No widespread dissemination of *Chlamydia trachomatis* diagnostic-escape variants and the impact of *Neisseria gonorrhoeae* positivity on the Aptima Combo 2 assay

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ABSTRACT

Objectives

A Finnish *Chlamydia trachomatis* (CT) new variant was detected in 2019 that escaped detection in the Hologic Aptima Combo 2 (AC2) assay due to a C1515T mutation in the CT 23S rRNA target region. Reflex testing of CT-negative/equivocal specimens, as well as those positive for *Neisseria gonorrhoeae* (NG) with the Hologic Aptima CT assay (ACT) was recommended to identify any CT-variants

Methods

From June to October 2019, specimens with discrepant AC2/ACT CT results were submitted to Public Health England and screened for detectable CT DNA using an in-house RT-PCR. When enough DNA was present, partial CT 23S rRNA gene sequencing was performed. Analysis of available relative light unit (RLU) and interpretive data were performed.

Results

A total of 317 discordant AC2/ACT specimens were collected from 315 patients. Three-hundred were tested on the RT-PCR; 53.3% (n=160) were negative and 46.7% (n=140) were positive. Due to low DNA load in most specimens, sequencing was successful for just 36 specimens. The CT 23S rRNA wild-type sequence was present in 32 specimens, and two variants with C1514T or G1523A mutation, were detected in four specimens from three patients.

Of the discordant specimens with NG interpretation; 36.6% of NG-negative/CT-negative AC2 specimens had detectable CT DNA in the in-house RT-PCR vs. 53.3% of NG-positive/CT-negative specimens.

Conclusions

No widespread dissemination of AC2 diagnostic-escape CT variants has occurred in England. We however identified the impact of NG positivity on the discordant AC2/ACT specimens; a proportion appeared due to NG positivity and the associated NG signal, rather than any diagnostic-escape variants or low DNA load. Several patients with gonorrhoea may therefore receive false-negative AC2 CT results. Single diagnostic targets and multi-plex diagnostic assays have their limitations such as providing selection pressure for escape mutants and potentially reduced sensitivity, respectively. These limitations must be considered when establishing diagnostic pathways.

KEY MESSAGES

- We detected four specimens from three patients with Aptima Combo 2 (AC2) diagnostic-escape *Chlamydia trachomatis* (CT) variants in England during 2019.
- There was no widespread dissemination of these CT variants, with a prevalence of <0.003%.
- *Neisseria gonorrhoeae* (NG) positivity appeared to reduce CT sensitivity; 37% of NG-negative vs. 53% of NG-positive AC2/ACT discordant specimens had detectable CT DNA in a RT-PCR.

INTRODUCTION

Chlamydia is the most common bacterial sexually transmitted infection globally[1] as well as in England.[2] In 2019, 229,411 cases of chlamydia were recorded in England, which encompassed a 5% increase from the previous year.[2] In addition to *Chlamydia trachomatis* (CT) testing in sexual health clinics across England, the National Chlamydia Screening Programme (NCSP) delivers opportunistic screening to sexually active young people in community settings resulting in a total of over 1.3 million CT tests performed among those aged 15 to 24 years in 2019.[2]

For CT testing, nucleic acid amplification tests (NAATs) are the diagnostic methods of choice due to their high levels of sensitivity and specificity. One such commonly used NAAT is the Aptima Combo 2 (AC2) assay (Hologic Inc., San Diego, USA) that detects both a CT target (23S rRNA) and a *Neisseria gonorrhoeae* (NG) target (16S rRNA). A CT variant that escaped detection with the AC2 was first described in Finland during February 2019.[3] These AC2 false-negative samples were correctly identified as CT-positive by alternative CT NAATs and then subsequently found to be CT-positive with the mono-plex Aptima CT (ACT) assay that has a different target (16S rRNA).[3] This Finnish CT new variant, now referred to as the FI-nvCT, escaped detection in the AC2 assay due to a mutation, C1515T, in the CT 23S rRNA gene target region.[3] Analysis of additional specimens discrepant in the AC2 and ACT showed that false-negative AC2 results were most likely to occur in specimens that had 20 to 84 relative light units (RLU; i.e. total RLU divided by 1000 as the Hologic Tigris and Panther report the RLU values) in the AC2 assay.[3] The C1515T mutation in the CT probe binding site results in lower RLUs and a subsequent false negative or equivocal result. Further analysis estimated the presence of the FI-nvCT in 6-9% of CT cases within three main AC2-using regions in Finland. The FI-nvCT was subsequently detected in Sweden,[4] Norway[5] and Denmark.[6]

