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Utility of Specimens Positive for *Neisseria gonorrhoeae* by the Aptima Combo 2 Assay for Assessment of Strain Diversity and Antibiotic Resistance

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In our jurisdiction, the Aptima Combo 2 assay (Gen-Probe, Inc.) is used to detect *Neisseria gonorrhoeae* from specimens collected at clinics for sexually transmitted infections (STI) and from select community patients. In addition, swabs are also collected for *N. gonorrhoeae* culture, susceptibility testing, and sequence typing (ST). Since only a small proportion of samples from provincial cases undergo culture, the available trends in antimicrobial susceptibility and predominant strain types may not be representative of all *N. gonorrhoeae* infections. Due to the limitations facing the use of *N. gonorrhoeae* culture to understand these trends in the general community, we performed a molecular analysis for markers of cephalosporin resistance and ST determination by using nucleic acid extracts of specimens sent for Aptima testing. Thirty-four samples submitted for both Aptima testing and *N. gonorrhoeae* culture from the same anatomic location (within 24 h) were included in the study. Sequence type was determined based on the sequence of the *por* and *tbpB* genes, and amino acid changes in the PBP 2 protein, encoded by the *penA* gene, were considered representative for the assessment of antimicrobial susceptibility. Sequence identity of 100% was observed between the sequences obtained from Aptima-analyzed samples and culture samples. Sequencing results showed an association between decreased susceptibility to extended-spectrum cephalosporins (ESC^{ds}), *tbp* allele 110, ST 1407, and amino acid changes (G545S, I312M, and V316T) in the PBP 2 protein. Our data, generated based on a few representative genes, suggest that gonococcal samples positive by Aptima testing can be used to determine single nucleotide polymorphisms associated with ESC^{ds} and the sequence type based on molecular strain typing. Confirmation of these findings may obviate the need for gonorrhea culture in the future.

Neisseria gonorrhoeae is a common sexually transmitted infection that affects mucosal surfaces and causes a spectrum of conditions, including urethritis, endocervicitis, pelvic inflammatory disease, and infertility (1). Over the years, gonococci have developed resistance to multiple classes of antibiotics, including penicillins, tetracyclines, macrolides, and quinolones (2). Of recent concern is a steady increase in the MICs to extended-spectrum cephalosporins, including cefixime and ceftriaxone, in several countries including Canada, with reported levels at 0.12 μg/ml, one doubling dilution away from the Clinical and Laboratory Standards Institute (CLSI) limit of 0.25 μg/ml for susceptibility (3–7; CLSI standard M100-S23, January 2013). According to a recent U.S. Centers for Disease Control and Prevention (CDC) update, untreatable gonorrhea could soon be a reality in the United States (http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6131a3.htm). The agency noted that *N. gonorrhoeae* seems to be developing resistance to the oral antibiotic cefixime, and it no longer recommends cefixime at any dose as a first-line regimen for treatment of gonococcal infections. The recent isolation of two extensively ceftriaxone-resistant strains in Japan and France (4, 8) intensified these concerns.

A comprehensive understanding of the prevalence of decreased susceptibility to extended-spectrum cephalosporins (ESC^{ds}) and the sequence types (ST) of circulating *N. gonorrhoeae* strains within patient populations may help to guide key decisions in planning for the management of *N. gonorrhoeae*. However, traditional susceptibility testing and strain analysis require culture-based methods and collection of specimens compatible with these methodologies. In many jurisdictions, *N. gonorrhoeae* testing is routinely done by molecular methods alone, and the lack of culture has made routine susceptibility testing and strain analysis impossible. In our laboratory, molecular testing for *N. gonorrhoeae* is performed using the FDA-approved Aptima Combo 2 assay (Gen-Probe Inc., San Diego, CA) (9). Our laboratory also receives specimens from the sentinel surveillance clinics in the province (two sexually transmitted infections [STI] clinics) where additional swabs are collected for culture, and the isolates are used for susceptibility testing and strain analysis. One key concern is that the shift to molecular testing for *N. gonorrhoeae* diagnosis and the availability of limited numbers of specimens for culture may lead to biases in our understanding of antimicrobial susceptibility patterns and circulating strains in the populations not attending sentinel surveillance sites or not having cultures prepared from clinical specimens. One approach is to utilize specimens that test positive by a molecular method for further analysis of antimicrobial resistance and strain typing.

