Epidemiol. Infect. (2001), **126**, 219–224. Printed in the United Kingdom © 2001 Cambridge University Press

Investigation of an outbreak of ciprofloxacin-resistant *Neisseria gonorrhoeae* using a simplified opa-typing method

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(*Accepted 30 November 2000*)

SUMMARY

Ciprofloxacin-resistant gonococci have been isolated from patients in the United Kingdom since 1993. Until recently, evidence has suggested that the majority of infections are not endemic but have been acquired abroad. In October 1999, increasing numbers of ciprofloxacin resistant isolates of the non-requiring auxotype were reported in Oldham and Rochdale (Northwest England). These and similar isolates from elsewhere in England and Wales were genetically characterized using a simplified opa-typing method (a non-radioactive PCR–RFLP method targeting the *opa* family of genes). Of 73 isolates studied, 24 had unique opa-types (10 from infections acquired abroad), whilst the remaining 49 were indistinguishable (none were known to be acquired abroad). This cluster included 31 isolates from Oldham and Rochdale, 16 from elsewhere in the north of England, and 2 from Southern England and South Wales with known epidemiological links to cases from Manchester and Rochdale respectively. This study illustrates the potential for spread of an antibiotic resistant clone of *N. gonorrhoeae* both locally and nationally and demonstrates that endemic acquisition of ciprofloxacin-resistant gonococci is now a significant problem in the United Kingdom.

INTRODUCTION

Fluoroquinolones such as ciprofloxacin are commonly used as a first line therapy for uncomplicated gonorrhoea [1–3]. However, following the introduction of this treatment, strains of *Neisseria gonorrhoeae* with reduced susceptibility (minimum inhibitory concentration or MIC $\geqslant$ 0·05 mg/l) and high level resistance (MIC $\geqslant$ 1 mg/l) to ciprofloxacin emerged in the Far East [4, 5] and are now isolated worldwide [6]. *N. gonorrhoeae* strains exhibiting reduced susceptibility and resistance were first reported in the United Kingdom in 1990 and 1994 respectively [7, 8]. Since 1997, the number of these isolates referred to the Gonococcus Reference Unit (GRU) of the PHLS Genitourinary Infections Reference Laboratory (GUIRL) has increased dramatically. Decreased susceptibility to ciprofloxacin was detected in 72 of 1339 gonococci referred to the GRU during 1997. Of these isolates, 42 (3·1%) were intermediate resistant (MIC 0·05–0·5 mg/l) to ciprofloxacin and 30 (2·2%) were resistant to ciprofloxacin (MIC $\geqslant$ 1 mg/l) [9]. In contrast, during 1999, of 2736 isolates referred to the GRU, 135 (4·9%) had intermediate resistance and 189 (6·9%) were resistant. A review of patient data showed that initially most strains were acquired abroad and predominantly from the Far East. Recently, there has been an increase in endemic acquisition of resistant strains and in 1999, 51% of all ciprofloxacin-resistant isolates referred to the GRU were acquired in the United Kingdom compared with 8% in 1997.

In October 1999, an increase in the number of cases

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https://doi.org/10.1017/S0950268801005209 Published online by Cambridge University Press

of ciprofloxacin-resistant gonorrhoea was detected in Rochdale and Oldham (NW England). This study reports the characterization of isolates from this outbreak in the context of other ciprofloxacin resistant isolates of the same phenotype submitted to the GRU from elsewhere in England and Wales. Opa-typing [10], a highly discriminatory PCR–RFLP method, was used to type the isolates. The method characterizes isolates by the amplification and restriction digestion of the *opa* family of genes which code for 11 copies of the outer membrane opacity protein [11]. The original published methodology is complex and requires steps that involve gel purification and the use of radioactive isotopes. We have simplified the method and used it to analyse isolates from the outbreak of ciprofloxacin-resistant *N. gonorrhoeae* in the north west of England.

