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Emergence of high-level azithromycin resistance in *Neisseria gonorrhoeae* in England and Wales

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***Objectives*: This study aimed to investigate the origin of high-level azithromycin resistance that emerged in isolates of *Neisseria gonorrhoeae* in England and Wales in 2007, and to establish methods for identifying high-level azithromycin resistance.**

***Methods*: The Gonococcal Resistance to Antimicrobials Surveillance Programme (GRASP) data from 2001–07 were examined for emerging trends in azithromycin susceptibility. Further to the identification of six high-level azithromycin-resistant isolates in GRASP 2007, an additional 102 isolates were selected on the basis of azithromycin susceptibility and geographic origin from the GRASP 2006 and 2007 collections. Susceptibility testing by Etest and disc diffusion was performed on all 108 isolates and 75 of these were typed by *N. gonorrhoeae* multiantigen sequence typing.**

***Results*: A slight drift towards higher MICs of azithromycin was observed in the gonococcal population since 2001. Of greater concern was the first example of a shift to high-level resistance observed in six isolates in 2007. All six isolates were sequence type 649, which was not observed in any of the lower-level azithromycin-resistant isolates from 2007 or in any isolates tested from the same geographical locations. Contact tracing data for one patient suggested a link with Scotland. Disc diffusion testing of all 108 isolates showed that azithromycin, but not erythromycin, discs can differentiate between low-level and high-level resistance.**

***Conclusions*: High-level azithromycin resistance has emerged in England and Wales. Contact tracing and typing data suggest this may have originated from Scotland. Surveillance of azithromycin resistance will be key in controlling its further dissemination.**

Keywords: disc diffusion, Etest, NG-MAST

Introduction

Effective treatment of *Neisseria gonorrhoeae* infection is essential, not only for individual patient management but also for control of gonorrhoea. The development of resistance to therapeutic antimicrobials presents a significant challenge to treatment, and has been monitored in England and Wales since 2000 by the Gonococcal Resistance to Antimicrobials Surveillance Programme (GRASP). Since 2001, GRASP has monitored gonococcal susceptibilities to azithromycin, an acid-stable macrolide with a long half-life (68 h). As azithromycin can be administered orally in a single dose, it is an attractive agent for the treatment of sexually transmitted infections (STIs) because it has activity against several agents. However, it is not recommended in the treatment of gonorrhoea[1,2] as at a dosage of 1 g azithromycin achieves variable, often inadequate, cure rates.[3] A higher dose of 2 g is more effective but is poorly tolerated, causing a range of gastrointestinal symptoms.[4,5] In contrast, a 1 g dose of azithromycin is recommended as an effective treatment for *Chlamydia trachomatis* infection[6] and is used widely. Co-infection with *C. trachomatis* occurs in ~20% of men and ~40% of women[1] with gonorrhoea, and so the gonococcal population may be frequently exposed to 1 g doses of azithromycin used in the treatment of chlamydia.

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It has been suggested that the use of azithromycin as part of a dual therapy could be effective in the treatment of pharyngeal gonorrhoea[7] and that azithromycin in combination with other antimicrobials, including third-generation cephalosporins, has a synergistic effect.[8] Combination therapies may become increasingly important in the future if resistance develops to currently recommended third-generation cephalosporin therapies.

The current study presents the first evidence of an upwards drift in azithromycin MICs as well as a major shift to high-level resistance in the gonococcal population in England and Wales. This study recommends the use of 15 μg azithromycin discs as an alternative to Etest, to facilitate the monitoring of azithromycin resistance in all laboratories.

Materials and methods

Study population

Following the identification of 6 *N. gonorrhoeae* isolates exhibiting high-level resistance to azithromycin as part of GRASP in 2007,[9] these 6 isolates plus a further 102 isolates were selected from the GRASP isolate collections for 2006 and 2007, to establish methods for identification of this new phenotype. Fifty-five isolates with an MIC of ≥1.0 mg/L, indicating azithromycin resistance,[10] and 53 isolates representing a range of MICs correlating with azithromycin susceptibility (<0.03–0.5 mg/L) were selected from the GRASP 2006 and 2007 collections. To examine the possible origin of the highly resistant isolates, 35/102 isolates were recovered from patients in Liverpool, England and 11/102 originated from Cardiff, Wales. Additionally, the azithromycin MIC data for all 11482 isolates collected from patients attending STI clinics and submitted to GRASP between 2001 and 2007 were analysed as described below, to determine if MIC drift had occurred over this testing period.