To ensure detection of the FI-nvCT, and other similar diagnostic-escape CT variants, and to avoid false-negative or equivocal results, Hologic recommended ACT reflex testing of specimens which were interpreted as CT-negative or CT-equivocal (RLU ≥ 15), or NG-equivocal/-positive (if CT-negative/equivocal) according to the RLU value on the AC2.[7] Approximately 44% (1,684,028/3,863,336) of all CT tests carried out in England in 2018 used the Hologic AC2 assay,[8] therefore ACT reflex testing as recommended by Hologic[7] was performed throughout England. In addition, during June-August 2019 Public Health England (PHE) undertook a case finding exercise to establish if this variant or any others, were circulating to inform whether patient recall for retesting was required.[8 9] This initial exercise did not find any FI-nvCT, however, two new CT variants escaping AC2 assay detection were identified in England; one had a C1514T mutation and the other a G1523A mutation in the 23S rRNA gene target region.[8] The prevalence of AC2 diagnostic-escape nvCTs in England was therefore estimated to be <0.003%, so a patient recall was not required. The study continued until October 2019, of which we present the final results and from additional

analysis highlight the impact of NG positivity on the discordant AC2/ACT specimens, which appeared to cause some of the AC2 CT false negative results.

METHODS

From June to October 2019, PHE requested laboratories to submit weekly AC2 testing data, along with specimens with discrepant AC2/ACT CT results to PHE, Colindale as previously described.[8]

Specimens processed by PHE were extracted using the Roche MagNA Pure 96 instrument (Burgess Hill, UK) and subsequently screened for detectable CT DNA using a real-time (RT)-PCR that incorporates a CT target of a 88-bp region of the cryptic plasmid and an internal control target of human RNase P[10] as well as a 36-bp deletion of the chromosomal polymorphic membrane protein H gene (*pmpH*).[11] Specimens which were CT positive with a cycle threshold <38 were then subjected to partial CT 23S rRNA gene sequencing.[12]

Limited patient data was available with the submitted specimens; gender, age and site of infection. AC2 RLU values, ACT RLU values and CT/NG interpretation data were also requested. According to instructions from Hologic, the RLU values and the subsequent CT interpretation for the AC2 assay are as follows; 'CT-negative' 1-24 RLUs, 'CT-equivocal' 25-99 RLUs and 'CT-positive' ≥100 RLUs.[13] if both CT and NG are present in the specimen then the RLU values determining CT results are; 'CT-negative' 1-84 RLUs, 'CT-equivocal' 85-249 RLUs and 'CT-positive' ≥250 RLUs.[13] Thus, it is important to emphasize that in the dual kinetic assay method used in the AC2 assay, there are one flasher acridinium ester-labelled DNA probe for CT and one glower-labelled DNA probe for NG. These probes result in different light kinetics and if both CT and NG are present in a sample, an intermediate RLU signal is generated. The luminometer should be able to distinguish between the different RLU signals and determine if CT, NG, or both pathogens are present in a sample. Accordingly, only one RLU signal and value is reported despite that both CT and NG are detected, and these are only distinguished based on the light kinetics.[14] Interpretation data was not available for CT from the ACT, however it was assumed they were all CT-positive. All samples were taken during routine diagnostics and PHE has authority to perform health surveillance activity.

RESULTS

Patient characteristics

A total of 334 discordant AC2/ACT specimens, which had a positive CT result using the ACT, were collected from 6 June 2019 to 14 October 2019. Seventeen (5.1%) specimens were excluded as they were either sent in error, leaked in transit, received from outside of England or received after the end of the study. The included 317 discrepant specimens were collected from 315 patients; four specimens were received from two patients.

Gender and age were known for 306 submitted specimens, of which 53.3% (n=163) were female. The age range was 0 – 65 years, with a median and mode of 25 and 19 years, respectively. Females were younger than males with a median and mode age of 22 and 20 years, versus 30 and 21 years in males.

Site of infection was known for 304 specimens, the remaining 13 specimens had unknown site (n=5) or were pooled (n=8). The majority (64.1%, n=195) of specimens were urogenital (urine, cervical, vaginal or urethral), followed by rectal (25.3%, n=77), pharyngeal (9.2%, n=28) and eye specimens (1.3%, n=4).

