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A *Chlamydia trachomatis* 23S rRNA G1523A variant escaping detection in the Aptima Combo 2 assay (Hologic) was widespread across Denmark in July-September 2019

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SUMMARY

Hadad R, Jensen JS, Westh H, Grønbæk I, Schwartz LJ, Nielsen L, Vang TM, Nielsen R, Weinreich LS, Skov MN, Olsen M, Møller JK, Kolmos B, Unemo M, Hoffmann S. A *Chlamydia trachomatis* 23S rRNA G1523A variant escaping detection in the Aptima Combo 2 assay (Hologic) was widespread across Denmark in July-September 2019

Chlamydia trachomatis infection is the most common bacterial sexually transmitted infection globally, and nucleic acid amplification tests (NAATs) are recommended for highly sensitive and specific diagnosis. In early 2019, the Finnish new variant of *Chlamydia trachomatis* (FI-nvCT) was identified. The FI-nvCT has a C1515T mutation in the 23S rRNA gene, making it escaping detection in the Aptima Combo 2 (AC2; Hologic) NAAT, and the FI-nvCT has been subsequently reported in Sweden and Norway. In the present study, we investigated the presence of the FI-nvCT

and other AC2 diagnostic-escape CT mutants in July-September 2019 in Denmark. The FI-nvCT was present but rare in Denmark. However, another AC2 diagnostic-escape CT mutant (with a 23S rRNA G1523A mutation) was found to be widespread across Denmark, accounting for 95% (76/80) of AC2 diagnostic-escape nvCT samples from five Danish CT-diagnostic laboratories. This nvCT-G1523A has previously only been detected in one single sample in the United Kingdom and Norway, respectively. It is vital to monitor the continued stability of the NAAT targets in local, national and international settings and monitor as well as appropriately analyse incidence, unexplained shifts in diagnostics rates, and/or annual collections of samples diagnosed as negative/equivocal using NAATs with different target(s). Furthermore, diagnostic CT NAATs with dual target sequences are crucial and fortunately, an updated Hologic AC2 assay including one additional target sequence is in advanced development.

Key words: *Chlamydia trachomatis*, FI-nvCT, New variant, Aptima Combo 2 assay, 23S rRNA, C1515T, G1523A, Denmark

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INTRODUCTION

Chlamydia trachomatis infection is the most common bacterial sexually transmitted infection globally (1), and nucleic acid amplification tests (NAATs) are recommended for highly sensitive and specific diagnosis (2,3). In early 2019, the Finnish new variant of *Chlamydia trachomatis* (FI-nvCT) was identified (4,5). The FI-nvCT has a C1515T mutation in the CT 23S rRNA gene, making it escaping detection in the Aptima Combo 2 (AC2; Hologic Inc., San Diego, CA, USA) NAAT that targets CT 23S rRNA (4-9). The FI-nvCT has been subsequently reported in Sweden (6) and Norway (7). Two additional very rare AC2 diagnostic-escape nvCTs were described in single specimens in Norway (7) and the United Kingdom (8). As the FI-nvCT, these two rare diagnostic-escape nvCTs also had a single nucleotide polymorphism (SNP) in the CT probe detection 23S rRNA sequence of AC2, that is, 23S rRNA C1514T or G1523A (7,8). The AC2 detection is measured in relative light units (RLUs) and the 23S rRNA mutations in these nvCTs result in low CT RLU values, mostly 15-99, that are interpreted as negative or equivocal in AC2 (4-9). The Aptima *C. trachomatis* NAAT (ACT; Hologic Inc.), which targets CT 16S rRNA, detects all these diagnostic-escape nvCTs, and reflex testing of AC2 samples with CT RLU values of 15-99 using ACT was implemented in May-June 2019 in many European countries (9,10).

Considering the spread of particularly the FI-nvCT in Finland (4,5), Sweden (6) and Norway (7); the close geographic proximity and frequent travelling between the Nordic countries; and the rapid risk assessment published by the European Centre for Disease Prevention and Control (ECDC) on 17 June 2019 (10), it was urgent to appropriately investigate if the FI-nvCT and/or additional AC2 diagnostic-escape nvCTs were spreading also in Denmark. In Denmark, with over 5.8 million inhabitants, the majority (62% in 2019) of CT samples are analysed using AC2 on Panther instruments (Hologic Inc.).

