: *E. coli*, animal, transfer, sulphonamide resistance, human gut

**Introduction**

Endogenous bacterial flora may play an important role as acceptor and donor of transmissible drug resistance genes. *Escherichia coli* is commonly found as a commensal in the intestinal tract, but may also be implicated in intestinal and extra-intestinal infections. Sulfamethizole is one of the first treatments of choice for uncomplicated urinary tract infections in Denmark.¹ The sul2 gene encodes sulphonamide resistance and is the most common gene found among sulphonamide-resistant *E. coli* from different animals, food, healthy humans and human infections.²–⁸

Resistance to sulphonamides caused by sul1 and sul2 was shown to be common among normal faecal flora *E. coli* of healthy fattening pigs in 10 herds with different antimicrobial use.⁹,¹⁰ The highest number of antimicrobial-resistant *E. coli* was from herds with a high use of antimicrobial agents. Non-pathogenic *E. coli* from pigs may thus represent a considerable reservoir of antimicrobial resistance genes that might be transferable to pathogens.

There have been numerous studies of resistance transfer *in vitro*, but few studies *in vivo* in humans. *In vitro* transfer of resistance genes is relatively easy to demonstrate and *in vivo* transfer in the intestine of germ-free or antibiotic-treated...
experimental animals has also been shown to occur.\textsuperscript{11–14} These transfers have been attained by modifying the normal flora of the intestinal tract.

There are very few reports of antimicrobial resistance transfer in man. In 1969, Smith\textsuperscript{15} carried out an experiment in which antibiotic resistance (R factors) was transferred to the resident \textit{E. coli} in the gut of a human from ingested cultures of \textit{E. coli} of animal and human origins. The amount of transfer was small and the resistant resident organisms did not persist in the gut for long. In 1970, Wiedemann et al.\textsuperscript{16} failed to demonstrate \textit{in vivo} transmission of drug resistance from an \textit{E. coli} to other enterobacteria in the gut of four human subjects.

There are several previous studies where antimicrobial agents were administered in parallel to the administration of \textit{E. coli} strains to study their colonization and transfer in the gut. In 1974, Burton et al.\textsuperscript{17} showed that the oral administration of tetracycline enhanced the colonization and the transfer of antibiotic resistance from an ingested \textit{E. coli} of animal origin to the resident \textit{E. coli} in the intestinal tract of humans. Transfer of the \textit{bla\textsubscript{TEM-1}} gene between two strains co-residing in the gut of a child treated with ampicillin was also demonstrated.\textsuperscript{18} \textit{In vivo} inter-bacterial transfer of the R factor was observed in two patients. It was transferred from \textit{E. coli} in the gut of two patients to a \textit{Shigella sonnei} and a \textit{Proteus mirabilis}, respectively.\textsuperscript{19} Indications of transfer of antimicrobial resistance in clinical cases have been observed, but controlled experiments with healthy humans showing transfer of resistance genes between \textit{E. coli} under natural conditions are rare and not recent.

Some of the above-mentioned studies were published many years ago, before genotyping was widely available. The results of such studies should be viewed within the limits of their experimental design. Since we had the possibilities of using the latest molecular typing techniques, this is one of the methodological strengths of our study and our human model can assess with certainty if transfer of resistance genes has taken place.

The objective of this study was to investigate whether the sulphonamide resistance gene \textit{sul2} could transfer the human gut between \textit{E. coli} bacteria; either from a pig-derived sulphonamide-resistant \textit{E. coli} to a recipient of human origin provided, which would shed light on the risk associated with resistant \textit{E. coli} in the food, or from \textit{E. coli} in the resident flora to the recipient. As a side observation, the study also allowed us to measure to what extent a pig-derived \textit{E. coli} could colonize the human gut and for how long. Such data are necessary to perform risk assessment of human intake of food containing resistant bacteria.

\section*{Materials and methods}

\subsection*{Study subjects}

The study was conducted with nine healthy human volunteers from February 2007 to February 2008. All were older than 18 years with normal intestinal functions. None had consumed antimicrobial agents 1 month prior to or during the study, and none took other medicines during the experiment. All volunteers were enlisted on an informed written consent basis. The study protocol for the healthy volunteers participating in this study was approved by the Scientific Ethics Committee for Copenhagen and Frederiksberg municipalities [(KF)01-324866].