METHODS

Bacterial isolates

Isolates of *N. gonorrhoeae* used in this study had been referred to the GRU for confirmation of antibiotic resistance. On receipt, isolates were confirmed as gonococci, auxotyped, and their quantitative susceptibility to ciprofloxacin and penicillin determined using methods previously described [12]. Opa-typing was carried out on all isolates of the non-requiring (NR) auxotype with an MIC for ciprofloxacin of $\geqslant 1$ mg/l (resistant) and for penicillin of 0·64–1·28 mg/l (intermediate resistant or resistant) that were submitted to the GUIRL between April 1999 and February 2000 from anywhere in England and Wales. There were 73 isolates of the designated phenotype during this period. In addition, six isolates from Oldham or Rochdale of different auxotypes and/or ciprofloxacin resistance status were opa-typed. The optimization of the opa-typing protocol was carried out using gonococcal strains A and E from the WHO control set. Epidemiological data were requested for each isolate as is the routine practice of the GUIRL.

DNA preparation

Bacteria grown on heated blood agar plates were suspended in 100 μl of sterile distilled water to give a visibly turbid suspension, and mixed with 50 μl of 15% w/v Chelex-100 resin slurry (Biorad Laboratories Ltd, Hemel Hempstead, United Kingdom). Samples were heated (95 °C for 10 min) and centrifuged (2 min at 13000 *g*). These crude DNA lysates were used for PCR, either immediately or after storage at −20 °C. Purified *N. gonorrhoeae* DNA was prepared from the bacterial growth harvested from one heated blood agar plate by the standard phenol/chloroform extraction method [13]. DNA was resuspended in 100 μl sterile distilled water and its concentration measured spectrophotometrically. All DNA preparations were made in duplicate from each isolate.

PCR analysis

Crude DNA lysate (2 μl) or purified DNA (200 ng) was added to a 50 μl reaction containing the following reagents: 1 × PCR buffer (Gibco–BRL, Paisley, UK), 1·5 mM $MgCl_2$, 200 μM dNTPs (Gibco–BRL), and 0·5 μM of each primer opa-up (forward) 5′-GCGATTATTTCAGAAACATCCG-3′ and opa-down (reverse) 5′-GCTTCGTGGGTTTTGAAGCG-3′ as previously published [10]. The reactions were overlaid with mineral oil and the PCR was performed using an Omnigene thermal cycler (Hybaid Ltd, Ashford, UK). Two thermal cycling programmes were evaluated. The originally reported programme [10] required an initial denaturation step at 95 °C for 5 min followed by cooling to 68 °C over 20 min. After the addition of 1·25 units *Taq* DNA polymerase (Gibco–BRL), 25 cycles of 95 °C for 1 min 68 °C for 2 min and 72 °C for 2 min were performed with a final extension at 72 °C for 5 min. In the second, simplified thermal cycling programme 1·25 units of Platinum *Taq* DNA polymerase (Gibco–BRL) was included in the reaction mix prior to the initial denaturation step which was 94 °C for 3 min. This was followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 7 min. PCR products were checked prior to digestion by electrophoresis in 1% agarose gel, stained with ethidium bromide (10 μg/ml) and visualized under UV transillumination. When required, PCR products were gel purified using Gene Clean, (Anachem, Luton, UK) as directed by the manufacturer. PCR products (22 μl) were digested overnight with 5 U of *Taq*I at 65 °C, or *Hha*I (neoshizomer of *HinP*I used previously [10]) or *Alu*I at 37 °C in the appropriate buffer. All restriction enzymes were obtained from Gibco–BRL.

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Polyacrylamide gel electrophoresis of PCR products

Unpurified and gel purified PCR products were compared by denaturing gel electrophoresis using a 6% (19:1 w/w acrylamide:bisacrylamide) polyacrylamide gel containing 1 × TBE (89 mM Tris-base, 89 mM boric acid, 2 mM EDTA and 6 M urea). 20 μl of sample was mixed with 5 μl of denaturing loading solution (formamide containing 0·25% cyanol blue and 0·25% bromophenol blue) and heated to 95 °C for 2 min before loading. Electrophoresis was carried out in 1 × TBE at 20 V/cm for 2 h at 65 °C in a temperature controlled tank (BioRad). Non-denaturing gel electrophoresis was used for the analysis of 8 μl of digested PCR products mixed with 4 μl loading solution (40% Ficoll-400, 0·25% cyanol blue, 0·25% bromophenol blue). This was performed using 10% polyacrylamide gels (37·5:1 w/w acrylamide:bisacrylamide for *Taq*I or *Alu*I digests and 29:1 w/w acrylamide:bisacrylamide for *Hha*I digests) in 1 × TBE at 13·3 V/cm for 3·5 h. Gels were subsequently stained for 15 min with 10 μg/ml ethidium bromide solution and photographed under UV transillumination using a Kodak DC120 digital camera.