Several studies have shown that accumulation of mutations in chromosomal genes can lead to the phenotype of ESC^{ds}. The genes studied include *penA*, which encodes penicillin binding protein 2

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(PBP 2); *porB1*, which encodes an outer membrane protein channel related to the entry of antibiotics; *ponA*, which encodes PBP 1; *pilQ*, which encodes the outer membrane secretin PilQ; and *mtrR*, a repressor of the MtrC-MtrD-MtrE efflux pump (5, 10, 11). Polymorphisms in the *penA* allele have been significantly associated with ESC^{ds} in *N. gonorrhoeae* (4, 12, 13). PBP 2 is a membrane-bound enzyme involved in cell wall synthesis, and mutations in different positions may result in various extents of increased MICs. Several reports have suggested that the resistant strains are largely clonal and contain specific PBP 2 mosaic patterns, such as type XXXIV (3). Sequence type 1407, based on the *por* and *tbpB* genes, has also been associated with ESC^{ds} (14, 15).

In this study, we evaluated samples submitted for *N. gonorrhoeae* screening by the Aptima Combo 2 assay to determine if such samples can be used to establish circulating STs and antimicrobial susceptibility patterns. As a proof of principle, we sequenced the *por* and *tbpB* genes for determination of the ST and the *penA* gene for cephalosporin resistance determinants.

MATERIALS AND METHODS

Amplification and detection of *Chlamydia trachomatis* and *N. gonorrhoeae* with the Gen-Probe Panther system. Urine or cervical swab specimens collected from patients visiting the STI clinic or a community clinic in Calgary, Alberta, Canada, were submitted to the Provincial Laboratory for Public Health (Provlab, Calgary, Canada) for the detection of *C. trachomatis* and *N. gonorrhoeae* by using the Aptima Combo 2 assay (Hologic Gen-Probe Inc., San Diego, CA). Specimen processing and testing were performed according to the manufacturer's protocol. Specimens analyzed in this validation had to fulfill two criteria: (i) they were from patients with a positive molecular result for *N. gonorrhoeae*, and (ii) a specimen was also collected from the patient for culture, susceptibility, and strain analysis from the same anatomic site (urethral swab from males and cervical swab from females) within 24 h of the molecular specimen collection. These criteria allowed for the analysis of 34 sample pairs.

Specimen extraction, amplification, and sequence analysis. Preliminary experiments comparing the extraction of nucleic acid from Aptima specimens using the easyMAG automated extractor (bioMérieux, Quebec, Canada) and QIAamp DNA minikit (Qiagen, Ontario, Canada) showed that better extraction results and an easier workflow were available with the QIAamp columns. Thus, for our study, nucleic acid extraction was performed directly from specimens submitted for Aptima testing and from suspensions of cultures prepared in saline using the QIAamp DNA minikit. These extracts were used for amplification and sequencing of the *penA*, *por*, and *tbpB* gene targets performed using previously described primers (7, 16, 17) for direct molecular characterizations. In a preliminary pilot study, mutations in the *ponA* gene, coding for PBP 1, *pilQ* coding for the pilus secretin protein, bla_{TEM-1} coding for β-lactamase, and the *Mtr* promoter and repressor regions in addition to the *penA*, *por*, and *tbpB* genes were also monitored using samples with elevated cefixime MIC values; however, a reproducible association was found between changes in *penA*, *por*, and *tbpB* and decreased susceptibility for the limited number of samples tested in this study (data not shown). Thus, for this study, samples were analyzed for antimicrobial susceptibility based on mosaic changes that had been correlated with ESC^{ds} in the PBP 2 protein encoded by the *penA* gene and on *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) based on the sequences of the *por* and *tbpB* genes (17).

