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Title: Applying molecular algorithms to predict decreased susceptibility to ceftriaxone from a report of strains of *Neisseria gonorrhoeae* in Amsterdam, The Netherlands

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Dear Editor,

In a recent article, de Korne-Elenbaas et al described a group of ceftriaxone-decreased susceptible and ceftriaxone-susceptible *Neisseria gonorrhoeae* strains in Amsterdam, The Netherlands, collected from 2014 to 2019 at a sexually transmitted infection (STI) clinic.[1] Resistance has been reported in every class of antibiotics used for gonococcal treatment. The prevalence of antimicrobial resistance in *N. gonorrhoeae* is rising globally.[2] The current treatment recommended by the World Health Organization is dual-therapy with ceftriaxone and azithromycin, although several countries have moved to ceftriaxone monotherapy.[3, 4] In The Netherlands, the recommended treatment for uncomplicated gonorrhea is monotherapy with ceftriaxone 500mg intramuscular as a single dose.[5] It is therefore critical to be able to predict the susceptibility of *N. gonorrhoeae* strains to ceftriaxone in order to guide treatment and reduce the spread of resistant strains.

The mechanisms for the development of resistance to ceftriaxone in *N. gonorrhoeae* are complex, making prediction of ceftriaxone susceptibility difficult.[4] The four primary genes that have been associated with resistance to ceftriaxone are *penA*, *ponA*, *penB*, and *mtrR*.[6] The *penA* gene encodes the penicillin-binding protein 2; multiple alterations in *penA* can result in resistance through decreased binding affinity of beta-lactam drugs such as ceftriaxone. The *ponA* gene encodes the penicillin-binding protein 1, and the amino acid alteration L421P results in a similar but less influential effect on drug binding affinity. The *penB* gene encodes the PorB porin protein, and amino acid alterations at the 120 and 121 position result in decreased

permeability of antimicrobials. Finally, the *mtrR* gene encodes the transcriptional repressor of the MtrCDE efflux pump, and the deletion of an adenine residue in the promoter region results in increased efflux of antimicrobials. Despite our knowledge of these genes, accurate prediction of ceftriaxone susceptibility phenotype has been impeded by the multiple mechanisms of resistance and genetic heterogeneity of *N. gonorrhoeae* strains globally.[4]

Coupling ceftriaxone susceptibility and genetic data in a global set of *N. gonorrhoeae* strains published through October 15th 2019, we proposed four molecular algorithms to predict decreased susceptibility to ceftriaxone using a MIC of >0.064 mg/L.[6] Our algorithms vary in whether they 1) include *penA* mosaicism, and 2) include *penA* or non-*penA* genes (*mtrR, penB, ponA*). Some of the algorithms resulted in high sensitivity or specificity with low to moderate complementary specificity and sensitivity, depending on the genetic targets used. The proposed algorithms could offer flexibility in the genes targeted, depending on the setting and prevalence of genetic markers.

In the report by de Korne-Elenbaas *et al.*, there were 318 *N. gonorrhoeae* strains with MICs ranging from <0.002 to 0.125 mg/L. Using the MIC breakpoint of >0.064 mg/L, there were 80 ceftriaxone decreased susceptible strains and 238 ceftriaxone susceptible strains.[1] Using the genomic sequence data in their report, we applied the previous molecular algorithms to these strains to determine their performance in predicting decreased susceptibility.

First, using the algorithm in Figure 1A, the arm with the highest sensitivity included wildtype *penA* A311 and non-wildtype *penA* A510V, resulting in a sensitivity and specificity of 94% and 25%, respectively. Applying that algorithm to the Amsterdam strains, all ceftriaxone decreased susceptible isolates were captured (100% sensitivity) with a specificity of 7%.

Using the algorithm in Figure 1B, the estimated sensitivity and specificity were 95% and 62% respectively in absence of mosaicism with non-wildtype *penA* L447V and presence of any of the following additional *penA* mutations: G542S, P551L/S, or A501V/T. Applying that algorithm to the Amsterdam strains, the sensitivity was 100% while the specificity was 77%.

