Meta-Analysis of Experimental Data Concerning Antimicrobial Resistance Gene Transfer Rates during Conjugation^{∇†}

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This paper presents the results of a meta-analysis of published transfer rates of antimicrobial resistance genes. A total of 34 papers were identified, of which 28 contained rates estimated in relation to either donor or recipient bacterial counts. The published rates ranged from 10^{-2} to 10^{-9} . Generalized linear modeling was conducted to identify the factors influencing this variation. Highly significant associations between transfer frequency and both the donor ($P = 1.2 \times 10^{-4}$) and recipient ($P = 1.0 \times 10^{-5}$) genera were found. Also significant was whether the donor and recipient strains were of the same genus ($P = 0.023$) and the nature of the genetic element ($P = 0.0019$). The type of experiment, in vivo or in vitro, approached statistical significance ($P = 0.12$). Parameter estimates from a general linear model were used to estimate the probability of transfer of antimicrobial resistance genes to potential pathogens in the intestine following oral ingestion. The mean logarithms of these probabilities are in the range of $[-7.0, -3.1]$. These probability distributions are suitable for use in the quantitative assessment of the risk of transfer of antimicrobial resistance genes to the intestinal flora of humans and animals.

Antimicrobial resistance poses a significant, serious, and increasing threat to modern health care (54). Patients infected with or even only colonized by resistant pathogens are reported to suffer greater mortality, require longer hospital stays, and cost the health service substantially more than patients with antimicrobial-sensitive infections (e.g., see references 21, 33, and 53). Although more attention has quite naturally been focused on hospitals as the primary reservoir and place of transmission of many antimicrobial-resistant pathogens (15, 17, 24, 25), recent years have seen increased interest in the role of the nonhospital community as a significant reservoir of such resistant pathogens (68).

When considering the epidemiology of antimicrobial-resistant pathogens outside of hospitals, the role of transmission in food has been highlighted by several authors (1, 59). The most interest has been concerned with the presence of antimicrobial-resistant pathogens such as *Salmonella* or *Campylobacter* in foods (1, 2, 5, 22, 28, 44, 48, 60). However, in recent years there has also been increasing concern about antimicrobial resistance in food bacteria that are not in themselves generally pathogenic or likely to be long-term colonizers of the gastrointestinal tract. The suggestion is that these bacteria may transmit antimicrobial resistance through conjugation that results in the host's own microflora becoming resistant (7, 32, 40, 57, 62). This issue has particular resonance for probiotics, where there is also some debate over whether antimicrobial resistance genes in probiotic bacteria could transfer to host commensal bacteria that would then pose a risk to health (27, 55, 63).

One of the problems when assessing the risk of antibiotic

resistance through gene transfer from food-borne bacteria is that there is no clear understanding of the transfer rates associated with various bacterial species. Existing studies give rates that are very different from each other, in part because of the different bacterial strains and different methodologies used. At present, it is not possible to construct second-order quantitative risk assessments for the transmission of antimicrobial resistance from organisms in food because the uncertainty in gene transmission rates is unknown. This study was undertaken to quantify this transmission frequency in order to inform a subsequent quantitative risk assessment.

MATERIALS AND METHODS

Electronic literature databases were searched up to March 2008. The ISI Web of Knowledge and PubMed databases were searched by using combinations of keywords including "bacteria," "antibiotic," "antimicrobial," "resistance," "gene," "transfer," "conjugation," "conjugal," "gastrointestinal," "intestine," "gut," "in vitro," "in vivo," "plasmid," and "transposon." In addition, all of the references for each paper included in subsequent analyses were searched for further publications. Finally, all included papers were identified in the ISI Web of Knowledge database for subsequent citations.

All values concerning the rate of resistance transfer were extracted from each relevant report, in addition to information about the bacterial species and strains used (donor and recipient); the type of antibiotic resistance element under study (plasmid, transposon, or other); whether the experiment was in vitro filter, in vitro other, or in vivo; details of the methods used (transfer medium in in vitro studies and animal species and intestinal flora colonization in in vivo studies); the time at which the transconjugant population was measured; and whether the experiment was carried out in the presence of antibiotics.

The logarithms of the transfer rates were analyzed for key parameters by using SPSS (version 14.0). Analysis with single predictor variables was conducted by one-way analysis of variance (ANOVA) and with multiple predictor variables within the framework of a univariate general linear model.

Estimated probability distributions for the logarithm of the transfer rates in the intestine were derived from the parameter estimates of the general linear model by using probabilistic modeling with @Risk4.5 (Palisade Corporation, Ithaca, NY). The intercept and predictor variables were entered as normal distributions into cells of an Excel @Risk spreadsheet. The cells were summed after appropriate modification, and this was then marked as the output cell by @Risk.