Chlamydia trachomatis DNA detection and sequencing

The first 17 specimens were referred to the World Health Organization Collaborating Centre for Gonorrhoea and Other STIs at Örebro University, Sweden and the remaining 300 were processed at PHE.

Sequencing was performed directly on the 17 specimens sent to Örebro University; 5 had a wild-type CT 23S rRNA gene sequence in respect to the AC2 probe target region and 12 specimens had no sequencing result available, most likely due to low-levels of CT DNA present.

Of the 300 specimens investigated at PHE, 53.3% (n=160) were negative and 24.0% (n=72) were low positive in the in-house RT-PCR (cycle threshold ≥38), so no further analysis was performed. Thirty-two (10.7%) specimens had cycle thresholds <38 but subsequent sequencing was unsuccessful. Of those remaining 36 (12%) specimens with a successful CT 23S rRNA sequence, 32 included CT 23S rRNA wild-type and four specimens with variants (from three patients) were detected. Two variants have been previously described;[8] a variant with a CT 23S rRNA C1514T mutation was detected from two different urine specimens from one male heterosexual in his 20s. The other variant was from a female in her 20s with a vaginal specimen harbouring a CT 23S rRNA G1523A mutation. An additional specimen with the CT 23S rRNA C1514T variant was detected in urine from a male heterosexual in his 20s (Figure).

AC2 RLU analysis

Of the 300 specimens processed at PHE, both RLU and CT interpretive data was available for 79.7% (n=239) specimens. Forty specimens (13.3%) had only interpretative CT (negative/equivocal) AC2 data available, nine (3%) had just AC2 RLU data and 12 specimens (4%) had neither data submitted with the specimens. Additionally, AC2 NG interpretative result was available for 254 (84.7%) specimens processed at PHE, of which 58.7% (n=149) were NG negative, 40.6% (n=103) NG positive and 0.8% (n=2) NG equivocal.

AC2/ACT discordant, NG-negative specimens

Of AC2/ACT discordant specimens with a known NG-negative interpretation, AC2 RLU data was available for 144 specimens with a range of 12-97 RLUs. There was an equal proportion of specimens in the 'CT-negative' AC2 RLU range (1-24) and in the 'CT-equivocal' AC2 RLU range (25-99); 49.3% and 50.7%, respectively (Table 1). Additionally, in both ranges there was an equal proportion of specimens that were detected by the in-house CT RT-PCR (40.8% vs. 41.1%, Table 1). Four wild-type and one C1514T 23S rRNA gene sequence was detected in the 1-24 RLU group, and 6 wild-type and two variants (C1514T and G1523A) were detected in the 25-99 RLU group.

Table 1. AC2 RLU values of AC2/ACT discordant, NG-negative specimens

AC2 RLU range	No. of specimens	No. CT RT-PCR positive	% CT RT-PCR positive
1-24	71	29	40.8
25-99	73	30	41.1
Total	144	59	41.0

Abbreviations: AC2 - Aptima Combo 2 assay, RLU – relative light units, ACT - Aptima *Chlamydia trachomatis* assay, CT - *Chlamydia trachomatis*, RT-PCR – Real-time PCR

In respect to available AC2 CT interpretative criteria when the specimens were known to be NG negative, most (77.2%) were regarded as CT negative with a RLU range of 12-83 (Table 2). A higher

proportion of the CT equivocal samples were, as expected, positive on the in-house CT RT-PCR assay (51.5%, Table 2).

Table 2. AC2 CT interpretative criteria of AC2/ACT discordant, NG-negative specimens

AC2 CT interpretation	No. of specimens	No. CT RT-PCR positive	% CT RT-PCR positive	RLU range
Negative	112	41	36.6	12-83
Equivocal	33	17	51.5	25-99
Total	145	58	40.0	

Abbreviations: AC2 - Aptima Combo 2 assay, CT - *Chlamydia trachomatis*, ACT - Aptima *Chlamydia trachomatis* assay, NG – *Neisseria gonorrhoeae*, RT-PCR – Real-time PCR, RLU – relative light units

AC2/ACT discordant, NG-positive specimens

Of the 103 NG-positive specimens processed at PHE, the CT results on the in-house CT RT-PCR were negative (48.5%, n=50), low positive (cycle threshold ≥38) (27.2%, n=28), or positive (24.3, n=25). Sequencing was attempted on 25 specimens with cycle thresholds <38, of which 13 were successfully sequenced and were all wild-type.