Susceptibility testing

All gonococcal isolates were tested for susceptibility to azithromycin and erythromycin by Etest and by disc diffusion methods.[10,11] Briefly, a gonococcal suspension of $\sim 10^4$ cfu/μL (equivalent in turbidity to that of a 0.5 McFarland standard) was inoculated onto the surface of a GC agar plate (BD Diagnostics, Oxford, UK) supplemented with 1% isovitalex. Etest strips (Bio-Stat Ltd, Stockport, UK) containing either azithromycin or erythromycin (concentration ranges 0.016–256 and 0.013–256 mg/L, respectively), or discs containing azithromycin (15 μg) or erythromycin (5 μg) (Oxoid Ltd, Basingstoke, UK) were applied to the plate surface. The susceptibility of six isolates to clindamycin was also tested by Etest (concentration range 0.016–256 mg/L). All results were read after 18–20 h of incubation in 5% CO_2 at 36°C. MICs were defined as the concentration point at which the zone of inhibition intercepted the Etest strip and the diameters of disc zones of inhibition were recorded. The MIC of azithromycin for the six highly resistant isolates was determined by agar dilution as described previously,[9] using medium containing doubling dilutions of azithromycin (Pfizer, Kent, UK) up to 8192 mg/L.

Sequence-based typing

A total of 75/108 isolates were typed by *N. gonorrhoeae* multiantigen sequence typing (NG-MAST), which differentiates strains on the basis of variation in two hypervariable alleles, *por* and *tbpB*, as described previously.[12] Thirty-four isolates from GRASP 2007 with an azithromycin MIC of ≥1.0 mg/L were selected to examine the types circulating in the azithromycin-resistant gonococcal population in England and Wales. The remaining 41 isolates from Liverpool ($n=30$) and Cardiff ($n=11$) were selected to examine the distribution of gonococcal genotypes circulating in areas of high-level azithromycin resistance.

Statistical analysis of azithromycin MIC data from GRASP 2001–07

An analysis was performed in Stata Statistical Software Release 10 (StataCorp LP, TX, USA) to identify potential trends in azithromycin MICs for gonococci recovered from patients attending STI clinics and referred to GRASP between 2001 and 2007. The natural logarithm of the MIC was regressed on year.

The command used was designed for linear regression and included weights to allow for the incomplete retrieval of all isolates sampled. The slope of this regression line was then exponentiated, resulting in an estimate of the geometric mean between successive years. The estimated ratio between 2007 and 2001 is the ratio between successive years multiplied by itself 6 times (the number of year intervals).

Results

Trends in azithromycin susceptibilities

Analysis of GRASP data for azithromycin MICs between 2001 and 2007 demonstrated a drift towards higher MICs in the gonococcal population in England and Wales over time (Figure 1). The ratio of geometric means of MIC data in 2001 compared with 2007 was 1.06 (95% CI: 1.00–1.13), indicating this trend was weakly significant ($P=0.05$).

Characterization of high-level azithromycin-resistant gonococcal isolates

Between 2001 and 2006, the highest MIC recorded in GRASP was 8 mg/L. In 2007, a major shift was observed in six isolates with azithromycin MICs of >256 mg/L by Etest. Full titration by agar dilution showed that the MIC was 4096 mg/L for all