\subsection*{Bacterial strains}

The donor strain was an \textit{E. coli} of animal origin, isolated from a Danish healthy pig in 2003.\textsuperscript{20} It was resistant to sulphonamides (MIC $>1024$ mg/L) and gentamicin (MIC $>32$ mg/L). Gentamicin was used as a second marker, due to the very low rate of resistance in the Danish healthy human population.\textsuperscript{20}

The recipient strain was \textit{E. coli} K-12 (MG1655); rifampicin-resistant (MIC $>256$ mg/L). It has a human origin.\textsuperscript{21} The original \textit{E. coli} strain K-12 was obtained from a stool sample of a diphtheria patient in Palo Alto, CA, USA, in 1922.

Both donor and recipient strains had a different morphology (the recipient especially had a very distinct one) and were able to grow in different selective plates (Table 1) showing a different resistance profile. In order to confirm the phenotype of the donor and recipient colonies isolated from each volunteer’s faecal sample, both the morphology and their ability to grow in the selective plates were investigated.

\subsection*{Media}

The selective plates used to count \textit{in vitro} transconjugants were: (i) LB agar (Sigma-Aldrich, Denmark A/S) added sulfamethizole (1024 mg/L), gentamicin (8 mg/L) and rifampicin (50 mg/L); (ii) sulfamethizole (1024 mg/L) and rifampicin (50 mg/L); and (iii) gentamicin (8 mg/L) and rifampicin (50 mg/L) (suppliers in Table 1). The plates were incubated at 35°C for 24 h, and the colonies were counted. Selected transconjugants were frozen at $-40^\circ$C in 10% glycerol for further investigation. The selective plates used for the \textit{in vivo} study are displayed in Table 1. Discovery agar is an agar that incorporates a chromogenic medium enabling detection of \textit{E. coli} by colonies displaying a salmon red colour.

\subsection*{In vitro matings}

\textit{In vitro} matings were performed to investigate transferability of the resistance genes \textit{sul2} and \textit{aac(3)-IV}, encoding sulphonamide and gentamicin resistance, respectively, between the donor and recipient strains. Broth mating experiments were performed as follows: donor and recipient strains from blood agar plates were suspended in 5 mL of 0.9% saline until OD (546 nm) of 0.13 ($=10^8$ cfu/mL). To 8 mL of pre-warmed LB broth, we added 800 µL of the recipient solution and 80 µL of the donor solution (ratio 10:1). The culture was incubated for 4 h at 37°C and shaken (200 rpm). Serial dilutions of the mixed culture were made until 10$^{-6}$ in 0.9% saline. From the undiluted and the six dilutions, we performed cfu counting (double measurements). All were done in triple replicas and the mating experiments were repeated twice.

\subsection*{Preparation and ingestion of \textit{E. coli} suspensions}

\textit{E. coli} suspensions of donor and recipient were prepared as follows. The strains were grown overnight at 35°C on 5% blood agar (SSI Diagnostica, Hillerød, Denmark). A suspension of the recipient strain was prepared to reach an OD (546 nm) of 1.0 in 0.9% saline equivalent to $10^9$ cfu/mL; 1 mL of this suspension was added to 250 mL of pasteurized whole milk to obtain a total count of $10^8$ bacteria in 250 mL of whole milk, which was immediately ingested by each volunteer. Three hours later, a suspension of the donor strain was prepared in 0.9% saline until it reached an OD (546 nm) of 0.15 ($=10^8$ cfu/mL). Donor suspension (100 µL) was added to 250 mL of milk to obtain a total count of $10^7$ bacteria, immediately after it was ingested by each volunteer. All bacterial
Antimicrobial-supplemented medium* | Day of collection of stool sample | Use
--- | --- | ---
Discovery agar base supplemented with 2 mg/L amphotericin B | 0 | for total count of the volunteers’ own E. coli before the study, two colonies were isolated from each for susceptibility testing
Discovery agar base supplemented with 1024 mg/L sulfamethizole and 2 mg/L amphotericin B | 0 (1, 2)* | as negative control to make sure that none of the volunteers was colonized by sulfonamide-resistant E. coli before the study
Discovery agar base supplemented with 1024 mg/L sulfamethizole, 8 mg/L gentamicin and 2 mg/L amphotericin B | 0–7, 14, 35 | for detection of the donor strain and transconjugants
Discovery agar base supplemented with 50 mg/L rifampicin and 2 mg/L amphotericin B | 0–7, 14, 35 | for detection of the recipient strain and transconjugants
Discovery agar base supplemented with 1024 mg/L sulfamethizole, 50 mg/L rifampicin and 2 mg/L amphotericin B | 0–7, 14, 35 | for detection of transconjugants only