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RESULTS

Altogether, 28 papers were included in the analysis (3, 4, 9, 10, 12–14, 16, 19, 23, 26, 29, 34, 36, 38, 39, 43, 45, 47, 49, 50, 51, 57, 58, 61, 64–66). Six papers were excluded because results were presented in a way that was not consistent with the main data set, for example, results presented as resistant organisms per gram of feces (20, 30, 35, 37, 41, 42); for a summary of these papers, see the supplemental material. Where transfer frequencies were presented as a range, the geometric mean was taken as the frequency. Some 128 data points were included in the analysis. An initial analysis of the transmission rates showed that the reported transfer rates exhibit a huge variation, ranging from about 10^{-2} to 10^{-9} /cell. Analysis showed that this data set could not be distinguished from a log normal distribution, and so all analyses were performed with the logarithms of the published transmission rates.

Table 1 shows the one-way ANOVA of all factors likely to influence transfer frequencies. In this initial analysis, all variables were statistically significant, with the exception of the recipient Gram reaction and whether or not transfer frequencies were calculated with reference to donor or recipient bacterial counts. These variables were dropped from further analysis. Of the other variables, the genus of the donor organism, the Gram status of the donor, the test method (whether on filter, other in vitro, or in vivo), the nature of the transferable element, and whether the transfer was to a recipient of the same genus as the donor were all highly significant ($P < 10^{-9}$).

All of the variables were then entered into a general linear model, except where variables would be highly correlated with other variables (i.e., Gram stain with genus). In this case, the most significant variable was placed into the model, except for the variable Gram direction, as this was correlated with several other variables. Within the general linear model, both full factorial and main effects models were included, but as none of the interaction terms were significant, only the main effects model is presented here. Table 2 shows the parameter estimates for the model. The R^2 value for this model was 0.676, showing that the model accounted for a substantial proportion (68%) of the observed variance of the dependent variable (the logarithm of the transfer frequency), and it was greater than for any other combination of the predictor variables. It can be seen that the main variables influencing transfer rates are the genera for the donors and recipients. The genetic element and whether the donor and recipients were members of the same genus also had significant impacts. Experiment type was not quite statistically significant in this model. Table 2 also shows the parameters for model 2, which was the same as model 1 except that the genetic element was not included, for reasons discussed below.

In order to test the predictive ability of this model, a series of jackknife estimates were done where the model was recalculated with each experiment being excluded and then the model used to predict the excluded transmission rate. The predicted transmission rates obtained by the jackknife approach were then compared with the predicted rates from the full model and with actual rates. There was no significant difference among the means of all three data (actual, 5.61; predicted from full model, 5.61; predicted from jackknife, 5.63) (one-way ANOVA; $P = 0.99$). The variance of the actual data

TABLE 1. Single-variable analysis of transmission rates

Variable	n^a	Mean log ^b	SD log ^c	P value
Donor organism				3.79×10^{-10}
<i>Escherichia coli</i>	25	−3.25	1.91	
Other gram-negative organisms	11	−5.21	2.06	
<i>Enterococcus</i> spp.	37	−6.97	1.88	
<i>Lactococcus</i> spp.	23	−5.22	2.25	
<i>Lactobacillus</i> spp.	17	−6.62	1.62	
Other gram-positive organisms	15	−5.94	1.34	
Donor Gram reaction				7.63×10^{-9}
Negative	36	−3.85	2.13	
Positive	92	−6.30	1.97	
Recipient organism				0.00118
<i>Escherichia coli</i>	32	−4.64	3.17	
Other gram-negative organisms	16	−7.13	1.77	
<i>Enterococcus</i> spp.	28	−5.62	1.84	
<i>Lactococcus</i> spp.	10	−4.15	2.44	
<i>Lactobacillus</i> spp.	17	−6.28	1.11	
Other gram-positive organisms	25	−6.01	1.34	
Recipient gram reaction				0.585
Negative	48	−5.47	3.01	
Positive	80	−5.70	1.74	
Donor and recipient same genus				3.05×10^{-11}
No	96	−6.33	1.98	
Yes	32	−3.46	1.76	
Gram reaction direction				1.73×10^{-14}
Positive to positive	74	−5.76	1.77	
Positive to negative	18	−8.53	0.82	
Negative to positive	7	−4.98	1.09	
Negative to negative	29	−3.58	2.24	
Test method				4.07×10^{-12}
In vitro, filter	84	−6.56	1.68	
In vitro, other	28	−3.46	2.14	
In vivo	16	−4.41	2.30	
Plasmid				9.16×10^{-13}
pAMβ1	42	−5.90	2.09	
RP1	12	−1.80	1.17	
Tn916	28	−7.21	1.82	
Other	46	−5.37	1.71	
Conjugates expressed as:				0.380
Donor	51	−5.43	2.90	
Recipient	57	−5.83	1.84	

^a n is the number of experiments (each study usually reported more than one experiment).