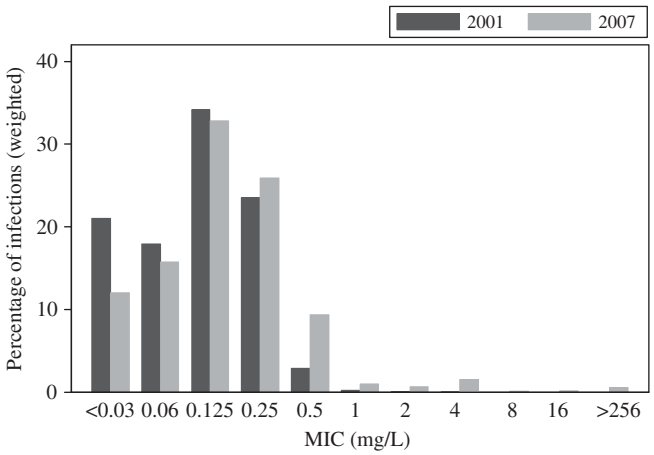

Figure 1. Azithromycin MICs for all gonococcal isolates from STI clinics referred to GRASP during 2001–07.

six isolates. Additionally, these isolates displayed high-level cross-resistance to other agents in the macrolide–lincosamide–streptogramin class of antimicrobials, with MICs of erythromycin and clindamycin >256 mg/L. Susceptibility testing by agar dilution as part of GRASP showed that all six isolates had similar resistance profiles, being susceptible to penicillin, ciprofloxacin, spectinomycin, ceftriaxone and cefixime, but with MICs of tetracycline (4–8 mg/L) consistent with chromosomally mediated resistance mechanisms.[9]

All six isolates were recovered from heterosexual patients, none of whom had received azithromycin. Five of the patients (three female, two male, mean age 26 years, range 21–32 years) were from Liverpool. The remaining patient was a 19-year-old male from Cardiff. NG-MAST typing showed that all six isolates were sequence type (ST) 649 (Table 1). ST649 was not observed in any of the other isolates tested over the same time period in Liverpool or Cardiff (Table 1), or in any of the lower-level azithromycin-resistant isolates recovered in 2007 (Table 1). Contact tracing information showed that the patients in Liverpool were linked in two clusters, while all contacts were untraceable for the patient from Cardiff (Figure 2).

Detection of high-level azithromycin resistance

Testing of 108 isolates in different azithromycin susceptibility categories by disc diffusion demonstrated that the zone of

Table 1. Distribution of gonococcal NG-MAST STs in Liverpool and Cardiff and in azithromycin-resistant isolates recovered as part of GRASP in 2007

	NG-MAST			Patient demographics	
Azithromycin MIC (mg/L)	ST[a]	*por*	*tbpB*	geographical location (*n*)	number of MSM[b]
<0.03–0.5	1182	767	26	Liverpool (9)	2
	225	4	4	Liverpool (3), Cardiff (2)	1
	51	39	27	Cardiff (3)	0
	25	18	27	Liverpool (2)	0
	359	301	29	Liverpool (1), Cardiff (1)	2
	1407	908	110	Cardiff (2)	0
	384	263	26	Liverpool (2)	0
	28	19	4	Liverpool (2)	0
	1746	1076	32	Liverpool (2)	0
	332	147	108	Liverpool (1)	0
	87	73	16	Liverpool (1)	0
	3373	2040	26	Liverpool (1)	1
	3374	2041	26	Liverpool (1)	0
	3375	2042	321	Liverpool (1)	1
	738	4	21	Cardiff (1)	0
	1440	105	4	Liverpool (1)	0
	2208	35	35	Liverpool (1)	0
	1105	39	4	Liverpool (1)	0
	2135	206	75	Liverpool (1)	0
	3313	2012	330	Cardiff (1)	0
	3315	2013	70	Cardiff (1)	0
≥1–8.0	359	301	29	Birmingham (5), London (3), Manchester (1), Liverpool (1)	5
	1195	702	29	London (3), Brighton (1)	4
	3150	1907	4	Bristol (2)	1
	225	4	4	London (1), Bristol (1)	2
	2449	1515	29	Newcastle (1)	unknown
	2322	1445	137	London (1)	0
	3302	922	137	London (1)	0
	437	14	4	London (1)	1
	738	4	21	Birmingham (1)	1
	822	555	4	Brighton (1)	0
	1782	1444	70	Birmingham (1)	0
	3314	2011	29	London (1)	1
	2616	1602	4	London (1)	1
	2	2	16	London (1)	0
>256	649	442	29	Liverpool (5), Cardiff (1)	0

[a]Sequence type defined on the basis of combined *por* and *tbpB* alleles.
[b]Men who have sex with men.

