Increasing Macrolide and Fluoroquinolone Resistance in *Mycoplasma genitalium*

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Escalating resistance to azithromycin and moxifloxacin is being reported for *Mycoplasma genitalium* in the Asia-Pacific region. Analyzing 140 infections, we found pretreatment fluoroquinolone-resistance mutations in *parC* (13.6%) and *gyrA* (5%). ParC S83 changes were associated with moxifloxacin failure. Macrolide/fluoroquinolone-resistance mutations were in 8.6% of specimens, for which recommended therapies would be ineffective.

Mycoplasma genitalium infection is a major cause of urethritis in men and is associated with cervicitis, pelvic inflammatory disease, preterm birth, and spontaneous abortion in women (*1*). In the United States, Australia, and Europe, the recommended first-line treatment for *M. genitalium* infection is the macrolide azithromycin. However, a recent meta-analysis documented a rapid decline in its efficacy, from 85% before 2009 to 67% after 2009; the highest levels of resistance were in the Asia-Pacific region (*2*). The second-line therapy recommended by the US Centers for Disease Control and Prevention (https://www.cdc.gov/std/tg2015/default.htm) is the fluoroquinolone moxifloxacin. Quinolones target the DNA gyrase (comprising GyrA and GyrB) and topoisomerase IV (ParC and ParE). Quinolone binding involves serine at position 83 (*Escherichia coli* GyrA numbering) and the acidic amino acid 4 positions away (D87 or E87) (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/23/5/16-1745-Techapp1.pdf) (*3*). Mutations affecting these residues or surrounding sequence (the quinolone resistance-determining region, QRDR) may confer resistance (*4*).

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Moxifloxacin treatment failure is being increasingly reported, particularly in the Asia-Pacific region (*5,6*), along with increasing detection rates of resistance mutations (*7*). Although several studies have reported the prevalence of QRDR mutations in *M. genitalium*, most mutations have not been linked with treatment outcomes. Our aims with this study were to report the prevalence of mutations in the *parC* and *gyrA* genes in patients with *M. genitalium* infection, to correlate specific mutations with moxifloxacin outcomes, and to determine the prevalence of dual (macrolide/fluoroquinolone) resistance.

The Study

From July 1, 2012, through June 30, 2013, samples were collected from consecutive *M. genitalium*–infected participants at the Melbourne Sexual Health Centre in Australia (*5*). Detection of *M. genitalium*, load quantitation, and sequence analyses were performed as described previously (*5*,*8*). Overall, 155 patients (112 men, 43 women) with PCR-confirmed *M. genitalium* infection were recruited, representing 90% of patients with infections diagnosed at the Centre over the study period. We obtained adequate samples from 140 of the 155 patients to generate baseline *parC* and *gyrA* gene sequences; these 140 formed the study group.

Patients were initially given a single dose of 1 g azithromycin. The 54 for whom this treatment failed (positive by PCR test-of-cure at day 28 or persistent symptoms before day 28, with no identified reinfection risk) were given moxifloxacin (400 mg/d for 10 d). The 6 for whom moxifloxacin treatment failed were given pristinamycin (1 g 4×/d for 10 d). This study was approved by The Alfred Hospital Ethics Committee (no. 150/12), and informed consent was obtained from patients.

In pretreatment specimens, various single-nucleotide polymorphisms (SNPs) were observed in the *parC* and *gyrA* QRDR (Table 1; online Technical Appendix). Of the 19 (13.6%) of 140 samples with ParC substitutions, 16 had S83 mutations (14 S83I, 2 S83R) and 3 had D87N substitutions.

We found a significant association between detection of ParC S83 mutations and treatment failure. *M. genitalium* from all 6 patients for whom moxifloxacin failed but from only 3 of the 48 patients for whom moxifloxacin was effective had the ParC S83 mutation ($p<0.0001$ by Fisher exact test) (Table 2, patients 1–9). The 3 infections successfully treated despite a change at ParC S83 are of interest. For

Table 1. Pretreatment *gyrA* and *parC* SNPs detected according to moxifloxacin treatment from 140 samples collected from patients with *Mycoplasma genitalium* infection, Melbourne, Australia, July 2012 through June 2013*

Gene, SNP†	Amino acid change	Moxifloxacin failure, no. (%), n = 6‡	Moxifloxacin success, no. (%), n = 48	Not treated with moxifloxacin, no. (%), n = 86	Total prevalence, no. (%), n = 140
gyrA					
A229G§	K77E	–	–	1 (1.2)	1 (0.71)
G240A§	Silent (R80)	–	–	1 (1.2)	1 (0.71)
G285A	M95I	2 (33)¶	2 (4.2)¶	–	4 (2.9)
G295A§	D99N	1 (17)¶	–	–	1 (0.71)
G295T§	D99Y	1 (17)¶	–	–	1 (0.71)
A296G§	D99G	1 (17)	–	–	1 (0.71)
parC					
C184T§	P62S	–	–	13 (15)	13 (9.3)
C234T	Silent (H78)	–	6 (12.5)	10 (12)	17 (12)
A247C	S83R	2 (33)	–	–	2 (1.4)
G248T	S83I	4 (67)	3 (6.3)	7 (8.1)	14 (10)
G259A	D87N	–	1 (2.1)	2 (2.3)	3 (2.1)
T269A§	I90N	–	–	1 (1.2)	1 (0.71)
C324T§	Silent (N108)	–	–	1 (1.2)	1 (0.71)
No change	–	–	38 (79)	60 (70)	98 (68.5)

*SNP, single-nucleotide polymorphism; –, mutation absent.
†The *M. genitalium* G37 genome sequence (NC_000908) was the reference sequence.
‡All corresponding postmoxifloxacin treatment-failure samples contained the same SNP profiles. Multiple SNPs were detected in some samples.
§Sequence variations not previously described in *M. genitalium.*
¶A mutation was combined with an S83 change.

these patients, low bacterial load may have contributed to therapeutic success (*9*), led to spontaneous clearance, or resulted in false-negative follow-up PCR (Table 2). However, in contrast, treatment failed for 1 patient with a low anal load of *M. genitalium* and S83 change. Similar to the findings for this study, in the parent cohort of 155 patients, organism load influenced apparent azithromycin cure; 7% of infections carrying markers of azithromycin resistance were cured by azithromycin, and organism load was significantly lower than that among those with resistant infections for whom azithromycin treatment failed (*5*).

The prevalence of S83 changes in this study is higher than that detected in a study at Sydney Sexual Health Centre (Sydney, New South Wales, Australia) (8.4%, n = 143) (*10*). Studies in Japan reported prevalence ranging from 3.6% (n = 28) to 29.4% (n = 51) and 36.8% (n = 19) (*7,8,11*), although 1 study involved a cohort at higher risk (female sex workers). A low prevalence of S83 mutation has been observed in Europe (1.5% in France, 5% in England and Germany) (*12–14*). This mutation has been associated with moxifloxacin failure in 3/3 cases in the Sydney-based study (*6*).

Table 2. Samples containing changes in key amino acids of ParC and GyrA from 140 patients *with Mycoplasma genitalium* infection, Melbourne, Australia, July 2012 through June 2013*

Patient no./sex	Sample	Log_{10} load, GEQ†	Treatment (moxifloxacin) outcome	SNPs in *parC*			SNPs in *gyrA*			
				A247C S83R	G248T S83I	G259A D87N	G285A M95I	G295A D99N	G295T D99Y	A296G D99G
1/F	Cervical swab	4.73	Failure	–	+	–	+	–	–	–
2/M	Urine	2.94	Failure	–	+	–	+	–	–	–
3/F	Anal swab	2.15	Failure	–	+	–	–	+	–	–
4/M	Urine	3.80	Failure	–	+	–	–	–	–	+
5/M	Urethral swab	5.10	Failure	+	–	–	–	–	+	–
6/M	Urine	4.66	Failure	+	–	–	–	–	–	–
7/F	Cervical swab	1.82	Success		+	–	+	–	–	–
8/M	Urine	2.47	Success	–	+	–	+	–	–	–
9/M	Urine	1.82	Success	–	+	–	–	–	–	–
10/M	Urine	4.07	Success	–	–	+	–	–	–	–
11/F	Urine	2.47	NT	–	+	–	–	–	–	–
12/M	Urine	3.46	NT	–	+	–	–	–	–	–
13/M	Urine	4.51	NT	–	+	–	–	–	–	–
14/M	Urine	3.51	NT	–	+	–	–	–	–	–
15/M	Urine	2.25	NT	–	+	–	–	–	–	–
16/M	Urine	1.11	NT	–	+	–	–	–	–	–
17/F	Urine	1.94	NT	–	+	–	–	–	–	–
18/M	Urine	2.50	NT	–	–	+	–	–	–	–
19/F	Cervical/swab	3.08	NT	–	–	+	–	–	–	–

*Dataset includes all failed treatments for moxifloxacin. GEQ, genome equivalent; NT, not treated with moxifloxacin because azithromycin treatment was successful; SNP, single-nucleotide polymorphism; +, sequencing of sample successful and SNP present; –, SNP absent.
†The log_{10} loads of *M. genitalium* were calculated per swab or 1 mL of urine. For all 155 patients in the parent cohort, loads varied from 0.84 to 6.17 (median 3.36).

SNPs that changed the ParC acidic residue (D87) were rare (2.1%) and because of low numbers could not be associated with treatment outcomes. Other studies found higher frequency of this change (3.5%–7.1%) (*7*,*8*,*10*,*15*); authors of 1 study reported an association with levofloxacin failure (*15*).

M. genitalium GyrA lacks the S83 residue common to GyrA of other bacteria, having instead a methionine at the equivalent position (M95). This enzyme is therefore probably partially resistant to quinolones. GyrA changes (at M95 or D99) occurred at a frequency of 5.0% (7/140) but could not be correlated with treatment outcome because they occurred concurrently with S83 changes in ParC. Previously, a GyrA M95I change was associated with *M. genitalium* treatment failure in 1 patient (*6*).

Patients who received moxifloxacin were followed up with a PCR test-of-cure at 14 and 28 days. For the 6 for whom treatment failed, the mutation profiles in follow-up specimens were unchanged from the initial premoxifloxacin sequence, suggesting lack of resistance selection in vivo after moxifloxacin.

A total of 60 (42.9%) of the 140 pretreatment samples had macrolide-resistance mutations (*5*). Both macrolide and *parC* fluoroquinolone mutations at S83 or D87 were present in 12 (8.6%) of the 140 samples. Prevalence of fluoroquinolone resistance markers was higher in samples with (20%, 12/60) than without (8.8%, 7/80) macrolide-resistance mutations, although this difference did not reach statistical significance ($p = 0.08$). This finding suggests that successive treatment failures with first-line, then second-line, antimicrobial drugs are generating strains resistant to 2 classes of drugs. Previous studies found lower levels of combined macrolide and fluoroquinolone mutations in men attending a urology clinic (3/51, 5.9%) (*7*) and higher levels in a high-risk population (female sex workers; 4/16, 25%) (*11*).

This study has limitations. The resistance profiles for the infecting strains of *M. genitalium* were not tested in in vitro culture. There may be other unknown changes in the genome that confer resistance to the drugs of interest. In addition, the resistance levels reported are probably underestimates because samples were collected in 2012–2013 and levels have probably risen since then (*7*).

Conclusions

We found high frequency of ParC S83 changes associated with fluoroquinolone resistance in a sexually transmitted infection clinic in urban Australia; these changes were associated with moxifloxacin failure. The high level of dual markers for macrolide/fluoroquinolone resistance suggests successive treatment failure after sequential monotherapy leading to the serious outcome that ≈10% of *M. genitalium* infections are not treatable with recommended or readily available antimicrobial drugs. In the absence of alternatives, treatment with pristinamycin cured all 6 patients with dual-class resistance infections (G.L. Murray et al., unpub data).

This study highlights the urgent need for antimicrobial drug resistance surveillance and the value of diagnostic assays that report the presence of resistance markers to optimize treatment. Our results suggest that it is time to reconsider the indications for azithromycin and invest in trials of different available as well as novel classes of antimicrobial drugs for *M. genitalium* treatment. They also raise serious concerns about sequential use of monotherapy and the need to evaluate combination therapies as we enter a new era of untreatable sexually transmitted infections.

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etymologia

Fluoroquinolone [floor"o-kwin'o-lōn]

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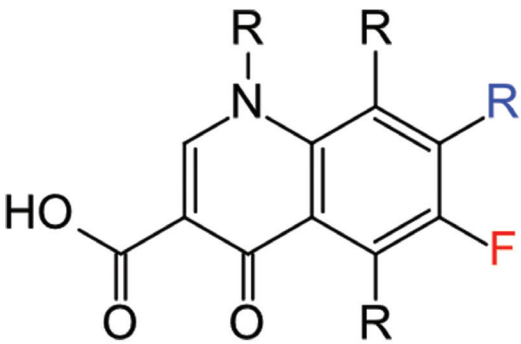
The first quinolone (*quinol*[ine] + *-one* [compound related to ketone]), nalidixic acid, was isolated as a byproduct of chloroquine (see "quinine," https://wwwnc.cdc.gov/EID/article/21/7/ET-2107_article) synthesis and was introduced in 1962 to treat urinary tract infections. In 1980, researchers at the Kyorin Pharmaceutical Company showed that the addition of a fluorine atom to the quinolone ring resulted in an antibiotic with broader antimicrobial activity, which was named norfloxacin, the first fluoroquinolone. In 1983, Bayer published data that showed adding a single carbon atom to norfloxacin—what would become ciprofloxacin—further increased activity. Fluoroquinolones are today among the most frequently used antimicrobial drugs to treat infections in humans and animals.



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Population Responses during the Pandemic Phase of the Influenza A(H1N1)pdm09 Epidemic, Hong Kong, China

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During August 2009–July 2010, we conducted 7 longitudinal telephone surveys among 503 adults in Hong Kong, China, to explore changes in their behavioral and psychological responses to the influenza A(H1N1)pdm09 virus epidemic. Trends were examined using generalized estimating equations models. Findings showed that responses varied with the course of the pandemic.

On June 11, 2009, the World Health Organization declared the influenza A(H1N1)pdm09 (pH1N1) virus outbreak a pandemic (*1*). Previous studies have investigated community responses to the pandemic in different countries during early stages of the epidemic (*2–5*). The studies investigated persons' risk perceptions and knowledge related to the virus, perceived efficacy of preventive measures, and psychological and behavioral responses. However, because of intersample variations, these cross-sectional studies did not capture within-person changes. We conducted a longitudinal cohort study to investigate changes in responses among the general Hong Kong, China, population during the pH1N1 pandemic.

The Study

A cohort sample of 18- to 60-year-old adults in Hong Kong participated in 7 rounds of telephone surveys during August 2009–July 2010, which covered almost the entire pH1N1 pandemic period in Hong Kong. At baseline, we invited 677 adults to participate; 503 (74.3%) consented and completed the survey (online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/23/5/16-0768-Techapp1.pdf). We measured the following variables: knowledge about the modes of pH1N1 virus transmission; risk perceptions associated with the virus (perceived susceptibility to and severity of infection); perceived efficacy and use of preventive measures (e.g., handwashing, using a facemask); psychological responses (worry about infection and emotional distress); and evaluations of the government's performance in pandemic control. Sample sizes for surveys 2–7 ranged from 452 to 481, yielding retention rates of 89.8%–95.6%.

Most participants were women (57.9%), 40–60 years of age (55.8%), employed full time (55.9%), and married (65%). Sex and age distributions were comparable to those in the local census data (*6*). We aimed to determine whether there were overall linear trends in participants' perceptions, psychological responses, and behavioral responses to the pandemic. We examined linear trends for these variables across the 7 time points by using generalized estimating equations (GEE) models. GEE models not only account for intracorrelated repeated measures data but also fit various data types using appropriate link functions. The analyses were conducted using PROC GENMOD (SAS Institute, Cary, NC, USA); 2-sided $p<0.05$ was considered significant.

Over time, >85% of the participants used a face mask and immediately visited a doctor when experiencing influenza-like symptoms. More than 50% of the participants washed their hands >10 times every day throughout the survey period ($p>0.05$). As the pandemic progressed, a decreasing percentage of participants wore masks in public areas; avoided touching their mouth, nose, and eyes; took antiviral drugs; and avoided crowded places ($p<0.001$) (online Technical Appendix Table 2). Percentages of participants feeling worried, depressed, or emotionally disturbed about pH1N1 virus decreased over time ($p<0.001$) (online Technical Appendix Table 3).

Over time, a decreasing percentage of participants recognized that touching infected persons or contaminated objects could result in virus transmission ($p<0.001$). Throughout the study period, a consistently high percentage of participants (>92%) recognized that the virus could be transmitted via respiratory droplets. Misconceptions about possible transmission through insect bites (26.1%) and water sources (34.5%) were prevalent. The percentage of participants reporting at least 1 misconception was stable over time ($p>0.05$). A consistently high percentage (>90%) of participants believed that using face masks in public areas, washing hands frequently, and avoiding crowded places could effectively prevent the spread of pH1N1 virus ($p>0.05$).

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The percentage of participants believing that pH1N1 virus would be more harmful than seasonal influenza in terms of fatality and bodily damage increased over time (p<0.001). The percentages of participants who believed the population was highly susceptible to pH1N1virus infection and who perceived a high chance of having a large-scale local outbreak in the coming year dropped significantly (p<0.001), but some fluctuations were observed; for example, the percentage peaked during survey round 2 (around the September influenza season).

Throughout the study period, ≈12% 21% of the participants gave a failing score (<5 on a 0- to 10-point scale) for the governments overall performance in controlling the pandemic (p>0.05). However, during survey rounds 2 7, an increasing percentage of participants believed in the governments ability to control the pandemic (p<0.001) (online Technical Appendix). The percentage of participants who believed that Hong Kong would not have enough vaccine or medication to deal with the pandemic decreased over time (p<0.001).

Conclusions

This study investigated changes in community perceptions over the course of the pH1N1 pandemic in Hong Kong. Findings were highly comparable to those from other local cross-sectional surveys (*5*,*7*) and a systematic review (*8*). Knowledge regarding preventive measures and adherence to such measures was, in general, higher among our participants than among the general population in other countries (e.g., Australia, India, and the Netherlands) (*9*–*11*). The prevalence of misconceptions about some incorrect modes of transmission (e.g., insect bites) gradually declined. However, ≈50% of participants still held at least 1 of the 4 misconceptions regarding transmission (i.e., airborne transmission over a long distance and transmission through insect bites, water sources, and well-cooked pork). Furthermore, over time, a lower percentage of participants avoided touching their eyes, nose, and mouth to prevent virus transmission. A 2015 systematic review suggested that health authorities should provide more updated information about the virus (*8*). We also recommend using health campaigns to increase public awareness about different routes of pH1N1 virus transmission.

Perceived severity of pH1N1 virus infection decreased over time, which may partially explain the decline in distress and avoidance behaviors; this pattern was also observed in a recent review (*8*). However, an increasing proportion of participants believed that, compared with seasonal influenza, pH1N1 resulted in more deaths and more severe body damage. Perceived susceptibility to infection declined substantially as the epidemic progressed, suggesting that the public gradually perceived fewer risks from pH1N1 virus. Avoidance behaviors and use of facemasks in the absence of influenza-like symptoms became less prevalent over time, similar to a trend seen in Malaysia (*12*). Mental distress among persons in Hong Kong was lower during the pH1N1 pandemic than during the SARS (severe acute respiratory syndrome) pandemic (*13*), possibly due to the milder consequences of pH1N1 infection. Persons in Hong Kong seemed to remain rational during the pandemic, thereby avoiding possible pandemic-associated economic threats.

Public support for the government declined over time. During survey round 5, a total of 20.6% of the participants gave a failing score to the government's performance, and 13.5% perceived that the government would not be able to control the pandemic. The poll was split as to whether the government should use the same response for pH1N1 influenza and seasonal influenza. Our findings suggest that the public should be advised of the pros and cons of pH1N1 control policies; a watchful step-down may be better accepted if the policies are understood.

This study has limitations. First, telephone surveys may be subject to self-selection bias. However, participants' demographics were comparable to those in local census data (*6*). Second, Hong Kong's unique experience with the SARS outbreak may have influenced the population's response to the pH1N1 pandemic; thus, our findings may not be fully generalizable to other countries. Third, we treated time as a continuous variable in the GEE models. Ideally, polynomials should be added to the linear time variable; however, given the small number of time points and absence of theoretical shapes, that was not feasible.