^b Mean log is the mean value of the logarithm of the transfer probability/cell.

^c SD log is the standard deviation of the logarithms of the transfer probability.

was greater, with marginal significance, but there was very little difference between that predicted by the jackknife process and the full model (5.25, 3.55, and 3.65, respectively) (Levene's test; $P = 0.046$). It is concluded that the model gives a good prediction of transmission rates but those experimental data

TABLE 2. Parameter estimates from the general linear model of the logarithm of the transfer probability

Parameter	Model 1 coefficient (B)	95% confidence interval of B		Significance	Model 2 coefficient (B)	95% confidence interval of B		Significance
		Lower bound	Upper bound			Lower bound	Upper bound	
Intercept	-3.85	-5.22	-2.48		-3.53	-4.93	-2.13	
Donor organism				0.00012				0.00013
<i>Escherichia coli</i>	0.48	-0.92	1.87		0.87	-0.59	2.32	
Other gram-negative organisms	1.53	-0.15	3.21		1.24	-0.44	2.91	
<i>Enterococcus</i> spp.	-0.94	-1.96	0.07		-1.20	-2.24	-0.15	
<i>Lactococcus</i> spp.	0.12	-1.84	2.08		-0.43	-2.08	1.23	
<i>Lactobacillus</i> spp.	-1.94	-3.67	-0.20		-2.09	-3.66	-0.53	
Other gram-positive organisms	0 ^a				0 ^a			
Recipient organism				0.00001				0.00072
<i>Escherichia coli</i>	-0.71	-1.79	0.37		-0.35	-1.34	0.64	
Other gram-negative organisms	-1.79	-3.00	-0.58		-1.54	-2.76	-0.32	
<i>Enterococcus</i> spp.	1.62	0.38	2.85		1.38	0.12	2.64	
<i>Lactococcus</i> spp.	1.99	0.47	3.51		1.82	0.25	3.40	
<i>Lactobacillus</i> spp.	0.34	-1.15	1.83		0.57	-0.98	2.12	
Other gram-positive organisms	0 ^a				0 ^a			
Donor and recipient same genus				0.023				0.000057
No	-1.05	-1.95	-0.15		-1.79	-2.64	-0.95	
Yes	0 ^a				0 ^a			
Genetic element				0.0019	NA ^b			
pAMβ1	-0.79	-2.10	0.52					
RP1	2.40	1.06	3.75					
Tn916	-0.59	-1.50	0.32					
Other	0 ^a							
Test method				0.12				0.033
In vitro, filter	-0.99	-1.98	0.01		-0.96	-1.97	0.04	
In vitro, other	-0.16	-1.14	0.83		0.40	-0.59	1.38	
In vivo	0 ^a				0 ^a			

^a Parameter set to zero as redundant.^b NA, not applicable.

are subject to greater variance, probably because of experimental error in individual experiments.

In order to estimate the probability distribution for other scenarios, the parameter estimates were derived for several distinct models. Model 2 excluded the genetic element from the list of variables on the basis that this parameter would not be known in most risk assessments. Table 3 shows the estimated probability of transfer to the human intestinal flora for several microorganisms. In estimating this probability distribution, we use parameter values that correspond to a recipient population that is an equal mixture of *Escherichia coli* and *Enterococcus*. For each organism, the predicted transfer frequency was calculated 10,000 times with parameter values being randomly sampled from the uncertainty distribution of each parameter from the general linear model. Estimates were calculated for both *E. coli* and *Enterococcus* as recipients, with the final transfer frequency being the arithmetic mean of the two. The parameter estimate for experimental design was taken to be in vivo transfer.

DISCUSSION

The results of experiments designed to quantify resistance gene transfer frequencies show a huge variation. The results presented here show that much of this variation is due to

TABLE 3. Estimated distribution parameters for the logarithm of the transfer frequencies^a

Donor	Mean log ^b	SD log ^c
<i>Escherichia coli</i>	-3.05	1.04
<i>Enterococcus</i> spp.	-4.33	0.94
<i>Lactococcus</i> spp.	-5.29	1.20
<i>Lactobacillus</i> spp.	-6.96	1.17
Other gram-negative bacteria	-3.63	1.21
Other gram-positive bacteria	-4.86	0.87

^a From model 2, based on the general linear model coefficients. These values are suitable for use in quantitative microbial risk assessments.