Amplification was performed using the AccuStart II GelTrack PCR SuperMix kit from Quanta Biosciences (Gaithersburg, MD) with 12.5 μl of 2× Supermix, a final concentration of 0.6 μM for the forward and reverse primers, and 5 μl of template nucleic acid in a total volume of 25 μl. The amplification protocol included an initial denaturing at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing for 30 s at 58°C for *por*, 69°C for *tbpB*, 55°C for the primer pair penA-Compl-F/penA-Compl-R, and 60°C for the primer pair penA-F/penA-R, and then extension at 72°C for 60 s. A final extension step was performed for 10 min at 72°C followed by cooling. Amplified products were sequenced in both directions on a 3130 genetic analyzer (Applied Biosystems [ABI], Foster City, CA). Contig assembly for the *penA* gene was performed using SeqScape v2.6 (ABI), and analyses of nucleotide and protein sequence alignments were performed using the sequence analysis software BioEdit v7.1.1 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

Susceptibility testing of isolates. Specimens were plated at the bedside on selective medium (Thayer Martin). Confirmation of *N. gonorrhoeae* was undertaken using Gram staining, an oxidase assay, and the AccuProbe assay (Gen-Probe, San Diego, CA). The MICs for 6 antibiotics (penicillin, tetracycline, ceftriaxone, cefixime, ciprofloxacin, and azithromycin) were determined by Etest (bioMérieux, France) on GC base agar. The interpretation of MIC values was based on Clinical and Laboratory Standards Institute (CLSI) guidelines (standard M100-S23; January 2013).

Assay specificity. To ensure the clinical specificity of the assay, a total of 30 specimens that had tested negative for *N. gonorrhoeae* by the Aptima Combo 2 assay, including cervical ($n = 12$), rectal ($n = 1$), and urine ($n = 17$) samples, were extracted, and amplifications of the *penA*, *tbp*, and *por* gene targets were attempted with the primers described above. Amplification of nonspecific targets was not observed, suggesting that the primers were 100% specific to the desired target in clinical specimens and that the assay would not generate false positives from these specimens.

In addition, to ensure analytic specificity, amplification products that were obtained as a result of the presence of closely related species in the collected specimen types were also analyzed. We tested representatives of such organisms, including *Neisseria lactamica*, *Neisseria sicca*, *Neisseria meningitidis*, and *Mycoplasma hominis* by using the primers described above for the *penA*, *tbp*, and *por* gene targets. Amplified products were obtained for the *tbp* and *por* genes from *N. lactamica* and *N. meningitidis*. Primers for the *penA* gene successfully amplified the template from *N. lactamica*, *N. meningitidis*, and *N. sicca*. Sequencing and BLAST comparison of the sequences to the NCBI database revealed correct designations of the organisms, suggesting that even if these organism sequences were amplified from patient specimens, interpretation of results would be simple.

RESULTS

Specimens tested. A total of 34 positive specimens, including 28 urine and 6 cervical specimens that gave relative light unit (RLU) values between 1,212 and 2,593 in the Aptima Combo 2 assay, could be matched to a specimen collected within 24 h for culture and susceptibility and strain analyses. Amplification and sequencing were successful from 30 specimens; four specimens (including three urine and one cervical specimen) failed to amplify the sequences of interest from the nucleic acid extracts of the Aptima specimens. This was likely the result of low bacterial loads in these samples, as confirmed with the Anyplex CT/NG real-time detection kit (v3.1) from Seegene (Seoul, South Korea). The crossing threshold values for these samples were consistently higher than 33, confirming this observation. These specimens were also tested for the presence of PCR inhibitors in spiking experiments, and the results of these experiments indicated the absence of inhibitors (data not shown). The MIC values as determined by Etest methodology for penicillin, tetracycline, ceftriaxone, cefixime, ciprofloxacin, and azithromycin for the paired culture specimens linked to the 30 Aptima specimens from which a sequence could be obtained are shown in Table 1. The *por* (490 bp) and *tbp* (390 bp) genes were used to determine the respective allele numbers as provided via the website ng-mast.net. These allele numbers were then used for sequence typing using NG-MAST.

