The Journal of Infectious Diseases

MAJOR ARTICLE

Fitness Costs of Drug Resistance Mutations in Multidrug-Resistant *Mycobacterium tuberculosis*: A Household-Based Case-Control Study

Phillip P. Salvatore,[1,4] Mercedes C. Becerra,[2] Pia Abel zur Wiesch,[5] Trevor Hinkley,[5] Devinder Kaur,[3] Alexander Sloutsky,[3] and Ted Cohen[5]

[1]Department of Epidemiology, Harvard School of Public Health, [2]Department of Global Health and Social Medicine, Harvard Medical School, and [3]Massachusetts Supranational TB Reference Laboratory, University of Massachusetts Medical School, Boston; [4]Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland; and [5]Epidemiology of Microbial Diseases, Yale School of Public Health, New Haven, Connecticut

Background. The projected long-term prevalence of multidrug-resistant (MDR) tuberculosis depends upon the relative fitness of MDR *Mycobacterium tuberculosis* strains, compared with non-MDR strains. While many experimental models have tested the in vitro or in vivo fitness costs of various drug resistance mutations, fewer epidemiologic studies have attempted to validate these experimental findings.

Methods. We performed a case-control study comparing drug resistance–associated mutations from MDR *M. tuberculosis* strains causing multiple cases in a household to matched MDR strains without evidence of secondary household cases.

Results. Eighty-eight multiple-case and 88 single-case household MDR strains were analyzed for 10 specific drug resistance–associated polymorphisms previously associated with fitness effects. We found that the isoniazid-resistant *katG* Ser315Thr mutation occurred more than twice as frequently in multiple-case households than in single-case households (odds ratio [OR], 2.39; 95% confidence interval [CI], 1.21–4.70), corroborating previous experimental findings. However, strains carrying both the *katG* Ser315Thr mutation and the *rpsL* Lys43Arg mutation were less likely to be found in multiple-case households (OR, 0.09; 95% CI, .01–.73), suggesting a negative epistatic interaction which contrasts previous findings.

Conclusions. The case-control design presents a useful approach for assessing in vivo fitness effects of drug resistance mutations.

Keywords. MDR *M. tuberculosis*; antibiotic resistance; fitness cost; transmission; case-control study; molecular epidemiology; *katG*; *rpsL*; epistasis; Lima, Peru.

Highly drug-resistant strains of *Mycobacterium tuberculosis* are a major obstacle to halting further spread of tuberculosis in many settings. Recent estimates produced by the World Health Organization report approximately 450 000 (possible range, 300 000–600 000) incident cases of multidrug-resistant (MDR) *M. tuberculosis* (defined as a strain with resistance to at least isoniazid and rifampicin) in 2012 [1]. While containing MDR tuberculosis is already a well-recognized problem in settings where drug-resistant tuberculosis constitutes a substantial fraction of tuberculosis cases or where there is a large absolute burden of MDR tuberculosis, the threat that these highly resistant strains of *M. tuberculosis* pose to global containment depends critically on the evolutionary fitness of these bacteria [2, 3].

Mutational events that result in antibiotic resistance are often associated with fitness costs [4–10]. While early studies of in vitro–generated resistance suggested that mutations associated with *M. tuberculosis* resistance impaired bacterial growth rates or virulence [4, 9, 11, 12], more recent evidence reveals that mutations observed among clinical drug-resistant *M. tuberculosis* strains differ from those among these laboratory-derived resistant mutants [13], often are not associated with a reduction in growth rate [7, 14], and often are equally as transmissible as their drug-susceptible counterparts [15, 16]. The absence of substantial deficits associated with these drug-resistant mutants may either reflect low-cost resistance-conferring mutations [17, 18] or higher-cost resistance-conferring mutations that occurred in so-called preadapted genetic backgrounds [7, 15] or that were later compensated by additional mutations [19, 20].

Evolutionary fitness, however, is a complex trait that requires the successful transmission of MDR *M. tuberculosis* to a secondary host in whom it establishes infection, replicates, and is subsequently transmitted [21, 22]. While laboratory assays performed with the intention of evaluating fitness provide invaluable controlled and reproducible data, findings from in vitro approaches may not always correlate with evolutionary fitness [23, 24] or epidemiological fitness (ie, transmissibility). Conversely, epidemiological fitness is typically investigated using cluster-based analysis, in which researchers evaluate the genetic similarities of sample isolates to identify potential clusters of transmission. In this study, we use a novel case-control

Received 3 March 2015; accepted 15 June 2015; published online 19 June 2015.

Correspondence: P. P. Salvatore, Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, 615 N Wolfe St, Baltimore, MD 21205 (psalvat1@jhu.edu).

The Journal of Infectious Diseases® 2016;213:149–55

DOI: 10.1093/infdis/jiv347

approach to investigate the association between mutations in 9 genes implicated in drug resistance and the probability of an MDR *M. tuberculosis* strain being transmitted and generating secondary cases within households in Lima, Peru.

METHODS

Study Population

The present analysis describes a study nested in a previously described cohort of patients with MDR *M. tuberculosis* and their household contacts in Lima [25–28]. Briefly, between 1996 and 2003, index patients with a diagnosis of active MDR tuberculosis in metropolitan Lima began treatment regimens tailored to their specific MDR tuberculosis profiles. At the start of each index patient's individualized treatment regimen, a record of each patient's household contacts was compiled and, along with specimen samples and medical histories, stored for future reference [27, 28]. Beginning in 2004, study personnel identified household contacts of these index patients with MDR tuberculosis who had subsequently received a diagnosis of active tuberculosis [25].