Using the algorithm in Figure 1C, the estimated sensitivity and specificity in the presence of *ponA* L421P and at least one of *penB* G120 or A121 were 92% and 61% respectively. Applying that algorithm to the Amsterdam strains was associated with 100% sensitivity and a specificity of 84%.

Finally, using the algorithm in Figure 1D, the estimated sensitivity and specificity were 89% and 72% respectively in absence of mosaicism and presence of *mtrR* promoter adenine deletion and *ponA* L421P. Once again, all decreased susceptible strains were captured (sensitivity 100%) with a specificity of 75%.

Next, using the clinic's reported prevalence of ceftriaxone decreased susceptibility at 1.1%, we estimated the positive and negative predictive values for each of the four algorithms. All

algorithms resulted in a positive predictive value <10% with a 100% negative predictive value. The algorithm utilizing non-*penA* genes without mosaicism resulted in the highest positive predictive value of 6.5%. Therefore, given the low prevalence of decreased susceptibility, the low positive predictive values indicate that these algorithms, if developed into molecular tests, would have limited utility within the Amsterdam STI clinic. However, developing tests to detect decreased susceptibility using a lower MIC breakpoint of >0.064 mg/L would help identify concerning isolates. For example, positive tests might help to identify isolates needing additional antibiotic susceptibility testing, modified treatment regimens (e.g. increased ceftriaxone dosage or alternative antimicrobials), or closer follow-up with a test of cure, ultimately reducing the chance for treatment failure, overall cost of treatment, and spread of resistant *N. gonorrhoeae* strains.

Furthermore, as the prevalence of ceftriaxone decreased susceptibility increases, so too will the positive predictive values of these algorithms. For example, at a prevalence of 30%, the positive predictive values of three out of the four algorithms are over 60% with no compromise in the negative predictive values. Therefore, while utilization of these algorithms as molecular tests would incur additional lab work, cost, and have minimal benefit at the current prevalence of ceftriaxone decreased susceptibility in the Amsterdam STI clinic, they would be significantly more useful as the rates of ceftriaxone resistant *N. gonorrhoeae* increase.

Using the report by de Korne-Elenbaas et al, we demonstrate the potential application of the proposed molecular algorithms for the prediction of decreased susceptibility to ceftriaxone in

N. gonorrhoeae. Not only were all strains of ceftriaxone decreased susceptible captured using all four algorithms, but it appears that three of four algorithms perform well in the Netherlands with higher specificity values than that predicted using a global set of isolates. Moreover, we recently validated two of our algorithms in a report of a ceftriaxone-resistant *Neisseria gonorrhoeae* strain from Portugal.[7, 8] The genetic distinctness of the Amsterdam and Portugal strains with respect to sequence-type combinations (MLST-MAST) of *N. gonorrhoeae* isolates and different countries of origin further illustrates the value of incorporating several genetic markers into molecular tests to predict decreased susceptibility to ceftriaxone. In light of the urgent global health threat posed by antibiotic resistance in *N. gonorrhoeae*, utilizing genetic targets for the rapid prediction of ceftriaxone resistance is critical. Continual global monitoring of genetic loci associated with ceftriaxone resistance will enable further improvements in molecular testing algorithms.