There is no international consensus on MIC levels that indicate decreased cephalosporin susceptibility in *N. gonorrhoeae*, but the

TABLE 1 Characterization of samples, based on conventional antibiotic susceptibility, mutation(s) in PBP 2, and NG-MAST[a]

Patient no.	Sample type	Susceptibility (MIC, in μg/ml)[b]						*penA* mutation[c]			Allele no.[d]		
		Penicillin	Tetracycline	Ceftriaxone	Cefixime	Ciprofloxacin	Azithromycin	I312M	V316T	G545S	*por*	*tbpB*	ST[e]
1	Urethra	2	1	0.06	**0.12**	>32	0.5	M	T	S	**908**	**110**	**1407**
2	Urethra	2	2	0.06	**0.12**	≥32	0.5	M	T	S	**908**	**110**	**1407**
3	Urethra	2	0.5	0.06	**0.12**	≥32	0.5	M	T	S	**3669**	**110**	**6200**
4	Cervix	2	0.5	0.06	**0.12**	>32	0.5	M	T	S	**2743**	**110**	**4461**
5	Urethra	2	1	0.06	**0.12**	16	0.5	M	T	S	**1914**	**110**	**3158**
6	Urethra	2	1	0.06	**0.12**	≥32	0.5	M	T	S	**908**	**110**	**1407**
7	Urethra	0.25	0.25	0.016	**0.06**	4	0.06	M	T	S	**908**	**110**	**1407**
8	Cervix	0.25	0.12	**0.25**	≤0.016	≥32	0.25	I	V	G	**1910**	**27**	**4637**
9	Urethra	0.5	1	0.016	≤0.016	8	0.25	I	V	G	1489	563	2400
10	Urethra	0.25	0.5	0.008	≤0.016	0.008	1	I	V	G	5108	29	8632
11	Urethra	0.25	0.5	0.016	≤0.016	0.008	0.5	I	V	G	908	29	3935
12	Urethra	0.5	1	0.016	≤0.016	0.008	1	I	V	G	908	29	3935
13	Urethra	0.25	8	0.004	≤0.016	≤0.002	0.06	I	V	G	2649	1066	5083
14	Urethra	0.12	0.5	0.008	≤0.016	≤0.002	0.25	I	V	G	1808	29	2992
15	Urethra	0.5	0.5	0.012	≤0.016	0.008	1	I	V	G	908	29	3935
16	Urethra	0.25	0.5	0.008	≤0.016	0.016	1	I	V	G	908	29	3935
17	Urethra	1	0.25	0.008	≤0.016	4	0.25	I	V	G	2852	25	4709
18	Cervix	1	0.25	0.008	≤0.016	4	0.25	I	V	G	2852	25	4709
19	Urethra	1	0.5	0.016	≤0.016	4	0.5	I	V	G	2852	25	4709
20	Urethra	1	1	0.016	≤0.016	4	0.5	I	V	G	53	10	657
21	Urethra	0.5	1	0.008	≤0.016	0.008	1	I	V	G	908	29	3935
22	Cervix	0.5	0.5	0.016	≤0.016	0.016	1	I	V	G	908	29	3935
23	Urethra	0.12	0.12	0.004	≤0.016	0.002	0.12	I	V	G	2155	16	3556
24	Urethra	0.12	0.25	0.004	≤0.016	0.004	0.125	I	V	G	852	19	1319
25	Urethra	0.25	0.5	0.008	≤0.016	0.008	0.25	I	V	G	1808	29	2992
26	Cervix	0.25	0.5	0.008	≤0.016	0.008	1	I	V	G	908	29	3935
27	Urethra	0.25	0.25	0.008	≤0.016	0.002	0.5	I	V	G	1808	29	2992
28	Urethra	0.03	0.12	≤0.016	≤0.016	≤0.016	0.12	I	V	G	55	4	69
29	Urethra	0.25	0.25	0.008	≤0.016	0.004	0.5	I	V	G	1808	29	2992
30	Urethra	1	1	0.03	0.03	>32	0.12	I	V	G	908	563	7574

[a] Samples with ESC[ds] phenotype, related allele numbers, and sequence types are shown in bold.
[b] Antibiotic susceptibility profiles were determined with Etest strips.
[c] Amino acid alteration(s) in the *penA* gene (I312M, V316T, and G545S).
[d] Allele number, based on the *por* and *tbpB* genes.
[e] ST, based on NG-MAST result.