We used 24-loci mycobacterial interspersed repetitive units–variable number of tandem repeats (MIRU-VNTR) analysis and spoligotyping to distinguish households in which the index patient may have transmitted *M. tuberculosis* to a household contact from households in which patients were infected by separate sources [26].

We used a matched case-control design in which cases were defined as strains likely to have been successfully transmitted and caused secondary disease within households and controls were defined as strains for which we had no evidence of secondary cases within households. Definitions of case strains and control strains and our matching criteria, which attempt to control for levels of resistance and opportunities for within-home transmission, are detailed below. This study was approved by the Committee on Human Studies of the Office of Research Subject Protection of Harvard Medical School.

Definitions of Case Strains and Eligible Control Strains

Case strains were MDR *M. tuberculosis* strains for which we had evidence of multiple within-household infections. We defined such a strain as one collected from an index patient that had a matching 24-loci MIRU and spoligotype pattern (see the Supplementary Methods for detailed genotyping methods) to an MDR strain subsequently obtained from at least 1 household contact [26]. Only strains collected from patients who received a diagnosis of prevalent or incident MDR tuberculosis between 9 September 1996 and 9 September 2003 that had also been tested for the extensive drug resistance phenotype were included in our study. Eighty-nine bacterial isolates met the above inclusion criteria for cases.

Eligible control strains were MDR *M. tuberculosis* strains for which we did not have any evidence of secondary within-household infections. For the purposes of this study, eligible control strains included all MDR strains from index patients for which we did not have a genotypically matching secondary case of MDR tuberculosis from within the same household. Genotyping was only performed for strains from households with at least 2 MDR tuberculosis diagnoses. Strains from index patients in households without any secondary MDR tuberculosis cases and from patients in households with ≥2 genetically distinct MDR *M. tuberculosis* strains were eligible to be selected as controls. As described above, only strains collected from patients who received a diagnosis of MDR tuberculosis between 1996 and 2003 that had been tested for the extensive drug resistance phenotype were eligible for inclusion. Four hundred and ninety bacterial isolates met the above inclusion criteria for eligible controls.

Selection of Control Strains

We conducted a matched case-control study in which a subset of eligible control strains was matched to cases, to examine the impact of particular resistance-conferring mutations on the generation of secondary cases. In an attempt to isolate the effects of these resistance mutations on successful transmission, we controlled for major factors likely to correlate with transmission potential in our matching procedure. These matching criteria included (1) the number of antibiotics (within 1) to which the index patient's infecting strain was resistant of 10 tested, (2) the number (within 2 persons) of household contacts of the index patient who were aged >14 years at the time of initiation of the MDR tuberculosis regimen by the index patient, and (3) a similar follow-up time (within 6 months), defined as the interval between initiation of a specialized MDR tuberculosis treatment regimen by the index patient and the date of the first household survey conducted (Figure 1). Follow-up time was defined to exclude the potential period of infectiousness before initiation of the MDR tuberculosis regimen for 2 reasons: index patients were not yet identified as having MDR tuberculosis during this period, and evaluation of household contacts was less rigorous during this period, suggesting a higher potential for the differential misclassification of cases as controls. The process for matching cases and controls is represented diagrammatically in Figure 1.

Laboratory Methods

We performed drug-susceptibility testing, genotyping by MIRU-VNTR, spoligotyping, and DNA sequencing of 9 genes implicated in drug resistance (*katG*, the *inhA-fabG1* locus, the *ahpC* promoter, *rpoB*, *gyrA*, *rrs*, *rpsL*, *embB*, and *pncA*) at the Supranational Reference Laboratory at the University of Massachusetts Medical School (Supplementary Methods).

Identification of Resistant Polymorphisms Previously Associated With Fitness Effects

To identify previously reported associations between resistance polymorphisms and evolutionary fitness costs, we performed a