Our findings provide valuable information regarding overall linear trends and changes in community responses toward the pH1N1 pandemic among a Hong Kong cohort. These findings should help inform other countries in formulating appropriate pandemic control plans for influenza and other emerging infectious diseases.

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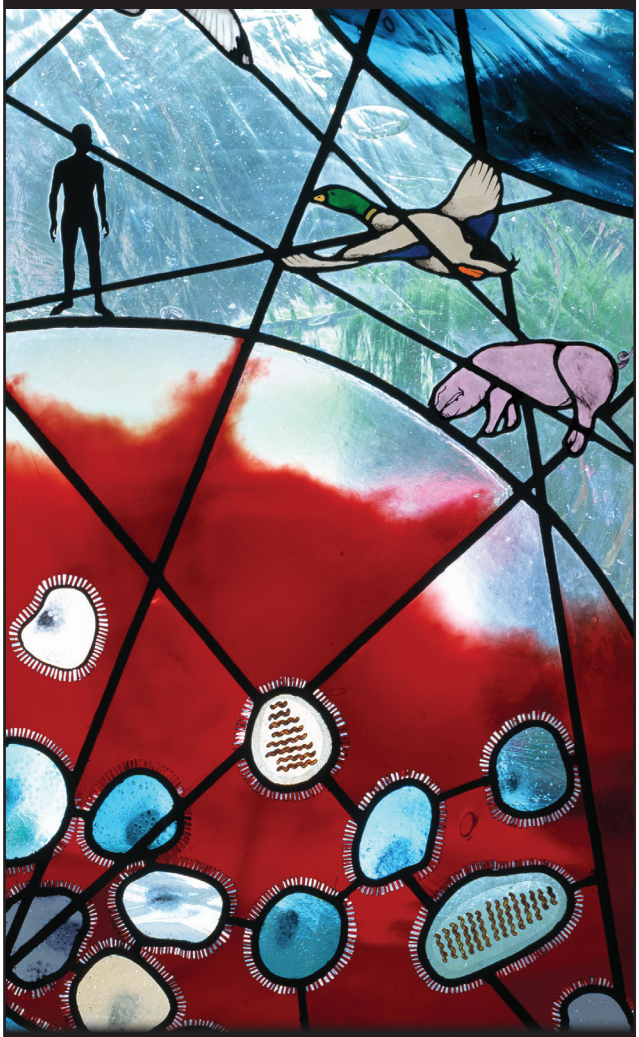
EID Podcast:
Stained Glass and Flu



The work of art shown here depicts the interrelationship of human, animal, and environmental health.

Stained-glass windows have been appreciated for their utility and splendor for more than 1,000 years, and this engaging work of art by stained glass artist Jenny Hammond reminds us that influenza A viruses—which can be easily spread between animals and human, use various host species, and exist in many different environments—remain an enduring and global health concern.

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EMERGING INFECTIOUS DISEASES

Survey of Treponemal Infections in Free-Ranging and Captive Macaques, 1999–2012

Amy R. Klegarth, Chigozie A. Ezeonwu, Aida Rompis, Benjamin P.Y.-H. Lee, Nantiya Aggimarangsee, Mukesh Chalise, John Cortes, M. Feeroz, Barbara J. Molini, Bess C. Godornes, Michael Marks, Michael Schillaci, Gregory Engel, Sascha Knauf, Sheila A. Lukehart, Lisa Jones-Engel

Survey results showed treponemal infection among pet macaques in Southeast Asia, a region with a high prevalence of human yaws. This finding, along with studies showing treponemal infection in nonhuman primates in Africa, should encourage a One Health approach to yaws eradication and surveillance activities, possibly including monitoring of nonhuman primates in yaws-endemic regions.

Yaws, an endemic tropical disease distinguished by bone and skin lesions, is caused by infection with *Treponema pallidum* subsp. *pertenue* treponemes. Successful yaws treatment campaigns during 1950–1965 were followed by a resurgence of disease, and the World Health Organization (WHO) consequently mounted a yaws eradication campaign (*1*). Although the agent of yaws is spread among humans via direct contact, research has shown that nonhuman primates (NHPs) may serve as mammalian host reservoirs with the potential for zoonotic transmission (*2*). Successful eradication campaigns depend on there being no reservoir shielding the agent from eradication efforts; thus, the role that NHPs play in yaws among humans must be determined (*3*).

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African Old World primates (OWPs) can be infected by *T. pallidum* and exhibit symptoms of yaws (*2*). Of note, the *Treponema* Fribourg-Blanc strain (isolated from a baboon in western Africa in 1966) exhibits remarkable genetic similarity to strains that cause yaws in humans (*4*) and in experiments, was shown capable of infecting humans (*5*). More recently, studies focusing on treponemal infections among NHPs in eastern Africa and the Republic of Congo showed that the NHP geographic range overlaps considerably with areas having a formerly high prevalence of yaws in humans (*2*).

Macaques (*Macaca* spp.), OWPs native to Asia and northern Africa, are susceptible to and have been experimentally infected with *T. pallidum* (*6*). After the initial WHO eradication efforts, yaws was believed to be largely eliminated from countries of mainland Asia, although reporting and active case detection have not been uniform throughout the region (*7*). Several island nations in Asia, however, continue to report active human yaws cases (*8*,*9*).

Macaques, the most widely distributed and numerous NHPs in the world, are sympatric with humans throughout Asia, thriving in human-altered environments and commonly kept as pets. To further characterize the role NHPs might play in the maintenance of *T. pallidum* subspecies, we screened an extensive archive of serum samples collected from free-ranging and captive macaques.

The Study

As part of a project characterizing the pathogen landscape among macaques and humans, we collected blood samples from NHPs during 1999–2012 and stored them at −80°C (*10*). We retrospectively screened samples from 734 macaques representing 13 species distributed throughout the animal's natural geographic range (Table 1). Study protocols were approved by the University of Washington Institutional Animal Care and Use Committee (no. 4233–01) and adhered to the American Society of Primatologists Principles for the Ethical Treatment of NHPs (https://www.asp.org/society/resolutions/EthicalTreatmentOfNonHumanPrimates.cfm).

We used a Macro-Vue RPR Card Test Kit (BD, Franklin Lakes, NJ, USA) to screen the 734 blood samples; 11 (1.5%) were positive (Table 2). The RPR (rapid plasma reagin) test, a lipoidal test (nontreponemal) for IgG and IgM typically associated with treponemal infection, can

Table 1. Number and species of free-ranging and captive macaques tested for treponemal infection, by location, 2000–2014*

Country, species	Year(s) sampled	Total no. sampled	No. captive	No. free-ranging
Nepal	2003			
Macaca mulatta		28	0	28
Bangladesh	2008–2012			
M. mulatta		137	14	123
Thailand	2003			
M. arctoides		2	2	0
M. assamensis		5	5	0
M. fascicularis		2	2	0
M. mulatta		9	9	0
M. nemestrina		4	4	0
Cambodia	2011			
M. fascicularis		39	0	39
M. leonina		5	0	5
M. nemestrina		1	0	1
M. spp. (hybrid)		3	0	3
Singapore	2003, 2005–2006, 2009			
M. fascicularis		76	0	76
Gibraltar	2004, 2009, 2013–2014			
M. sylvanus		124	0	124
Indonesia				
Bali	2000–2003			
M. fascicularis		157	0	157
Java	2002			
M. fascicularis		25	25	0
Sulawesi	2000–2002			
M. balantak		5	5	0
M. fascicularis		5	5	0
M. hecki		7	7	0
M. maura		9	9	0
M. nemestrina		2	2	0
M. nigra		22	14	8
M. nigrescens		11	11	0
M. ochreata		1	1	0
M. tonkeana		40	40	0
Macaca spp. (hybrid)		15	15	0
Total	2003–2014	734	170	564

*The 734 tested macaques represented 13 species. Captive category included pets, macaques used in performances, and macaques in zoos; free-ranging included wild macaques, urban macaques, and those at temples, shrines, and reserve parks.

occasionally elicit nonspecific responses. To confirm RPR-positive samples, we used ESPLINE TP (Fujirebio, Tokyo, Japan), an enzyme immunoassay for measuring reactivity to 2 recombinant *T. pallidum* antigens, Tp47 and Tp17. ESPLINE TP and RPR tests have been validated for use in OWPs (*11*). Of the 11 RPR test–positive samples, 1 was from Singapore; 2 from Bali, Indonesia; and 8 from Sulawesi, Indonesia. Six samples (all from Sulawesi) yielded confirmatory positive results on the ESPLINE TP assay. Of note, in Sulawesi, the only positive macaques were pets sampled from South Sulawesi and West Sulawesi Provinces, which make up the island's southwestern peninsula (Figure). We also used ESPLINE TP to test the 28 RPR test–negative samples from Sulawesi's southwestern peninsula; none tested positive.

At the time of sampling, the macaques underwent a physical examination, including close inspection of head, trunk, extremities, oral cavity, and genitals. We conducted a retrospective review of the data and found that none of the macaques had lesions typical of treponemal infection (*4*). Of the 734 macaques, 13, including 2 seropositive macaques from Sulawesi's southwestern peninsula, had hypopigmentation on the palms of their hands, feet, or both. Hypopigmentation is rarely seen in yaws but is a common manifestation of pinta, which is caused by infection with *T. carateum*, a close relative of *T. p. pertenue*.

Conclusions

Our findings show that pet macaques in Southeast Asia can be infected with *Treponema* spp. related to those that infect humans. The overall prevalence of infection was low in our survey, but the pocket of infection detected among pets in Sulawesi's southwestern peninsula is noteworthy. The demonstration of reactivity in the serologic tests provides unequivocal evidence that the macaques had been infected with *T. pallidum* or a highly related pathogen. We had hoped to amplify a portion of *tp0548*, a locus in the *T. pallidum* genome used for molecular typing, but no amplifiable pathogen DNA was found in the whole-blood samples that had been held in storage for >10 years. Therefore, we could not determine whether the treponemal strains from NHPs in Sulawesi resembled strains that cause human yaws.

Table 2. Treponemal infections in blood samples from free-ranging and pet macaques, by geographic location, 1999–2012*

Location	No. macaques positive/no. negative (% reactive)†	No. macaques tested/no. positive‡
Indonesia		
Bali	2/155 (1.3)	2/0
Java	0/25 (0)	NA
Sulawesi	8/109 (7.3)	8/6
Nepal	0/28 (0)	NA
Singapore	1/75 (1.3)	1/0
Bangladesh	0/137 (0)	NA
Thailand	0/22 (0)	NA
Cambodia	0/48 (0)	NA
Gibraltar	0/124 (0)	NA
Total	11/734 (1.5)	11/0

*NA, indicates that samples in the region were not tested.
†Determined by using the Macro-Vue RPR (rapid plasma reagin) test (BD, Franklin Lakes, NJ, USA).
‡Determined by using ESPLINE TP (Fujirebio Inc., Tokyo, Japan), a reagent for the detection of *Treponema pallidum* antibodies.

Sulawesi, the third largest island in the Indonesian archipelago, has a population of ≈17 million persons and 7 endemic macaque species. The seropositive samples from South Sulawesi and West Sulawesi Provinces were collected in July and August of 2000, immediately predating an active yaws outbreak among humans in the region that caused 241 documented cases in the neighboring southeastern peninsula during 2001–2011 (WHO, http://apps.who.int/iris/bitstream/10665/75528/1/WHO_HTM_NTD_IDM_2012.2_eng.pdf) (Figure). During that outbreak, WHO characterized the South Sulawesi and West Sulawesi Provinces as "data deficient" regions in regard to the status of yaws among the human population. Most macaques whose samples were used in this study were free-ranging, but all of the macaques sampled in South Sulawesi and West Sulawesi Provinces had been captured at a young age for use as pets. The association between humans and pet macaques is often intimate, with the sharing of food; space; and physical contact through grooming, play, or aggression (*12*). Two of the *Treponema* spp.–infected pets were owned by the same person and housed together. Studies of pet macaques in Sulawesi and their owners have indicated that infectious agents can move between these populations (*12*,*13*). Although the treponemal serologic status of the pet owners in this study is unavailable, the fact that seropositive pet NHPs from a region neighboring an area with a high number of human yaws cases suggests that the NHP cases resulted from treponeme transmission from humans to pets.

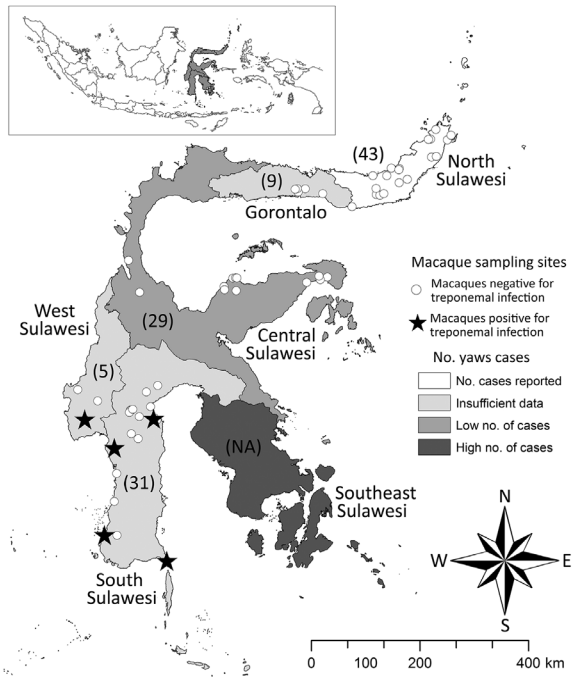

Figure. Individual sampling sites where macaques were tested for infection with *Treponema* spp. during 1999–2012 and the number of human yaws cases during 2001–2011, Sulawesi, Indonesia. Numbers in parentheses indicate number nonhuman primates sampled in each of the 6 provinces. ESPLINE TP (Fujirebio Inc., Tokyo, Japan) reagent for the detection of *T. pallidum* antibodies was used to determine whether macaque samples were positive for treponemal infection. The number of human yaws cases was determined by the World Health Organization (*1*). Inset map shows the location of Sulawesi in Indonesia (gray shading). NA, not available.

All macaques in this study, with the exception of *M. sylvanus* from Gibraltar, were from historically yaws-endemic areas where WHO conducted past yaws eradication campaigns. Much of Asia has a rich tradition of human–NHP commensalism, and macaques are common in villages, often as pets (*10*). Moreover, we previously showed that macaques can harbor an array of mammalian picornaviruses, astroviruses, and mycobacteria (*13*–*15*), underscoring the role of macaques in the ecology of these pathogens. However, as with our current study of treponemal infections, definitive evidence for transmission and the direction of transmission have not been established for these pathogens.

Our findings of treponemal infections among macaques in Southeast Asia, along with published work showing infection in NHPs in Africa (*4*), should encourage holistic and One-Health approaches to eradication and surveillance activities, including consideration of monitoring NHPs in yaws-endemic regions. Such approaches are particularly relevant for pet NHPs, which can easily be assessed and treated. The human–NHP interface is ancient and complex, and continued research, particularly in yaws-endemic regions, can help to ameliorate concerns as a second WHO yaws eradication campaign moves forward.

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Dr. Klegarth is a postdoctoral research associate in the Evolutionary Emergence of Infectious Diseases Laboratory at the University of Washington. Her research is focused on human–wildlife conflict, with an emphasis on urban nonhuman primates, and the infectious agents that are transmitted at this interface.

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Phenotypic and Genotypic Shifts in Hepatitis B Virus in Treatment-Naive Patients, Taiwan, 2008–2012

Chau-Ting Yeh, Kung-Hao Liang, Ming-Ling Chang, Chao-Wei Hsu, Yi-Cheng Chen, Chih-Lang Lin, Wey-Ran Lin, Ming-Wei Lai

We examined the characteristic changes of hepatitis B virus (HBV) in antiviral drug treatment–naive patients referred for pretreatment evaluation in Taiwan during 2008–2012. Over time, we observed substantial decreases in the prevalence of HBV e antigen (HBeAg) and increasing prevalence of the precore G1899A mutation and HBV-DNA levels in HBeAg-positive patients.

Hepatitis B virus (HBV) replication is dependent on the activity of its reverse transcriptase, an error-prone enzyme, which results in the accumulation of genomic mutations. In the natural course of chronic hepatitis B, the prevalence of some mutations (e.g., precore stop codon mutations, basal core promoter mutations, deletions in the pre-S gene region) gradually increases with the progression of disease through the different clinical stages (*1*,*2*). This increase is largely caused by longer duration of chronic HBV infection, during which HBV has to adapt to environmental changes for better survival. Because of the availability of antiviral drug therapies, HBV replication can now be completely suppressed in most patients (*3*,*4*). However, drug resistance and suboptimal or no responses to antiviral drugs can occur (*5*), and there is a lag of few months between the start of treatment and complete virologic suppression. In addition, treatment noncompliance and intermittent treatment almost always result in virologic, or even clinical, relapses. In such cases, antiviral drug therapy imposes an iatrogenic selection pressure on HBV, and the selected viruses carrying mutations can cause infections.

HBV has been infecting humans for a long time; thus, it is possible that genotypic or phenotypic changes have occurred over time, especially since the introduction of HBV vaccine and antiviral drugs. We examined serologic changes and genotypic alterations of HBV in treatment-naive patients during 2008–2012 in Taiwan, where a universal vaccination program was launched in 1986.

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The Study

During January 2008–December 2012, we reviewed the clinical and virologic data for 1,224 treatment-naive patients with chronic hepatitis B who were referred to our clinic at Chang Gung Memorial Hospital, Taoyuan, Taiwan, from all parts of Taiwan for pretreatment evaluation. We obtained patients' age and sex and assessed platelet count, cirrhosis status, HBV e antigen (HBeAg) and HB e antibody (anti-HBe) status, HBV genotype, HBV DNA level, basal core promoter mutations, and precore stop codon mutations. We conducted the study using previously described methods (*6*,*7*). The study was approved by the Institutional Review Board of Chang Gung Memorial Hospital.

Univariate regression analysis indicated a significant increase in patient age ($p = 0.001$) and a significant decrease in the number of HBeAg-positive patients ($p<0.001$) over the 5-year year period. Three factors were significantly associated with HBeAg status: an increased prevalence of anti-HBe ($p = 0.004$) and increased prevalence of precore G1896A ($p = 0.003$) and G1899A ($p = 0.019$) mutations. However, no significant changes occurred in the prevalence of 9 of 10 basal core promoter mutations (online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/23/5/16-1894-Techapp1.pdf). We noted a mild, but significant, decrease in the prevalence of G1730C mutations ($p = 0.034$).

Multivariate analysis showed that patient age and changes in the prevalence of HBeAg were independent of each other (adjusted $p = 0.025$ and 0.021, respectively). However, multivariate analysis that included G1730C, HBeAg, and age showed that the changing prevalence of G1730C was not independent of HBeAg and age (adjusted $p = 0.222$, 0.049, and 0.027, respectively).

These data indicate that the decreasing prevalence of HBeAg was not due to the increasing age of treatment-naive patients but due to an authentic phenotypic change of HBV over the years. It was unclear why patient age increased over the 5-year period; one possibility is the gradual acceptance of antiviral drug therapy by older patients, who may have been worried about possible side effects of new drugs. The cause of the decreasing prevalence of HBeAg over time was possibly due to the fact that a higher proportion of HBeAg-positive patients were treated in the early era of antiviral drug use and more HBeAg-negative patients received treatment at a later time. Alternatively,

because HBV infection has occurred in and expanded among the human population over the past decades, the serologic alterations may be attributed to a changing trend in the mode of transmission on a population scale; for example, in Taiwan, horizontal transmission has increased because of injection drug abuse and sexual transmission, and vertical transmission has decreased because of the neonatal vaccination program.