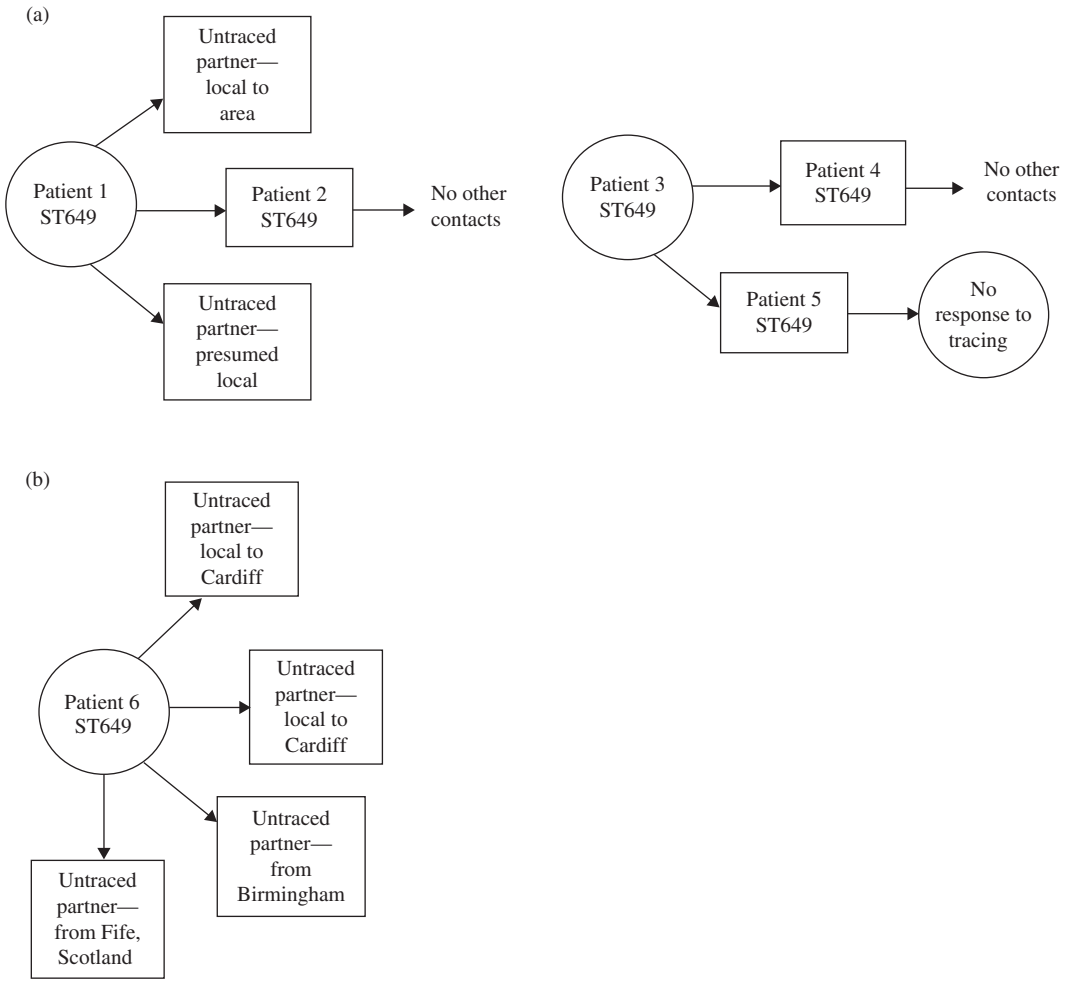

Figure 2. Schematic representation of sexual networks of patients infected with high-level azithromycin-resistant gonococcal genotype ST649. Circles (men) or squares (women) represent sexual contacts in (a) Liverpool and (b) Cardiff. Arrows indicate patients had identified the other as a contact.

inhibition for erythromycin discs decreased as azithromycin and erythromycin MICs increased (Table 2), but no zone was observed in many strains ($n=33$) with low to moderate azithromycin resistance (Table 2). In contrast, zones of inhibition for discs containing 15 μg of azithromycin were observed for all isolates with azithromycin MICs ranging from 1.0 to 8.0 mg/L (Table 2). Only high-level azithromycin-resistant isolates showed no inhibitory zone (Table 2).

