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Strain Typing and Antimicrobial Resistance of Fluoroquinolone-Resistant *Neisseria gonorrhoeae* Causing a California Infection Outbreak▽

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Antimicrobial-resistant *Neisseria gonorrhoeae* is an emerging public health problem as a result of the alarming limitation in treatment options. We examined an outbreak in California of fluoroquinolone-resistant *Neisseria gonorrhoeae* (QRNG) by evaluation of a combination of routine isolates from the Gonococcal Isolate Surveillance Project and isolates collected by expanded surveillance performed between April 2000 and June 2002. QRNG isolates were characterized by two methods: (i) determination of a combination of antibiogram, auxotype, serovar, Lip type, and patterns of amino acid alteration in the quinolone resistance-determining region of GyrA and ParC (ASLGP) and (ii) pulsed-field gel electrophoresis (PFGE). Strain typing was used to describe the QRNG outbreak strains and the associated antimicrobial resistance profiles. Among 79 isolates that were completely characterized, we identified 20 different ASLGP strain types, and 2 of the types were considered to belong to outbreak strains that comprised 65% (51/79) of the isolates. By PFGE typing, there were 24 different strain types, and 4 of these were considered outbreak types and comprised 66% (52/79) of the isolates. The overall agreement between the typing methods in distinguishing outbreak strains and non-outbreak strains was 84% (66/79). The most common QRNG ASLGP strain type had chromosomally mediated resistance to penicillin and tetracycline and an azithromycin MIC of 0.5 μg/ml. The occurrence of an outbreak caused by QRNG strains that could fail to be eradicated by most antibiotic classes reinforces the serious problem with antimicrobial resistance in *Neisseria gonorrhoeae* that the public health system faces. Adherence to a regimen with the recommended antibiotics at the appropriate dose is critical, and monitoring for antimicrobial susceptibility needs to be actively maintained to adapt treatment guidelines appropriately.

Neisseria gonorrhoeae antimicrobial susceptibility in the United States has been monitored by the Gonococcal Isolate Surveillance Project (GISP) at the Centers for Disease Control and Prevention (CDC), Atlanta, GA, since 1986. The need for routine surveillance for antimicrobial susceptibility was established with the emergence of chromosomal resistance to penicillin and tetracycline in *Neisseria gonorrhoeae* in the United States in the 1970s and 1980s (32). With the development of plasmid-mediated resistance to penicillin and tetracycline, the treatment recommendations were changed to ceftriaxone, with cefixime and ciprofloxacin being oral alternatives (29). Fluoroquinolone-resistant *Neisseria gonorrhoeae* (QRNG) was first isolated in Hawaii in 1991, but it was not until after 1998 that the frequency of QRNG started to escalate (5). In California, the frequency of QRNG isolates increased from less than 1% in 2000 to 25.4% in 2005 (2). At the onset of the occurrence of QRNG in California, expanded surveillance suggested an outbreak among men who have sex with men (MSM) in Southern California (1). A transmission network was constructed from the epidemiological connections determined from the sexual partnerships and venues where sexual partners met and could be supported by strain typing of the isolates from these cases among MSM (18). In this paper, we report on the complete strain typing and examine the antimicrobial resistance profiles among the isolates identified in this surveillance. This should provide important information on the origins of evolving *Neisseria gonorrhoeae* antimicrobial resistance in the United States by characterizing the strains that became established on the continent.

MATERIALS AND METHODS

QRNG surveillance and case identification. A QRNG case was defined as an infection caused by an isolate exhibiting a ciprofloxacin MIC of ≥1.0 μg/ml (ciprofloxacin resistant) (14, 19). From April 2000 through December 2000, cases were identified in the GISP sentinel sexually transmitted disease (STD) clinics in California (Long Beach, Orange County, San Diego, and San Francisco). As part of the GISP, each month gonococcal isolates were collected from the first 25 men with symptomatic urethral infections. Beginning in January 2001 and extending through June 2002, we identified women and men with QRNG infections at various anatomic sites through enhanced surveillance at STD clinics in Southern California. For a short time (February 2001 through April 2001), all gonococcal isolates from Kaiser Permanente Southern California were tested for fluoroquinolone resistance. The components of this investigation were determined by the CDC to be nonresearch public health activities not requiring CDC Institutional Review Board approval.