We subsequently separated patients into HBeAg-positive (n = 398) and HBeAg-negative (n = 826) groups. In the HBeAg-positive group, we saw a significant increase over time in the prevalence of precore G1899A mutations (p = 0.039) and level of HBV DNA (p = 0.013); however, these 2 factors were independent of each other (adjusted p = 0.009 and 0.003, respectively). Of note, we found no change in age over time in this subgroup (p = 0.281) (online Technical Appendix Table 2). Furthermore, because these patients were all HBeAg-positive, we could not explain the data by a differential proportion of HBeAg-positive patients being treated in the early and later periods of the antiviral drug era. Instead, other factors (e.g., changes in transmission routes, altered predominant risks of exposure, changes of HBV prevalence in different subpopulations) could be responsible. Alternatively, dependent on the scale of antiviral drug treatment received in this population, the therapeutic methods might also partly contribute to selection of mutation G1899A and HBV with higher replication efficiency.

In the HBeAg-negative treatment-naive patients (n = 826), we found a borderline increase in patient age (p = 0.046) and a borderline decreased prevalence of male patients (p = 0.044) over time. In addition, we noted a significant decrease in the prevalence of mutation A1752G over the 5-year period (p = 0.022). Multivariate analysis showed that these 3 changes were not independent (adjusted p = 0.062 for age, 0.067 for male sex, and 0.201 for mutation A1752G; data not shown).

Conclusions

Our findings showed a shift in the phenotypic and genotypic characteristics of HBV in treatment-naive patients in Taiwan, an area where chronic hepatitis B is endemic, after the widespread use of antiviral drugs. In Taiwan, because of a limited budget for national health insurance and a high prevalence of chronic hepatitis B, insurance coverage for hepatitis B treatment is not lifelong. Under the insurance plan, patients are provided with continuous nucleotide and nucleoside antiviral treatment for 3 years, after which the drugs are withdrawn to observe for durability of clinical remission. About half of patients have clinical relapses, and almost all have virologic relapses (*8*). Only patients with clinical relapses are retreated. After 3 years of retreatment, the antiviral drugs are withdrawn again to observe for durability; subsequent retreatments (for 3 years) are given only to patients with clinical relapses. The procedure is repeated until no clinical relapse has occurred. It is conceivable that the characteristics of HBV in these antiviral drug–treated, hepatitis B–relapsed patients have been selected by antiviral drugs. Antiviral drug–selected HBVs could potentially spread to treatment-naive patients and cause new infection or superinfection. However, the contribution of this therapeutic factor to the phenotypic and genotypic alterations of HBVs is unclear. Other environmental and social factors, such as altered transmission routes, changes of exposure risks, variations of HBV prevalence in subpopulations over time, and effects of neonatal vaccination, could lead to the changes in HBV characteristics.

Over the 5 years of this study, we found an increasing prevalence of G1899A mutations and an increasing concentration of serum HBV DNA in treatment-naive, HBeAg-positive patients. The mean HBV DNA concentrations increased from 7.7 to 8.3 $\log_{10}$/mL and then stabilized without further increases during the last 3 years of the study. On the other hand, prevalence of G1899A mutations increased from 2.5% to 17.4%. The clinical significance of this mutation in HBeAg-positive patients is unclear and requires further study. In conclusion, this study revealed that, in Taiwan, HBV characteristics have been changing in the era after introduction of antiviral drug treatment and HBV vaccination.

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C.T.Y. designed and supervised the study; C.T.Y. and K.-H.L. drafted the manuscript; and M.-L.C., C.-W.H., Y.-C.C., C.-L.L., W.-R.L., and M.-W.L. collected the samples, performed all assays, and interpreted the data.

Dr. Yeh is director of the Liver Research Center and an attending physician in the Department of Hepato-gastroenterology, Chang Gung Memorial Hospital, Taoyuan, Taiwan. His primary research interests include molecular virology of hepatitis viruses and oncogenic mechanisms of hepatocellular carcinoma.

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- Tickborne Relapsing Fever, Bitterroot Valley, Montana, USA
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- Evidence for *Elizabethkingia anophelis* Transmission from Mother to Infant, Hong Kong
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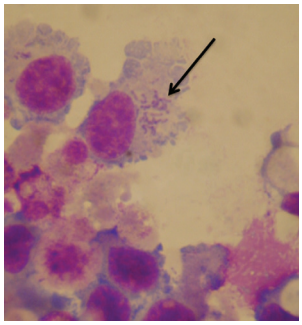

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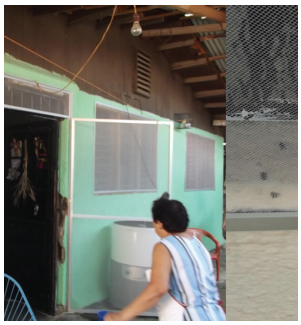

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Reassortant Clade 2.3.4.4 Avian Influenza A(H5N6) Virus in a Wild Mandarin Duck, South Korea, 2016

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A reassortant clade 2.3.4.4 avian influenza A(H5N6) virus was isolated from a fecal sample of a Mandarin duck (*Aix galericulata*) in South Korea during October 2016. This virus was genetically similar to H5N6 subtype virus isolates from China, Vietnam, Laos, and Hong Kong, including human isolates.

Highly pathogenic avian influenza viruses (HPAIVs) have caused major economic losses in poultry industries and represent a serious threat to public health. The H5N1 subtype of these viruses was first detected in 1996 from a domestic goose in Guangdong, China (Gs/GD), and its H5 hemagglutinin (HA) gene has subsequently evolved into 10 genetically distinct virus clades (0–9) and multiple subclades (*1*). Since 2008, novel reassortant HPAIVs bearing the HA gene of the Gs/GD lineage H5 clade 2.3.4 and neuraminidase (NA) gene subtypes N1, N2, N5, N6, N8, and N9 have been identified in China (*2*).

Although clade 2.3.4 of influenza A(H5N8) virus caused influenza outbreaks in eastern Asia and was subsequently disseminated into Europe and North America by wild aquatic birds in late 2014 (*3*,*4*), clade 2.3.4.4 of this virus has caused continuous outbreaks in China since 2013 (*5*). This virus disseminated into Laos and Vietnam in 2014 and Hong Kong in 2015 (*6*,*7*). Since the first influenza case in Sichuan Province, China, 15 human cases of influenza caused by this subtype have been reported in China during April 2014–May 2016 (*8*).

We report detection of an H5N6 subtype HPAIV in a fecal sample obtained from a wild bird sampled in South Korea during the fall 2016. We sequenced and genetically analyzed the complete genome of this virus isolate.

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The Study

On October 28, 2016, we isolated an H5N6 subtype HPAIV from 1 of 391 fecal samples collected from wild birds in Gokgyo-cheon, South Korea (36°45′12.3″N, 127°07′12.7″E). Gokgyo-cheon is a wild bird habitat for wintering of migratory waterfowl, including mallard (*Anas platyrhynchos*), spot-billed duck (*Anas poecilorhyncha*), Mandarin duck (*Aix galericulata*), and common teal (*Anas crecca*). The species of the positive fecal sample was identified as Mandarin duck on the basis of DNA barcoding technique as described (*9*). There were no detectable clinically ill or dead wild birds at the sampling site.

Full-length genome sequencing and phylogenetic analysis were conducted to trace the origin of A/Mandarin_duck/Korea/K16-187-3/2016(H5N6) virus, hereafter referred to as MD/KR/2016. Methods used are detailed in online Technical Appendix 1 (https://wwwnc.cdc.gov/EID/article/23/5/16-1905-Techapp1.pdf). We entered genome sequences in the GISAID (Global Initiative on Sharing All Influenza Data) EpiFlu database (https://www.gisaid.org) under accession nos. EPI861480–EPI861488. Strains used in analysis are shown in online Technical Appendix 2 (https://wwwnc.cdc.gov/EID/article/23/5/16-1905-Techapp2.xlsx).

The isolate was identified as an HPAIV on the basis of multiple basic amino acids at the HA proteolytic cleavage site (PLRERRRKR/G). GISAID BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) searches indicated that H5 and N6 genes had high nucleotide identity in HA (99.17%) and NA (99.24%) with A/great_egret/Hong_Kong/00032/2016 (H5N6) (Table 1). Internal gene segments, except the polymerase basic 1 (PB1) gene, had high nucleotide identity with other H5N6 subtypes isolated in Guangdong and Jiangxi, China (PB2, 99.09%; polymerase acidic, 98.96%; nucleoprotein, 99.16%; matrix, 98.98%; and nonstructural protein [NS], 98.31%). However, the PB1 gene had high nucleotide identity (97.01%) with H4 low pathogenicity avian influenza viruses (LPAIVs).

In previous phylogenetic analyses, the HA gene of clade 2.3.4.4 viruses was divided into 4 distinct subgroups (online Technical Appendix 1 Figure 1) (*10*). Group intercontinental A (icA) contains H5N8 subtype virus and

[1]These authors contributed equally to this article.

Table 1. Nucleotide identities between reassortant clade 2.3.4.4 avian influenza A(H5N6) virus isolated from a wild Mandarin duck, South Korea, 2016, and nearest virus homologs in the GISAID database*

Gene	Virus	GISAID accession no.	% Identity
PB2	A/feline/Guangdong/2/2015(H5N6)	EPI760095	99.09
PB1	A/duck/Guangdong/S4040/2011(H4N2)	EPI692414	97.01
PA	A/Syrrhaptes paradoxus/Guangdong/ZH283/2015(H5N6)	EPI839169	98.96
HA	A/great_egret/Hong_Kong/00032/2016(H5N6)	EPI687156	99.17
NP	A/Syrrhaptes paradoxus/Guangdong/ZH283/2015(H5N6)	EPI839171	99.16
NA	A/great_egret/Hong_Kong/00032/2016(H5N6)	EPI687157	99.24
M	A/feline/Guangdong/2/2015(H5N6)	EPI760101	98.98
NS	A/duck/Jiangxi/NCDZT1123/2014(H5N6)	EPI590810	98.31

*GISAID, Global Initiative on Sharing All Influenza Data (http://www.gisaid.org); HA, hemagglutinin; MP, matrix; NA, neuraminidase; NP, nucleoprotein; NS, nonstructural protein; PA, polymerase acidic; PB1, polymerase basic 1; PB2, polymerase basic 2.

its reassortant viruses identified in China, South Korea, Japan, Taiwan, Canada, the United States, and countries in Europe during 2013–2016. Group B contains H5N8 subtype viruses identified in China and South Korea during 2013–2014, and in Russia in late 2016. Group C contains H5N1 and H5N6 subtype viruses identified in China, Vietnam, Laos, and Hong Kong, including isolates from humans in Guangdong, Yunnan, and Hunnan Provinces, China. Group D contains H5N6 subtype viruses identified in China and Vietnam, including an isolate from a human in Sichuan Province, China. The HA gene of MD/KR/2016 virus belonged to group C and clustered with H5N6 subtype viruses isolated from humans, cats, and the environment in Guangdong during 2014–2015 and a migratory aquatic bird in Hong Kong during January 2016 (A/great_egret/Hong_Kong/00032/2016 [H5N6]) (online Technical Appendix 1 Figure 1).

A previous study reported that A/environment/Guangdong/GZ693/2015 (H5N6), hereafter referred to as GZ693/2015(H5N6), is a 7:1 gene reassortant virus between H5N6 HPAIV and LPAIVs found in southern China (*7*). MD/KR/2016 clustered with GZ693/2015(H5N6) virus for all 8 genes (online Technical Appendix 1 Figure 2). In particular, the HA, NA, PB2, polymerase acidic, nucleoprotein, matrix, and NS protein genes clustered with GZ693/2015(H5N6) and other clade 2.3.4.4 group C H5N6 viruses. The PB1 gene clustered with GZ693/2015(H5N6) (nucleotide identity 92.79%) and LPAIVs, such as H3N2 and H4N2 subtype viruses, from southern China. Phylogenetic analysis and BLAST search collectively sug-

Table 2. Amino acid analysis of avian influenza A(H5N6) virus from a wild mandarin duck, South Korea, 2016, and reference strains of clade 2.3.4.4 H5N6 subtype virus*

	HA (H5 numbering)†							PB2‡			NA del,§	NS¶		
													80–84	
Group, strain	123	126	129	133	156	222	224	591	627	701	59–69	42	del	PDZ
South Korea H5N6 subtype and closely related avian isolates														
A/Mandarin_duck/Korea/K16-187-3/2016	P	E	Del	S	A	Q	G	Q	E	D	Yes	S	Yes	ESEV
A/great_egret/Hong_Kong/00032/2016	P	E	Del	A	A	Q	G	Q	?	?	Yes	?	?	No
A/environment/Guangdong/GZ693/2015	P	E	L	A	A	Q	G	Q	E	D	No	S	No	ESEV
C, human isolates														
A/Shenzhen/1/2016	P	Del	S	A	A	Q	G	Q	K	D	Yes	S	No	No
A/_Guangdong_/ZQ874/2015H5N6	P	E	L	A	A	Q	G	Q	E	D	Yes	S	Del	ESEV
A/_Guangdong_/SZ872/2015H5N6	P	Del	S	A	A	Q	G	Q	E	D	Yes	S	No	No
A/Shenzhen/1/2015	P	Del	S	A	A	Q	G	Q	E	D	Yes	S	No	No
A/Yunnan/14563/2015	P	Del	S	A	A	Q	G	Q	K	D	Yes	S	No	No
A/Yunnan/14564/2015	P	Del	S	A	A	Q	G	Q	K	D	Yes	S	No	No
A/Yunnan/0127/2015	P	Del	S	A	A	Q	G	Q	K	D	Yes	S	Yes	No
A/Guangzhou/39715/2014	P	E	L	A	T	Q	G	Q	K	D	Yes	S	Yes	ESEV
A/Changsha/1/2014	P	Del	S	A	A	Q	G	Q	E	D	Yes	S	Yes	ESEV
D, human isolate														
A/Sichuan/26221/2014	T	E	L	A	A	Q	G	Q	E	N	No	S	Yes	ESEV
C, mammalian isolates														
A/swine/Guangdong/1/2014	P	E	L	A	A	Q	G	Q	E	D	Yes	S	Yes	EPEV
A/swine/Guangdong/2/2014	P	E	L	A	A	Q	G	Q	E	D	Yes	S	Yes	EPEV
A/feline/Guangdong/1/2015	P	E	L	A	A	Q	G	Q	E	D	Yes	S	Yes	ESEV
A/feline/Guangdong/2/2015	P	E	L	A	A	Q	G	Q	E	D	Yes	S	Yes	ESEV

*Del, deletion; HA, hemagglutinin; NA, neuraminidase; NS, nonstructural protein; PB2, polymerase basic 2; PDZ, PDZ binding motif.
†S123P, S133A, T156A, Q222L, and G224S mutations in HA have been associated with increased binding to human-like receptor (α-2–6 sialic acid).
‡Q591K, E627K, and D701N mutations have been associated with improved replication of avian influenza virus in mammals.
§NA stalk deletion has been associated with enhanced pathogenicity in mice.
¶42S, 80–84 deletion, and ESEV PDZ binding motif have been associated with increased virulence in mice.

gest that MD/KR/2016 virus had an identical genotype to GZ693/2015(H5N6).

Most of clade 2.3.4.4 group C viruses have leucine or serine at position 129 (H5 numbering) in HA protein. However, MD/KR/2016 had a single amino acid deletion at position 129 (Table 2), as did A/great_egret/Hong_Kong/00032/2016 (H5N6). This deletion at position 129 and phylogenetic network analysis suggested that MD/KR/2016 is closely related to H5N6 subtypes isolated from wild birds in Hong Kong in 2016 (online Technical Appendix 1 Figure 3). MD/KR/2016 contained the mutation associated with increased virulence in mammals and mammalian transmissibility (S123P and T156A mutations in the HA gene; P42S and D92E mutation, and elongated C-terminus with PDZ binding motif in NS gene). However, this isolate lacked the Q226L and G228S mutations in HA, which have been associated with increased binding to human-type receptor (α-2,6–linked sialic acid) and lacked Q591K, E627K and D701N mutations in PB2, which have been associated with enhanced pathogenicity and adaptation to mammalian hosts (*11*). All of the 9 H5N6 subtype human isolates of group C lacked the Q226L and G228S mutations in HA, but 5 viruses contained the E627K mutation in PB2 (Table 2), suggesting that some purported mammalian adaptation amino acid substitutions were not necessary for sporadic virus infection of H5N6 HPAIV in humans.

Conclusions

Wild aquatic birds have been suspected to play a key role in dissemination of HPAIVs to various regions, as seen with clade 2.2 H5N1 HPAIV in 2005, clade 2.3.2.1 H5N1 HPAIV in 2009, and clade 2.3.4.4 H5N8 HPAIV in 2014 (*4*,*12*). Some populations of Mandarin ducks are year-round residents in South Korea and Japan; others populations migrate between Russia and eastern Asia (*13*). In South Korea, HPAIV was detected from Mandarin duck samples in 2010 (H5N1) and 2014 (H5N8) (*14*,*15*) and again in 2016 during this study, suggesting that Mandarin ducks are a major host species for clade 2.3.4.4 H5 HPAIV and can disseminate the virus throughout South Korea and into other countries. Detection of the H5N6 HPAIV clade 2.3.4.4 in a migratory bird species in South Korea; reports of H5N6 outbreaks in poultry from China, Laos, and Vietnam; and diagnosis of lethal human cases of highly homologous H5N6 viruses in China raise a concern over the potential for broad geographic dissemination of zoonotic H5N6 HPAIV by wild birds outside eastern Asia.

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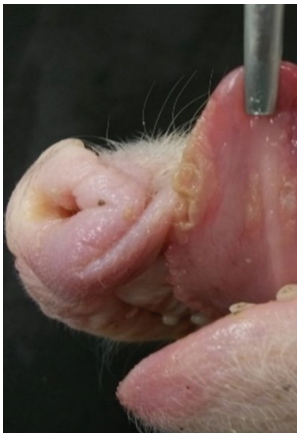

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- Infection with Possible Novel Parapoxvirus in Horse, Finland, 2013
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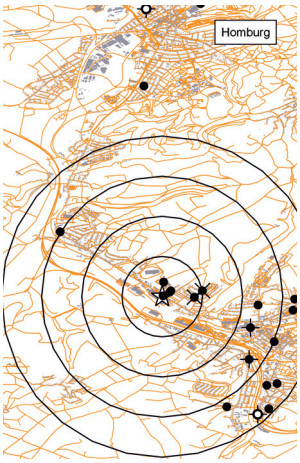

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Amoxicillin and Ceftriaxone as Treatment Alternatives to Penicillin for Maternal Syphilis

Yuichi Katanami, Takehiro Hashimoto, Saho Takaya, Kei Yamamoto, Satoshi Kutsuna, Nozomi Takeshita, Kayoko Hayakawa, Shuzo Kanagawa, Norio Ohmagari

There is no proven alternative to penicillin for treatment of maternal syphilis. We report 2 case-patients with maternal syphilis who were successfully treated without penicillin. We used amoxicillin and probenecid for the first case-patient and amoxicillin, probenecid, and ceftriaxone for the second case-patient.

Syphilis is caused by the bacterium *Treponema pallidum*. Untreated maternal syphilis can lead to serious complications, including congenital syphilis, stillbirth, and neonatal death (*1*). In 2012, the estimated numbers of worldwide cases of maternal and congenital syphilis were 930,000 and 350,000, respectively (*1*). Recently, the United States reported increasing rates of congenital syphilis (*2*).

Penicillin G is recommended for treatment of maternal syphilis (*3*). Benzathine penicillin G is used in many countries but is unavailable in others, such as Japan. As a result, guidelines in Japan of treatment sexually transmitted diseases recommend benzylpenicillin benzathine hydrate (1.2 million units/d) and oral amoxicillin or ampicillin (1.5 g/d) as alternatives (*4*). However, there is little evidence to support use of these regimens. We report 2 case-patients with maternal syphilis who were successfully treated without penicillin.

The Study

Case-patient 1 was a 20-year-old woman who came to the hospital at the National Center for Global Health and Medicine (Tokyo, Japan) because of positive results for treponemal and nontreponemal tests in December 2014. Eight months earlier, she was given a diagnosis of trichomonal vaginitis, which resolved after a course of metronidazole. Three months before coming to the hospital, she was examined in a clinic because of a rash on her abdomen and back, for which she was given topical treatment, which resulted in resolution of the rash within a few weeks. A rapid plasma reagin (RPR) and *T. pallidum* hemagglutination assay (TPHA) were not performed at that time.