MATERIALS AND METHODS

The numbers of mandatorily reported CT cases in Denmark in 2018 and 2019 were obtained at the Statens Serum Institut, Copenhagen, Denmark. Consecutive clinical AC2 specimens obtained at

five CT-diagnostic clinical microbiology laboratories across Denmark, i.e. in Hvidovre, Herlev, Odense, Vejle and Aalborg (all AC2 laboratories in Denmark and together responsible for 62% of the CT diagnostic testing in 2019), from 1 July to 30 September 2019 were assessed. Samples with AC2 CT RLU values of 15-97 were reflex tested using the ACT. AC2 and ACT were performed in accordance with the instructions from the manufacturer (Hologic Inc., San Diego, CA, USA). Confirmation of AC2 diagnostic-escape nvCTs (one sample per patient) was performed by sequencing of the V2 region of the 23S rRNA gene at the WHO Collaborating Centre for Gonorrhoea and other STIs, Sweden, as described previously (5).

RESULTS

Diagnostics and incidence of *Chlamydia trachomatis* in Denmark

The number of CT cases in Denmark in 2019 compared to 2018 was similar for each month until ACT reflex testing of AC2 samples with CT RLU values of 15-99, in accordance with the ECDC rapid risk assessment (10), was initiated in mid- to late-June 2019 (FIG. 1). After ACT reflex testing started, an increase in the number of CT positive samples was seen compared to the corresponding months in 2018, i.e. an increase by 461 CT positive cases in July 2019, 486 cases in August and 364 cases in September (FIG. 1). However, the significance of these findings is not clear due to the fluctuations of number of positive samples, particularly in different months and seasons, such as summer, and over the years.

Possible cases of AC2 diagnostic-escape nvCTs in Denmark

Consecutive clinical AC2 specimens obtained at five CT-diagnostic clinical microbiology laboratories across Denmark, i.e. in Hvidovre, Herlev, Odense, Vejle and Aalborg, from 1 July to 30 September 2019 were assessed. During these months, 66552 samples (Hvidovre: 34737, Herlev: 12717, Odense: 6850, Vejle: 4184, Aalborg: 8064) were examined and 6117 samples (9.2%) were positive for CT in AC2 (Hvidovre: 2726, Herlev: 1210, Odense: 687, Vejle: 504, Aalborg: 990). In total, 707 (1.1%) samples with AC2 CT RLU values of 15-97 were reflex tested using the ACT. Of these samples (n=707), 204 (28.9%) were positive in ACT (RLUs 94-8891) (FIG. 2) and accordingly possible cases of AC2 diagnostic-escape nvCTs (Hvidovre: 107, Herlev: 35, Odense: 37, Vejle: 6, Aalborg: 19).

Confirmed cases of AC2 diagnostic-escape nvCTs in Denmark

In total, samples from 150 of the above mentioned possible cases of AC2 diagnostic-escape nvCTs (Hvidovre: 85, Herlev: 20, Odense: 28, Vejle: 4, Aalborg: 13) were available for sequencing (the vast majority from July-August 2019). Eighty (53.3%) of these samples contained an nvCT with a SNP in the AC2 CT probe detection sequence of the 23S rRNA gene (12 (8.0%) contained CT wild type and 58 (38.7%) had too low bacterial load for sequencing). Notably, nine (6%) of the 150 possible cases of AC2 diagnostic-escape nvCTs were also positive for *Neisseria gonorrhoeae.* Of the 80 AC2 diagnostic-escape nvCT samples, 95% (n=76) contained nvCT with the G1523A SNP in the 23S rRNA gene (RLUs: 16-86), 2.5% (n=2) 23S rRNA C1514T (RLUs: 17 and 25), and 2.5% (n=2) 23S rRNA C1515T (RLUs: 21 and 26), that is, the FI-nvCT. The AC2 diagnostic-escape nvCT G1523A was widely spread across Denmark and detected in Hvidovre (n=43), Herlev (n=15), Odense (n=7), Vejle (n=4), and Aalborg (n=7). The nvCT C1514T and the FI-nvCT was found in one sample each in Hvidovre and Odense, respectively. Gender distribution was 97 females, 50 males and three unknown. No information regarding the age, sexual orientation or recent travel of the patients was available for the present study.