*Sul2: 5’-GTG TGT GCG GAT CAA GTC AG-3’ (start position at 747). The multiplex PCR kit from Qiagen (Ballerup, Denmark) was used with the following amplification conditions: heating for 15 min at 94°C, followed by 30 cycles at 94°C for 30 s, 48°C for 90 s and 72°C for 90 s, followed by 72°C for 10 min. E. coli NCTC 50020 was used as positive control and E. coli DH5α plasmid pUVP4401 as negative control.6

The sul2 (727 bp) and blaTEM (918 bp) PCR products were purified using the MinElute® PCR purification kit (Qiagen) and sequenced at Macrogen (Seoul, Korea). For the blaTEM gene, two extra primers were designed inside the gene for better sequencing quality: blaTEM-m-R (AAA GCG GTT AGC TCC TTC GG) and blaTEM-m-F (TCA CCA GTC ACA GAA AAG CAT CCT AC). These primers were synthesized by Macrogen and used for sequencing. The sequences were analysed with Vector NTI software and compared by multiple alignment (ClustalW) with those of the animal donor or the volunteer’s own E. coli, if positive to these genes (only in one volunteer).

**Table 1.** Selective media used to detect the donor strain, the recipient strain and transconjugants in the in vivo transfer experiments
Transfer of sul2 in the human intestine

2 μL of SpeI). Only 50 μL of restriction enzyme mixture was added to each tube for incubation. The digested DNA was electrophoresed in a 1% agarose gel in 0.5× TBE using a CHEF DR II apparatus (Bio-Rad, Herlev, Denmark). The programme was 4–12 s for the first 8 h and then 15–25 s for 16 h, at 150 mA. Lambda Ladder PFG Marker NO340 (>50 kb) (New England BioLabs) was used as a molecular size marker.

Plasmid profiles

Small-scale preparations of purified E. coli plasmid DNA were performed using the High Pure Plasmid Isolation Kit (Roche Diagnostics, Mannheim, Germany). Purified plasmids were run in a 1% electrophoresis gel together with E. coli V517 and 39R861 used as size markers. V517 has eight plasmids (54, 7.2, 5.6, 5.1, 4.4, 3.0, 2.7 and 2.0 kb) and 39R861 has four (147, 63, 36 and 7 kb).25,26

Southern blot hybridization of the sul2 gene

Plasmid DNA was transferred to a nylon membrane (Hybaid-N filter, Amersham Biosciences, UK).27 A digoxigenin (DIG)-labelled DNA probe for sul2 was prepared by PCR amplification, using primers and conditions reported by Kerr et al.6 and the PCR DIG Probe Synthesis Kit (Roche Diagnostics). Hybridization was carried out at 64°C and detection was performed using the DIG Wash and Block Buffer Set (Roche Diagnostics).27

Results

Colonization by the E. coli of animal origin

The variations of the concentrations of the donor and recipient strains in faeces during the study are shown in Figure 1. Prior to the study (day 0), the volunteers were not colonized with sulphonamide-resistant E. coli (except for the volunteer marked in red in Figure 1).

The animal donor strain colonized the gut of the volunteers for more than 2 weeks but <1 month, and reached a maximum of 1.4×10⁴–2.1×10⁶ cfu/g faeces on days 1–6, depending on the subject. The human recipient strain was detected for up to a maximum of 7 days (the majority were not shed in the faeces after day 5). It reached a maximum of 2.1×10¹–7.5×10⁵ cfu/g faeces on day 1 or 2 (only one subject on day 6). At the end of the study (day 35), no sulphonamide-resistant E. coli were detected in the stools of any volunteer (Figure 1). One of the volunteers did not shed the donor and recipient strains after their ingestion, and they appear as zero in both graphs.

Transfer of resistance

In vitro broth mating showed that sul2 transferred easily from the pig-derived E. coli strain to the human recipient with a mean transfer rate of 1×10⁻² transconjugants/recipient. Transfer of the sul2 gene to the recipient strain occurred at detectable levels in the intestines of one volunteer (Figure 1). A transconjugant was recovered on day 2. This subject already had sulphonamide-resistant E. coli in the gut before the start of the experiment. The transfer rate found was 5×10⁻¹⁴ transconjugants/recipient.

There was also a co-transfer of the blαTEM-1c gene encoding ampicillin resistance.