Strain typing. The QRNG isolates were characterized by the Neisseria Reference Laboratory, Division of STD Prevention, National Center for HIV/AIDS, Viral Hepatitis, STD, and TB Prevention, CDC, by their auxotype, serovar, Lip type, and amino acid alteration patterns in the quinolone resistance-determining regions (QRDRs) of GyrA and ParC (hereafter abbreviated ASLGP). Susceptibilities to penicillin, tetracycline, spectinomycin, ceftriaxone, cefixime, ciprofloxacin, ofloxacin, and azithromycin were determined by agar dilution on GC II agar base medium (Becton Dickinson, Sparks, MD) supplemented with 1% IsoVitaleX (Becton Dickinson) (22). Susceptibilities to penicillin, tetracycline,

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spectinomycin, ceftriaxone, cefixime, ciprofloxacin, and ofloxacin were interpreted according to the criteria of the CLSI (11). CLSI has not established interpretive criteria for the susceptibilities of gonococcal isolates to azithromycin. An azithromycin MIC of ≥1.0 μg/ml was defined as a critical MIC. Serovars were determined by using a modified panel of PorIA-specific and PorIB-specific monoclonal antibodies in coagglutination tests (16). Auxotyping and Lip typing were performed as described previously (23, 28). Amino acid alterations in the QRDRs of GyrA and ParC were determined by PCR amplification, followed by DNA sequencing on a CEQ8000 apparatus (Beckman Coulter, Inc., Fullerton, CA) (12). The strain types of the isolates were characterized by ASLGP and then numbered sequentially. Subtypes were defined by differences in antibiotic susceptibility.

The isolates were analyzed by pulsed-field gel electrophoresis (PFGE) at the Orange County Public Health Laboratory. Agarose plugs containing genomic DNA were restricted with NheI (10 U/μl; catalogue number 885851; Roche Diagnostics, Indianapolis, IN), according to the CDC protocol for the typing of *Neisseria meningitidis* strains (8). Electrophoresis was performed on a contour-clamped homogeneous electric fields device (CHEF MAPPER; Bio-Rad, Hercules, CA), with the ramped pulse times beginning with 2.2 s and ending with 35 s at 6 V cm at 14°C for 18 h. Tagged image file format images were normalized with the use of BioNumerics software (Applied Maths Kortrijk, Belgium), and cluster analysis was performed by the use of the Dice coefficient and the unweighted pair group method with arithmetic means. The band position tolerance and optimization were set at 1.5% and 0.5%, respectively. Fingerprint patterns were assigned a pattern number to differentiate among indistinguishable patterns and different patterns. Isolates whose profiles did not differ by any bands were considered indistinguishable and were assigned the same pattern number. Isolates that differed by one or more bands were considered different and were assigned a new number.

Statistical analysis was done for the correlates of an antimicrobial susceptibility profile of resistance to fluoroquinolones, penicillin, and tetracycline and an MIC of 0.25 μg/ml or higher for azithromycin. All analyses used SAS (version 9.2) software for the testing of significance, which was a P value of 0.05, by the chi-square test.

RESULTS

QRNG cases. From April 2000 through June 2002, a total of 82 QRNG isolates were identified in California from routine GISP sites and through expanded surveillance; these isolates represented 1 isolate per patient. Of these isolates, 79 were typed by all laboratory methods. Three isolates were not fully typed because the organisms were not sustained in culture and all testing could not be completed. Of the 79 typed isolates, most were from San Diego (n = 32), followed by Orange County (n = 21), San Francisco (n = 12), Long Beach (n = 12), and Los Angeles (n = 2). The majority of cases were male (95%); the median age was 33 years (range, 18 to 64 years); and 50% of the cases identified themselves as white, 9% as African American, 22% as Hispanic, and 15% as Asian/Pacific Islander.

