Antimicrobial susceptibility testing of historical and recent clinical isolates of in the United Kingdom using the Etest method

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Title: Antimicrobial susceptibility testing of historical and recent clinical isolates of Bordetella pertussis in the United Kingdom using the Etest method

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Abstract: Reports of the development of antimicrobial resistance by Bordetella pertussis to macrolides in the United States and Taiwan, together with a recent increase in pertussis notifications and laboratory confirmed cases in England and Wales in 2008, prompted the examination of historical and recent clinical isolates from patients for evidence of such resistance in our collection. Isolates submitted to our laboratory as part of the enhanced surveillance scheme for pertussis, from 2001 to 2009, were tested against three agents, erythromycin, clarithromycin and azithromycin, by the Etest (bioMérieux) method. All isolates (n = 583) were fully susceptible to all three agents tested (Minimum Inhibitory Concentrations, ≤0.125 μg/ml). All but one strain (582/583) had MICs of ≤0.064 for all three agents. The control strain of B. pertussis A228 (from Centers for Disease Control and Prevention, USA) with a resistant phenotype had an MIC of >256 μg/ml. Although no evidence of resistance was found in the strains tested from the United Kingdom, screening for antimicrobial resistance of B. pertussis may be warranted in cases that are unresponsive to macrolide treatment and to provide early warning of such emergence in the future.

Response to Reviewer 1

This approach, which includes isolates with different serotypes, from different age groups, from geographically diverse locations within UK, and distinct genotypic types would be adequate for surveillance purposes, but taking into account that occurrence of B. pertussis resistance to macrolides is estimated to be low, the recommendation would be that a given B. pertussis isolate should be tested for resistance only if there is therapeutic failure.

Authors response: This is indeed what we have stated 'sensitivity testing for B. pertussis may be warranted in cases that are unresponsive to macrolide treatment '

But we believe that the additional clause 'and to provide early warning of for emergence of such resistance of in the future.' Is fully warranted.

In addition we would argue that evidence showing that B.pertussis isolates are sensitive to macrolides is still noteworthy in order to prevent the over-reporting of the occurrence of resistance. Certainly these data have not been previously reported for the UK, however, to accommodate this reviewer's view we have re-formatted the manuscript to a brief report, but would argue that a medical microbiology journal is the most appropriate.

Response to Reviewer 2

This study should be published. It confirms that macrolides are still antibiotics of choice for pertussis infection. The methodology is correct; data are clear and well presented.

Authors reposnse: We thank this reviewer for these positive comments.

Why are the authors using glycerol? The consensus technique was published by Mooi et al., Eur. J. Clin. Microbiol. Infect. Dis. 2000. The chromosome of B. pertussis being dynamic and containing many IS, glycerol might induce rearrangements.

Authors response: The use of glycerol as a cryoprotectant for preservation of suspensions of microorganisms at low temperature is well-established and is widely used in both commercial and in-house preparations. Our cultures are held at -80°C so these are effectively inert. Strains tested for genotypic markers such as pertactin, pertussis toxin and MLVA type before and after storage show no difference. The mechanism for possible induction of rearrangements of the B.pertussis genome due to glycerol is not clear and in the absence of evidence to this effect we cannot comment further. We note that the method suggested by the reviewer uses saccharose, but we are not aware of any data comparing different preservation media on the pertussis genome and any effects of genome rearrangement.

BRIEF REPORT

Antimicrobial susceptibility testing of historical and recent clinical isolates of *Bordetella pertussis* in the United Kingdom using the Etest method

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Abstract Reports of the development of antimicrobial resistance by *Bordetella pertussis* to macrolides in the United States and Taiwan, together with a recent increase in pertussis notifications and laboratory confirmed cases in England and Wales in 2008, prompted the examination of historical and recent clinical isolates from patients for evidence of such resistance in our collection. Isolates submitted to our laboratory as part of the enhanced surveillance scheme for pertussis, from 2001 to 2009, were tested against three agents, erythromycin, clarithromycin and azithromycin, by the Etest (bioMérieux) method. All isolates (*n* = 583) were fully susceptible to all three agents tested (Minimum Inhibitory Concentrations, ≤0.125 µg/ml). All but one strain (582/583) had MICs of ≤0.064 for all three agents. The control strain of *B. pertussis* A228 (from Centers for Disease Control and Prevention, USA) with a resistant phenotype had an MIC of >256 µg/ml. Although no evidence of resistance was found in the strains tested from the United Kingdom, screening for antimicrobial resistance of *B. pertussis* may be warranted in cases that are unresponsive to macrolide treatment and to provide early warning of such emergence in the future.

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Susceptibility testing of *Bordetella* spp. including *B*. *pertussis* clinical isolates is not routinely performed and prior to 1994 resistance to erythromycin in *B. pertussis* had not been observed [1]. However, since then there have been a few reports presenting the emergence of erythromycin-resistant *B. pertussis* isolates in the USA [2,3] and one in Taiwan [4].