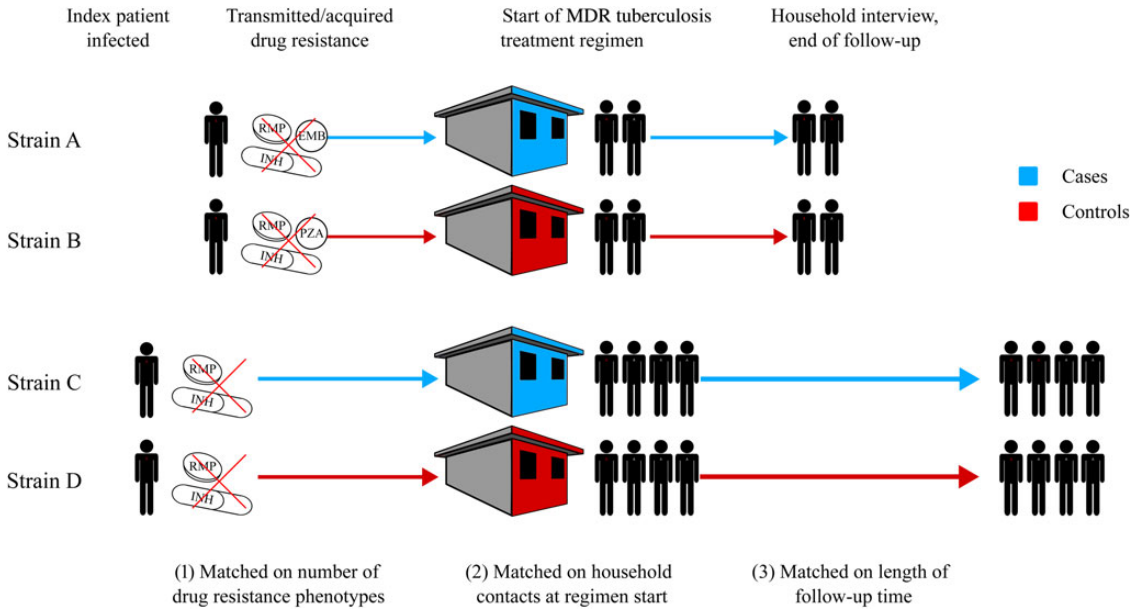

Figure 1. Methods for the selection of cases and controls. Cases were strains of multidrug-resistant (MDR) *Mycobacterium tuberculosis* that successfully infected (and produced disease in) a secondary patient in the same household as the index patient following the index patient's initiation of a tailored MDR tuberculosis treatment regimen. Controls were strains of MDR *M. tuberculosis* that were not successfully transmitted to any known household contact of the index patient during follow-up. To ensure that cases and controls had similar opportunities to be transmitted, matches were formed, using 3 criteria: (1) the number of antibiotics (±1), of 10 tested (capreomycin, ciprofloxacin [or levofloxacin], cycloserine, ethambutol, ethionamide, isoniazid, kanamycin, rifampicin, streptomycin, and pyrazinamide), to which the index patient's infection was resistant; (2) the number of household contacts (±2) of the index patient who were older than 14 years at the time of initiation of the MDR tuberculosis regimen by the index patient; (3) follow-up time (±6 months), defined as days elapsed between initiation of a specialized MDR tuberculosis treatment regimen by the index patient and the date of the first household survey conducted. See "Methods" and "Results" sections for further details on the matching procedure. Abbreviations: EMB, ethambutol; INH, isoniazid; PZA, pyrazinamide; RMP, rifampin.

systematic search of the literature. We used the PubMed.gov database and included the following terms: "mycobacterium" or "tuberculosis", "resistance", "mutation" or "polymorphism", "fitness" or "compensatory" or "biological cost", and any of the 9 genes under investigation in the present study ("ahpC" or "embB" or "gyrA" or "katG" or "inhA" or "fabG1" or "pncA" or "rpoB" or "rpsL" or "rrs"). To be included, each article must have explicitly reported a measure of mycobacterial survival, growth, or transmission due to a specified polymorphism relative to wild-type or another relevant negative control in the absence of the relevant antibiotic.

Statistical Analysis

We used 2 statistical strategies in this investigation. First, a priori hypotheses were generated to describe the relationships between known resistance polymorphisms and transmission fitness (identified in the literature search described above). A conditional logistic regression model was fitted to account for matching and included a dichotomous parameter for each mutation identified. Backward elimination was used to iteratively remove the least significant term in each model until all remaining parameters had $P < .05$.

Second, to investigate individual polymorphisms or epistatic interactions not previously associated with bacterial fitness or transmission, we performed a separate data-mining procedure (Supplementary Methods). Serial hypothesis corrections [29] were conducted in R, version 3.0.3 (R Foundation for Statistical Computing, Vienna, Austria).

Household demographic and epidemiologic characteristics were entered and stored in SAS, version 9.1 (SAS, Cary, North Carolina), while analysis of gene sequence data was performed in Stata SE, version 12.0 (StataCorp, College Station, Texas).

RESULTS

Characteristics of Case Strains and Matched Control Strains

On average, eligible case strains were detected in households with longer follow-up times and with more contacts than eligible control strains (Table 1). Controls were selected randomly from eligible strains and were matched to cases on the basis of factors described in "Methods" section. We were not able to find a suitable match for 1 case, leaving 88 cases and 88 matched controls. Characteristics of cases and controls before and after matching are presented in Table 1. Since it is not possible to observe the onset of infectiousness, finding proxies for the duration of infectiousness is challenging. In addition to controlling for potentially differential opportunities to generate secondary cases during follow-up by matching, we also calculated the time between the first recorded tuberculosis diagnosis and

Table 1. Matching Characteristics of Cases and Controls

Characteristic	Eligible Cases (n = 89)	Eligible Controls (n = 490)	Matched Cases (n = 88)	Matched Controls (n = 88)
Follow-up time, d	1492 ± 543	1399 ± 467	1473 ± 517	1476 ± 517
Adults per household, no.	5.3 ± 2.4	4.3 ± 2.5	5.2 ± 2.3	5.1 ± 2.4
Resistance phenotypes, no.[a]	5.0 ± 1.4	4.9 ± 1.6	5.0 ± 1.4	5.0 ± 1.4

Data are mean ± SD.