In 2000, six male urethral isolates from the GISP in Orange County were found to be QRNG, and four of these were typed. Subsequently, 37 male urethral QRNG isolates from the GISPs in the Southern California sites of Long Beach, Orange County and San Diego collected from January 2001 to June 2002 were fully typed. The majority (n = 30) of these GISP isolates were from MSM; the remainder were from men who had sex with women (n = 6), and one patient with a partner whose gender was unknown. Expanded surveillance at Southern California GISP sites identified 24 additional QRNG isolates, all of which were typed. Of these, 20 isolates were from self-reported MSM and 4 were from women. The QRNG isolates from men were isolated from the rectum (n = 16) and the pharynx (n = 4). The QRNG isolates from the four women were isolated from the cervix (n = 3) and the pharynx (n = 1). Also in Southern California, two of three QRNG isolates from men found through Kaiser Permanente Southern California in Los Angeles were typed: one was from the pharynx of an MSM and one was from the urethra of a man who had sex with women. Twelve cases of QRNG infection were identified among standard GISP male urethral isolates from San Francisco between April 2000 through June 2002; of these, five cases involved MSM.

Strain typing and antibiotic resistance. Twenty strain types were identified by ASLGP typing; these included three subtypes (subtypes ASLGP-04a, ASLGP-05a, and ASLGP-08a) that differed from their corresponding type by their antibiograms (Table 1). Two strains, ASLGP-05 and ASLGP-04, were outbreak strains; these strains accounted for 42 (53%) and 9 (11%) of the 79 cases of QRNG infection, respectively. Both the ASLGP-05 and the ASLGP-04 strain types required proline, were Lip type 17c, and exhibited amino acid alterations of a change to Phe at position 91 (91>Phe) in GyrA and 87>Arg in ParC. ASLGP-05 belonged to a distinctly different serovar (serovar IB-3C8), whereas ASLGP-04 belonged to serovar IB-2H7, 2G2.

Twenty-four unique PFGE patterns were identified by comparison of the band differences for the 79 isolates (Fig. 1). Similar to ASLGP typing, two dominant strain patterns were identified. These strains, labeled gon001 and gon003, accounted for 32 (41%) and 14 (18%) of the 79 isolates, respectively. An additional six isolates of strains labeled gon002 or gon012 had greater than 95% similarity to each other, according to their PFGE patterns, suggesting a third outbreak strain, making 52 (66%) total potential outbreak strains as determined by PFGE. Comparison of PFGE-identified outbreak strains with ASLGP typing-identified outbreak strains showed agreement for 47 of 55 (85%) of the outbreak isolates and 19 of 24 (79%) of the non-outbreak isolates.

Resistance to penicillin was found in 58 (73%) of the isolates; 50 isolates exhibited chromosomally mediated resistance, and 8 were penicillinase-producing *N. gonorrhoeae* (PPNG) isolates. Tetracycline resistance was found in 55 (69%) of the isolates, with 49 of those isolates exhibiting chromosomally mediated resistance and 6 having high MICs, suggestive of plasmid-mediated resistance. Among the 79 QRNG isolates, 54 isolates had combined penicillin and tetracycline resistance: 47 *N. gonorrhoeae* isolates with chromosomally mediated resistance to penicillin and tetracycline (CMRNG); 5 isolates had plasmid-mediated resistance to both penicillin and tetracycline; and 2 were PPNG with chromosomally mediated tetracycline resistance. The MICs for azithromycin ranged from 0.03 to 0.5 μg/ml, with 9 isolates having MICs of 0.06 μg/ml or less (7 of these were sensitive to all antimicrobials other than fluoroquinolones) and 27 isolates having MICs of 0.125 or 0.25 μg/ml. One isolate, typed as ASLGP-17, had an azithromycin MIC of 0.5 μg/ml and was also a PPNG strain and tetracycline resistant. The main outbreak strain detected by ASLGP strain typing, ASLGP-05, was CMRNG and had an azithromycin MIC of 0.5 μg/ml. The other outbreak strain, ASLGP-04, was susceptible to penicillin and tetracycline and had an azithromycin MIC of 0.125 μg/ml.