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Two months before coming to the hospital, she missed her menstrual period and showed a positive result for a home pregnancy test. Serologic tests for treponemal and nontreponemal antibodies were performed at a nearby clinic, and she was referred to our hospital 13 weeks into her pregnancy. Her RPR titer was 1:16, and TPHA showed positive results. The patient was given a diagnosis of early latent syphilis and prescribed a 14-day course of amoxicillin (6 g/day) and probenecid (1 g/d). The RPR titer decreased to 1:8 and 1:4 at 3 and 6 months after treatment, respectively. RPR was the last test performed before delivery.

She gave birth to a boy at 41 weeks gestation. The baby did not have any signs or symptoms of congenital syphilis, and his serum RPR and TPHA titers were 1:1 and 1:640, respectively (Table). The baby was not treated for congenital syphilis and has not shown any signs of congenital syphilis infection. At 15 months of age, results for RPR and TPHA were negative for a serum sample from the infant.

Case-patient 2 was a 31-year-old woman who came to the same hospital because of a fever in July 2015. One month earlier, she went to another hospital for investigation of a genital ulcer. The day before coming to our hospital, she was given a diagnosis of syphilis and prescribed amoxicillin (1.5 g/d).

A few hours after she took the first dose of amoxicillin, a fever developed, and the patient came to the emergency department of our hospital, where she was given a diagnosis of a Jarisch–Herxheimer reaction. She was also found to be 6 weeks pregnant. Her RPR titer was 1:32 and TPHA titer was 1:160, and she was given a diagnosis of primary syphilis. Three days later, she again came to our hospital for additional evaluation. Treatment was changed to amoxicillin (3 g/d) and probenecid (750 mg/d). Three days after this change in treatment, she could no longer tolerate the medication because of hyperemesis gravidarum, and she was admitted to our hospital. She was given ceftriaxone because she could not tolerate frequent administration of penicillin. Intravenous ceftriaxone (2 g/d) was given for 8 days. Her RPR titer decreased to 1:4 and 1:4 at 6 and 7 months after treatment, respectively. RPR was the last test performed before delivery.

In March 2016, she gave birth to a girl at 39 weeks gestation. The baby did not have any signs or symptoms of congenital syphilis, her RPR titer was negative, and the TPHA titer was 1:320 in a serum sample (Table). The baby was not treated for congenital syphilis, and RPR and TPHA results at 4 months of age showed negative results.

Table. Characteristics of 2 newborns of women with maternal syphilis, Japan*

Characteristic	Newborn for case-patient 1	Newborn for case-patient 2
Leukocytes, ×1,000 cells/μL	13.78	22.88
Neutrophils, %	76.0	68.0
Lymphocytes, %	13.0	18.0
Monocytes, %	7.0	4.0
Eosinophils, %	3.0	7.0
Hemoglobin, g/dL	14.7	19.2
Hematocrit, %	41.6	54.3
Platelets, ×10,000/μL	18.8	30.2
Total bilirubin, mg/dL	6.1	3.0
Direct bilirubin, mg/dL	0.1	0.2
Blood urea nitrogen, mg/dL	7.7	8.3
Serum creatinine, mg/dL	0.64	0.50
Aspartate aminotransferase, U/L	37	49
Alanine aminotransferase, U/L	10	23
Lactate dehydrogenase, U/L	595	560
Alkaline phosphatase, U/L	315	493
C-reactive protein, mg/dL	0.35	<0.01
Rapid plasma reagin titer	1:1	Negative
Chest radiograph	Unremarkable	Unremarkable
Bone radiograph	ND	Normal
Hepatomegaly	No	No

*ND, not done.

Conclusions

In 2012, the World Health Organization estimated that 930,000 cases of maternal syphilis resulted in cases of 350,000 congenital syphilis (*1*). In Japan, the National Institute of Infectious Diseases reported that the number of patients with syphilis is increasing (*5*). As the incidence of women with syphilis increases in Japan, incidence of congenital syphilis also increases (*6*). The efficacy of penicillin for treatment of syphilis is well established by clinical experience and is the only treatment option with documented efficacy (*3*).

Both case-patients described in this report were given amoxicillin and probenecid. A pharmacokinetic study reported that oral amoxicillin and probenecid could attain treponemicidal concentrations in cerebrospinal fluid; therefore, these drugs were considered alternative agents for treatment of neurosyphilis (*7*). Tanizaki et al. (*8*) reported that treatment with oral amoxicillin (3 g) and probenecid (750 mg) was highly effective in and well tolerated by syphilis patients with HIV infection. However, in their report, all patients were men.

For case-patient 2, we changed treatment to ceftriaxone, which is active against *T. pallidum* and has an effective concentration in cerebrospinal fluid. Marra et al. (*9*) reported that ceftriaxone is an alternative to penicillin for treatment of neurosyphilis or early syphilis among HIV-infected patients. US Centers for Disease Control and Prevention guidelines recommend ceftriaxone as an alternative treatment of syphilis in nonpregnant women (*3*). However, data regarding the use of ceftriaxone for treatment of maternal infections and prevention of congenital syphilis are insufficient (*3*).

Because RPR titers for both case-patients became nonreactive, treatment with amoxicillin plus probenecid and ceftriaxone successfully prevented syphilis in both fetuses. Amoxicillin and probenecid are not routinely prescribed for pregnant women because of little evidence of their efficacy in preventing congenital syphilis. Because benzathine penicillin G is not available in Japan, intravenous penicillin G is used to treat maternal syphilis. However, this treatment option requires hospitalization for frequent administration; admission of all maternal syphilis patients is not feasible.

Although ceftriaxone can be administered once a day, it requires daily hospital visits. Azithromycin is not recommended for use during pregnancy (*3*), and treatment failures for fetuses have been reported (*10*). Tetracyclines are contraindicated during pregnancy (*3*). Therefore, we used amoxicillin in accordance with guidelines for Japan (*4*).

One study reported the effect of probenecid during pregnancy on fetal outcomes (*11*). Because probenecid can cross the placental barrier, its use in pregnancy must follow careful consideration of anticipated benefits and possible hazards (*12*). Probenecid was prescribed to increase serum levels of penicillin. Amoxicillin monotherapy might be considered for treatment maternal syphilis if an appropriate dose is given.

The World Health Organization estimates that 5.6 million doses of 2.4 million units of benzathine penicillin are needed annually to treat all syphilis cases, and 930,000 doses are needed to prevent all cases of congenital syphilis (*13*). In May 2016, the 69th World Health Assembly reported that benzathine penicillin had been in short supply for several years (*14*). Therefore, during shortages of penicillin, it is prudent to consider alternative treatment regimens.

In conclusion, amoxicillin and ceftriaxone should be considered as alternatives to penicillin for treatment of maternal syphilis. Further studies evaluating the efficacy of amoxicillin and ceftriaxone are warranted.

Dr. Katanami is a physician at the National Center for Global Health and Medicine, Disease Control Prevention Center, Tokyo, Japan. His primary research interest is tropical infectious diseases.

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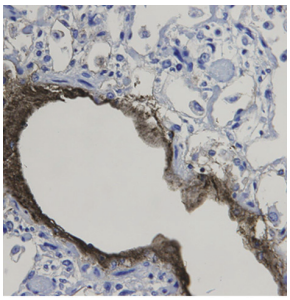

- Multifacility Outbreak of Middle East Respiratory Syndrome in Taif, Saudi Arabia
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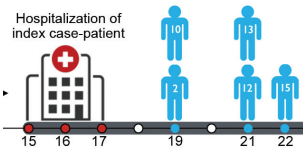
- Human Papillomavirus Prevalence and Herd Immunity after Introduction of Vaccination Program, Scotland, 2009–2013



- Autochthonous *Nocardia cerradoensis* Infection in Humans, Spain, 2011 and 2014
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Azithromycin Resistance and Decreased Ceftriaxone Susceptibility in *Neisseria gonorrhoeae*, Hawaii, USA

John R. Papp, A. Jeanine Abrams, Evelyn Nash, Alan R. Katz, Robert D. Kirkcaldy, Norman P. O'Connor, Pamela S. O'Brien, Derek H. Harauchi, Eloisa V. Maningas, Olusegun O. Soge, Ellen N. Kersh, Alan Komeya, Juval E. Tomas, Glenn M. Wasserman, Gail Y. Kunimoto, David L. Trees, A. Christian Whelen

During 2016, eight *Neisseria gonorrhoeae* isolates from 7 patients in Hawaii were resistant to azithromycin; 5 had decreased in vitro susceptibility to ceftriaxone. Genomic analysis demonstrated a distinct phylogenetic clade when compared with local contemporary strains. Continued evolution and widespread transmission of these strains might challenge the effectiveness of current therapeutic options.

Neisseria gonorrhoeae is a sexually transmitted pathogen that has progressively developed resistance to the antimicrobial agents recommended for treatment (*1*). Third-generation cephalosporins are among the last class of antimicrobial agents that are still effective, and ceftriaxone is the foundation of treatment options recommended by the United States (*2*) and other countries. The diminished cache of drugs to treat gonorrhea has led most countries to recommend a combination of ceftriaxone and azithromycin in an attempt to ensure effective therapy and slow the emergence of resistance by decreasing the likelihood that a *N. gonorrhoeae* isolate would survive concomitant exposure to 2 antimicrobial agents with distinct mechanisms of action (*2*). However, sporadic treatment failures have been reported (*2*), and gonorrhea is considered a global health concern by the World Health Organization and the Centers for Disease Control and Prevention (CDC) because of the few remaining treatment options.

Surveillance for antimicrobial susceptibility of *N. gonorrhoeae* was established by the CDC in the United States in 1986 as penicillin and tetracycline resistance was becoming widespread. The CDC Gonococcal Isolate Surveillance Project (GISP; Division of STD Prevention, National Center for HIV/AIDS, Viral Hepatitis, STD, and TB Prevention) collects ≈5,000 isolates per year from men with urethritis seeking care at sexually transmitted disease clinics across the United States and assesses the isolates for antimicrobial susceptibility (*3*). The findings are used by CDC to formulate national treatment recommendations and develop research and disease intervention priorities. Ceftriaxone remains highly effective in treating gonorrhea in the United States; 99.9% of isolates were inhibited by <0.125 μg/mL in 2014 (*4*). However, the percentage of isolates with decreased azithromycin susceptibility (azithromycin MIC ≥2 μg/mL) rose sharply from 0.6% in 2013 to 2.5% in 2014. Fortunately, none of the 2014 isolates demonstrated clinical resistance or decreased susceptibility to both azithromycin and ceftriaxone.

The Hawaii Department of Health (HDOH) State Laboratories Division maintains nucleic acid amplification, culture, and antimicrobial drug susceptibility testing by Etest for *N. gonorrhoeae*. During 2016, the HDOH and CDC became aware of several *N. gonorrhoeae* isolates with high-level resistance to azithromycin and decreased susceptibility to ceftriaxone in Hawaii as a result of routine laboratory testing and jointly initiated an enhanced laboratory investigation of the isolates.

The Study

The HDOH confirmed the identification of 61 isolates of *N. gonorrhoeae*, collected during February 2016–May 2016, and antimicrobial drug susceptibility testing was performed on all of them. Isolates were identified as *N. gonorrhoeae* by using the API NH test kit (bioMérieux, Marcy l'Etoile, France), and the MICs for azithromycin, ceftriaxone, and cefixime was assessed by Etest (bioMérieux) on GC II agar supplemented with 1% IsoVitaleX (bioMérieux). Etest carried out at the HDOH State Laboratories Division found that 8 *N. gonorrhoeae* isolates had extremely high MICs (>256 μg/mL) for azithromycin and MICs of 0.125–0.25 μg/mL for ceftriaxone and cefixime (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/23/5/17-0088-Techapp1.pdf). The 8 isolates,

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which were collected from 7 patients and included 2 isolates (urethral and urine) from the same patient (GCWGS_0182 and GCWGS_0322), were sent to CDC for confirmatory testing using agar plate dilution (*5*).

All 61 *N. gonorrhoeae* isolates were sequenced (paired-end; 2 × 250-bp read length) on an Illumina MiSeq sequencer (Illumina Denmark ApS, Copenhagen, Denmark) at the HDOH State Laboratories Division. De novo assembly was conducted at CDC by using SPAdes 2.5.1 (http://www.cab.spbu.ru/software/spades), and the core genome single-nucleotide polymorphism alignment was generated by using Parsnp 1.2 (http://www.cbcb.umd.edu/software/harvest), with the FA19 genome (GenBank accession no. CP012026) as the reference. The maximum-likelihood phylogeny was reconstructed by using RAxML 8.0.0 (http://sco.h-its.org/exelixis/web/software/raxml) with 1,000 bootstrap replicates. Whole-genome sequencing data were also used to determine the multilocus sequence typing (MLST) and *N. gonorrhoeae*–multiantigen sequence typing (NG-MAST) allelic profiles for the targeted isolates.

Results of the phylogenetic analysis indicated that the 8 isolates were closely related and formed a single clade (Figure) with 223 single-nucleotide polymorphism differences. MLST analysis revealed 1 unique profile, sequence type (ST) 1901 (online Technical Appendix), which is a highly successful lineage associated with multidrug resistance that probably originated in Japan (*6*). The results of the NG-MAST analysis indicated that all 8 isolates shared 1 novel profile, ST14121. Epidemiologic investigations did not associate sexual network transmission among the 7 patients, although 2 patients reported sex with the same partner. However, the consistent MLST and NG-MAST profiles, in combination with the strongly supported clade, suggest the circulation of a single strain within the population.

To assess the contribution of known mutations to macrolide and cephalosporin resistance, we examined mutations in *penA*, *ponA*, *mtrR*, and 23S rRNA genes. Regarding azithromycin resistance, a deletion in the *mtrR* promoter associated with low-level resistance (*7*) and 4 mutated 23S rRNA copies with the A2059G mutation that confers high-level resistance (*8*) were identified in all 8 isolates. The *ponA* L421P mutation and mosaic *penA* alleles have been associated with reduced susceptibility to cephalosporins (*7*,*9*). The *ponA* L421P mutation was found in all 8 isolates; however, only the nonmosaic *penA* XVIII allele was detected.

The first *N. gonorrhoeae* isolate (H11S8) with high-level azithromycin resistance (HL-AziR) in the United States was identified in Hawaii in 2011 (*10*). More recently, Public Health England characterized 7 *N. gonorrhoeae* HL-AziR isolates that were collected in northern England during November 2014–March 2015 (*11*). Isolate H11S8 and those from England were more susceptible to ceftriaxone (MIC range 0.004–0.03 µg/mL) than the cluster of *N. gonorrhoeae* HL-AziR isolates identified in Hawaii. Genetic comparisons of the 2011 Hawaii isolate placed it in a distinct clade on the phylogenetic tree (Figure). The NG-MAST of H11S8 was ST649, and those from England were ST9768. Three HL-AziR *N. gonorrhoeae* strains were

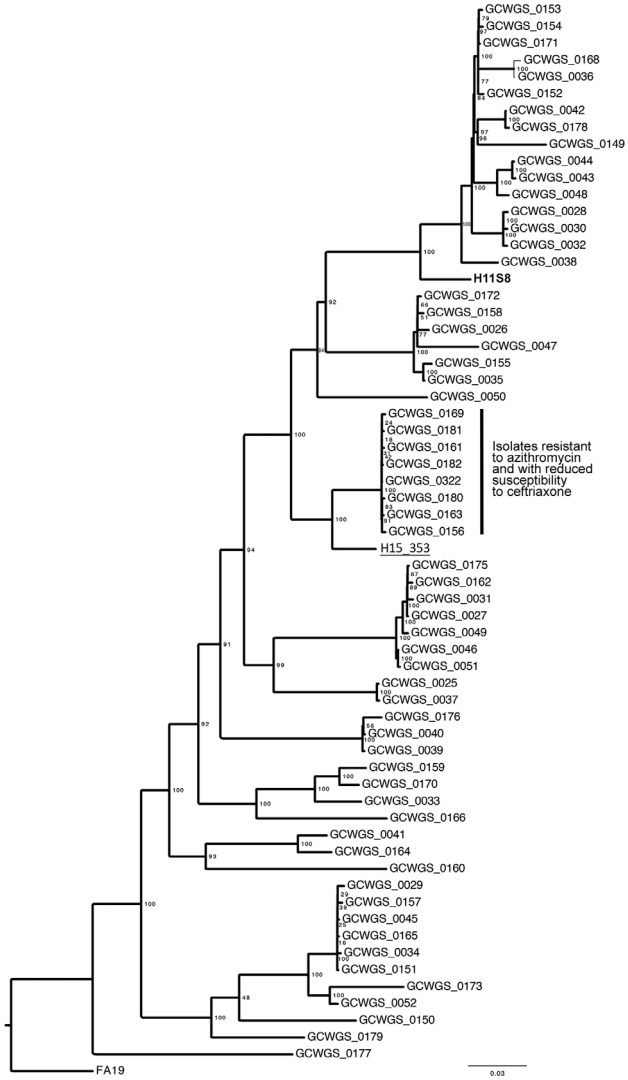

Figure. Maximum-likelihood phylogeny of *Neisseria gonorrhoeae* samples (N = 62) collected in Hawaii during February–May 2016, 1 isolate collected in Hawaii in 2011, and 1 isolate collected in the United Kingdom in 2015. The clade denoted with the black vertical bar contains 8 samples that exhibited resistance to azithromycin (MIC ≥256 µg/mL by Etest) and reduced susceptibility to ceftriaxone (MIC range 0.094–0.125 µg/mL). The 2011 isolate from Hawaii (H11S8, bold) also exhibited resistance to azithromycin. The United Kingdom isolate (underlined) was associated with failed dual antimicrobial therapy of ceftriaxone and azithromycin. The phylogeny is based on the core genome single nucleotide polymorphism alignment of the 62 genomes and the FA19 reference genome. Values on the nodes of the phylogeny (based on 1,000 bootstrap replicates) represent the support for each node and the corresponding clade. Scale bar indicates substitutions per site.

isolated in 2011 and 2012 in Sweden with slightly higher ceftriaxone MICs (range 0.032–0.064 µg/mL) and were identified as either NG-MAST ST285 or ST8727 (*12*).

All patients infected with the HL-AziR isolates in our study were successfully treated with 250 mg ceftriaxone plus 1 g azithromycin. In contrast, a recent pharyngeal *N. gonorrhoeae* isolate, resistant to azithromycin and ceftriaxone, was recovered from a patient in the United Kingdom following treatment with dual antimicrobial therapy of 500 mg ceftriaxone plus 1 g azithromycin (*13*). Although the isolate was genetically distinct from the 8 isolates in Hawaii, it was more closely related to those 8 isolates than to the other 53 contemporary isolates from Hawaii.

Conclusions

The combination of ceftriaxone and azithromycin remains the hallmark for the treatment of gonorrhea worldwide on the basis of surveillance data that monitors antimicrobial susceptibility (*2*,*14*,*15*). Slight fluctuations have been observed in ceftriaxone MICs, but rarely have isolates been recovered with a MIC >0.5 µg/mL. However, a growing body of evidence suggests that azithromycin is becoming less effective and should not be used as a monotherapeutic agent for gonorrhea. The observation of increased MICs for ceftriaxone and azithromycin in a cluster of strains from Hawaii might be the harbinger that the effectiveness of current treatment options will be challenged. It is critical that countries expand systematic surveillance for drug-resistant *N. gonorrhoeae* and that laboratories maintain culture capacity to support rapid response activities to confirm suspected treatment failures and mitigate transmission through contact tracing. Expansion of laboratory capacity to conduct genetic analysis in real time would further benefit clinicians and sexually transmitted disease public health programs by identifying novel mechanisms of resistance that could be used to develop nonculture antimicrobial resistance tests and rapidly identify resistant *N. gonorrhoeae* strains in sexual networks.

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Dr. Papp is a lead research microbiologist at the National Center for HIV/AIDS, Viral Hepatitis, STD, and TB Prevention, Centers for Disease Control and Prevention. He is the senior author for CDC recommendations for the laboratory detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae*.