[a] Ten antibiotics tested for phenotypic drug resistance: capreomycin, ciprofloxacin (or levofloxacin), cycloserine, ethambutol, ethionamide, isoniazid, kanamycin, rifampicin, streptomycin, and pyrazinamide.

the beginning of follow-up for MDR tuberculosis (during which index patients may have been intermittently infectious). We found that patients contributing case strains received their initial diagnosis of tuberculosis a median of 892 days (95% confidence interval [CI], 765–974) before the diagnosis of MDR tuberculosis, while patients contributing control strains received their initial diagnosis a median of 1420 days (95% CI, 1015–1739) before the diagnosis of MDR tuberculosis. This suggests that the cumulative infectious period of case strains may have been shorter than that for control strains, which implies that our analysis may be conservative: case strains should not have had an unbalanced opportunity to generate secondary cases.

Table 2. Index Patient and Household Characteristics of Cases and Matched Controls

Characteristic	Cases (n = 88[a])	Controls (n = 88[a])
Index patient age, y	29.1 (88)	32.2 (88)
Bedrooms per household, no.	3.2 (82)	3.4 (78)
Previous treatment regimens, no.	2.9 (88)	3.0 (87)
Index patient was female	40.9 (88)	40.9 (88)
Index patient infected with HIV	2.3 (88)	0.00 (88)
Index patient had history of substance abuse	4.9 (61)	7.3 (55)
Index patient had pulmonary tuberculosis	100 (88)	100 (88)
Index patient had cavitary lesions	75.0 (88)	63.6 (88)
Select resistance phenotype[b]		
RIF + INH only	5.7 (87)	2.3 (87)
RIF + INH + EMB	83.8 (87)	85.1 (87)
RIF + INH + PZA	67.9 (81)	65.1 (83)
RIF + INH + STR	71.3 (87)	72.4 (87)
RIF + INH + EMB + PZA	58.0 (81)	57.8 (83)
RIF + INH + EMB + STR	63.2 (87)	63.2 (87)
RIF + INH + PZA + STR	53.1 (81)	45.8 (83)
RIF + INH + EMB + PZA + STR	46.9 (81)	42.2 (83)

Data are mean value or % (no.) of strains.

Abbreviation: HIV, human immunodeficiency virus.

[a] No. may be <88 because of missing data.

[b] Select phenotypes presented here include rifampicin (RIF), isoniazid (INH), ethambutol (EMB), pyrazinamide (PZA), and streptomycin (STR). Data on all tested drugs are presented in Supplementary Figure 1.

Descriptive characteristics of index patients and households from which cases and controls were sampled are presented in Table 2, and drug-susceptibility characteristics are presented in Supplementary Figure 1. Data on the genetic diversity and allele frequencies of the 176 samples are presented in the Supplementary Results and Supplementary Tables 1–9, respectively. Genotyping results and lineage assignments are reported in Supplementary Tables 10–11.

Literature Search Results: Previously Identified Resistance-Conferring Mutations

To establish hypotheses to describe previously reported associations between resistance polymorphisms and evolutionary fitness costs, a systematic literature search was performed. Our search parameters returned 31 research articles (see the Supplementary Results for detailed results and annotations). One article, published only in Chinese, 2 articles reporting fitness costs in *Salmonella*, and a symposium chapter unavailable electronically were disregarded.

Of the 27 remaining articles, only 6 described epidemiological or phylogenetic investigations (none of which were case-control studies), while the remainder described in vitro or in vivo experimentation. Five studies did not test or sufficiently report specific resistance-associated polymorphisms, 9 did not report a comparison with a suitable negative control, and 6 did not test for fitness in the absence of drug or did not test a phenotype related to survival, growth, or transmission. Our criteria for inclusion were satisfied by 10 studies, with 8 presenting data on 10 statistically significant low-cost or no-cost resistance polymorphisms. These 10 polymorphisms consisted of the *inhA* promoter mutation −15;c-t; the *katG* mutations Ser315Thr, Ala139Val, and Gly300Trp; the *rrs* mutations A1408G, T1406A, G1491T, and C1409T; the *rpoB* mutation Ser531Leu; and the interaction allele of both *katG* Ser315Thr and the *rpsL* mutation Lys43Arg.

Fitness Effects of Previously Identified Resistance-Conferring Mutations

The results of the conditional logistic regression model for the 10 mutations identified a priori are summarized in Table 3. In

Table 3. Conditional Logistic Regression (CLR) Results for Significant Terms

Reference(s)	Gene(s)	Allele	OR (95% CI)[a]	*P* Values[a]
[15, 18]	*katG*	Ser315Thr	2.39 (1.21–4.70)	.012
[30]	*katG* + *rpsL*	Interaction[b]	0.09 (.01–.73)	.025

The initial model included 10 terms, one for each of the alleles identified through the literature search: the *inhA* promoter mutation −15;c-t; the *katG* mutations Ser315Thr, Ala139Val, and Gly300Trp; the *rrs* mutations A1408G, T1406A, G1491T, and C1409T; the *rpoB* mutation Ser531Leu; and the interaction allele of *katG* Ser315Thr and the *rpsL* allele Lys43Arg. An eleventh term for the *rpsL* Lys43Arg mutation in the absence of *katG*315 was also included. The results above are shown for significant terms after backward elimination.

Abbreviations: CI, confidence interval; OR, odds ratio.