CLSI standard (M100-S23 [January 2013]) defines decreased susceptibility to cefixime and ceftriaxone as a MIC of ≥0.5 μg/ml. In 2012, the World Health Organization (WHO) released a global action plan to control the spread and impact of antimicrobial-resistant *N. gonorrhoeae* and published new recommendations and criteria for decreased susceptibility to cephalosporins, including cefixime (a MIC of ≥0.25 μg/ml) and ceftriaxone (a MIC of ≥0.125 μg/ml) (http://whqlibdoc.who.int/publications/2012/9789241503501_eng.pdf). A resistance threshold of >0.12 μg/ml for cefixime in the treatment of *N. gonorrhoeae* has been also supported by other studies (18). The MIC for ceftriaxone in our study samples ranged from 0.004 to 0.25 μg/ml, and the MIC for cefixime ranged from <0.016 to 0.12 μg/ml. In the absence of a universal threshold for resistance to cephalosporins, we defined strains with MICs of ≥0.06 μg/ml to cephalosporins as having decreased susceptibility to extended-spectrum cephalosporins (ESC[ds]).

***por* and *tbpB* alleles and sequence types detected in samples with ESC[ds].** A sequence identify of 100% was observed between the sequences obtained with the Aptima and linked positive culture isolates, suggesting that direct molecular characterization can be performed from Aptima specimens. Of the cultures tested, six specimens exhibited ESC[ds], with a ceftriaxone MIC of 0.06 μg/ml and cefixime MIC of 0.12 μg/ml. All six pairs of primary specimens and isolates showed the presence of allele number 110 for the *tbpB* gene. Three of the six pairs exhibited allele number 908 for the *por* gene, and other paired specimens and isolates had alleles 1914, 2743, and 3669, corresponding to sequence types 1407, 3158, 4461, and 6200, respectively. Sequence comparisons between allele number 908 and allele numbers 1914, 2743, and 3669 revealed less than 0.5% variation at the nucleotide level. Allele 1914 showed the presence of two nucleotide changes (G628A and A649G), allele 2743 showed a change at nucleotide 632 from A to T, and allele 3669 had two base pair changes (A649G and G767A). One isolate had a ceftriaxone MIC of 0.016 μg/ml, its MIC for cefixime was slightly elevated at 0.06 μg/ml, and the *por* allele, *tbpB* allele, and ST for this sample were 908, 110, and 1407, respectively. One sample had a ceftriaxone MIC of 0.25 μg/ml and a cefixime MIC of ≤0.016 μg/ml, with 1910, 27, and 4637 as the *por*, *tbpB*, and ST results, respectively. Thus, an association was observed between an elevated MIC to cefixime and *tbp* allele 110. The other common *por* alleles detected were 1808 and 2852, and the frequently detected *tbpB* alleles were 29 and 25.

Mutations in the *penA* gene and ESC^{ds}. Mutations in a range of genes, including alterations in *penA*, have been implicated in the development of resistance (5, 10, 11). Amino acid changes in the PBP 2 protein have been reported to have a strong correlation with ESC^{ds}. Here, we sequenced the *penA* gene and analyzed the following amino acid changes: G545S, I312M, and V316T. A sequence identify of 100% was observed between the sequences obtained from the paired Aptima and culture samples, suggesting that direct molecular characterization can indeed be performed from the nucleic acid extracts of the Aptima specimens. Of the eight samples with ESC^{ds}, the MICs for cefixime were 0.12 μg/ml for six samples and 0.06 μg/ml and ≤0.016 μg/ml for one sample each; the corresponding ceftriaxone MICs were 0.06 μg/ml, 0.016 μg/ml, and 0.25 μg/ml. Cultures from seven of the paired samples with elevated cefixime MICs showed the presence of changes at the positions mentioned above: 545S, 312 M, and 316T. All other paired specimens and isolates ($n = 23$), including the sample with an MIC of 0.25 μg/ml for ceftriaxone, showed the presence of wild-type amino acids at these positions.

DISCUSSION

With the widespread use of high-throughput molecular techniques as front-line methods for the detection of *N. gonorrhoeae*, specimens for culture and susceptibility testing are frequently collected only from select patients who are often from a high-risk population and attending an STI clinic. The current gold standard method to detect ESC^{ds} is by isolation and phenotypic susceptibility testing, with strain typing performed on cultured isolates. Molecular characterization can be performed on isolates by sequencing of genes that exhibit single nucleotide polymorphisms (SNPs) correlated with decreased susceptibility or resistance to antibiotics (10, 19) and genetic determinants for rapid strain typing. Due to the higher sensitivity offered by molecular methods, nucleic acid-based testing is now considered the gold standard for detection of *N. gonorrhoeae* (20). With the decline in the use of culture for routine diagnosis, fewer isolates are available for susceptibility testing and strain analysis. In this study, the utility of samples submitted for high-throughput testing in the identification of SNPs that are correlated with ESC^{ds} and for strain typing was investigated.