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Regional Transmission of *Salmonella* Paratyphi A, China, 1998–2012

Xin Lu, Zhenpeng Li, Meiying Yan, Bo Pang, Jialiang Xu, Biao Kan

To explore transmission patterns and genetic relationships of *Salmonella enterica* serovar Paratyphi A in China, we conducted a genome-wide single-nucleotide polymorphism analysis on the strains in the 4 provinces in which incidence was highest during 1998–2012. Markedly phylogeographic clustering suggested regional virus circulation after introduction from areas in southeastern China.

In Asia, incidence of paratyphoid fever remains high (*1*). In the mid-1990s, the number of paratyphoid fever cases in Asia caused by *Salmonella enterica* serovar Paratyphi A started to increase (*2–4*). In 2000, an estimated 5.41 million cases occurred; areas where incidence was highest (i.e., >100 cases/100,000 population per year) included south-central and Southeast Asia (*5*). Since 1998, the incidence of paratyphoid fever in Asia and the world has been highest in China, ranging from 0.08 to 192.5 cases/100,000 population annually (*6*); the provinces in which incidence is highest are Guangxi, Guizhou, Yunnan, and Zhejiang (*7*).

Information about the transmission routes and risk factors for infection could be used to improve the control strategies and measures for paratyphoid fever. Laboratory-based pathogen molecular subtyping, particularly genome-wide single-nucleotide polymorphism (SNP) analysis, can markedly improve outbreak detection, source tracing, and understanding of the epidemic modes. In this study, we analyzed genome-wide SNP and epidemiologic data from *Salmonella* Paratyphi A strains isolated from the China provinces where incidence was highest over a long period (1998–2012) and detected region-limited clone expansion in the epidemic provinces.

The Study

In 1998, the incidence of typhoid/paratyphoid fever in China was 4.82 cases/100,000 population (60,146 cases reported); this measure has since decreased annually to 0.88/100,000 (11,890 cases) in 2012 (China Information System for Disease Control and Prevention, unpub. data). Typhoid/paratyphoid fever cases in Guizhou, Yunnan, Zhejiang, and Guangxi Provinces accounted for 45.8% (in 1998) to 76.5% (in 2001) of all cases in China (online Technical Appendix Figure, https://wwwnc.cdc.gov/EID/article/23/5/15-1539-Techapp1.pdf).

To analyze the genomic epidemiology of paratyphoid fever in these provinces, we first selected 96 *Salmonella* Paratyphi A strains circulating in 15 provinces in China during 1998–2011 (online Technical Appendix Table 1). Strains were isolated from hospitalized patients suspected of having typhoid/paratyphoid fever and were maintained in the strain bank of the Chinese Center for Disease Control and Prevention. We then conducted genome-wide SNP genotyping by using the iPLEX Gold assay (Sequenom Inc., San Diego, CA, USA) with 2,343 SNPs obtained from 7 genomes sequenced in a previous study (*8*) and 17 genomes of *Salmonella* Paratyphi A strains sequenced in this study. We obtained 112 phylogenetically informative SNPs (including 57 nonsynonymous SNPs) (online Technical Appendix Table 2), which were further analyzed in 335 *Salmonella* Paratyphi A strains (online Technical Appendix Table 1) isolated from the provinces where incidence was highest (i.e., Guangxi, Guizhou, Yunnan, and Zhejiang) during 1998–2012 by using the iPLEX Gold assay. The population history of *Salmonella* Paratyphi A was estimated by using BEAST version 2.1.3 (http://beast.bio.ed.ac.uk/), and the maximum clade credibility tree was summarized by using TreeAnnotator and visualized by using FigTree version 1.4.2 (both within BEAST). The consensus tree (Figure 1) showed that all strains fell into 2 main clades: clade 1 consisted of 16 strains isolated from Yunnan, Guizhou, and Guangxi Provinces during 1998–2007; clade 2 consisted of the strains that were most common and widespread in these 4 provinces during 1998–2012. In clade 2, at least 3 subclades were formed, which were markedly characterized by geographic clustering according to province (Figure 1), suggesting intraprovince transmission of the different clones. In addition, the earlier strains in the root of each major subbranch were isolated mainly from Zhejiang, and in the years before 2005, some strains from Guangxi were also mixed in the Guizhou branch.

On the basis of the trees, we further determined from/to transmission of *Salmonella* Paratyphi A by using

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Circos (*9*) (Figure 1). The same SNP genotypes of *Salmonella* Paratyphi A strains were preferentially transmitted within a single province from year to year, whereas the strains from Zhejiang were frequently transmitted to Guizhou and Guangxi, particularly during 1998–2002. The transmission between Guangxi and Guizhou was markedly more frequent before 2004 and decreased after 2005. After 2005, we found no transmission from Yunnan to other provinces.

We also extracted information for 112 SNPs from 127 genomes of the worldwide *Salmonella* Paratyphi A isolates in GenBank (*10*) and constructed a phylogenetic tree by combining these data with data from the 335 strains from China obtained in this study (Figure 2). The

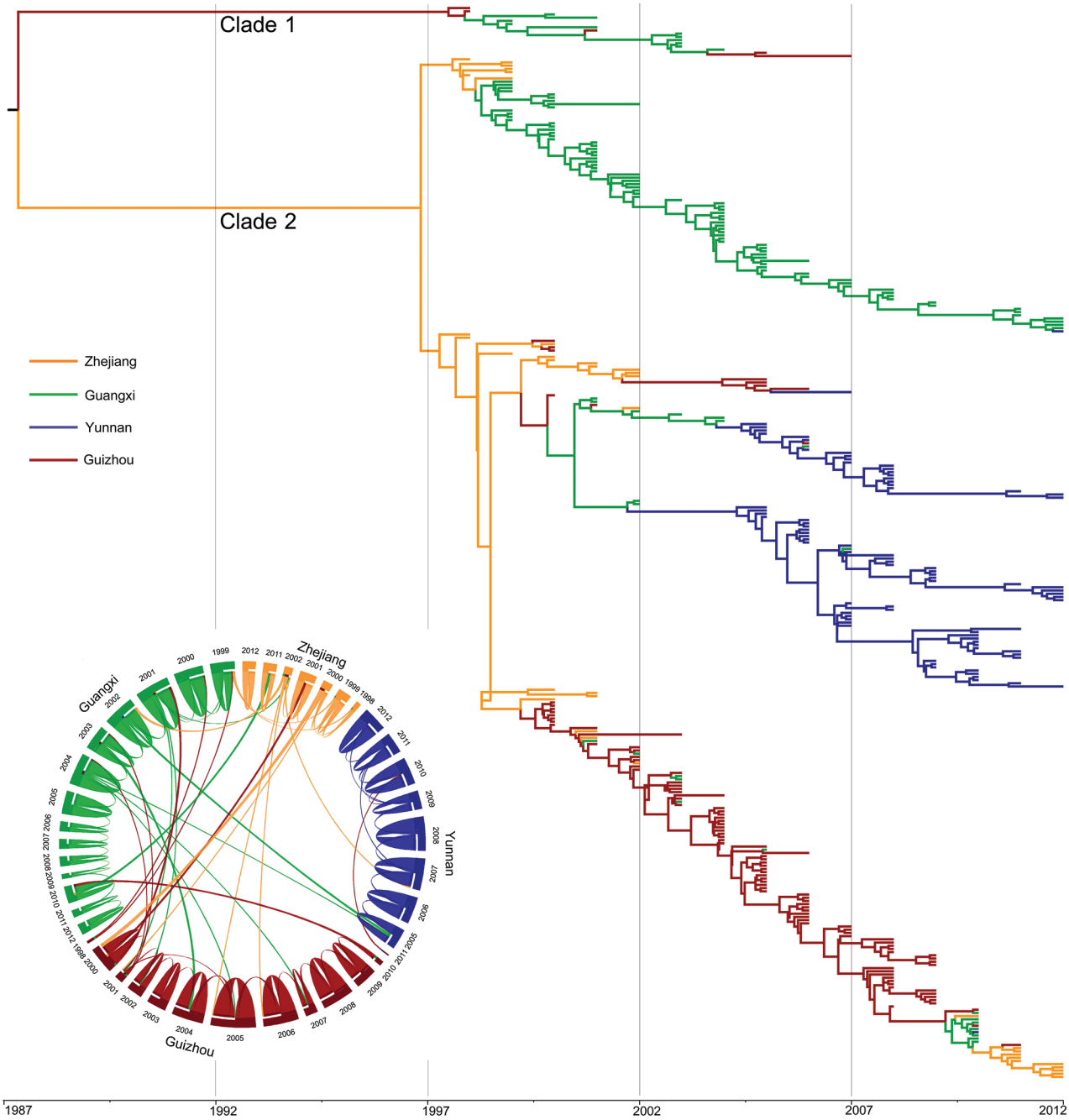

Figure 1. Phylogenetic tree of *Salmonella enterica* serovar Paratyphi A strains isolated from China, 1998–2012. The branches are colored according to inferred location. Inset: potential transmission of *Salmonella* Paratyphi A strains isolated from 4 provinces (Zhejiang Guangxi, Guizhou, and Yunnan). The flow bars indicate the source of transmission; 1 end of the bar directly touches the province of origin, and the other end of the bar exhibits a small gap before the province of destination.

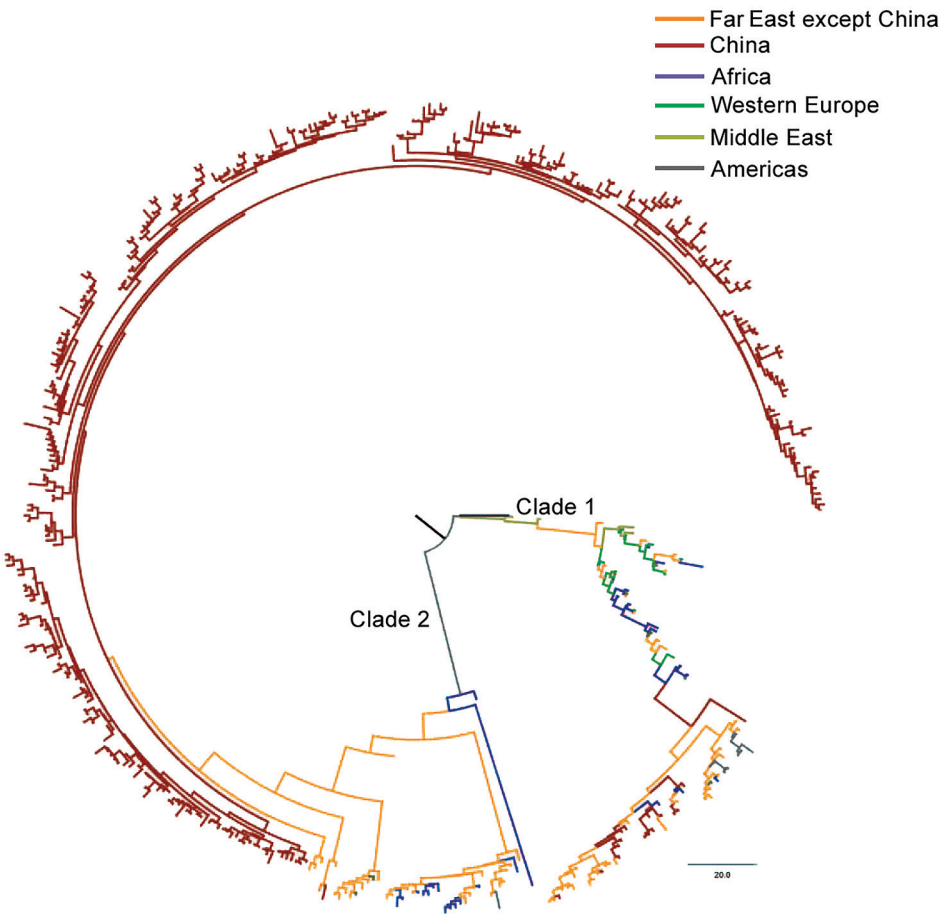

Figure 2. Phylogenetic tree of *Salmonella enterica* serovar Paratyphi A strains in China and worldwide. The branches are colored according to the inferred location. Scale bar indicates number of years.

international strains fell into 2 clades, and the strains from Southeast and southern Asia were positioned much closer to the root of the strains from China, suggesting that the potential source of *Salmonella* Paratyphi A in China might be India or Indonesia.

Conclusions

The genome-wide SNP phylogeny provided more accurate insights into the variation of *Salmonella* Paratyphi A strains in China. In Guizhou, Guangxi, and Yunnan Provinces, which are geographically adjacent, *Salmonella* Paratyphi A has existed for many years. Although we had speculated that the organism might show a mixture of genetic patterns, the phylogenetic tree showed that epidemic strains from different provinces gradually accumulated their own mutations to evolve and form obvious geographic branches. In earlier years of the study period (1998–2002), the epidemic strains from Guangxi and Guizhou Provinces might have originated from early epidemic strains from Zhejiang Province. The level of economic development in Zhejiang Province is high, whereas in Guangxi, Guizhou, and Yunnan Provinces it is lower; the rural population from these 3 provinces migrates frequently to work in the economically developed southeastern coastal areas in China, including Zhejiang (*11*), Jiangsu, and Guangdong Provinces. According to the fifth national census conducted in 2000 (http://www.stats.gov.cn/tjsj/pcsj/rkpc/5rp/index.htm) and the sixth conducted in 2010 (http://www.stats.gov.cn/tjsj/pcsj/rkpc/6rp/indexch.htm), the migration data within the 4 provinces showed this population movement trend (online Technical Appendix Table 3). At irregular intervals, migrant workers, mainly those who are young and middle-aged, return to their hometown for family reunions.

In the mid-1990s, paratyphoid fever became an emerging problem in Zhejiang Province; during 1997–2005, incidence was 8.61 cases/100,000 population (*12*). In those years, managing ex situ healthcare and medical treatments in China was problematic. When migrant workers got ill, they seldom sought medical treatment at the hospital in the city in which they worked; rather, they bought medicine at a chemist's shop or returned to their hometown for treatment (Zhang Q. The study on the health seeking behavior of migrant workers [master's thesis]. China: Shaanxi Normal University; 2012).

Because of lack of medical treatment in hospitals, migrant workers who become infected with *Salmonella* Paratyphi A easily become chronic carriers. Therefore, *Salmonella* Paratyphi A might be transmitted to Guangxi, Guizhou, and Yunnan Provinces via a migrating infected population, including patients and carriers. In addition, these 3 provinces are mainly mountainous, and the population flow among these provinces is limited by their lower economic development and inaccessibility. Therefore, the transmission pattern in these regions could be closely associated with the southeastern coastal areas, where the level of economic development is higher, and transmission among these 3 provinces could be absent. Moreover, in these paratyphoid-epidemic provinces, most of the overall population lives in rural agricultural areas. Given the combination of poor water and food hygiene with a hot and humid climate, the epidemic clones of *Salmonella* Paratyphi A could persist for a long time after being introduced into these areas.

In summary, we identified the evolution and transmission mode of paratyphoid fever in the China provinces where incidence is highest. Populations migrating to southeastern China probably mediated the transmission of *Salmonella* Paratyphi A. Considering the obvious regional clone expansion in these provinces, the local natural, social, and economic conditions need to be investigated for their potential roles in the spread of paratyphoid fever and for the development of intervention strategies.

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Exposure Risk for Infection and Lack of Human-to-Human Transmission of *Mycobacterium ulcerans* Disease, Australia

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We conducted epidemiologic and genetic analyses of family clusters of *Mycobacterium ulcerans* (Buruli ulcer) disease in southeastern Australia. We found that the incidence of *M. ulcerans* disease in family members was increased. However, the risk for exposure appeared short-term and not related to human-human transmission.

Mycobacterium ulcerans is a slow-growing organism that causes necrotizing infections of skin and soft tissue, often requiring reconstructive surgery and resulting in long-term disability (*1*,*2*). Prevailing opinion is that humans are infected from the environment; insects, such as mosquitoes (*3*,*4*), and water-residing biting arthropods (*5*,*6*), have been proposed as vectors for transmission. In Victoria, Australia, there is evidence that native opossums might be involved in transmission (*7*). However, despite extensive research, the environmental reservoir of the organism and mode of transmission remain unknown.

We postulated that examination of *M. ulcerans* disease (Buruli ulcer) family clusters might provide useful new information about disease epidemiology. Theoretically, genetically related first-degree relatives have similar susceptibility to disease, and families share the same environment and therefore a similar exposure risk. Thus, we examined the epidemiology of *M. ulcerans* disease in family clusters managed in a large prospective observational cohort from the Bellarine Peninsula in southeastern Australia. We used data collected from all confirmed *M. ulcerans* cases managed during January 1, 1998–April 12, 2016, at Barwon Health, a tertiary referral hospital in Geelong, Australia (*8*).

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The Study

For this study, only initial *M. ulcerans* lesions were analyzed. A family cluster was defined as multiple family members independently given a diagnosis of *M. ulcerans* disease who were living at the same residence at the time of diagnosis. Data was collected by using Epi Info 6 (Centers for Disease Control and Prevention, Atlanta, GA, USA) and analyzed by using Stata 12 (StataCorp LLC, College Station, TX, USA).

To determine the genetic relatedness of isolates derived from family clusters, we performed whole-genome sequencing and single-nucleotide polymorphism (SNP) analysis for 6 isolates derived from 3 family cluster pairs (Tables 1, 2). We sequenced DNA as 300-bp paired-end reads by using an MiSeq Sequencer (Illumina, Inc., San Diego, CA, USA). Resulting reads were mapped against the *M. ulcerans* Agy99 genome (*9*), including plasmid pMUM001 (*10*), by using Bowtie2 (*11*). Raw sequence reads for the 6 isolates have been deposited in the National Center for Biotechnology Information (Bethesda, MD, USA) Sequence Read Archive under BioProject accession no. PRJNA321660. We also performed whole-genome SNP analysis for 6 additional unrelated previously sequenced human *M. ulcerans* isolates (Sequence Read Archive accession no. SRP004497) obtained from the same disease-endemic region.

A total of 324 patients with *M. ulcerans* disease from the Bellarine Peninsula, Victoria, Australia, were managed in the Barwon Health observational cohort during January 1, 1998–April 12, 2016. Median age was 57 years (IQR 34–74 years), and 164 patients (50.6%) were men. For the whole cohort, a combined time of 1,968.5 years had elapsed from diagnosis of the initial *M. ulcerans* lesions until the time of study analysis (April 12, 2016). The median duration elapsed from initial diagnosis until study analysis was 4.7 years (IQR 2.8–9.7 years).

Twenty-one (6.5%) patients were part of a family cluster (Table 1), 9 genetically related and 12 related by marriage. All family clusters were diagnosed after the beginning of 2008. We found that significantly fewer family clusters were diagnosed during the first half of the study period (0 of 92 cases during 1998–2007) than in the second half (21 of 232 cases during 2008–2016) ($p<0.01$). The median time between diagnoses of *M. ulcerans* lesions in an additional family member, after the initial family

Table 1. Characteristics of 21 patients associated with family clusters of *Mycobacterium ulcerans* disease, Bellarine Peninsula, Victoria, Australia, 1998–2016*

Cluster	Isolate	Date of diagnosis	Time between lesions, mo	Location	Relationship	Patient age at diagnosis, y,/sex	Site of lesion	Type of lesion	WHO stage
1a	mu179	2008 Jul 21	0.4	PTL	Mother	54/F	Right thigh	Ulcer	1
1b	mu180	2008 Aug 4		PTL	Daughter	26/F	Left calf	Ulcer	1
2a	mu248	2010 Oct 24	20.6	PTL	Husband	84/M	Right forearm	Ulcer	1
2b	mu394	2012 Jul 4		PTL	Wife	84/F	Right forearm	Ulcer	1
3a	NT	2011 Jul 25	0.1	QUE	Husband	76/M	Right ankle	Ulcer	3
3b	NT	2011 Jul 28		QUE	Wife	75/F	Right elbow	Ulcer	1
4a	mu294	2011 Aug 22	1.3	PTL	Wife	65/F	Right knee	Ulcer	1
4b	mu308	2011 Sep 29		PTL	Husband	65/M	Left calf	Ulcer	1
5a	NT	2011 Aug 25	1.1	BH	Father	56/M	Right leg	Ulcer	1
5b	NT	2011 Sep 26		BH	Son	26/M	Right leg	Ulcer	1
6a	NT	2012 Jun 19	22.7	PTL	Wife	34/F	Left knee	Ulcer	1
6b	NT	2014 Apr 30		PTL	Husband	37/M	Right ankle	Ulcer	1
7a	NT	2012 Aug 14	22.9	QUE	Wife	74/F	Left ankle	Ulcer	1
7b	NT	2014 Jul 3		QUE	Husband	76/M	Left leg	Ulcer	1
8a	NT	2012 Oct 16	15.9	BH	Sister	20/F	Right foot	Ulcer	1
8b	NT	2014 Feb 14		BH	Brother	18/M	Left leg	Ulcer	1
9a	NT	2013 Apr 27	12.7	QUE	Wife	85/F	Right ankle	Ulcer	1
9b	NT	2014 May 12		QUE	Husband	90/M	Left forearm	Ulcer	1
10a	NT	2013 Dec 10	2.8	PTL	Father	34/M	Left hand	Ulcer	1
10b	NT	2014 Mar 4		PTL	Daughter	4/F	Right knee	Nodule	1
10c	NT	2014 Mar 5	0.0	PTL	Son	7/M	Right ankle	Nodule	1

*BH, Barwon Heads; NT, not tested; PTL, Point Lonsdale; QUE, Queenscliff; WHO, World Health Organization.

member was given a diagnosis, was 2.8 months (IQR 1.1–20.6 months). The rate of new diagnosis of an *M. ulcerans* lesion in another family member was 5.69/1,000 person-years (95% CI 3.15–10.29/1,000 person-years). We determined the cumulative proportion of patients given a diagnosis who had an affected family (Figure 1).