[a] Data were calculated from conditional likelihood ratio model parameters (ie, the exact conditional scores test).

[b] Defined as the concurrent presence of both *katG* Ser315Thr and *rpsL* Lys43Arg.

Table 4. Distribution of Significant Alleles Amongst Matched Pairs

	rpsL Lys43Arg		*katG* Ser315Thr		*rpsL* + *katG* Interaction[a]	
Variable	+Control	−Control	+Control	−Control	+Control	−Control
+Case	0	3	40	28	0	1
−Case	12	72	14	5	8	78

Each cell represents a unique type of case-control pair with regard to a given allele of interest. In concordant pairs (+case/+control or −case/−control), both members possess the same genotype at the allele; in discordant pairs (+case/−control or −case/+control), only 1 member possesses the allele.

[a] Defined as the concurrent presence of both *katG* Ser315Thr and *rpsL* Lys43Arg.

the final iteration, only 2 parameters remained statistically significant: the Ser315Thr mutation in *katG* and the combination of *katG* Ser315Thr and the *rpsL* mutation Lys43Arg. The distributions of these mutations among matched pairs are detailed in Table 4. In the absence of prior information, this study was not sufficiently powered to identify additional alleles significantly associated with the generation of secondary cases after correcting for multiple testing (see the Supplementary Results for details).

The *katG*315 mutation was widely prevalent in strains sequenced in this study, occurring in 78% of cases and 62% of controls. After accounting for matching, we found that strains harboring Ser315Thr had more than twice the odds of generating a secondary case as strains without the mutation (odds ratio [OR], 2.39; 95% CI, 1.21–4.70).

Strains carrying both the *katG*315 allele and the *rpsL*43 allele were previously reported to demonstrate shorter lag phase growth and higher replicative fitness in vitro than their drug-susceptible counterparts [30]. In our data, however, strains carrying both mutations had an odds of generating a secondary case that was <0.10 times the odds for strains carrying only one or neither mutation (OR, 0.09; 95% CI, .01–.73).

Interaction Between *katG* Ser315Thr and *rpsL* Lys43Arg

Irrespective of genetic background, the *rpsL*43 mutation was limited in prevalence among sequenced isolates. This mutation was found in only 3% of cases and 14% of controls, and in most matched pairs (83%) neither case nor control had the mutation. In pairs discordant for the allele, the odds of generating a secondary case for strains with the mutation were 0.25 times the odds for strains without the mutation (95% CI, .05–.93).

However, a substantial proportion of these mutations occurred together with the widely prevalent *katG*315 allele. There were insufficient numbers of the *rpsL*43 allele in the absence of *katG*315 to determine whether it has significant negative fitness effects in the absence of *katG*315 (OR, 0.5; 95% CI, .045–3.49). Conversely, the *katG*315 allele in the absence of *rpsL*43 has further beneficial fitness effects and was substantially more likely to generate secondary cases (OR, 4.5; 95% CI, 1.82–13.33). Thus, there appeared to exist negative epistasis between the 2 alleles. We cannot exclude the possibility that the overall detrimental effect of *rpsL*43 is largely due to negative epistasis with the *katG*315 allele.

DISCUSSION

Numerous previous studies have attempted to identify specific drug resistance mutations that limit or compensate the reproductive fitness of *M. tuberculosis* [31–33]. Much of this work has been experimental in nature, with fewer studies attempting to corroborate in vitro measurements of fitness with observed fitness for transmission in human populations [15, 16, 34].

In this analysis, 2 drug resistance–associated alleles were found to be significantly associated with increased or decreased probabilities of generating secondary cases: the *katG* Ser315Thr substitution (increased probability) and the simultaneous combination of *katG*315 and the *rpsL* Lys43Arg substitution (decreased probability). The *katG*315 allele is a well-characterized low-cost mutation conferring high-level resistance to isoniazid [18]. The findings presented here are consistent with experimental data indicating that strains with this mutation grow faster in competitive environments and may be equally virulent in vivo [18], with a magnitude of effect similar to those calculated from 2 previous cohort studies [15, 16].

Mutations in the *rpsL* gene, which codes for the ribosomal protein S12, have long been known to confer aminoglycoside resistance on *Escherichia coli* and *Salmonella* species and more recently have been associated with the same mechanism in *M. tuberculosis* [35, 36]. The Lys43Arg mutation is unique among known *rpsL* mutations, allowing nonrestrictive ribosomal elongation and growth rates comparable to those of wild-type *M. tuberculosis* [37]. Nearly all experimental studies to date in *M. tuberculosis* and other organisms have indicated that the Lys43Arg substitution is a low-cost resistance mutation that may compensate in *cis* for higher-cost *rpsL* mutations and may be more virulent in vivo than other aminoglycoside resistance mutations [23, 38–40].

In this and other studies, the Lys43Arg mutation was frequently associated with the *katG*315 allele mentioned above [30]. When considering the co-occurrence of resistance mutations, genetic epistasis may be observed. Epistasis occurs when the phenotypic impact (eg, fitness) of 2 polymorphisms in combination is greater than or less than those of the expected effects of the individual polymorphisms [41]; that is, when allele phenotypes interact in a nonadditive fashion [42]. Examples of epistasis between resistance mutations have been well-characterized experimentally in *E. coli* and *Pseudomonas aeruginosa* [43, 44]. So-called sign epistasis is the special case of epistasis in which the direction of the phenotypic effect of an allele changes conditionally on the basis of the presence of another polymorphism [45].