Opa-types were considered to be distinguishable if they differed by two or more bands in *Taq*I and *Hha*I digests. Isolates that demonstrated only a single band difference were further investigated by digestion with a third enzyme (*Alu*I). Isolates were considered to be indistinguishable from each other if no additional band differences were found.

RESULTS

Validation of the simplified opa-typing protocol

Crude lysates and purified DNA preparations of two strains gave identical opa-types (data not shown), so crude lysates were used subsequently. The opa-types from strains amplified by either thermal cycling regime were similar (data not shown), although the second, simpler cycling programme gave stronger bands and two additional bands were visible in WHO strain A and one band in WHO strain E. The second cycling programme was adopted as the standard. PCR products separated on agarose gels typically resolved as a 550 bp product with a smear of DNA of reduced mobility, but both the gel purified 550 bp band and unpurified PCR product resolved as a single band under denaturing conditions (data not shown). In addition, *Taq*I digested gel purified and unpurified product gave the same banding pattern (data not shown), so gel purification of PCR products was considered unnecessary.

Typing of ciprofloxacin-resistant isolates

Phenotypic analysis of the ciprofloxacin-resistant isolates received in October 1999 from Oldham and Rochdale showed that these were of the NR auxotype and they exhibited intermediate resistance to penicillin and resistance to ciprofloxacin. Of the isolates submitted to the GUIRL during April 1999–February 2000, 73 were of this phenotype comprising 31 isolates (42%) from patients attending GUM clinics in Rochdale or Oldham and the remainder from clinics across the United Kingdom. Nine infections were contracted abroad (predominantly in the Far East), 42 were acquired in the United Kingdom and no information was available for the remaining 22 isolates.

Opa-typing of the 73 ciprofloxacin-resistant isolates revealed a total of 25 opa-types, 24 of which were represented by single isolates. A group of four isolates initially appeared similar (with only one band difference in each case) but *Alu*I digestion distinguished between each of them. A further two isolates also differed by only a single band after *Taq*I and *Hha*I digests but they were also discriminated further by *Alu*I digestion. Of the 24 isolates with unique opa-types, none were known to be from sexual contacts, 10 were known to be infections acquired abroad, 7 were infections acquired within the United Kingdom and for 7 no details were available.

The remaining 49 isolates were indistinguishable and were designated opa-type 1. Where information was given (38 of 49), all were reported to have been infections contracted within the United Kingdom. The opa-type 1 cluster included 15 isolates from Oldham and 16 from Rochdale. A further 16 isolates were from patients attending other clinics in the north of England (Manchester, Bury, Huddersfield, Preston, Halifax, Burnley, Liverpool, Ashton-under-Lyme and Southport). Epidemiological data revealed that 10 isolates that were opa-type 1 were from known sexual contacts: 1 pair of contacts from Huddersfield, 2 pairs from Oldham, 1 pair from Rochdale and 1 contact pair with 1 patient diagnosed at Rochdale and the other at Preston. Five opa-type 1 isolates were isolated from known commercial sex workers attending clinics in Rochdale (3), Oldham (1) and Manchester (1) and