Core SNPs based on common variable nucleotide positions were identified for the 6 examined family isolates by whole-genome sequencing. A total of 4,918 core SNPs ascribed to the African Agy99 reference genome were identified according to strict filtering criteria. Only 8 SNPs were specific to ≥1 of the 6 isolates (Table 2). Of the 8 SNPs that differed among the isolates, only 3 were nonsynonymous substitutions. The remaining 5 SNPs were either intergenic or synonymous mutations.

Pairwise comparisons of family cluster isolates showed that isolates from the 4a/4b pair were genetically identical. In contrast, isolates from the 2a/2b and 1a/1b pairs contained several isolate-specific SNPs (Table 2; Figure 2). SNP analysis of unrelated *M. ulcerans* isolates from the same disease-endemic area showed that 3 of the 6 isolates were also genetically identical (Figure 2), which demonstrated that unrelated isolates can share a common genotype. The remaining 3 isolates contained 1–3 unique SNPs. Thus, family cluster isolates were not any more closely genetically related than 6 random isolates from the same geographic region.

Table 2. Description of 8 single-nucleotide polymorphisms specific to ≥1 of 6 family cluster isolates of *Mycobacterium ulcerans* disease, Bellarine Peninsula, Victoria, Australia, 1998–2016*

Position	Loci	Protein	Substitution	Amino acid change	Isolate	Coverage statistics
398430	Intergenic	–	G/A	–	mu179	T: 0, A: 35, G: 0, C: 1
398430	Intergenic	–	G/A	–	mu180	T: 0, A: 67, G: 0, C: 0
398430	Intergenic	–	G/A	–	mu248	T: 0, A: 100, G: 0, C: 1
398430	Intergenic	–	G/A	–	mu294	T: 0, A: 75, G: 0, C: 0
398430	Intergenic	–	G/A	–	mu308	T: 0, A: 58, G: 0, C: 0
1758272	MUL_1618	Membrane protein	C/T	Synonymous	mu248	T: 91, A: 1, G: 0, C: 0
2153447	MUL_1947	Thiamine pyrophosphate	A/G	Lys→Arg	mu294	T: 0, A: 1, G: 58, C: 0
2153447	MUL_1947	Thiamine pyrophosphate	A/G	Lys→Arg	mu308	T: 0, A: 0, G: 40, C: 0
2462577	MUL_2205	Hypothetical protein	T/C	Asp→Gly	mu179	T: 1, A: 1, G: 0, C: 47
4359638	MUL_3902	Membrane protein	C/A	Ala→Ser	mu180	T: 0, A: 60, G: 1, C: 0
4359638	MUL_3902	Membrane protein	C/A	Ala→Ser	mu248	T: 0, A: 108, G: 0, C: 1
5189291	Intergenic	–	G/T	–	mu248	T: 76, A: 0, G: 4, C: 0
5354966	MUL_4830	Putative GTPase	T/C	Synonymous	mu180	T: 2, A: 0, G: 1, C: 18
5354966	MUL_4830	Putative GTPase	T/C	Synonymous	mu248	T: 0, A: 0, G: 0, C: 20
5577431	MUL_5032	Immunogenic protein mbt64	A/G	Synonymous	mu394	T: 0, A: 0, G: 28, C: 0

*A total of 4,918 core single-nucleotide polymorphisms were identified for all 6 isolates compared with the African Agy99 reference genome. –, not applicable (mutations were not within a coding region).

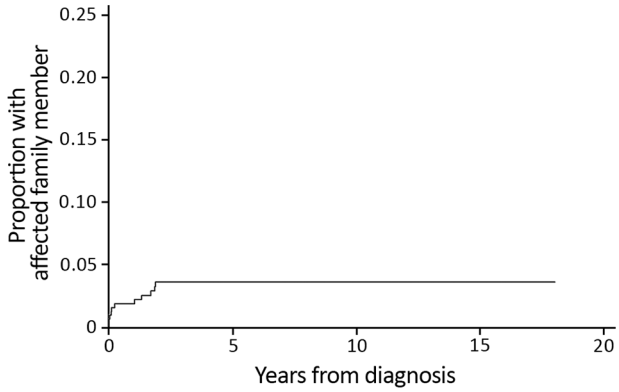

Figure 1. Cumulative proportion of patients with a family member affected by *Mycobacterium ulcerans* disease, Barwon Health cohort, Bellarine Peninsula, Victoria, Australia, 1998–2016.

Conclusions

Our examination of family clusters of *M. ulcerans* disease provides useful insights into the environmental reservoir and mode of transmission of this organism. First, the median time to diagnosis between family members was short (2.8 months), and no family members were given a diagnosis of an *M. ulcerans* lesion >23 months apart in a cohort spanning 18 years and nearly 2,000 combined years of elapsed time since diagnosis. This finding suggests that family members have been exposed to a source in the family's environment that persists only for a short period.

Second, with an incubation period for *M. ulcerans* disease estimated to be a median of 4.5 months (*12*), the observation that the median time between diagnoses in family clusters was <3 months suggest that infections were not being transmitted between family members. Further evidence against human-to-human transmission is apparent from whole-genome SNP analysis, which showed that pairs of isolates from 2 (2a/2b and 1a/1b) of 3 family clusters were not genetically identical. These findings support previous suggestions that *M. ulcerans* is unlikely to be transmitted from person to person (*13*).

Unknown is the type of short-term exposure that leads to the close temporal relation of family clustered infections. Opossums have been proposed as a source, either through contamination of the environment by infected feces or by an intermediate vector, such as mosquitoes, which transfer the infection from infected opossums to humans by a bite (*7*). Infected opossum(s) in the family environment might cause cases of human infection, then subsequently die of the disease (*14*), removing the source of infection. Alternatively, transmission could be related to a short-term change in the environment involving soil or foliage as a result of such events as home construction and renovation, or planting and removing trees or grasses (*13*). Mosquitoes in the area might be transiently infected/contaminated with *M. ulcerans* and infect humans through bites during this time (*15*).

In summary, the incidence rate of lesions in another family member (5.69/1,000 person-years) was higher than reported incidence rates during 2005–2009 in the general population of the Bellarine Peninsula (0.85–4.04 cases/year/1,000 population) (*7*). This finding suggests that genetic susceptibility or, more likely, localized exposure risk increases the likelihood of infection.

The incidence of *M. ulcerans* disease family clusters in an observational cohort in southeastern Australia was higher than in the general population of the disease-endemic area. However, when clusters occur, they are closely temporally related, which suggests a short-term risk for exposure and infection. Epidemiologic and genetic evidence suggests human-to-human transmission is not the source of infection.

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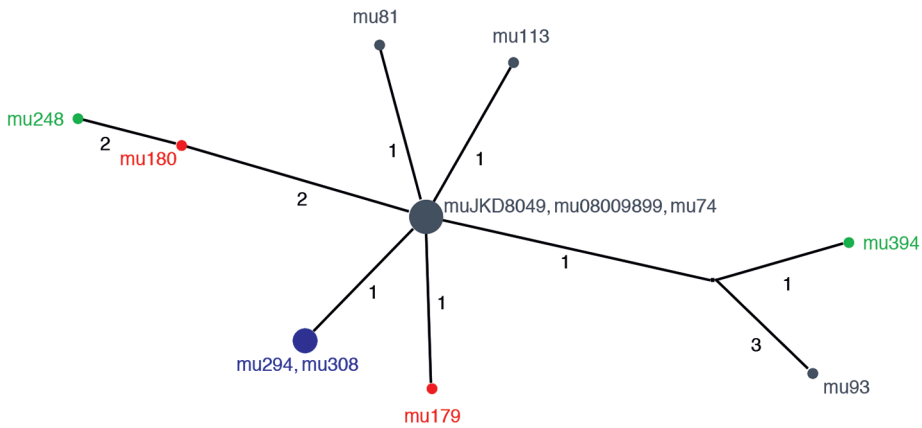

Figure 2. Median joining network of 12 SNPs of 12 *Mycobacterium ulcerans* isolates from patients with *Mycobacterium ulcerans* disease, Barwon Health cohort, Bellarine Peninsula, Victoria, Australia, 1998–2016. Node colors indicate clusters. Blue, cluster 4a/4b; red, cluster 1a/1b; green, cluster 2a/2b. Black nodes represent 6 unrelated isolates. The size of each node is proportional to the number of genetically identical isolates with identical genotypes. Values indicate number of SNPs between each node. SNP, single-nucleotide polymorphism.

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Estimated Incubation Period for Zika Virus Disease

Elisabeth R. Krow-Lucal, Brad J. Biggerstaff, J. Erin Staples

Information about the Zika virus disease incubation period can help identify risk periods and local virus transmission. In 2015–2016, data from 197 symptomatic travelers with recent Zika virus infection indicated an estimated incubation period of 3–14 days. For symptomatic persons with symptoms >2 weeks after travel, transmission might be not travel associated.

Zika virus is a mosquito-borne flavivirus transmitted primarily through the bite of infected *Aedes* spp.mosquitoes. Transmission can also occur through occupational laboratory exposure and by intrauterine, intrapartum, or sexual routes (*1*–*3*).

In May 2015, Zika virus disease cases were identified in Brazil, representing the first local transmission in the Americas (*4*). Subsequently, Zika virus spread rapidly, resulting in >463,000 suspected and laboratory-confirmed cases in the Americas as of June 30, 2016 (*5*). This rapid expansion highlighted key knowledge gaps, including incubation period. Characterizing the incubation period for Zika virus is needed for defining periods of risk and identifying local virus transmission. To estimate the incubation period, we used data from symptomatic persons who had traveled to an area with ongoing Zika virus transmission and for whom laboratory evidence indicated recent infection.

The Study

We included in our analysis persons for whom samples tested at the Centers for Disease Control and Prevention from January 1, 2015, through June 23, 2016, gave positive results, indicating recent Zika virus infection (defined as Zika virus RNA positivity by real-time reverse transcription or Zika or dengue virus positivity by IgM capture ELISA and confirmed by plaque reduction neutralization test with a Zika virus–specific neutralizing antibody titer ≥10 and Zika virus titer ≥4-fold higher than dengue virus titer) (*6*,*7*). We restricted our analysis to persons who were symptomatic, had known symptom onset date (onset of first symptom), had known travel dates from/to the continental United States, and were probably infected through a mosquito bite. We excluded from analysis those for whom disease was congenital or sexually transmitted and those reporting illness onset >2 months after travel (because of the typically shorter incubation periods for other flavivirus diseases).

To estimate the incubation period distribution, we first defined the exposure period as either the duration of travel if a person experienced illness after return from travel or the time from beginning of travel to the onset of illness if the traveler became ill during travel (Figure 1, panel A). We then fit various probability distributions in R (https://cran.r-project.org/) by using the dic.fit function in the coarseDataTools package, which uses methods detailed by Reich et al. (*8*). We selected the best model by using the Akaike information criterion. In addition to reporting fitted cumulative distribution function and associated 95% CIs, we reported certain quantiles and means. All analyses were conducted by using R.

For our primary analysis, we used all persons with evidence of a recent Zika virus infection (primary case set). We then performed a secondary analysis of persons with confirmed Zika virus infection and <2 weeks of travel (secondary case set), enabling evaluation of our estimates by using more stringent case definition requirements. A confirmed case of Zika virus disease was illness in a symptomatic person with a sample that was either Zika virus RNA positive or Zika or dengue virus IgM positive with neutralizing antibodies against Zika virus only.

From January 1, 2015, through June 23, 2016, we identified 337 persons with evidence of recent Zika virus infection. Of these, we excluded 140 (42%) because they did not meet the study criteria (Figure 2). Among the remaining 197 persons, median age was 42 (range 1–81) years, most (119/197; 60%) were female, and 11 (6%) were pregnant (Table). Median length of travel was 11 (range 2–177) days. The diagnosis of recent Zika virus infection was made by serologic testing for 134 (68%) persons, by molecular testing for 57 (29%), and by molecular and serologic testing for 6 (3%).

The Weibull distribution fit our data best (parameter estimates in online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/23/5/16-1715-Techapp1.pdf). For the primary case set, our estimates for incubation period were median 6.2 (95% CI 5.7—6.6) days (Figure 1, panel B) and mean 6.4 (95% CI 5.7–7.0) days. We estimated that, among persons in whom symptoms would develop,

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they would develop in 5% by 2.1 (95% CI 1.7–2.4) days and in 99% by 13.6 (95% CI 13.0–14.2) days (Figure 1, panel B; online Technical Appendix Table 2).

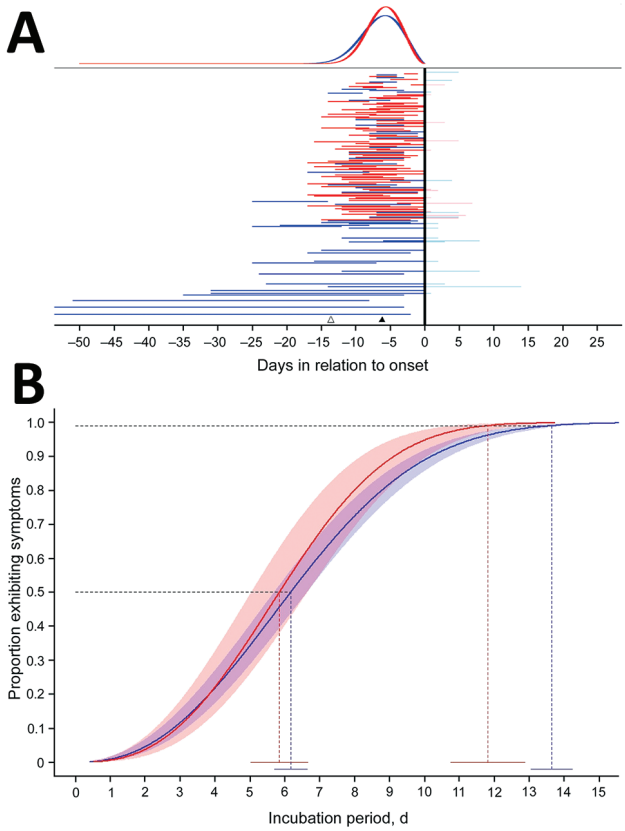

Figure 1. Estimated distribution of incubation period in days since infection for persons with evidence of recent Zika virus disease. A) Representation of individual interval censored travel data based on time of exposure relative to symptom onset (n = 197). Horizontal lines represent exposure times relative to onset. Vertical black line indicates symptom onset; red indicates persons with confirmed Zika virus disease; blue indicates all persons with Zika virus diseases; pink indicates exposure durations after symptom onset; and light blue indicates that these times did not contribute to the analysis. Individual data are sorted from bottom to top by exposure duration; to ease visible interpretation, we truncated long durations. The black triangle marks the estimated median incubation period for all Zika virus disease cases; the white triangle marks the estimated 95th quantile. The top panel shows the fitted Weibull density function; the blue line represents the distribution for all Zika virus disease cases; and the red line represents only those with confirmed Zika virus disease. B) Estimated distribution of time from infection to symptom onset (incubation period) for 197 persons with evidence of recent Zika virus infection (blue) and with confirmed Zika virus disease (red). The heavy line represents the estimated Weibull cumulative distribution function for the incubation period; 95% confidence bands are shown in red and blue shading. The 2 dotted lines represent the 50th and 99th quantiles; blue represents all cases; and red represents confirmed cases only. The solid horizontal line near the *x*-axis gives the point estimates and 95% CIs for the quantiles. Additional quantiles and CIs are shown in online Technical Appendix Table 2 (https://wwwnc.cdc.gov/EID/article/23/5/16-1715-Techapp1.pdf).

Of the 112 (57%) persons who had traveled for <2 weeks, cases were confirmed for 79 (71%). The age and sex distributions for these patients did not differ significantly from those of the primary case set (p = 0.67 and 0.44, respectively) (Table). The median length of travel was 8 (range 3–13) days. Zika virus diagnosis was confirmed by serologic testing for 47 (59%) patients, by molecular testing for 31 (39%), and by both methods for 1 (1%).

For patients with confirmed cases, we estimated the median incubation period to be 5.8 (95% CI 5.0–6.7) days (Figure 1, panel B; online Technical Appendix Table 2) and the mean to be 6.0 (95% CI 5.2–6.8) days. The quantile estimates (5%–95%) for these patients were similar to those for all travelers; however, among travelers with shorter travel durations and confirmed Zika virus infections, symptoms developed within 11.8 (95% CI 10.8 –12.9 days) days for 99%, compared with 13.6 days for all travelers.

On the basis of our analysis, we estimate that the incubation period for Zika virus is 3–14 days. We expect symptoms to develop within 1 week of infection for 50% and within 2 weeks for 99%. Our estimates for Zika virus incubation period are similar to those reported for other flaviviruses (*9–12*). The incubation period for Zika virus has

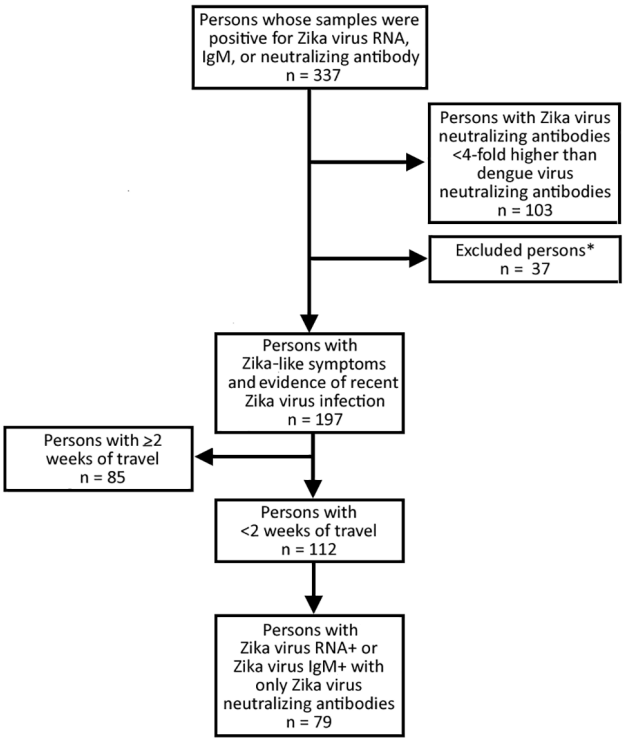

Figure 2. Persons with Zika virus–like symptoms and positive test results for Zika virus infection identified from samples received and tested for Zika virus infection at the Centers for Disease Control and Prevention. *Excluded for being asymptomatic, having congenital infection, having sexually transmitted infection, history of travel originating outside the United States, no date of symptom onset, symptom onset >2 months after travel return.