In the present analysis, strains harboring both mutations were significantly less likely to generate secondary cases, despite

the beneficial effect of *katG*315 on its own. These represent the first ever data indicating that the Lys43Arg mutation may confer fitness and transmissibility costs to drug-resistant bacteria. In the absence of the *katG*315 allele, strains carrying *rpsL*43 were somewhat less likely to generate secondary cases, although these data were not powered to detect a statistically significant association. Our findings suggest that *katG*315 and *rpsL*43 do not interact in an additive compensatory relationship but rather that *katG*315 exhibits sign epistasis when present in combination. The surprising nature of this finding warrants a cautious interpretation, in light of existing in vitro and in vivo evidence indicating the low-cost nature of the Lys43Arg mutation. Simultaneously, these findings underscore the importance of considering epistasis between alleles conferring resistance to different drugs.

Several explanations may account for the observed disparities between experimental and epidemiological data on the fitness costs of *rpsL* mutations, one of which concerns study design and inference. Evolutionary fitness has many dimensions, of which transmissibility is one [21, 22]. By design, this study was structured to only identify associations between bacterial genetics of highly resistant strains of *M. tuberculosis* and within-household transmission in a specific high-incidence setting in Peru [25–28].

To our knowledge, this study represents one of the first attempts to investigate the association between specific resistance mutations and the transmission potential of an infectious agent by using a case-control design. Prior studies have attempted to address this subject through more-traditional genetic cluster analysis [15, 34, 46]. Although useful, such studies may be unwieldy (requiring thousands of clinical isolates in low-incidence MDR tuberculosis settings), require robust population surveillance for several years [47], and may be prone to cluster misclassification or biases of incomplete sampling [48, 49]. Rather than disentangling the OR of a mutation appearing in genetically linked clusters, the case-control method allows direct calculation of the OR of transmission of a mutation by use of relatively few clinical isolates in high-incidence settings where surveillance systems may be unreliable. However, one critical caveat is that this design assumes that all secondary infections within a household are identified and sampled during follow-up. Thus, associations detected in this study should be interpreted not as the relative odds that a strain will be transmitted, but rather as the relative odds of a strain being transmitted, causing symptomatic tuberculosis, and then being detected in a secondary patient.

The validity of our findings depends on the household index patient being accurately identified and the ability of our genotyping methods to identify epidemiologically linked cases within homes. If an index patient infected a household contact and then relocated or died prior to 1996, a case strain could erroneously have been misclassified as a control strain, shifting estimated associations toward the null. If secondary household cases were not actually infected by our identified index case, we may have classified transmitted cases erroneously. Furthermore, as a result of including both prevalent and incident MDR tuberculosis diagnoses in the cohort, patients carrying case strains form a "survivor cohort," in that both index patients and secondary patients must have survived long enough to have their disease diagnosed and recognized as having been caused by a strain originating from the same household. In this event, mutations positively associated with the generation of secondary cases could actually be indicators for less lethal strains of MDR *M. tuberculosis*. In an attempt to ensure that index patients were comparable with respect to the longevity and duration of infectiousness, cases and controls were matched on the basis of the household's follow-up time and number of susceptible adult contacts. Thus, mutations that generate more secondary cases through increasing the average period of infectiousness would not be identified by this design. Additionally, the magnitude of fitness costs associated with some polymorphisms are dependent on the strain backgrounds in which they appear [7]; as genotyping was not performed on all controls, we were unable to control for strain backgrounds in this study. This may partially account for the observed coincidence of *katG*315 and *rpsL*43 among strains circulating in Russia [50]: while at least 64% of Russian strains descend from the highly transmissible Beijing lineage, <7% of genotyped strains in our study belong to this lineage. Furthermore, as sequencing was only performed for isolates from index patients, we are unable to confirm that index patients and patients with secondary cases who were infected with case strains harbored identical drug resistance mutations. However, in 91% of case households, the index patient had a strain with the same number of or more drug resistance phenotypes (of ethambutol, pyrazinamide, and streptomycin) than the corresponding secondary patient, suggesting high correspondence between resistance profiles of household contacts.

Despite this study's limitations, our finding that the *katG* Ser315Thr mutation—one of the most commonly cited low-cost mutations—was significantly associated with the increased generation of secondary cases serves as a useful positive reference for the validity of the study design [18]. Moreover, the difficulties in controlling for confounders may be outweighed by the opportunity to study resistance costs in naturally transmitted MDR *M. tuberculosis* strains in a population with a high disease burden. We suggest that other researchers further evaluate the finding that *rpsL*43 mutations are associated with fitness costs in clinical isolates.

Supplementary Data

Supplementary materials are available at http://jid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

Disclaimer. The content is solely the responsibility of the authors and does not necessarily represent the official views of the respective funding organizations. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Financial support. This work was supported by the Harvard School of Public Health (Mary E. Wilson and Harvey V. Fineberg Fellowship in Infectious Disease to P. P. S.); the Charles H. Hood Foundation (to M. C. B.); the National Heart, Lung, and Blood Institute (grant K01 HL080939 to M. C. B.); the Swiss National Science Foundation (grant PBEZP3_140163 to P. A. z. W.); the German Academic Exchange Service (grant D/11/45747 to P. A. z. W.); and the National Institute of General Medical Sciences (grant U54GM088558 to T. C.).

Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. World Health Organization (WHO). Global tuberculosis report 2013. Geneva: WHO, **2013**.
2. Blower SM, Chou T. Modeling the emergence of the "hot zones": tuberculosis and the amplification dynamics of drug resistance. Nat Med **2004**; 10:1111–6.
3. Cohen T, Murray M. Modeling epidemics of multidrug-resistant *M. tuberculosis* of heterogeneous fitness. Nat Med **2004**; 10:1117–21.
4. Barnett M, Busby SR, Mitchison DA. Tubercle bacilli resistant to isoniazid: virulence and response to treatment with isoniazid in guinea-pigs and mice. Br J Exp Pathol **1953**; 34:568–81.
5. Burgos M, Deriemer K, Small PM, Hopewell PC, Daley CL. Effect of drug resistance on the generation of secondary cases of tuberculosis. J Infect Dis **2003**; 118:1878–84.
6. Davies AP, Billington OJ, Bannister BA, Weir WR, McHugh TD, Gillespie SH. Comparison of fitness of two isolates of *Mycobacterium tuberculosis*, one of which had developed multi-drug resistance during the course of treatment. J Infect **2000**; 41:184–7.
7. Gagneux S, Long CD, Small PM, Van T, Schoolnik GK, Bohannan BJM. The competitive cost of antibiotic resistance in *Mycobacterium tuberculosis*. Science **2006**; 312:1944–6.
8. Li Z, Kelley C, Collins F, Rouse D, Morris S. Expression of katG in *Mycobacterium tuberculosis* is associated with its growth and persistence in mice and guinea pigs. J Infect Dis **1998**; 177:1030–5.
9. Middlebrook G, Cohn M. Some observations on the pathogenicity of isoniazid-resistant variants of tubercle bacilli author. Science **1953**; 118:297–9.
10. van Soolingen D, Borgdorff MW, de Haas PE, et al. Molecular epidemiology of tuberculosis in the Netherlands: a nationwide study from 1993 through 1997. J Infect Dis **1999**; 180:726–36.
11. Billington OJ, McHugh TD, Gillespie SH. Physiological cost of rifampin resistance induced in vitro in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother **1999**; 43:1866–9.
12. Wilson TM, Lisle GW, Collins DM. Effect of inhA and katG on isoniazid resistance and virulence of *Mycobacterium bovis*. Mol Microbiol **1995**; 15:1009–15.
13. Bergval IL, Schuitema ARJ, Klatser PR, Anthony RM. Resistant mutants of *Mycobacterium tuberculosis* selected in vitro do not reflect the in vivo mechanism of isoniazid resistance. J Antimicrob Chemother **2009**; 64:515–23.
14. Ordway DJ, Sonnenberg MG, Donahue SA, Belisle JT, Orme IM. Drug-resistant strains of *Mycobacterium tuberculosis* exhibit a range of virulence for mice. Infect Immun **1995**; 63:741–3.
15. Gagneux S, Burgos MV, DeRiemer K, et al. Impact of bacterial genetics on the transmission of isoniazid-resistant *Mycobacterium tuberculosis*. PLoS Pathog **2006**; 2:e61.
16. van Doorn HR, de Haas PEW, Kremer K, Vandenbroucke-Grauls CMJE, Borgdorff MW, van Soolingen D. Public health impact of isoniazid-resistant *Mycobacterium tuberculosis* strains with a mutation at amino-acid position 315 of katG: a decade of experience in The Netherlands. Clin Microbiol Infect **2006**; 12:769–75.
17. Lee J, Ammerman N, Nolan S, et al. Isoniazid resistance without a loss of fitness in *Mycobacterium tuberculosis*. Nat Commun **2012**; 3:753.
18. Pym AS, Saint-joanis B, Cole ST. Effect of katG Mutations on the Virulence of *Mycobacterium tuberculosis* and the Implication for Transmission in Humans. Infect Immun **2002**; 70:4955–60.
19. Comas I, Borrell S, Roetzer A, et al. Whole-genome sequencing of rifampicin-resistant *Mycobacterium tuberculosis* strains identifies compensatory mutations in RNA polymerase genes. Nat Genet **2012**; 44:106–10.
20. Sherman D, Mdluli K, Hickey M, Barry CI, Stover C. AhpC, oxidative stress and drug resistance in *Mycobacterium tuberculosis*. Biofactors **1999**; 10:211–7.
21. Day T, Alizon S, Mideo N. Bridging scales in the evolution of infectious disease life histories: theory. Evolution **2011**; 65:3448–61.
22. Mideo N, Alizon S, Day T. Linking within- and between-host dynamics in the evolutionary epidemiology of infectious diseases. Trends Ecol Evol **2008**; 23:511–7.
23. Björkman J, Nagaev I, Berg O, Hughes D, Andersson DI. Effects of environment on compensatory mutations to ameliorate costs of antibiotic resistance. Science **2000**; 287:1479–82.
24. Bull J, Levin B. Mice are not furry petri dishes. Science **2000**; 287:1409–10.
25. Becerra MC, Appleton SC, Franke MF, et al. Tuberculosis burden in households of patients with multidrug-resistant and extensively drug-resistant tuberculosis: a retrospective cohort study. Lancet **2011**; 377:147–52.