In total, 52 isolates (66%) were resistant to fluoroquinolones, penicillin, and tetracycline and had azithromycin MICs of 0.25 μg/ml or higher. The presence of this antimicrobial

TABLE 1. Strain typing of QRNG in California by ASLGP

ASLGP designation	No. of isolates	Auxotype[a]	Serovar	Genotype(s)[b]			MIC_{90} for susceptibility[c] (μg/ml)			
				Lip	GyrA	ParC	Pen	Tet	Cip	Azm
ASLGP-01	1	PAHUM	IB-3C8	16b	91>Phe, 95>Gly	86>Asn	0.5	1	4	0.25
ASLGP-02	1	Pro	IB-2G2	17c	91>Phe, 95>Gly	87>Arg	4	1	4	0.25
ASLGP-03	1	Pro	IB-2H7, 2G2	16b	91>Phe, 95>Gly	87>Arg	1	0.25	2	0.125
ASLGP-04	9	Pro	IB-2H7, 2G2	17c	91>Phe, 95>Gly	87>Arg	1	0.25	4	0.125
ASLGP-04a	2	Pro	IB-2H7, 2G2	17c	91>Phe, 95>Gly	87>Arg	1	1	4	0.125
ASLGP-05	42	Pro	IB-3C8	17c	91>Phe, 95>Gly	87>Arg	4	2	8	0.5
ASLGP-05a	2	Pro	IB-3C8	17c	91>Phe, 95>Gly	87>Arg	0.25	0.125	1	0.03
ASLGP-06	2	Pro	IB-3C8, 2H7	17c	91>Phe, 95>Gly	87>Arg	1	0.25	1	0.03
ASLGP-07	1	Proto	IB-2H7, 2G2, 2D4	12b	91>Phe, 95>Gly	87>Arg	0.25	1	2	0.03
ASLGP-08	2	Proto	IB-3C8	14d	91>Phe, 95>Gly	None	0.25	1	4	0.03
ASLGP-08a	1	Proto	IB-3C8	14d	91>Phe, 95>Gly	None	4	4	8	0.25
ASLGP-09	1	Proto	IB-3C8	15g	91>Phe, 95>Ala	91>Gly	4	4	8	0.25
ASLGP-10	2	Proto	IB-3C8	17c	91>Phe, 95>Ala	None	>64+	>32	4	0.25
ASLGP-11	1	Proto	NT[d]	16b	91>Phe, 95>Gly	86>Asn	32	0.125	1	0.03
ASLGP-12	2	Proto	IB-3C8	16b	91>Phe, 95>Gly	86>Asn	2	1	8	0.25
ASLGP-13	1	Pro	IB-2G2, 2D4	13f	91>Phe, 95>Gly	87>Arg	8	2	16	0.25
ASLGP-14	1	Pro	NT	17c	91>Phe, 95>Gly	87>Arg	4	2	8	0.25
ASLGP-15	1	Proto	IA-2F12, 5D1, 9D2	16b	91>Phe, 95>Gly	86>Asn	>64+	32	4	0.06
ASLGP-16	1	Proto	IB-2H7, 2G2, 2D4	16b	91>Phe, 95>Asn	None	4	2	2	0.25
ASLGP-17	1	Proto	IB-3C8	16b	91>Phe, 95>Gly	86>Asn	>64+	2	8	0.5
ASLGP-18	1	Proto	IB-3C8	18d	91>Phe, 95>Gly	86>Asn	>64+	2	8	0.25
ASLGP-19	1	Proto	IA-2F12, 4G5, 5G9, 5D1	13c	91>Phe, 95>Ala	87>Asn, 91>Gln	0.5	16	4	0.25
ASLGP-20	2	Proto	IB-3C8	18b	91>Phe, 95>Ala	0	>64+	>32	4	0.25

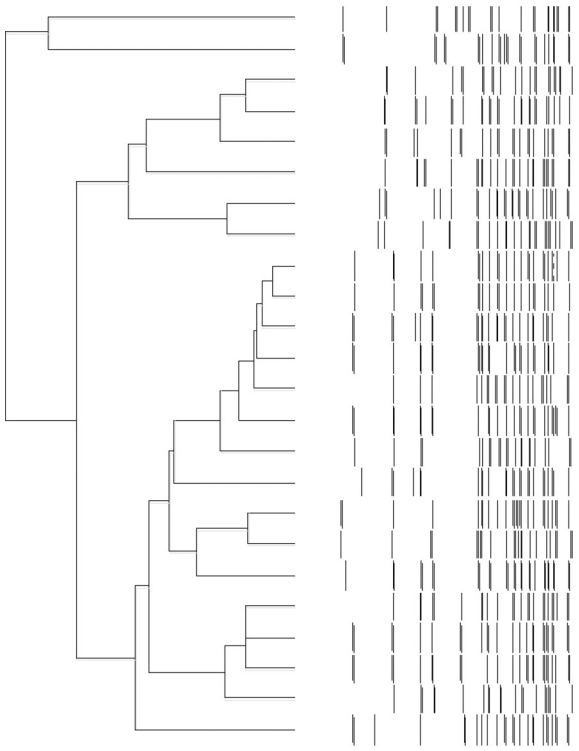
[a] PAHUM, required proline, arginine, hypoxanthine, uracil, and methionine for growth; Pro, proline; Proto, prototrophic.
[b] Lip polymorphisms encode a surface lipoprotein, GyrA and ParC genotype variants confer fluoroquinolone resistance and are defined by amino acid substitutions in the protein. Phe, phenylalanine; Gly, glycine; Ala, alanine; Arg, arginine; Asn, asparagine.
[c] Pen, penicillin (a plus sign indicates evidence to suggest β-lactamase production); Tet, tetracycline; Cip, ciprofloxacin; Azm, azithromycin.
[d] NT, not able to be typed.