These reports and a recent increase in notifications [5], prompted this examination of our collection of historical and recent clinical isolates of *B. pertussis*. Clinical isolates of *B. pertussis* are submitted to our laboratory, the Respiratory and Systemic Infection Laboratory (RSIL) as part of the enhanced surveillance scheme for pertussis for England and Wales. Our laboratory also receives isolates from Ireland, Northern Ireland and Scotland, and these were also included in this analysis. In this study, we report the susceptibility testing to erythromycin, clarithromycin and azithromycin by the Etest (bioMérieux) method for 583 *B. pertussis* clinical isolates that represent the majority (≥95 %) of isolates submitted to the RSIL between January 2004 and December 2009, and a selected number of isolates from September 2001 to December 2003. The years of isolation and numbers of strains isolated were as follows: 2001, 2; 2002, 40; 2003, 17; 2004, 68; 2005, 110; 2006, 53; 2007, 69; 2008, 134; and 2009, 90. Prior to testing isolates were initially subcultured into Charcoal blood agar (Media Services, Central Public Health Laboratory, London) to check for purity and incubated at 37°C for 24-72 hrs. Mueller Hinton agar plates supplemented with 10% horse blood (Oxoid, CPHL Media Services), 25 ml final volume, 90 mm triple-vented Petri dish (Greiner Bio-One Ltd), were inoculated by resuspension of bacterial growth as recommended for *Bordetella* spp. testing, but modified to use a higher MacFarland equivalent of 8, to achieve the semi-confluent growth required. E-strips (bioMérieux) were equilibrated to room temperature then carefully applied to the centre of each agar plate. Inoculated plates were incubated for 48 h to 7 days in a moist atmosphere at 37°C. The minimum inhibitory concentrations (MICs) were determined by using the Etest methodology. The following compounds were tested erythromycin (Cat. No. 1058), clarithromycin (Cat. No. 0878), and azithromycin (Cat. No. 0168). Control strains of erythromycin resistant *B. pertussis* A228 (a gift from Garry Sanden, Centers for Disease Control and Prevention, Atlanta, Georgia, USA) and the Oxford *Staphlyloccus aureus* NCTC6571 (National Collection of Type Cultures, London) were included in each batch.

The MIC values of test and control strains of *B. pertussis* were recorded at 72 h, following the manufacturer's instructions. In addition the plates were examined at 7 days to check for the appearance of the heterogenous phenotype for erythromycin resistance [6]. Isolates of *B. pertussis* with MICs of >256 µg/ml after 72 h were considered be resistant [2].

The *B. pertussis* isolates tested (from 2001-2009) included different serotypes (11 serotype 1,2; 569 serotype 1,3; and 11 untypable) from different age groups (<3 month, 348; 3-5 months, 90; 6-11 months, 23; 1-4 years, 1; 5-9 years, 5; 10-14 years, 10; ≥15 years, 15; unknown, 24) with a male:female ratio of 1:1.1. Isolates were received from geographically diverse locations within the United Kingdom and were known to include a number of distinct genotypic types, i.e., pertactin, pertussis toxin, Multi-locus Variable Number of Tandem Repeats Analysis [7], and authors' unpublished data. All isolates (523/523) were susceptible to all three agents tested (MICs ≤0.125 µg/ml) with the Etest method. An example of the results obtained is shown in the Figure. All isolates had MICs of ≤0.064 µg/ml with both erythromycin and azithromycin and one strain had an MIC of 0.125 µg/ml with clarithromycin. These results are comparable to those obtained with these agents by a study of 36 strains from Northern California by the Etest method [8]. No evidence of heterogeneous resistance was found. The erythromycin resistant *B. pertussis* A228 strain always yielded an MIC value of >256 µg/ml with all three agents, which is consistent with that previously reported [2]. The control strain NCTC6571 gave results consistent with target MICs for all three of these agents i.e., 0.12 µg/ml [9].

Macrolides (especially erythromycin) remain the current antibiotics of choice for post-exposure prophylaxis and treatment of pertussis infection [10]. Azithromycin is also recommended for prophylaxis of neonates as it is associated with fewer adverse effects [11]. The data from this study confirm that the current guidelines for macrolide treatment remain appropriate for *B. pertusiss* [11-13]. There are few documented occurrences of *B. pertussis* resistance to macrolides to date and the frequency of occurrence is estimated to be low (<1%) [6]. Although no evidence of macrolide resistance was found in this study of a large (*n* = 583) number of clinical isolates from the UK, sensitivity testing for *B. pertussis* may be warranted in cases that are unresponsive to macrolide treatment and to provide early warning of for emergence of such resistance of in the future. The mechanism of resistance

was proposed to be due to a mutation of the erythromycin binding site in the 23S rRNA gene, and identification of this and other potential resistance mechanism should allow genotypic testing and facilitate more rapid screening of large numbers of isolates or clinical specimens in the future.

Acknowledgments We thank Dr Garry Sanden for his kind gift of the erythromycin resistant *B. pertussis* A228 strain, Zak Prior for assistance with the Figure, and staff from all laboratories who have submitted *B. pertussis* isolates.

Conflicts of interest statement

None

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Figure. Example of results from a UK clinical *B. pertussis* strain subcultured on Mueller Hinton agar plate supplemented with 10% horse blood challenged with the erythromycin Etest strip after 72 h incubation, showing zone of clearance.