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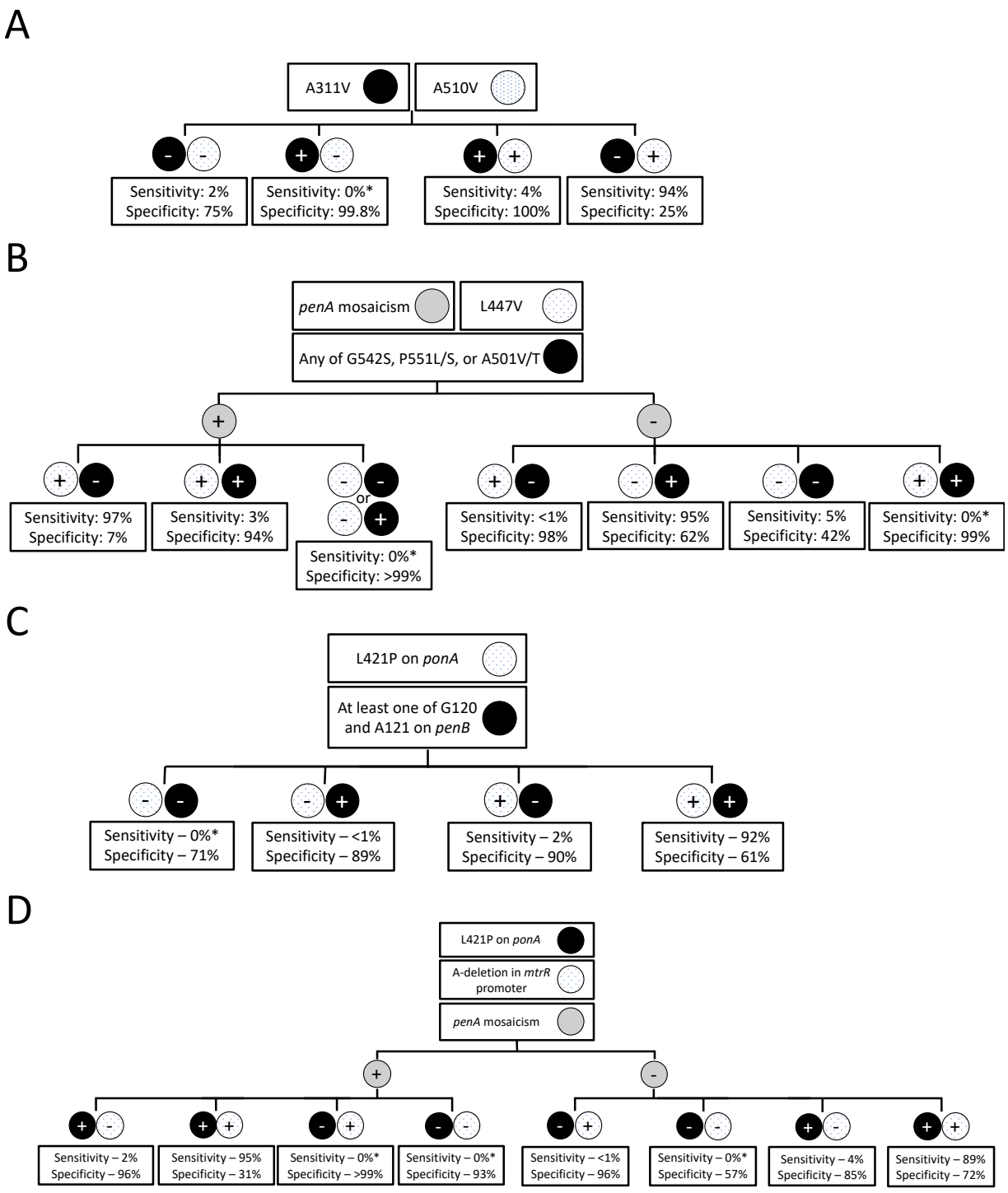

Figure 1. Four molecule algorithms predicting decreased susceptibility to ceftriaxone: Panel A is an algorithm utilizing *penA* amino acid alterations without mosaicism determination; Panel B is an algorithm utilizing *penA* amino acid alterations with mosaicism determination; Panel C is an

algorithm utilizing non-*penA* (*ponA*, *penB*) amino acid alterations without mosaicism determination; Panel D is an algorithm utilizing non-*penA* (*ponA*, *mtrR*) amino acid alterations with mosaicism determination. “+” indicates the presence of the corresponding genetic alteration, while “-“ indicates the absence of the corresponding genetic alteration. Sensitivity and specificity values are for decreased susceptibility to ceftriaxone. Testing for all genetic loci in each algorithm are intended to be done simultaneously, and not necessarily in a step-wise fashion. * indicates that no decreased susceptible strains have been found with this combination of genetic alterations.