^b Mean log is the mean of the distribution of the logarithms of the transfer frequency.

^c SD log is the corresponding standard deviation.

differences in experimental design, choice of donor and recipient organisms, and choice of transferable element. Indeed, these factors alone account for almost 70% of the observed variation. Within the final model, the choice of donor and recipient strains had the most important impact on the transmission rate, with the genetic element also having an important impact. In the model with the genetic element variable, experimental design did not achieve statistical significance, though in model 2 this was significant, with in vivo rates being about an order of magnitude larger than those for filter mating.

Live-animal (in vivo) experiments successfully target the bacterial environment and cannot be replaced, but they prove costly to run, difficult to initiate, and hard to control absolutely (11). The usual species used in these investigations are laboratory mice; however, a few studies have investigated bacterial transfer in other host species, such as rats (6), chickens (8), pigs (31, 67), and dogs (46). Rodents typically harbor a flora very different from that of humans and farm animals, especially ruminants (18). Gnotobiotic mice are raised under sterile conditions and thus have a limited diversity of intestinal microbes. Germ-free mice may be dosed with the fecal flora of humans, pigs, or calves (18). Human fecal flora-associated mice are often used in an attempt to mimic the potential interactions between bacterial species present in the human gastrointestinal tract, but germ-free mice are sometimes used so that the indigenous microbes do not interfere and thus the "worst-case scenario" in terms of gene or plasmid transfer may be estimated (45, 47, 61). However, given the relatively small number of different in vivo experiments reported in the literature, it was not possible to analyze for these different variables in the models.

Also, because of limited experimental data, this meta-analysis was not able to include variables relating to the impact of various host factors on rates of transfer between bacteria. Tuohy et al. detected significantly fewer transconjugants in male as opposed to female human flora-associated rats and suggested that differences in microbiota composition may be caused by hormonal effects or subtle differences in microbial adhesion sites on the mucosa (61). The same study also found plasmid transfer rates to vary with diet and proposed mechanisms by which nutritional components could influence plasmid transfer. Higher-fiber diets may provide more solid surfaces upon which bacterial pairs may form, or the increased fermentation may stimulate microbial activity. A higher fat content in the diet may also have influenced plasmid transfer either directly or through increased bile production and subsequent effects on microbial colonization (61).

The mammalian intestinal tract is an ideal environment for horizontal gene transfer to occur between bacteria (34, 50, 52). In this paper, we have provided estimates of the probability of antimicrobial gene transfer from various donors within intestinal tracts. However, in natural situations, any bacteria first need to pass through the acidic stomach and small intestine in order to reach the more favorable conditions in the colon. The ability to withstand low pH levels in the upper gastrointestinal tract has previously been demonstrated in human volunteers with *Enterococcus faecium* (56). However, many bacterial species are not as acid tolerant and so will not survive passage through the stomach to be able to act as gene donors in the intestine. Clearly, therefore, the estimates we have provided

are only applicable to bacterial species able to reach the intestines.

The colon contains very dense populations (approximately 10^{12} cells/g of feces) of commensal bacteria (52) and a high diversity of species (for example, *E. coli*, *Lactococcus* sp., *Enterococcus* sp., and *Bacteroides* sp.) (61). This raises issues about how to account for bacterial diversity when including recipient genus and equal genus terms in estimates of transfer rate probability distributions. When estimating the in vivo rate of transfer to the human intestine, only *E. coli* and *Enterococcus* were modeled as recipients. Clearly, the human intestine contains a wide variety of bacterial genera but for most fecal bacteria model parameters could not be estimated from existing data. *E. coli* and *Enterococcus* were included as they represent gram-negative and gram-positive organisms, respectively, and are the most clinically relevant of the genera with sufficient experimental data to give reasonable estimates. This would not introduce much of a bias, as all gram-positive recipient rates were more similar to each other than to gram-negative rates and vice versa. Nevertheless, in situations where transfer to specific elements of the gut microflora is of particular interest, parameter estimates for these genera could be used instead. Similarly for the modeling presented here, we were only interested in the transfer of resistance genes to the more clinically relevant groups. There is insufficient experimental evidence to state how much difference there would be in rates of transmission into mixed cultures of recipients compared to monocultures.

Conclusions. Published rates of transfer of antimicrobial resistance genes vary substantially from one study to another. However, more than two-thirds of the variance can be explained by a limited number of variables, namely, the genus of the donor, the genus of the recipient, the nature of the genetic transferable element, whether or not the donor and recipient were of the same genus, and the experimental design. Regression models can be used to derive probability distributions of rates of transfer to host bacteria in the human or animal intestine.

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