Since gentamicin and sulfamethizole did not co-transfer in vivo as in vitro, we tried to see if the gentamicin resistance gene aac(3)-IV would transfer alone in the intestines of three human volunteers. No transfer was detected (data not shown).

Confirmation of the transconjugant

Antimicrobial susceptibility testing showed that the transconjugant was resistant to sulfamethoxazole (MIC >1024 mg/L). The presence of the sul2 gene was confirmed by PCR. The transconjugant was also resistant to ampicillin (MIC >32 mg/L), and

Figure 1. Faecal excretion of E. coli strains of animal and human origins by the nine volunteers. (a) Animal donor strain. (b) Human recipient strain. Each curve shows the results for one subject. Each volunteer is represented by one colour. One of the volunteers did not shed the donor and recipient strains after their ingestion and they appear as zero in both graphs. Results from stool samples obtained within 24 h before ingestion of the bacteria are plotted as day 0. The minimal detectable level is shown by the broken line. The black open squares (days 0, 14 and 35) represent the superposition of results from all volunteers. The transconjugant excreted on day 2 is represented in (b) by a red asterisk and was isolated from the red volunteer. The gene transfer occurred from the volunteer’s own E. coli which on day 2 was 10⁷ cfu/g faeces.
the presence of the blaTEM-1c gene was confirmed by PCR. Sequences of both genes (sul2 and blaTEM-1c) were compared with those from the animal donor and the volunteer’s own sulphonamide-resistant \textit{E. coli}. The sul2 and blaTEM-1c genes in the transconjugant showed 100% identity to the genes from the volunteer’s own \textit{E. coli} from day 0. Both genes differed in 1 bp from those of the animal donor. PFGE typing of the \textit{in vivo} and \textit{in vitro} transconjugants showed the same profile as the recipient. Furthermore, after comparing the plasmids profiles, the volunteer’s resident \textit{E. coli} donor and the transconjugant contained a plasmid of the same size (>63 kb) (Figure 2a). The sul2 gene was localized on that plasmid (Figure 2b). This demonstrates that a plasmid bigger than 63 kb containing the sul2 gene was transferred from an \textit{E. coli} of the volunteer’s own flora to the recipient.

**Discussion**

This study shows that resistance genes can transfer among \textit{E. coli} residing in the human intestines. The sul2 gene was transferred together with blaTEM-1c gene coding for ampicillin resistance. This indicates that co-transfer of several resistance genes at the same time is possible. The transfer most probably occurred from this person’s own \textit{E. coli} to the recipient, due to the same identity of both genes sul2 and blaTEM-1c from the resident and transconjugant strains.

In the volunteer where the transfer took place on day 2, both the resident and recipient strains reached high concentrations (>106 cfu/g). This suggests that in order for the transfer to occur, high numbers of both donor and recipient should coincide at the same time in the gut, as suggested in previous observations.28

Our study was carried out without any selective pressure and the duration of colonization and transfer frequency would probably have been increased with the administration of antimicrobial agents. In support of this assumption, Anderson et al.29 did not obtain R-factor transfer in the absence of chemotherapy in four subjects. On the contrary, they proved R-factor transfer in the gut of three subjects when a 5 day course treatment with antibiotic was applied after ingestion of donor and recipient suspensions. We did not do this because of the ethical implications. \textit{In vitro} transfer results cannot be directly extrapolated to the \textit{in vivo} situation. In this study, the \textit{in vitro} transfer rate was higher than the \textit{in vivo} rate, but there are several explanations for this, e.g. the competitive flora of the gut, lack of selective pressure and less chances for donor and recipient to meet. Netherwood et al.30 showed that \textit{in vitro} methods, such as forced filter mating and liquid mating, underestimate the \textit{in vivo} rate of gene transfer. The methods used to detect transfer \textit{in vivo} have severe sensitivity limitations and tend to underestimate the true rate of transfer \textit{in vivo}.

In the present study, the volunteer in whom the transfer took place was colonized by sulphonamide-resistant \textit{E. coli} and was positive for the sul2 gene before the start of the experiment. The sul2 gene in the animal donor strain differed from the transconjugant by a mutation to T instead of a G at position 672 (data not shown). The type detected in the transconjugant has previously been associated mainly with poultry isolates based on a sequence comparison of isolates from different sources (M. Trobos, H. Christensen, M. Sunde, S. Nordentoft, Y. Agersø, G. S. Simonsen, A. M. Hammerum and J. E. Olsen, unpublished results). The association is made possible by the fact that the sul2 gene is conserved with mainly two varying positions. The volunteer had a mostly vegetarian diet, eating chicken only sporadically. Since the transconjugant had the sul2 sequence found mostly among chicken, a chicken origin of the \textit{E. coli} is likely, but it cannot be ruled out that other sources for this sequence type have remained undiscovered.