Discussion

This is the first study to describe the emergence of high-level azithromycin resistance in *N. gonorrhoeae* recovered from patients attending STI clinics in England and Wales, and to present evidence that the susceptibility of the whole gonococcal population examined as part of GRASP has decreased since 2001. While GRASP does not sample the entire gonococcal

Table 2. Comparison of azithromycin and erythromycin susceptibilities of 108 gonococcal isolates determined by Etest and by disc diffusion methods

Azithromycin MIC[a] (mg/L)	Number of isolates	Modal erythromycin MIC, mg/L (range)	Mean zone of inhibition diameter, mm (range)	
			erythromycin	azithromycin
<0.03–0.5	53	0.25 (0.047–1)	27 (20–39)	36 (28–45)
1.0–2.0	16	1 (0.75–16)	15 (0–22)	28 (22–35)
3.0–8.0	33	24 (3–>256)	0 (0–13)	21 (15–25)
>256	6	>256	0	0

[a]Azithromycin MICs were grouped into the following susceptibility categories, in ascending order: susceptible; decreased susceptibility; moderate resistance; and high-level resistance.

population in England and Wales, it has previously provided information that is considered sufficiently representative to guide treatment policy.[1] Resistance can develop in *N. gonorrhoeae* during patient treatment using a 1 g dose of azithromycin.[13] Patient treatment data collected by GRASP from 2001 to 2007 demonstrated a dramatic rise in the proportion of patients receiving azithromycin and a concurrent decrease in the prescribing of tetracyclines, suggesting a change in the management of concurrent chlamydia infection.[9] The use of a suboptimal dose of azithromycin in the treatment of chlamydia may exert a selection pressure on the gonococcal population and account for the observed upwards drift in MICs, particularly if the gonorrhoea is not diagnosed or treated specifically at the time of chlamydia treatment. Surveillance programmes in other countries, including the USA[14] and Scotland,[15] have reported similar drifts in azithromycin MICs in the gonococcal population.

The emergence of high-level azithromycin resistance in gonococci is of significant public health concern as it could limit future therapeutic options for the treatment of gonorrhoea. Examination of the contact tracing data for patients from Liverpool showed they were linked in two clusters that were apparently discrete, possibly suggesting that this highly resistant genotype is already endemic in the heterosexual population in Liverpool. While ST649 was not observed in any of the other isolates tested over the same time period in Liverpool or Cardiff, it would be necessary to type all isolates from these regions over a longer period to fully understand the distribution and prevalence of this type. Furthermore, none of the lower-level azithromycin-resistant isolates recovered in 2007 was type ST649, suggesting the highly resistant phenotype did not emerge from a pre-existing, lower-level resistance circulating in England and Wales. As none of the contacts for the patient from Cardiff was traceable, it is not known if this genotype had disseminated further from the index case. However, the possible contact with a patient from Scotland is potentially significant. High-level azithromycin resistance first emerged in the gonococcal population in Scotland in 2004 and by 2007 had disseminated to the extent that 75% of all azithromycin resistance observed in Scotland (5.2% overall) was high-level.[15] Sequence typing by NG-MAST showed high-level resistance was confined to six closely related types, of which ST649 was the most prevalent in 2007.[15] It seems reasonable to speculate that the resistance observed in England and Wales was imported from Scotland, which is supported by the contact tracing data for the patient from Cardiff. Lower-level azithromycin-resistant *N. gonorrhoeae* have been documented in various European countries[16–18] and other countries such as the USA,[14] Brazil[19] and Cuba.[20] However, as azithromycin resistance in gonococci is not monitored routinely in many countries, the origin of the high-level resistance that emerged in Scotland remains unknown. A recent report from Italy of five isolates with azithromycin MICs of 128 and 256 mg/L suggests that this high-level resistance has already disseminated to some extent within Europe at least.[21]