susceptibility profile was more often associated with isolates from MSM than with those from heterosexual men and women ($P = 0.03$). MSM were also strongly associated with the most common strain with this antimicrobial susceptibility, ASLGP-05 ($P < 0.0001$). Other predictors did not reach statistical significance for an association with this reduced susceptibility to multiple agents, including antibiotic use in the past 3 months ($P = 0.5$), travel history ($P = 0.4$), multiple sex partners ($P = 0.8$), and having a partner who was a commercial sex worker ($P = 0.7$). Isolates with reduced susceptibilities to multiple agents were more likely to come from San Diego (84% of isolates) and San Francisco (75%) than from Orange County (43%) and Long Beach (42%) ($P = 0.006$).

DISCUSSION

Gonorrhea is the second most frequently reported infectious disease in the United States, and gonococcal infections remain a challenge to public health because of a resurgence of the number of cases and rising antimicrobial resistance (6, 33). Our data from surveillance of QRNG isolates in California has elucidated how new antimicrobial-resistant strains can enter a country and become endemic, similar to what has been reported in Australia and Canada (21, 25). In a further examination of these data, we found evidence of resistance to multiple antimicrobial agents within the main endemic strains and found that individuals infected with these strains also had the potential to fail at least lower-dose azithromycin treatment regimens. The failure of azithromycin to eradicate *Neisseria gonorrhoeae* has been reported with 1-g single doses, and the isolates from those cases demonstrated MICs of 0.125 to 0.25 μg/ml, although that study used methods different from those used by reference laboratories in the United States (27). Although specific criteria have not been defined by the CLSI for azithromycin resistance in *N. gonorrhoeae* (11), isolates recovered in Kansas City, MO, in 1999 and 2000 had MICs as high as 4.0 μg/ml, but again, standard methods were not used (30).

Our findings do not affect treatment recommendations in the United States, where oral cefixime or intramuscular ceftriaxone are the first-line therapies for the treatment of gonorrhea (10). The manufacture of cefixime in tablet form was discontinued by the original manufacturer for many years in the United States, but it is now available again and can be used as first-line therapy when it is so indicated (4). Despite this gain, antimicrobial resistance in *N. gonorrhoeae* remains a serious problem that requires a comprehensive approach to prevention because of the potential for multidrug resistance (33), as demonstrated by the independent identification of *N. gonorrhoeae* strains in the United States with MICs that support decreased susceptibility to cefixime or the potential for treatment failure with suboptimal doses of azithromycin, the alternative oral agent for the treatment of gonorrhea (17, 30, 31). Cefixime and ceftriaxone still remain dependable first-line treatments because no failure of therapy with these agents for *N. gonorrhoeae* infections has been reported in the United States, although isolates with decreased cefixime susceptibility were found in Hawaii (30). In Japan, infections caused by strains exhibiting decreased susceptibility to cefixime have failed to respond to therapy with a 200-mg dose of cefixime, which is one-half of the dose recommended in the United