26. Cohen T, Murray M, Abubakar I, et al. Multiple introductions of multidrug-resistant tuberculosis into households, Lima, Peru. Emerg Infect Dis **2011**; 17:969–75.
27. Mitnick C, Bayona J, Palacios E, et al. Community-based therapy for multidrug-resistant tuberculosis in Lima, Peru. N Engl J Med **2003**; 348:119–28.
28. Mitnick C, Shin S, Seung K, et al. Comprehensive treatment of extensively drug-resistant tuberculosis. N Engl J Med **2008**; 359:563–74.
29. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Ser B (Methodological) **1995**; 57:289–300.
30. Spies FS, von Groll A, Ribeiro AW, et al. Biological cost in *Mycobacterium tuberculosis* with mutations in the rpsL, rrs, rpoB, and katG genes. Tuberculosis **2013**; 93:150–4.
31. Borrell S, Gagneux S. Infectiousness, reproductive fitness and evolution of drug-resistant *Mycobacterium tuberculosis*. Int J Tuberc Lung Dis **2009**; 13:1456–66.
32. Cohen T, Sommers B, Murray M. The effect of drug resistance on the fitness of *Mycobacterium tuberculosis*. Lancet Infect Dis **2003**; 3:13–21.
33. Müller B, Borrell S, Rose G, Gagneux S. The heterogeneous evolution of multidrug-resistant *Mycobacterium tuberculosis*. Trends Genet **2013**; 29:160–9.
34. Metcalfe JZ, Kim EY, Lin S-YG, et al. Determinants of multidrug-resistant tuberculosis clusters, California, USA, 2004–2007. Emerg Infect Dis **2010**; 16:1403–9.
35. Finken M, Kirschner P, Meier A, Wrede A, Böttger EC. Molecular basis of streptomycin resistance in *Mycobacterium tuberculosis*: alterations of the ribosomal protein S12 gene and point mutations within a functional 16S ribosomal RNA pseudoknot. Mol Microbiol **1993**; 9:1239–46.
36. Funatsu G, Wittmann HG. Ribosomal proteins. Location of amino-acid replacements in protein S12 isolated from *Escherichia coli* mutants resistant to streptomycin. J Mol Biol **1972**; 68:547–50.
37. Tubulekas I, Hughes D. Suppression of rpsL phenotypes by tuf mutations reveals a unique relationship between translation elongation and growth rate. Mol Microbiol **1993**; 7:275–84.
38. Björkman J, Hughes D, Andersson DI. Virulence of antibiotic-resistant Salmonella typhimurium. Proc Natl Acad Sci U S A **1998**; 95:3949–53.
39. Sander P, Springer B, Prammananan T, et al. Fitness cost of chromosomal drug resistance-conferring mutations. Antimicrob Agents Chemother **2002**; 46:1204–11.
40. Miskinyte M, Gordo I. Increased survival of antibiotic-resistant *Escherichia coli* inside macrophages. Antimicrob Agents Chemother **2013**; 57:189–95.
41. Borrell S, Gagneux S. Strain diversity, epistatis and the evolution of drug resistance in *Mycobacterium tuberculosis*. Clin Microbiol Infect **2011**; 17:815–20.
42. Chang H-H, Cohen T, Grad YH, Hanage WP, O'Brien TF, Lipsitch M. Origin and proliferation of multiple-drug resistance in bacterial pathogens. Microbiol Mol Biol Rev **2015**; 79:101–16.
43. Trindade S, Sousa A, Xavier KB, Dionisio F, Ferreira MG, Gordo I. Positive epistasis drives the acquisition of multidrug resistance. PLoS Genet **2009**; 5:e1000578.
44. Ward H, Perron GG, MacLean RC. The cost of multiple drug resistance in Pseudomonas aeruginosa. J Evol Biol **2009**; 22:997–1003.
45. Weinreich DM, Watson RA, Chao L. Perspective: Sign epistasis and genetic constraint on evolutionary trajectories. Evolution **2005**; 59:1165–74.
46. Van Soolingen D, De Haas PEW, Van Doorn HR, Kuijper E, Rinder H, Borgdorff MW. Mutations at amino acid position 315 of the katG gene are associated with high-level resistance to isoniazid, other drug resistance, and successful transmission of *Mycobacterium tuberculosis* in The Netherlands. J Infect Dis **2000**; 182:1788–90.
47. Van Soolingen D. Molecular epidemiology of tuberculosis and other mycobacterial infections: main methodologies and achievements. J Intern Med **2001**; 249:1–26.
48. Glynn JR, Vynnycky E, Fine PEM. Influence of sampling on estimates of clustering and recent transmission of *Mycobacterium tuberculosis* derived from DNA fingerprinting techniques. Am J Epidemiol **1999**; 149:366–71.
49. Murray M, Alland D. Methodological problems in the molecular epidemiology of tuberculosis. Am J Epidemiol **2002**; 155:565–71.
50. Casali N, Nikolayevskyy V, Balabanova Y, et al. Evolution and transmission of drug-resistant tuberculosis in a Russian population. Nat Genet **2014**; 46:279–86.