Table. Demographics, travel data, and laboratory testing results for Zika virus disease patients, United States, January 1, 2015, through June 23, 2016

Patient characteristic	All cases, no. (%), n = 197*	Confirmed cases, no. (%), n = 79†
Age, y		
0–19	19 (10)	10 (13)
20–39	71 (36)	29 (37)
40–59	79 (40)	32 (40)
≥60	27 (14)	7 (9)
Unknown	1 (<1)	1 (1)
Sex		
M	77 (39)	26 (33)
F	119 (60)	52 (66)
Unknown	1 (1)	1 (1)
Pregnant		
Yes	11 (6)	2 (3)
No	161 (82)	60 (76)
Unknown	25 (13)	17 (22)
Travel duration, d		
<7	24 (12)	15 (19)
7–13	88 (45)	64 (81)
14–20	31 (16)	0
21–27	12 (6)	0
≥28	42 (21)	0

*Persons with Zika virus–like symptoms and positive results for Zika virus RNA by real-time reverse transcription PCR or positive results for Zika or dengue virus IgM and Zika virus plaque reduction neutralization test (PRNT) results ≥10 and Zika virus titer ≥4-fold higher than dengue virus titer.
†Persons who traveled <2 weeks, experienced Zika virus–like symptoms, and had positive Zika virus RNA results by real-time reverse transcription PCR or positive Zika or dengue virus IgM results and PRNT ≥10 and dengue PRNT <10.

been estimated by Lessler et al., who reported data from 25 patients with variable exposure and laboratory evidence of infection (*13*). Their estimated median incubation period was similar to ours, 5.9 days, but the upper limit from that study was 18 days, which is 6 and 7 days longer than our estimates for the primary and secondary case sets, respectively. The difference in the upper limit was probably the result of the lower number of cases and higher variability in travel durations for their cohort.

Our analysis has several limitations. First, samples were submitted to the Centers for Disease Control and Prevention for all patients in this analysis, although guidance for testing recommended testing only persons with symptom onset <2 weeks after travel (*14*). Testing of all patients could have biased our sample population. Second, we included persons who were Zika virus IgM positive, considered as having recent infection. However, because the duration of IgM after Zika virus infection is not known, we might have included persons who had a prior infection unrelated to their most recent travel. Third, our analysis does not include other modes of transmission, such as sexual or congenital, for which incubation periods might differ. Fourth, we cannot be sure that all cases included in the analysis were caused by vector transmission because sexual transmission may have occurred during travel. Similarly, our primary case set included 11 pregnant women. Data suggest that the immunologic response to Zika virus infection might differ during pregnancy (*15*); however, in our analysis, the incubation periods of the pregnant women did not differ qualitatively from those of nonpregnant travelers.

Conclusions

According to our analysis, among Zika virus–infected travelers who will become symptomatic, 99% will experience symptoms within 2 weeks of exposure and 50% within 1 week. Persons for whom symptoms develop >2 weeks after travel and test results for a recent Zika virus infection are positive should be evaluated for alternative modes of transmission (e.g., sexual transmission) or local vectorborne transmission.

Acknowledgments

We thank the Zika Virus Response Epidemiology and Laboratory Teams and vectorborne disease surveillance coordinators in state and local health departments for their efforts with collection and sharing of data used in this analysis.

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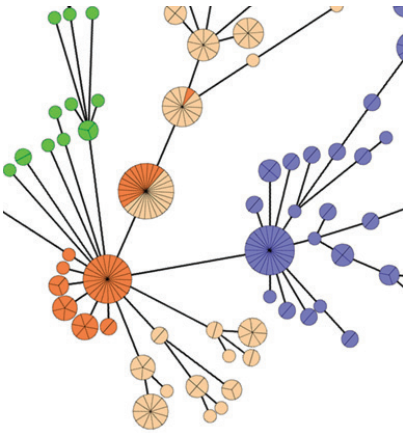

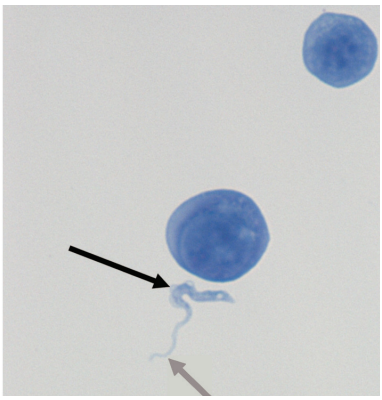

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Virulence Analysis of *Bacillus cereus* Isolated after Death of Preterm Neonates, Nice, France, 2013

Romain Lotte, Anne-Laure Hérissé, Yasmina Berrouane, Laurène Lotte, Florence Casagrande, Luce Landraud, Sabine Herbin, Nalini Ramarao, Laurent Boyer, Raymond Ruimy

After the deaths of 2 preterm neonates with *Bacillus cereus* systemic infection in the same intensive care unit, we investigated the pathogenic potential of this bacterium. Genetic and virulence analysis indicated the neonates were infected with 2 different strains with a virulence potential similar to environmental strains, indicating likely patient immune response failure.

Bacillus cereus is a gram-positive, spore-forming bacterium that is widespread in the environment. In adults, *B. cereus* is involved mainly in gastrointestinal infection and is the third most common cause of food poisoning (*1*). Rarely, this bacterium causes invasive or fatal infections in high-risk patients, such as immunocompromised adult patients and preterm neonates who have an immature immune system that is mostly restricted to innate immunity (*2–4*).

In 2013, two preterm infants with *B. cereus* infection died in the same intensive care unit. As part of the investigation of these deaths, we conducted genetic and virulence analyses of *B. cereus* strains from the patients and from the environment.

The Study

In September 2013, tracheobronchial aspiration and blood cultures positive for *B. cereus* were obtained from 2 premature newborns hospitalized in the same intensive care unit. An unfavorable outcome led to the infants' deaths despite an appropriate treatment with wide-spectrum antibiotic drugs.

The first premature infant was female, born at 27 weeks and 2 days of gestation, and weighed 880 g. An emergency cesarean delivery was performed because of the mother's preeclampsia. The Apgar score at birth was 1-2-10, with bagging ventilation and intubation at 5 min after birth. No evidence of maternal–fetal transmission of infection was retrieved. On day 4, signs of infection were noted in the newborn, including respiratory distress, tachycardia, and a gray skin complexion. Investigations revealed elevated inflammatory markers (C-reactive protein level 88 mg/L). Empirical intravenous antimicrobial drug therapy (cefotaxime, gentamicin, and vancomycin) was started.

Tracheobronchial aspiration was performed and, a sample grew 10^6 CFU/mL of *B. cereus* identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MicroFlex LT; Bruker Daltonics, Billerica, MA, USA) (log score value of 2.07 matching with *B. cereus* reference strain DSM 31T, MALDI Biotyper v2.3). The blood culture remained sterile after 14 days. During her stay, the neonate had refractory hypoxemia due to a diffuse pulmonary lung parenchymal necrosis that required high-frequency ventilation and continuous thoracic drain. Despite an appropriate antimicrobial drug treatment (15 days of vancomycin followed by fluoroquinolone), the neonate had chronic hypoxemia and died at 26 days of age.

The second premature neonate, born 2 days after the first, was male, born at 29 weeks and 4 days of gestation, and weighed 1,480 g. A cesarean section was performed to enable the mother to start chemotherapy for a maternal malignancy, diagnosed at 26 weeks of gestation. The Apgar score at birth was 10. Physical examination indicated no sign of maternal or neonatal infection. On day 4, signs of infection were observed in the newborn, along with respiratory distress. The infant was reintubated, and antimicrobial drug therapy (cefotaxime, gentamicin, and vancomycin) was started.

Blood cultures were positive after 9 hours, and subcultures grew with *B. cereus* (log score 2.02). Catheter cultures were positive and grew 10^6 CFU/mL of *B. cereus* (log score 2.1). On day 5, despite appropriate care and sepsis control, the newborn showed severe neurologic impairment. Control cranial ultrasound revealed brain empyema, cerebral necrosis, and cranial hemorrhages (Figure 1). An unfavorable outcome led to the patient's death at 8 days of age from multiple organ failure and cerebral abscesses.

The hospital's infection control team looked for environmental reservoirs as potential sources of contamination

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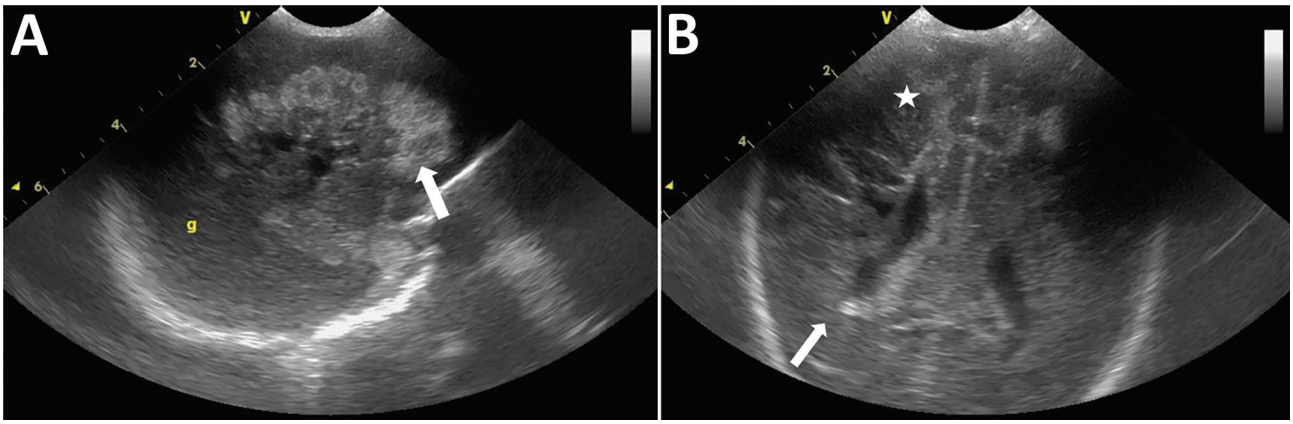

Figure 1. Standard echography cranial ultrasound of premature infant with *Bacillus cereus* sepsis, Nice, France, 2013. A) Left sagittal section showing large hemorrhagic hyperechogenic area of white material (white arrow). B) Frontal section showing right periventricular kystic hypoechogenic lesions (white arrow) with associated bilateral hemorrhagic hyperechogenic lesions (white star).

of the 2 newborns. Therefore, ventilation equipment, balloons used in manual ventilation, intravenous umbilical catheters, ultrasonic probes, linens (including towels and bedsheets), breast milk, and freeze-dried breast milk were collected and sent for microbiological analysis (Table 1). *B. cereus* cultures were positive for 5 environmental samples, including the surface of the incubator used for the first newborn (3 samples), ultrasonic probes (5 samples), and a bench surface used for bottle-feeding (5 samples). We compared all *B. cereus* strains, including those isolated from the 2 newborns, by using M13-PCR methods (*5*). This analysis revealed that the patients were infected by 2 different strains and that the environmental strains were different from strains isolated from patients. These data excluded a clonal transmission between the 2 patients and the hypothesis of a nosocomial outbreak caused by an emerging virulent strain (Figure 2, panel A). Nevertheless, a common source of infection for the 2 newborns by polyclonal strains cannot be excluded.

Table 1. Microbiological results of environmental sampling after deaths of 2 preterm neonates with *Bacillus cereus* infection, Nice, France, 2013*

Environmental site	No. positive/no. samples tested
Incubator, first newborn	3/7
Incubator, second newborn	0/7
Ultrasonographic probe	1/1
Bench surface used for bottle feeding	1/1
Control incubator 1	0/4
Control incubator 2	0/4
Control ultrasonographic probe	0/1
Heated humidifier for 2 control incubators	0/2
Wet bulb for 2 control incubators	0/2
Heating ramp for control incubator 1	0/1
Control incubator mattresses	0/2
Babies' sheets	0/1
Towels	0/1
Sterile field	0/1
Ultrasound gel dispenser bottles	0/2
Used ultrasound gel	0/1
Air filtration/ventilation equipment	0/1
Air flow sensor for ventilator	0/1
Heating unit for ventilator	0/1
Food refrigerator	0/1
Various cutlery	0/2

We screened the isolated strains for *B. cereus* main virulence factor genes hemolysin BL, nonhemolytic enterotoxin, cytotoxin K, and hemolysin II (Table 2) by using PCR and toxin production assay methods (*6–9*). Both patient and environmental isolates produced toxins. We further assessed the virulence potential using an in vivo model of *Drosophila melanogaster* infection. To validate the capacity of this model to detect the virulence of various bacterial strains, we first infected wild-type flies with *Escherichia coli* CIP 102181, *Staphylococcus aureus* CIP 110856, and *B. cereus* CIP 66.24T. We grew bacteria in Luria-Bertani broth overnight at 37°C and subcultured them up to an optical density of 0.8 at 600 nm. We dipped a tungsten needle into an equal volume of bacterial suspension or phosphate-buffered saline (control) and used it to prick 20–30 adult male flies (*10*). All flies infected with *S. aureus* and *B. cereus* died after 20 and 12 hours, respectively. Flies infected with *E. coli* displayed a survival rate similar to that of control flies, enabling us to validate *D. melanogaster* as a model for evaluating the strains' virulence potential (Figure 2, panel B).

We used the same protocol to compare all *B. cereus* strains. We found no statistical difference in survival between flies infected with the different *B. cereus* strains, including *B. cereus* CIP 66.24T (Figure 2, panel C). These data correlate with the absence of a specific virulence signature for those strains (Table 2).

Conclusions

Considering the fatal outcome of the 2 infections despite appropriate antimicrobial drug therapy, we addressed the question of a high virulence potential of the patients'

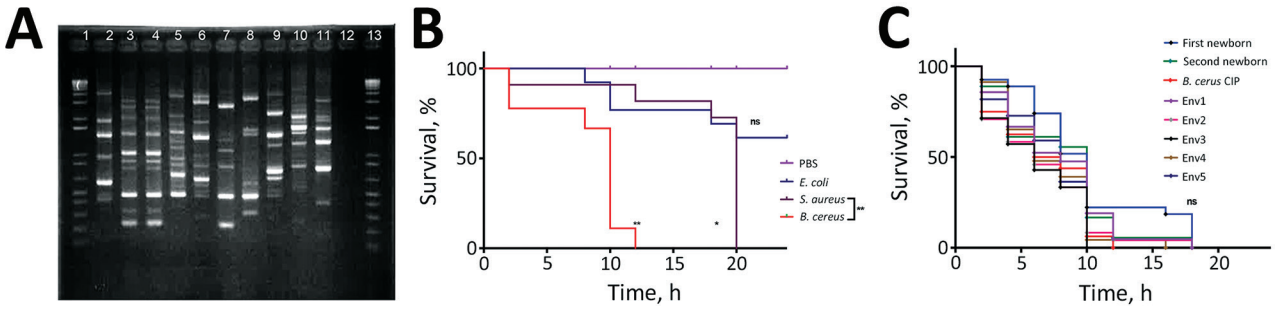

Figure 2. Genetic and virulence analyses of *Bacillus* spp. strains isolated from 2 preterm neonates with *B. cereus* infection and environmental sampling from intensive care unit, Nice, France, 2013. A) Molecular typing using M13-PCR methods, as described by Guinebretiere et al. (*5*). Lane 1, DNA ladder; lane 2, tracheobronchial fluid, first newborn; lane 3, blood culture, second newborn; lane 4, catheter, second newborn; lane 5, incubator surface, first newborn (Env1); lane 6, incubator surface, first newborn (Env2); lane 7, incubator surface, first newborn (Env3); lane 8, ultrasonographic probe (Env4); lane 9, bench surface used for bottle feeding (Env5); lane 10, incubator surface, second newborn (1); lane 11, incubator surface, second newborn (2); lane 12, negative control; lane 13, DNA ladder. B) Survival of flies infected with *Escherichia coli* CIP 102181, *Staphylococcus aureus* CIP 110856, and *B. cereus* CIP 66.24 T, compared with survival of control flies injected with phosphate-buffered saline (PBS). *p <0.01; **p <0.001; ns, not significant (all by Gehan-Breslow-Wilcoxon χ^2 test). C) Survival of flies infected with the different strains of *B. cereus* tested. Env, environmental; ns, not significant by Gehan-Breslow-Wilcoxon χ^2 test.

B. cereus strains by testing for the presence of virulence factor genes and expression levels. We found a similar virulence factor profile in the patients and in the environmental strains. This profile suggested that the outcome of the infection was probably not linked to the virulence potential of the strains.

We then used *D. melanogaster* as an infection model. We chose this model because flies rely only on innate immunity to survive infections, similar to preterm newborns, who have immature immune systems. We found that all isolated *B. cereus* strains (patient and environmental strains) displayed a similar killing potential, suggesting that the fatal outcome in both newborns was due not to the emergence of a hypervirulent strain but rather to a similar pathogenic potential for all *B. cereus* strains toward at-risk patients.

Given that *B. cereus* is ubiquitous in the environment and potentially fatal in preterm neonates, it appears critical to determine how these 2 neonates were infected and why they died, as well as why other preterm neonates hospitalized concurrently in the same room of the intensive care unit remained uninfected. Further investigations would be necessary to determine whether the deaths were a consequence of an innate immune defect, a high bacterial load at time of contamination, or a combination of both parameters.

Our study, along with previous ones (*3,4,11–13*), reinforces the idea that *B. cereus* is an underestimated emerging pathogen that can be involved in fatal healthcare-associated infections in premature newborns. Our results indicate that all *B. cereus* strains display potentially pathogenic properties toward at-risk patients. Considering that *B. cereus* is ubiquitous in the environment, it is essential to emphasize the necessity of strict hygiene measures and protocols to prevent bacterial transmission. Evaluating an immune response capacity in at-risk patients must be considered to avoid a fatal outcome from *B. cereus* infection.

Table 2. Virulence factor analysis of strain characteristics of bacteriologic samples obtained after deaths of 2 preterm neonates with *Bacillus cereus* infection, Nice, France, 2013*

Tested strain	Source of sample	Gene				Genotype group (%)	Nhe production inde)†	Hbl detection limit
		cytK1	*cytK2*	*ces*	*hlyII*			
First newborn	Tracheobronchial aspiration	–	+	–	–	III (99.72)	+++	–
Second newborn	Blood culture	–	+	–	–	IV (100)	+	1/64
Second newborn	Catheter	–	+	–	–	IV (100)	+	1/64
Environmental isolate 1	Incubator surface, first newborn	–	–	–	–	II (97.71)	++	–
Environmental isolate 2	Incubator surface, first newborn	–	+	–	–	III (99.72)	+++	–
Environmental isolate 3	Incubator surface, first newborn	–	+	–	+	IV (100)	+++	1/64
Environmental isolate 4	Ultrasonographic probe	–	+	–	–	IV (100)	+++	1/32
Environmental isolate 5	Bench surface used for bottle feeding	–	–	–	–	III (100)	+++	–

*Hbl, hemolytic BL toxin; Nhe, nonhemolytic enterotoxin; +, positive; –, negative.
†Nhe production level: +, low; ++ moderate; +++, high.

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The Discovery of Penicillin—New Insights After More Than 75 Years of Clinical Use

Robert Gaynes

After just over 75 years of penicillin's clinical use, the world can see that its impact was immediate and profound. In 1928, a chance event in Alexander Fleming's London laboratory changed the course of medicine. However, the purification and first clinical use of penicillin would take more than a decade. Unprecedented United States/Great Britain cooperation to produce penicillin was incredibly successful by 1943. This success overshadowed efforts to produce penicillin during World War II in Europe, particularly in the Netherlands. Information about these efforts, available only in the last 10–15 years, provides new insights into the story of the first antibiotic. Researchers in the Netherlands produced penicillin using their own production methods and marketed it in 1946, which eventually increased the penicillin supply and decreased the price. The unusual serendipity involved in the discovery of penicillin demonstrates the difficulties in finding new antibiotics and should remind health professionals to expertly manage these extraordinary medicines.

According to British hematologist and biographer Gwyn Macfarlane, the discovery of penicillin was "a series of chance events of almost unbelievable improbability" (*1*). After just over 75 years of clinical use, it is clear that penicillin's initial impact was immediate and profound. Its detection completely changed the process of drug discovery, its large-scale production transformed the pharmaceutical industry, and its clinical use changed forever the therapy for infectious diseases. The success of penicillin production in Great Britain and the United States overshadowed the serendipity of its production and the efforts of other nations to produce it. Information on penicillin production in Europe during World War II, available only in the last 10–15 years, provides new insights into penicillin's story.