AC2 CT interpretation was available for 100 NG-positive specimens, of which 92 (92%) had a CT-negative interpretation and 49 (53.3%) of these had detectable CT DNA in the in-house CT RT-PCR. The remaining eight (8%) had a CT equivocal result, of which six were negative on the in-house CT RT-PCR.. So, of the AC2/ACT discordant specimens with known NG interpretation; 36.6% of NG-negative/CT-negative AC2 specimens had detectable CT DNA in the in-house RT-PCR vs. 53.3% of NG-positive/CT-negative specimens. As previously stated, it is not possible to distinguish between the individual CT and NG RLUs in the AC2 assay as both RLU outputs are combined.

AC2 RLU values were available for 83 (80.6%) of the 103 NG-positive specimens, of which 81 had available AC2 interpretation; 74 CT-negative and 7 CT-equivocal. The most common RLU range was between 1251-1500 (45.8%, n=38), majority of which were positive with the in-house CT PCR assay (73.7%) (Table 3). Seventy specimens had RLUs >250 and were therefore within the AC2 CT-positive range when an NG signal is also present; 67 were interpreted as CT-negative, 1 as CT-equivocal and interpretation was not known for two specimens. Overall, 60% (42/70) of specimens with RLUs >250 had detectable CT DNA in the in-house CT RT-PCR. Eight specimens had no NG status with AC2 RLUs >250 (range 702-2651); all but one of these were positive on the in-house CT PCR and of the two specimens with enough DNA for 23S rRNA gene sequencing, both were wild-type.

Table 3. RLU values of NG positive and CT negative/equivocal specimens on the AC2 assay

AC2 RLU range	No. of specimens	No. CT RT-PCR positive	% CT RT-PCR positive
15-250	13	1	7.7
251-1000	6	2	33.3
1001-1250	12	3	25.0
1251-1500	38	28	73.7
1501-1750	12	8	66.7
>1750	2	1	50.0
Total	83	43	51.8

Abbreviations: RLU – relative light units, NG – *Neisseria gonorrhoeae*, CT - *Chlamydia trachomatis*, AC2 - Aptima Combo 2 assay, RT-PCR – Real-time PCR

DISCUSSION

Four specimens from three patients with nvCTs evading detection in the AC2 were found in England from June to October 2019, resulting in a prevalence of <0.003%. The FI-nvCT, with a 23S rRNA C1515T mutation, was not found, but instead three specimens harbouring nvCT-C1514T and one with nvCT-G1523A were detected. The nvCT-G1523A was found to be the most dominant (95%) nvCT in Denmark, resulting in diagnostic escape using the AC2, along with the nvCT-C1514T (2.5%) and the FI-nvCT (2.5%).[6] The nvCT-G1523A and nvCT-C1514T were also detected in one specimen each in Norway, whereas the FI-nvCT was much more widespread; present in 84% of discordant AC2/ACT specimens.[5] In England, there has been no decrease in CT positivity rate since 2018,[2] further confirming our findings that no widespread dissemination of AC2 diagnostic-escape CT variants has occurred.

Depending upon specimen type and combining symptomatic and asymptomatic patients, the AC2 sensitivity for CT ranges from 94.2-97.9%,[13] whereas the ACT ranges from 94.3-98.3.[15] This difference, albeit small, may explain some of the discrepancies not due to CT variants between the AC2 and ACT, but certainly not all. It should also be noted that if we were to select specimens based on a CT-negative/-equivocal ACT result, then with a large enough sample size, we could likely expect a small number of these specimens to be CT-positive in the AC2 assay. We further investigated whether NG positivity was impacting on the sensitivity of CT detection in the AC2. As described previously, if both CT and NG are present then the RLU values determining a CT-positive result (≥250) are higher than if just CT is present (≥100).[13] However, despite that only one RLU value ('intermediate RLU signal') is reported for specimens with both CT and NG the luminometer and subsequent interpretative algorithm used in the AC2 assay should be able to distinguish between the CT and NG RLU signals based on their different light kinetics.[14] Interestingly, we found that 84.3% (70/83) of specimens which were CT-positive in the ACT but were NG-positive/CT-negative in the AC2 had >250 RLUs and most (45.8%) had RLUs between 1251-1500. A positive CT RT-PCR result was established in 73.7% of AC2/ACT-discordant specimens within the 1251-1500 RLU range. This is concerning, as the in-house CT RT-PCR assay is generally less sensitive than the AC2 assay and we fail