PFGE Type	ASLGP Designation																						Total
	01	02	03	04	04a	05	05a	06	07	08	8a	09	10	11	12	13	14	15	16	17	18	19	20	
GON031												1												1
GON026																			1					1
GON005	1														2					1				4
GON007																		1						1
GON006														1							1			2
GON011									1															1
GON009										2														2
GON010											1											1		2
GON001		1	1	3	2	23	1										1							32
GON002				2		2		1																5
GON012						1																		1
GON003				2		11		1																14
GON014							1																	1
GON013																							1	1
GON015						1																		1
GON025						2																		2
GON023				1																				1
GON024																1								1
GON016						1																		1
GON018													1											1
GON019				1																				1
GON017																						1		1
GON020													1											1
GON021						1																		1
Total	1	1	1	9	2	42	2	2	1	2	1	1	2	1	2	1	1	1	1	1	1	2	1	79

FIG. 1. Comparison of PFGE and ASLGP analysis for typing of QRNG isolates in California. The highlighted area represents agreement among PFGE types with a similarity coefficient of 95% with outbreak strains identified by ASLGP.

States; these infections did respond to treatment with a 1-g intramuscular dose of ceftriaxone (36). If resistance to the currently recommended doses of cephalosporins emerges in the gonococcus, potential second-line agents are not readily available. Spectinomycin, one of the alternative antibiotics recommended for the treatment of *N. gonorrhoeae* infections, is no longer on the market in the United States (7), and azithromycin is not recommended for use for the primary treatment of uncomplicated gonococcal infections because of the gastrointestinal intolerance of the 2-g dose (9). In addition, isolates with azithromycin MICs exceeding 1.0 μg/ml have been isolated in the continental United States and Cuba, which suggests that this agent should not be recommended for use for the primary treatment of gonorrhea (17, 24).

Our study describes a QRNG outbreak in California that started in 2000 in which the main outbreak isolate was also CMRNG and had azithromycin MICs of 0.5 μg/ml. These isolates were primarily identified at STD clinics, and overall, the sample reported here represents a small proportion of the total number of gonorrhea cases in California. However, the study suggests that infections with strains that could fail to be treated by the use of low-dose azithromycin may occur frequently. The presence of resistance to fluoroquinolones, penicillin, and tetracycline and azithromycin MICs of 0.25 μg/ml or higher were correlated epidemiologically with MSM and were largely related to the presence among MSM of a common strain with this susceptibility profile. Through epidemiological methods, we have previously shown that many of these cases among MSM in Southern California could be connected to the same social and sexual network (18). However, we cannot fully rule out the possibility that these are subclones. None of our isolates had a critical MIC of 1.0 μg/ml, but it would be possible for azithromycin therapy to fail if the subtherapeutic dose of 1 g, which is recommended for the treatment of uncomplicated chlamydial infections (9), was used. This finding may have important implications in the overall strategy to control *N. gonorrhoeae* and *Chlamydia trachomatis*. It is imperative that the CDC-recommended regimen of treatment with a cephalosporin for gonorrhea be used; and if a chlamydial infection alone is being treated, appropriate screening for *N. gonorrhoeae* infection at all exposure sites, which may include the pharynx and the rectum, should be done. When patients with gonorrhea are allergic to cephalosporins, specimens should be obtained for susceptibility testing and to guide the choice of treatment, or, alternatively, arrangements should be made to desensitize the patient to cephalosporins. The choice of azithromycin is possible, but as the findings of our study suggest, the full 2-g dose recommended in California and the United States is required (3, 9).

We performed strain typing of *N. gonorrhoeae* isolates by two different reliable methods, ASLGP and PFGE, which were similarly effective in defining outbreak strains but were not in complete agreement. The auxotyping and serovar typing methods of characterizing *N. gonorrhoeae* strains have demonstrated utility in monitoring strain types in a community (15). When these methods are used in combination with Lip typing and determination of the amino acid alteration patterns in the QRDRs of GyrA and ParC, they provide an additional ability to discriminate strain types. The PFGE technology has also been used to characterize *N. gonorrhoeae* strains, including QRNG, and is commonly available in many public health laboratories (34, 35), although the use of two restriction enzymes, such as NheI and SpeI, would be recommended for surveillance for gonococcal strains (13, 20). Ideally, methods for the monitoring of strain types with resistance should be standardized internationally to allow global surveillance, because antimicrobial-resistant *N. gonorrhoeae* is an international public health threat that needs to be monitored as part of a coordinated control plan (26, 33). In the United States, surveillance for gonorrhea should remain a priority to detect the emergence of resistance to all classes of antibiotics to inform treatment recommendations in a timely manner.

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