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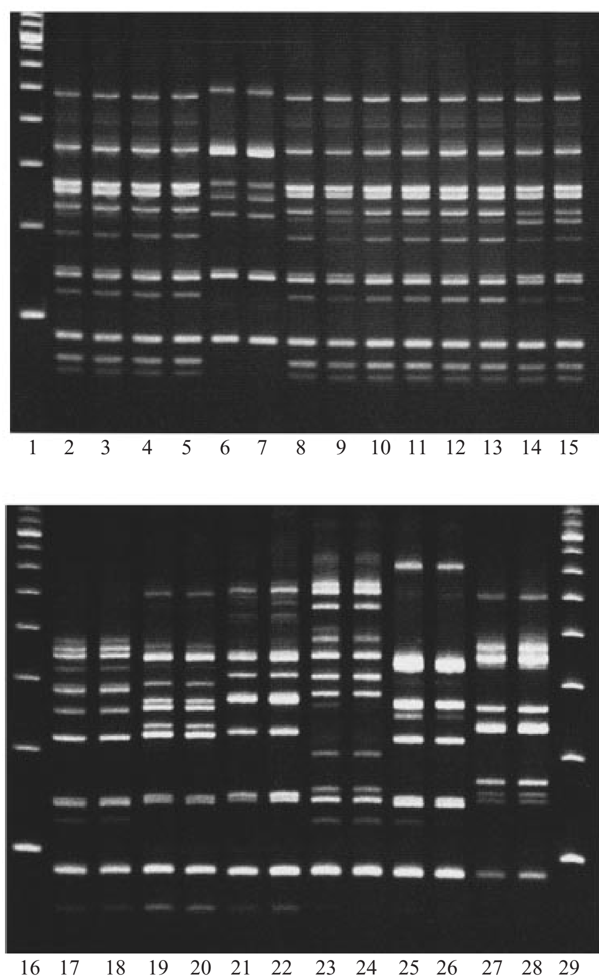

Fig. 1. Examples of opa-types identified in the study. Lanes 1, 16 and 29 100 bp ladder. Lanes 2–5 and 8–15, duplicate samples of six NR ciprofloxacin-resistant isolates of opa-type 1. Lanes 6–7, a NR ciprofloxacin-sensitive isolate of a different opa-type; lanes 17–28, duplicate samples of six NR ciprofloxacin-resistant isolates of different opa-types.

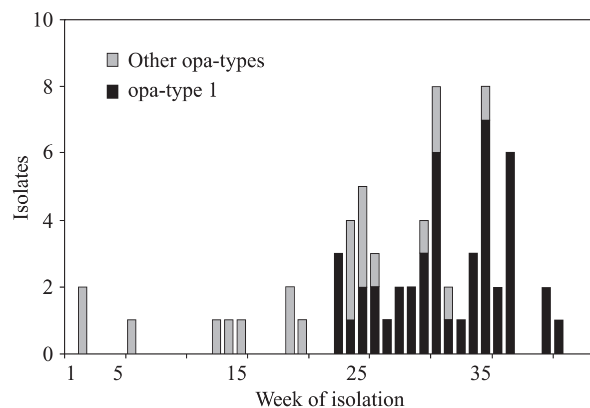

Fig. 2. Epidemic curve for *N. gonorrhoeae* isolates with intermediate resistance to penicillin, resistance to ciprofloxacin, and of the NR auxotype. April 1999–February 2000. Week 1 = 5–11 April 1999.

3 isolates were reported to have been acquired from contact with commercial sex workers in Rochdale. One isolate from Swansea and 1 from Gosport belonged to opa-type 1; both were found to have epidemiological links with Rochdale and Manchester respectively.

Two NR auxotype ciprofloxacin sensitive isolates, one arginine-requiring auxotype ciprofloxacin-sensitive isolate and 1 proline-requiring auxotype ciprofloxacin-resistant isolate from patients attending Oldham or Rochdale clinics during the same period were all clearly distinguishable and represented a further 4 unique opa-types. Examples of the opa-types are given in Figure 1.

The epidemic curve of ciprofloxacin-resistant NR isolates of *N. gonorrhoeae* reveals increasing numbers of isolates of opa-type 1 over a 19-week period (Fig. 2). The earliest date of isolation of opa-type 1 strains was 6 and 7 September 1999 when 3 isolates were isolated independently in clinics located in Bury, Manchester and Rochdale. Two of the 3 isolates were acquired in Manchester, but details were not available for the third.