The *in vitro* MIC of azithromycin does not necessarily correlate with outcome of treatment at a dose of 1 g, with treatment failure occurring in apparently susceptible isolates.[22] While little is known about the relationship between azithromycin resistance and treatment outcome of a 2 g treatment regimen, it seems unlikely that this would successfully eradicate a gonococcal infection as highly resistant as reported in the current study. Patient treatment data referred to GRASP in 2007 showed that azithromycin alone was prescribed in a minority of patients (data not shown), suggesting that in spite of the treatment recommendations this was used to treat gonococcal infection in addition to chlamydia. The emergence of high-level azithromycin resistance highlights the importance of always specifically treating gonorrhoea using the recommended third-generation cephalosporins to prevent treatment failure and to successfully intercept chains of transmission.

It should be noted that the current recommendations for treatment of non-gonococcal urethritis are a 1 g dose of azithromycin.[23] Microscopy can diagnose gonorrhoea in 90%–95% of symptomatic males, indicating that as many as 10% of symptomatic male patients with gonorrhoea may receive azithromycin as their only therapy. It is therefore essential that resistance to any agent that is being used therapeutically or could have potential in future therapeutic regimens is controlled as far as possible. In the case of azithromycin this could be achieved not only by ensuring that gonorrhoea is always treated appropriately, but also by monitoring azithromycin resistance through national and international surveillance schemes and by testing at the individual laboratory level. The European Surveillance of Sexually Transmitted Infections (ESSTI) showed in a survey of European laboratory practices in 2004[24] that azithromycin susceptibility testing is infrequent, with many laboratories favouring erythromycin susceptibility testing by disc diffusion or Etest (C. A. Ison, on behalf of the ESSTI network, unpublished data). While MIC breakpoints are defined for azithromycin resistance, disc diffusion is a more cost-effective alternative to Etest for routine diagnostic laboratories, as additional discs could be incorporated into an existing panel of antimicrobials. However, previously it was not known if either azithromycin or erythromycin discs could be used to differentiate between different levels of azithromycin resistance. The current study indicates that azithromycin, but not erythromycin, discs can differentiate between low to moderate and high-level azithromycin resistance. We thus recommend the use of 15 μg azithromycin discs to test gonococcal isolates for azithromycin resistance. A zone diameter of $\leq$27 mm to define azithromycin resistance has been proposed previously, to correlate with the suggested resistance breakpoint MIC of $>$1 mg/L.[25] The current study used the same lower breakpoint ($\geq$1 mg/L) as GRASP, which was proposed by European Committee on Antimicrobial Susceptibility Testing (EUCAST)[10] and tentatively recommended by CDC. Isolates with an MIC of $>$1 mg/L correlated with the recommendations of BSAC, with no overlap in zone size between resistant and susceptible organisms. In contrast, isolates with a lower MIC of 1 mg/L gave larger zone diameters (ranging from 28 to 35 mm) that overlapped with the zone sizes of susceptible isolates (MICs $<$1 mg/L). Disc diffusion therefore may not be suitable to identify isolates that are only just resistant according to EUCAST guidelines; however, the clinical relevance of isolates displaying this level of susceptibility is uncertain.

In conclusion, high-level azithromycin resistance has emerged in England and Wales. This increases the risk of treatment failure in cases of gonorrhoea inappropriately treated with azithromycin and poses a threat to the development of future therapeutic regimens containing this agent. It is essential that all patients with gonorrhoea are treated appropriately using the current recommended therapies. Surveillance of azithromycin resistance will be key in controlling further dissemination of this emerging phenotype and we recommend the inclusion of a

15 μg azithromycin disc in existing panels of antimicrobials used in laboratories to monitor gonococcal resistance.

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There are no conflicts of interest to declare.

Disclaimer

The views expressed in the publication are those of the authors and are not necessarily those of the Department of Health.

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