Linton et al.31 studied whether resistant \textit{E. coli} strains from commercially purchased chickens were transferred and established as part of the flora of human consumers. One of five volunteers became colonized by five of the 15 resistant \textit{E. coli} strains found in one chicken after thawing. The strains stayed in the faecal flora for 10 days. A resistant faecal normal flora in meat-producing animals may thus have an impact on the resistance of the normal flora of human consumers. In a study by Johnson et al.,32 molecular typing methods matched four turkey-source isolates to selected human clinical and faecal isolates representing the clonal group A of extra-intestinal pathogenic \textit{E. coli} (ExPEC). Meats purchased in grocery stores, particularly turkey products, were frequently contaminated with antimicrobial-resistant \textit{E. coli} and ExPEC.

Smith33 concluded that \textit{E. coli} strains of animal origin were poorer colonizers than strains of human origin. In this study, we showed the opposite, that the strain of animal origin was more persistent in the gut than the human strain. A possible explanation could be that our recipient strain, \textit{E. coli} K12, has been cultivated in the laboratory for so long that it has lost the ability to colonize humans.33 The poor survival of the recipient strain might have been the reason why transfer was low. This corresponds to previous survival studies of \textit{E. coli} K12 where it only survived for few days (maximum six) in the human intestine, but was ultimately eliminated.34,35 In these two studies, they could, however, transfer hybrid plasmids from \textit{E. coli} K12 to the resident \textit{E. coli}.

In addition, the strain of animal origin reached higher numbers in the gut, even though it was administered in 100-fold

![Figure 2.](image-url)
lower numbers than the recipient. This was done because we wanted to mimic the situation of healthy humans eating meat containing sulphonamide-resistant *E. coli* and see for how long they would colonize the gut. If the strain had persisted for more than 2 weeks after one ingestion only, we could expect a longer persistence in the gut when people consume meat several times per week. This would provide a continuous flow of resistance genes that could transfer to the human indigenous flora.

One of the volunteers did not shed the donor and recipient strains after their ingestion, and they appear as zero in Figures 1 and 2. A possible explanation could be that this person did not ingest enough food with the bacterial suspensions and the gastric acids killed them, or they were at a competitive disadvantage with the indigenous flora.

To investigate how well our recipient strain colonized, we performed a pilot study where three human volunteers ingested only the recipient strain. It colonized for a period of 3–5 days (data not shown). Furthermore, during the follow-up of these three volunteers, in one person at days 3 and 35, *E. coli* resistant to tetracycline from his own flora were detected (6 × 10⁴ cfu/g and 10⁶ cfu/g, respectively), while another person contained >5 × 10⁵ cfu/g *E. coli* resistant to tetracycline, and to both sulphonamide and gentamicin (>10³ cfu/g) on days 14 and 35. *E. coli* resistant to these antibiotics were below the detection level at the start of the experiment. This underlines that *E. coli* of unknown origin (food, animals or environment), which colonize the gut for specific days may represent a pool of resistance genes that might transfer to other *E. coli* of the gut flora. It seems that the stability of the *E. coli* intestinal flora is dependent on what is swallowed. In 1970, Cooke *et al.* found 63 samples of food out of 873 containing *E. coli* numbers between 25 and 10⁶ cfu/g in a hospital. An identical faecal serotype of *E. coli* from food and five patients was isolated. All the serotypes found in the food and in the patients were also isolated from meat and poultry on or before its arrival in the hospital.

Even with the low concentration of the recipient strain in the volunteers’ gut in the current study, a transconjugant was detected. There would be a higher possibility of transfer to the resident *E. coli* (10⁶–10⁷ cfu/mL) that are present in the human gut. If food containing a resistant bacterial strain is ingested in sufficient amounts, and the strain is able to colonize the human gut, we have demonstrated the possibility of transfer of resistance genes to the resident bacteria in the intestinal flora. However, studies with larger numbers of individuals are required to ascertain the actual transmission rate between resistant animal donor strains and resident recipient flora.

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**Transparency declarations**

None to declare.

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