In this preliminary study, we observed 100% sequence identity between the *por*, *tbpB*, and *penA* sequences obtained from the Aptima samples and linked isolates, suggesting that direct molecular characterization of *N. gonorrhoeae* can be performed with Aptima specimens. Furthermore, based on the sequence data, an association was observed between ESC^{ds}, *tbpB* allele 110, ST 1407, and amino acid changes (G545S, I312M, and V316T) in the PBP 2 protein. All isolates with elevated cefixime resistance showed the presence of *por* allele 908 or another highly related allele (less than 0.5% variation at the nucleotide level). An association of ST 1407 with decreased cefixime susceptibility and the wide circulation of this clone has been extensively reported (3, 8, 14, 14, 21–23). A study comparing antimicrobial resistance surveillance with molecular typing of gonococcal isolates from Europe showed that the genogroup (G1407) accounted for 23% of the overall isolates and predominated in several countries. That study's results also suggested that this isolate first emerged in 2007 and spread globally, causing most of the treatment failures with third-generation cephalosporins. There appear to be no isolates of G1407 that are highly sensitive to cefixime, with 96% of isolates showing MICs of ≥0.06 mg/liter, suggesting that sequence typing may be a valuable predictor of antimicrobial resistance (14). High-level cefixime- and ceftriaxone-resistant strains from patients with clinical failures identified in France and Spain showed the presence of ST 1407 and the *penA* mosaic pattern XXXIV (8, 24). The circulation of this strain has been reported to cause treatment failures in Canada (3), suggesting that monitoring for these changes is critical. Sequencing from closely related species, such as *N. lactamica*, *N. sicca*, *N. meningitidis*, and *M. hominis*, in the collected specimen types revealed that correct designation of the organisms is possible. As the advances in molecular testing identify positive *N. gonorrhoeae* samples in different anatomic locations, such as the pharynx, appropriate control organisms will need to be tested to ensure analytic specificity. Our study suggests that genito-urinary tract specimens sent for Aptima testing can be used instead of culture to characterize single nucleotide polymorphisms associated with cephalosporin resistance and for strain typing.

Although the small convenience sample size used in this study was likely not representative of our overall cases, it provides proof of principle that specimens submitted for high-throughput molecular detection methods may become valuable tools for the surveillance of genetic markers that lead to antimicrobial resistance and the relevant sequence types in *N. gonorrhoeae*. Such a strategy has the potential to provide more comprehensive surveillance data on circulating *N. gonorrhoeae* strains and may allow for a more accurate estimation of the prevalence of resistance markers in the general population. Furthermore, these methods can be further modified in the future to provide timely sequence-based determinations from clinical specimens. A decrease in turnaround times will be key, as the prompt provision of test results has the potential to impact patient care by reducing disease complications and transmission.

However, there are still some concerns about relying solely on molecular techniques for the surveillance of resistance determinants to characterize *N. gonorrhoeae* isolates. The potential disadvantages include the inability to (i) differentiate between intermediate and high-level resistance to predict the MIC, (ii) consider the multiple mechanisms by which *Neisseria* attains resistance to the different antibiotics, (iii) correlate the genotypic results with the expressed phenotype, and (iv) detect novel resistance mechanisms. Thus, culture and susceptibility testing will remain the gold standard method until these issues are studied further. In the future, we envision that nucleic acid-based assays will be used more extensively to detect known mutations that lead to decreased cephalosporin resistance. However, further studies will be required to fully understand the complex interactions between SNPs, their correlations to changes in MIC values, and patient outcomes. The molecular approaches identified in this paper still need to be modified to make them amenable to high-throughput testing and automation, which could enable a more widespread surveillance of changes in sequence types or cephalosporin susceptibility that might allow these approaches to be adapted as point-of-care tests (25).

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