DISCUSSION

Opa-typing was applied to NR auxotype, ciprofloxacin-resistant *N. gonorrhoeae* isolates submitted to the GUIRL between April 1999 and February 2000. Several simplifications of the original opa-typing method were introduced to make the methodology more broadly accessible and allow a timely response in outbreak situations or other applications. The detection of 24 unique opa-types from phenotypically indistinguishable isolates with no known epidemiological link demonstrates a high level of discrimination. However, a direct comparison of the original method (using a radioactive label) and the simplified methods was not carried out, so the relative sensitivities are unknown.

Opa-typing revealed that most isolates from geographically separate locations around the country were distinguishable, as would be anticipated for strains with no recent epidemiological links. A high proportion of the strains with unique opa-types were known to be acquired abroad, particularly from the Far East, which is consistent with the epidemiology of ciprofloxacin-resistant strains isolated in the United Kingdom over previous years [14, 15]. However, the cluster of opa-type 1 isolates observed in the North of England is consistent with an emerging trend towards

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the endemic spread of ciprofloxacin-resistant *N. gonorrhoeae* [16]. The earliest isolates of this cluster occurred on 6 and 7 September 1999, when 3 isolates were collected at 3 separate clinics in the north west of England. Attempts were made to identify an index case for this outbreak, i.e. a patient with a strain of *N. gonorrhoeae* which had been acquired outside the United Kingdom and which had an opa-type typical of the outbreak strain. However, despite analysing all ciprofloxacin-resistant NR isolates submitted to the GUIRL over the previous 5 months, no such index case was detected. So it is probable that the isolate from the index case was not submitted for analysis.

Although the majority of ciprofloxacin-resistant isolates of opa-type 1 were identified in Oldham and Rochdale, a significant number were found over a much wider geographical area. Related isolates were recovered from nine other urban centres in both NW and NE England and in addition 2 cases were detected in Southern England and South Wales which were epidemiologically linked with Manchester and Rochdale respectively. The identification of sexual networks within large geographical areas is not easily achieved in a timely manner by the use of contact tracing data which is time-consuming and is invariably incomplete. Opa-typing may be very useful to help identify related isolates of *N. gonorrhoeae* that originate from sexual networks. This is clearly of practical use where the circulating strain has a resistance determinant. Timely and responsive dissemination of the information to GUM clinics may help to reduce episodes of treatment failure and the resultant size of an outbreak.

The existence of 49 isolates indistinguishable by opa-typing over a period of 6 months in the north of England suggests that the opa-type can be stable over several transmission links in a chain, contrary to the original hypothesis [10]. Clusters of *N. gonorrhoeae* strains with indistinguishable opa-types were first demonstrated in Sheffield where a longitudinal study of consecutive isolates identified two clusters of 64 and 31 isolates [17]. The population structure of *N. gonorrhoeae* is described as non-clonal and, despite the apparent contradiction, this is not inconsistent with the data provided by opa-typing. The non-clonal nature of *N. gonorrhoeae* is based on the analysis of a collection of strains which spanned 3 decades and were isolated from several different countries [18]. Our study and that of Ison and colleagues [17] were directed at a local level and over shorter periods of time where the population structure of *N. gonorrhoeae* may best be described as 'epidemic' as predicted by O'Rourke and Stevens [18]. Results from the Sheffield study together with our data reveal that opa-typing is capable of tracking clonal expansion of *N. gonorrhoeae* populations over epidemiologically relevant time periods. The reason why 1 clone emerges and dominates while similar strains do not may be host related (behavioural, demographic or biological) or strain related. Such issues are difficult to investigate, but the application of opa-typing to epidemiological studies may contribute valuable information.

ACKNOWLEDGEMENTS

We are grateful to Dr H. Lacey, Dr T. D. I. Cartmill, Dr A. Ahamed and Dr L. Benons for their contribution, together with all the Departments of Microbiology and Genitourinary Medicine that have provided us with gonococcal isolates and patient information. We also thank Mrs K. Gough, Ms M. Hemming and Miss C. Weall for excellent technical assistance and Dr A. J. Herring for critical review of the manuscript.

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