Dawn of Chemotherapy and the "Magic Bullet"

At the beginning of the 20th century, Paul Ehrlich pioneered the search for a chemical that would kill a microorganism and leave the host unaltered—the "magic bullet." Ehrlich also coined the term chemotherapy: "There must be planned chemical synthesis: proceeding from a chemical substance with recognizable activity, making derivatives from it, and then trying each to discover the degree of its activity and effectiveness. This we call chemotherapy" (*2*). After extensive testing, he found a drug with activity against the bacterium *Treponema pallidum*, which causes syphilis. The introduction of this drug, arsphenamine (Salvarsan), and its chemical derivative neoarsphenamine (Neosalvarsan) in 1910 ushered in a complete transformation of syphilis therapy and the concept of chemotherapy. Unfortunately, despite exhaustive searches, the promise of more magic bullets for microbial therapy remained elusive. For 20 years, Salvarsan and Neosalvarsan were the only chemotherapy for bacterial infections.

Alexander Fleming's Discovery

A chance event in a London laboratory in 1928 changed the course of medicine. Alexander Fleming, a bacteriologist at St. Mary's Hospital, had returned from a vacation when, while talking to a colleague, he noticed a zone around an invading fungus on an agar plate in which the bacteria did not grow. After isolating the mold and identifying it as belonging to the *Penicillium* genus, Fleming obtained an extract from the mold, naming its active agent penicillin. He determined that penicillin had an antibacterial effect on staphylococci and other gram-positive pathogens.

Fleming published his findings in 1929 (*3*). However, his efforts to purify the unstable compound from the extract proved beyond his capabilities. For a decade, no progress was made in isolating penicillin as a therapeutic compound. During that time, Fleming sent his *Penicillium* mold to anyone who requested it in hopes that they might isolate penicillin for clinical use. But by the early 1930s, interest had waned in bringing to life Paul Ehrlich's vision of finding the magic bullet.

Discovery of Prontosil and Sulfa Drugs

This dismal outlook on chemotherapy began to change when Gerhard Domagk, a German pathologist and bacteriologist, found bacteriologic activity in a chemical derivative from oil dyes called sulfamidochrysoïdine (also known as Prontosil). This compound had bacteriologic activity in animals, but strangely, none in vitro. Prontosil had limited but definite success when used to treat patients with bacterial infections, including Domagk's own child. A German company patented the drug, and ultimately, Domagk won a Nobel Prize in 1939. The paradox of Prontosil's in vivo success but lack of success in vitro was explained in 1935, when French scientists determined that only part of Prontosil was active: sulfanilamide. In animals, Prontosil was metabolized into sulfanilamide. Within 2 years, sulfanilamide

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and several derivative sulfa drugs were on the market. The success of sulfanilamide changed the cynicism about chemotherapy of bacteria (*1*).

Isolation of Penicillin at Oxford University

The success of sulfa drugs sparked interest in finding other agents. At Oxford University, Ernst Chain found Fleming's 1929 article on penicillin and proposed to his supervisor, Howard Florey, that he try to isolate the compound. Florey's predecessor, George Dreyer, had written Fleming earlier in the 1930s for a sample of his strain of *Penicillium* to test it for bacteriophages as a possible reason for antibacterial activity (it had none). However, the strain had been saved at Oxford. In 1939, Howard Florey assembled a team, including a fungal expert, Norman Heatley, who worked on growing *Penicillium* spp. in large amounts, and Chain, who successfully purified penicillin from an extract from the mold. Florey oversaw the animal experiments. On May 25, 1939, the group injected 8 mice with a virulent strain of *Streptococcus* and then injected 4 of them with penicillin; the other 4 mice were kept as untreated controls. Early the next morning, all control mice were dead; all treated mice were still alive. Chain called the results "a miracle." The researchers published their findings in The Lancet in August 1940, describing the production, purification, and experimental use of penicillin that had sufficient potency to protect animals infected with *Streptococcus pyogenes, Staphylococcus aureus,* and *Clostridium septique* (*4*).

After the Oxford team had purified enough penicillin, they began to test its clinical effectiveness. In February 1941, the first person to receive penicillin was an Oxford policeman who was exhibiting a serious infection with abscesses throughout his body. The administration of penicillin resulted in a startling improvement in his condition after 24 hours. The meager supply ran out before the policeman could be fully treated, however, and he died a few weeks later. Other patients received the drug with great success. The Oxford team then published their clinical findings (*5*). At the time, however, pharmaceutical companies in Great Britain were unable to mass produce penicillin because of World War II commitments. Florey then turned to the United States for assistance.

Penicillin and US Involvement

In June 1941, Florey and Heatley traveled to the United States. Concerned about the security of taking a culture of the precious *Penicillium* mold in a vial that could be stolen, Heatley suggested that they smear their coats with the *Penicillium* strain for safety on their journey. They eventually arrived in Peoria, Illinois, to meet with Charles Thom, the principal mycologist of the US Department of Agriculture, and Andrew Jackson Moyer, director of the department's Northern Research Laboratory. Thom corrected the identification of Fleming's mold to *P. notatum*; it was initially identified as *P. rubrum* (*1*).

Thom also recognized the rarity of this *P. notatum* strain because only 1 other strain in his collection of 1,000 *Penicillium* strains produced penicillin. The strain that was eventually used in mass production was a third strain, *P. chrysogenum*, found in a moldy cantaloupe in a market, which produced 6 times more penicillin than Fleming's strain. When a component of the media that Heatley used to grow the mold in England was unavailable, A.J. Moyer suggested using corn steep liquor, a waste product from the manufacture of cornstarch that was available in large quantities in the midwestern United States. With corn steep liquor, the investigators produced exponentially greater amounts of penicillin in the filtrate of the mold than the Oxford team had ever produced. Heatley remained in Peoria for 6 months to work on methods of growing *Penicillium* strains in large quantities. Florey headed east to interest the US government and multiple drug companies in penicillin production. The US government took over all penicillin production when the United States entered World War II. Researchers at drug companies developed a new technique for producing enormous quantities of penicillin-producing *Penicillium* spp.: deep-tank fermentation. This process adapted a fermentation process performed in swallow dishes to deep tanks by bubbling air through the tank while agitating it with an electric stirrer to aerate and stimulate the growth of tremendous quantities of the mold. Unprecedented United States/Great Britain cooperation for penicillin production was incredibly successful. In 1941 the United States did not have sufficient stock of penicillin to treat a single patient. At the end of 1942, enough penicillin was available to treat fewer than 100 patients. By September 1943, however, the stock was sufficient to satisfy the demands of the Allied Armed Forces (*6*).

Public Awareness: The Fleming Myth

Early in 1942, Florey and Heatley went back to England. Because of the shortage of penicillin supplies coming from the United States, the Oxford group still had to produce most of the penicillin they tested and used. In August 1942, Fleming obtained some of the Oxford group's supply and successfully treated a patient who was dying of streptococcal meningitis. When the patient recovered, the cure was the subject of a major article in The Times newspaper in Great Britain, which named Oxford as the source of the penicillin. However, neither Florey nor Fleming was acknowledged in the article, an oversight quickly corrected by Fleming's boss, Sir Almroth Wright. He wrote a letter to

The Times expounding on Fleming's work and suggested that Fleming deserved a "laurel wreath." Fleming happily talked to the press. Florey not only did not speak with the press but prohibited any member of the Oxford team from giving interviews, leading many to erroneously believe that Fleming alone was responsible for penicillin.

Secrecy in Wartime England

The British government went to great lengths to prevent the means for producing penicillin from falling into enemy hands. However, news about penicillin leaked out. A Swiss company (CIBA, Basal, Switzerland) wrote to Florey requesting *P. notatum*. Concerned about responding, Florey contacted the British government. Agents attempted to track down where Fleming's *Penicillium* cultures had been distributed. Fleming wrote, "During the past 10 years I have sent out a very large number of cultures of *Penicillium* to all sorts of places, but as far as I can remember NONE have gone to Germany" (*7*). Florey believed that, without the mold, no one in Germany could produce penicillin even though his publication had provided a "blueprint" for its small scale manufacture. Florey was wrong, and so was Fleming.

Fleming had sent a culture of *Penicillium* strains to "Dr. H. Schmidt" in Germany in the 1930s. Schmidt was unable to get strain to grow, but even though the Germans did not have a viable strain, other Europeans did.

Production during World War II

France

Someone at Institut Pasteur in France, had Fleming's strain. In 1942, efforts began at Institut Pasteur and Rhone-Poulenc to produce penicillin. Eventually, German officials found out and, in early 1944, the Germans asked the French for their *P. notatum*. They were given a false strain that did not produce penicillin. With limited supplies, the French produced only enough penicillin to treat ≈30 patients before the wars end.

The Netherlands

The situation in the Netherlands was different. The Centraalbureau voor Schimmelcultures (CBS) near Utrecht had the largest fungal collection in the world. A published list of their strains in 1937 included *P. notatum*. A letter found at CBS shows that in February 1942 the Nazis asked CBS to send their strain of *P. notatum* to Dr. Schmidt in Germany, mentioning penicillin in the letter. CBS told the Germans they did not have Fleming's strain of *P. notatum*. In fact, they did. In the 1930s, Fleming had sent his strain to Johanna Westerdijk, the CBS director. Westerdijk could not refuse the German request for their strain of *P. notatum* but sent them the one that did not produce penicillin.

Efforts to produce penicillin in the Netherlands went underground at a company in Delft, the Nederladsche Gist-en Spiritusfabriek (the Netherlands Yeast and Spirit Factory, NG&SF). After the German occupation in 1940, NG&SF was still allowed to function. Because Delft was not bombed in the war, NG&SF's efforts were unaffected. In early 1943, NG&SF's executive officer, F.G. Waller, secretly wrote to Westerdijk at CBS, asking for any *Penicillium* strains that produced penicillin. In January 1944, Westerdijk sent all of CBS' *Penicillium* strains to NG&SF.

Four reports in NG&SF records detailed their efforts (*8*). In the first report, NG&SF scientists tested 18 *Penicillium* strains from CBS; they found 1 strain with the greatest antibacterial activity, which was coded P-6 and was identified as *P. baculatum.* The second report discussed how NG&SF scientists then isolated an extract from P-6. They gave the substance in the extract the code name Bacinol after the species from which it was derived and to keep the Germans unaware of what they were doing (Figure). As Waller wrote, "When we first started looking, in 1943, only one publication was available, that of Fleming in 1929. It was on that basis we started our research" (*6*). NG&SF researchers then had help from an unanticipated source. In 1939, Andries Querido was employed by NG&SF as a part-time advisor. By January 1943, however, his Jewish background limited his visits. On his last visit in the summer of 1944, Querido met someone in Amsterdam's Central Train Station who gave him a copy of the latest Schweizerische Medizinische Wochenschrift (Swiss Medical Journal), which he passed on to the NG&SF scientists. The June 1944 issue contained an article entirely devoted to penicillin, showing the results that the Allies had achieved, including details of penicillin growth in corn steep extract, the scaling up of penicillin production, the measurement of strength by the Oxford unit, results of animal and human studies, and identification of the bacteria known to be susceptible to penicillin. The third report described how NG&SF scientists isolated Bacinol from the extract using the information supplied secretly by Querido.

Large-scale production would be difficult to do and to keep secret from the Germans, especially with a German guard on site. However, NG&SF scientists used an obvious ploy to keep the German guard, who knew nothing about microbiology, at bay: they kept him drunk. "We did have a German guard whose job it was to keep us under surveillance, but he liked gin, so we made sure he got a lot. He slept most afternoons" (*6*). NG&SF scientists used milk bottles for growing large quantities of *Penicillium* mold. From July 1944 until March 1945, production of Bacinol continued, as detailed in the fourth report. At the end of the war, the NG&SF team still did not know if Bacinol was actually penicillin until they tested it

Figure. Bacinol 2, building named in honor of the site of efforts in the Netherlands to produce penicillin during World War II and the drug produced by the Netherlands Yeast and Spirit Factory in Delft. Bacinol was a code name for penicillin. Source: https://commons.wikimedia.org/wiki/File:Delft_-_Gevel_Bacinol_2.jpg

against some penicillin from England, proving it to be the same compound. NG&SF began marketing the penicillin they produced in January 1946. Although the original building where Bacinol was produced was demolished, NG&SF named a new building in honor of their WWII efforts (Figure).

The Nazis eventually succeeded in making penicillin by October 1944. However, Allied air raids crippled mass production of the drug (*9*).

Patents

The issue of a patent for penicillin was a controversial problem from the beginning. Chain believed that obtaining a patent was essential. Florey and others viewed patents as unethical for such a life-saving drug. Indeed, penicillin challenged the basic notion of a patent, considering it was a natural product produced by another living microorganism. The prevailing view Great Britain at the time was that a process could be patented, but the chemical could not. Merck (New York, NY, USA) and Andrew Jackson Moyer each filed patents on the process of penicillin production with no opposition. Eventually, at war's end, British scientists were faced with paying royalties for a discovery made in England. The penicillin production at NG&SF turned out to be more than of historical interest. Because NG&SF had researched and developed their own penicillin using their own mold culture, *P. baculatum*, and used their own production methods, they were not embroiled in any patent clash; the marketing of their penicillin eventually increased penicillin supply and decreased prices.

Nobel Prize in 1945

Penicillin's colossal effects led to the awarding of the Nobel Prize in Medicine and Physiology in 1945 to Fleming, Chain, and Florey. Penicillin was isolated from other microorganisms, which led to a new term, antibiotics. Using similar discovery and production techniques, researchers discovered many other antibiotics in the 1940s and 1950s: streptomycin, chloramphenicol, erythromycin, vancomycin, and others.

Conclusions

Lessons can be learned from the circumstances surrounding the discovery of penicillin. The US government's successful takeover of penicillin's production and the unprecedented cooperation among drug companies (and nations) should strongly encourage public/private partnerships as we search for additional effective antimicrobial drugs. In addition, despite their essential value in modern medicine, antibiotics are also the only class of drugs that lose their efficacy with large-scale use as bacteria develop antibiotic resistance. We now are struggling with resistant bacteria that cause infections that are virtually untreatable. Infections such as those occurring after transplantation and surgical procedures, caused by these highly antibiotic-resistant pathogens, are threatening all progress in medicine. Yet, drug companies, some of the same companies that helped develop penicillin, have nearly abandoned efforts to discover new antibiotics, finding them no longer economically worthwhile. The dry pipeline for new antibiotics has led

the Infectious Diseases Society of America and others to call for a global commitment to the development of new agents (*10*). We also must expertly manage the drugs that are currently available. The noteworthy serendipity involved in the discovery of penicillin should remind us that new antibiotics are difficult to find and, more important, should make us mindful when using these limited medical treasures.

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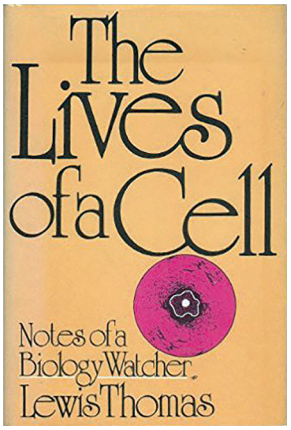
EID Podcast:
Lives of a Cell: 40 Years Later, A Third Interpretation



In 1974, Lewis Thomas (1913–1993), physician, professor, and dean, published The Lives of A Cell, the first of 2 books subtitled Notes of a Biology Watcher. The phrase "lives of a cell" refers to the independent yet interrelated parts of a human cell—including mitochondria, centrioles, and basal bodies—that once led independent lives. Without these previously independent lives working together, we would not have the capacity for thought, communication, and movement. Dr. Thomas wrote, "Our membranes hold against equilibrium, maintain imbalance, bank against entropy…. We are shared, rented and occupied."

Our human lives do not depend just on the lives in our individual cells. Our lives depend fully on the earth, including the atmosphere, and the many other human and nonhuman lives that occupy it. In explaining this complex interdependence, Dr. Thomas observed that the earth is "most like a cell." This second interpretation of lives of a cell refers to the many interrelated earthly entities, such as plants, whales, humans, and even viruses, that "dart rather like bees from organism to organism, from plant to insect to mammal to me and back again," all protected by the sky—a membrane that "works, and for what it is designed to accomplish it is as infallible as anything in nature."

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EMERGING INFECTIOUS DISEASES

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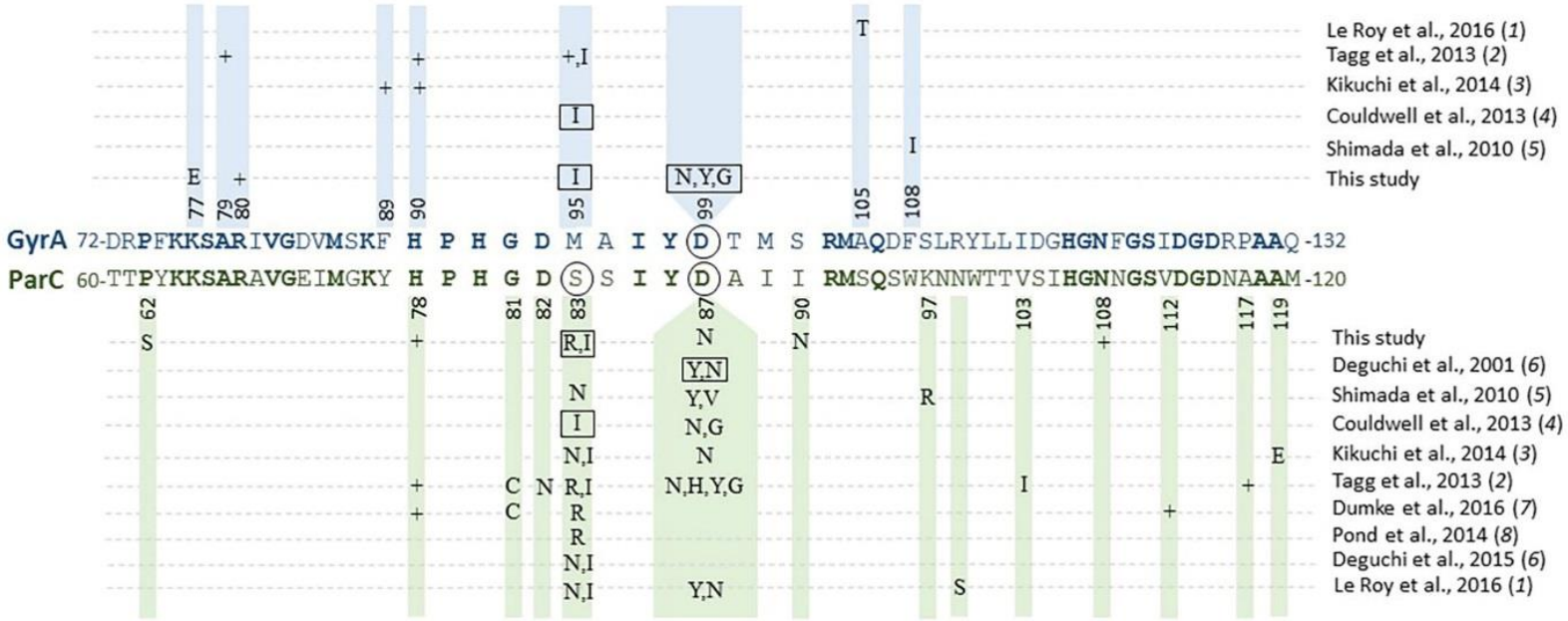
Macrolide and Fluoroquinolone Resistance in *Mycoplasma genitalium*

Technical Appendix



Technical Appendix Figure. Comparison of amino acid sequence changes in *Mycoplasma genitalium* ParC and GyrA. The amino acid sequences from strain G37 were aligned, and the relevant portion of the sequence is presented, with identical residues indicated in boldface. Numbers refer to the amino acid sequence number from the *M. genitalium* sequence. Changes in sequence at specific residues are indicated by

letters enclosed by shading, either above the alignment (GyrA) or below (ParC). The relevant study is indicated on the right. A boxed letter indicates that the change has been linked with fluoroquinolone treatment failure. Circled amino acids in the primary sequences indicate residues that may be directly involved in fluoroquinolone binding. + indicates silent mutation.

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