to confirm approximately 10% of specimens positive on commercial assays (unpublished data). So if we consider the RLUs, our data suggests that 84.3% of NG-positive specimens in this sample set of discordant AC2/ACT specimens, had a missed CT-positive result when the AC2 was used alone. In addition, 36.6% of NG-negative/CT-negative AC2 specimens had detectable CT DNA in the in-house RT-PCR vs. 53.3% of NG-positive/CT-negative specimens, which further suggests that a proportion of the CT wild type AC2/ACT discordant results are due to NG positivity and the associated NG RLU signal, resulting in decreased CT sensitivity. A number of patients with gonorrhoea may therefore receive false-negative AC2 CT results and accordingly concurrent CT infections will be missed, which is especially concerning now when gonorrhoea treatment in England no longer includes empirical 1 g azithromycin[16] which would have cleared most of the undetected CT cases. Fortunately, within England, many patients return for a gonorrhoea test-of-cure (ToC), so any on-going CT infections should be subsequently detected. However, this time delay, along with no ToC in some settings, increases the chances of further sequelae associated with CT infections as well as further transmission. Interestingly, the study in Denmark is the only other country to mention dual NG positivity, which was quite high at 6% of the 150 possible cases of AC2 diagnostic-escape nvCTs.[6] It should be noted however that some patients would have multi-site CT infections which would subsequently result in appropriate treatment, and a limitation of this study is no access to data on multi-site infections in the patients within our dataset.

The presence of diagnostic-escape mutants when a single-target NAAT is used for molecular diagnostics is not a new phenomenon, as so clearly illustrated by the Swedish nvCT. This nvCT harbours a 377-bp deletion within its cryptic plasmid enabling it to escape detection in both the Roche and Abbott CT assays used at that time.[17] The logistical and financial advantages to the laboratory of dual CT/NG testing as well as not testing equivocal samples on an alternate assay are clear. However the risk of using a single diagnostic assay that may offer a clear selective advantage to an escape mutant of that assay should be appropriately assessed when choosing the diagnostic pathway. Within England, a number of different CT assays/platforms are in use and many (although not all) laboratories routinely retest equivocal samples on alternative assays. This make-up of different platforms and correct equivocal retesting, may at least partly explain why no AC2 diagnostic-escape mutants managed to distribute more widely. We should also highlight that with robust CT-equivocal retesting practices in place then many discrepancies would hopefully be resolved, and if not, a second sample requested. In any setting, laboratory and epidemiological data should be monitored carefully to identify any unusual trends and appropriate quality assurance, especially external quality assessments (EQAs) should be robust enough to detect diagnostic-escape variants.

To date there does not seem to be any patient characteristic associated with those harbouring these diagnostic-escape CT variants. Whole-genome sequencing should give us more insight into whether there is any genetic predisposition that enables the variants to transmit more successfully in the population and it will be interesting to understand their phylogenetic relationship to other sequenced CT strains. A study to investigate the European-wide prevalence of the FI-nvCT is currently underway using an Aptima-format FI-nvCT-specific assay on the Panther platform (Hologic).[18] This assay will not however detect additional diagnostic-escape nvCTs, so discordant specimens will still need to be sequenced. Hologic has recently developed an updated version of the AC2 assay, which includes an additional CT 23S rRNA target that should provide detection coverage for the nvCTs identified to date and this revised AC2 assay has replaced the previously used assay in Europe.[19] Ideally all diagnostic platforms should employ more than one diagnostic target to prevent mutants from emerging. Further work is required to assess the true impact NG positivity has

on specimens which co-harbour CT and are tested on the AC2, as well as considering the impact of sensitivity on other multi-plex platforms.

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COMPETING INTERESTS

None declared

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CONTRIBUTORS

MJC, GSD, HF, JS, DJR, PM, PH, NOG and KF were involved in the design of the study. MJC, MU, RH, MAF, MD, JM were responsible for the laboratory investigations. MJC and GSD analysed the data. MJC, GSD, HF, JS, MU and KF drafted the paper, which was commented on and approved by all the authors. MJC is responsible for the overall content of the manuscript.

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LEGEND

Figure 1. Flow diagram representing number of discordant *Chlamydia trachomatis* AC2/ACT specimens received and sequencing results