DISCUSSION

In the present study, we identified in Denmark all three previously described AC2 diagnostic-escape nvCTs causing false-negative or equivocal AC2 results (4-8). The FI-nvCT was initially published in late-May 2019 in Finland (4) and the prevalence of FI-nvCT appeared to be relatively high in particular in South-West Finland (4,5). FI-nvCT was subsequently reported to be present in Sweden (6) and widely spread in Norway (7). Two additional AC2 diagnostic-escape nvCTs, with 23S rRNA C1514T or G1523A gene mutation, were also found in one sample each in Norway (7) and the United Kingdom (8), respectively. Already in mid-June 2019, recommendations to ACT reflex test all specimens with AC2 CT RLU values of 15-99 (possible AC2 diagnostic-escape nvCT) were published (9,10). Most worryingly, in Denmark 0.3% of all samples and 3.3% of all CT positive samples examined with AC2 from July to September 2019 were possible cases of an AC2 diagnostic-escape nvCT. Furthermore, 95% (n=76) of the confirmed AC2 diagnostic-escape nvCT samples, corresponding to 0.2% of all examined AC2 samples or 1.9% of all true AC2 CT positive samples (likely underestimated because 39% of samples could not be sequenced) from July to August 2019, contained the 23S rRNA G1523A nvCT. Surprisingly, this widely spread nvCT-1523A has still not been detected in the neighbouring country Sweden (6,unpublished) and only two samples in Denmark contained the FI-nvCT despite that this variant is widely spread in

Finland (4,5) and Norway (7), and not exceedingly rare in Sweden (6,unpublished). This indicates that the spread of CT strains between Denmark and the additional Nordic countries is more limited than anticipated, which was also indicated as one of the contributing factors more than 10 years ago when the Swedish nvCT was widely spreading in Sweden but very rare in Denmark (11-13). An Aptima-based FI-nvCT specific assay has now been developed and will be used in a pan-European surveillance study (9). Based on the present results from Denmark, it is important to not only use the Aptima-based FI-nvCT specific assay in the pan-European study, but additionally perform 23S rRNA gene sequencing on AC2/ACT discordant samples from countries where other diagnostic-escape CT variants are widespread, such as in Denmark, where the proportion of AC2/ACT discordant samples negative in the FI-nvCT specific assay is high, or where the distribution of diagnostic-escape CT variants or AC2/ACT discordant samples is completely unknown. The results of the present study show that the ACT reflex testing currently recommended for AC2 samples with CT RLUs of 15-99 should be continuously performed (9,10), and the CT RLU values caused by the nvCT-1523A monitored, i.e. to ensure that the RLU≥15 cut-off captures also all nvCT-1523A samples. Ultimately, a validated and quality assured revised version of AC2 that detects all the diagnostic-escape CT variants is very important, and such NAAT is fortunately in advanced development (14).

Whole genome sequencing of the different AC2 diagnostic-escape nvCTs is in progress and this work will contribute to the understanding on how CT is evolving including the emergence, clonality, spread, and fitness advantages/disadvantages of these nvCTs. The presence of several different reported AC2 diagnostic-escape nvCTs illustrates the vulnerability of using a single target for detection of an infectious agent with nucleic acid amplification tests (NAATs). This was seen already with the experience of the Swedish nvCT initially detected in 2006 in Sweden where a deletion in the cryptic plasmid that included the target sequence for the Roche and Abbott CT NAATs led to diagnostic escape (11-13). Previous genomic, phenotypic and epidemiological studies on the Swedish nvCT did not reveal any altered biological fitness and instead showed that the rapid spread was most likely mainly due to the advantage of a strong diagnostic selection, introduction in a high-frequency transmitting population, and the lack of subsequent tracing and treatment of sexual contacts (11).

In conclusion, it is vital to monitor the continued stability of the NAAT targets in local, national and international settings and monitor as well as appropriately analyse incidence, unexplained shifts in diagnostics rates, and/or annual collections of samples diagnosed as

negative/equivocal using NAATs with different target(s). Furthermore, diagnostic CT NAATs with dual target sequences are crucial and fortunately, an updated Hologic AC2 assay including one additional target sequence is in advanced development (14). Finally, international and national surveillance programmes capturing NAAT diagnostic-escape variants for CT and other infectious agents are imperative.

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Figure legends

FIG 1. Number of *Chlamydia trachomatis* (CT) cases reported in Denmark from January to September 2018 and 2019, respectively. Reflex testing using the Aptima CT assay started in mid-June 2019, which is indicated by a black line.

FIG 2. Number of samples tested positive for *Chlamydia trachomatis* (CT) in Aptima Combo 2 assay (AC2; black), samples that qualified for reflex testing by Aptima CT assay (ACT; dark grey), and samples that tested positive in ACT (light grey). The number of samples examined in AC2 was 18499 in July, 24555 in August, and 23498 in September.

Figure 1

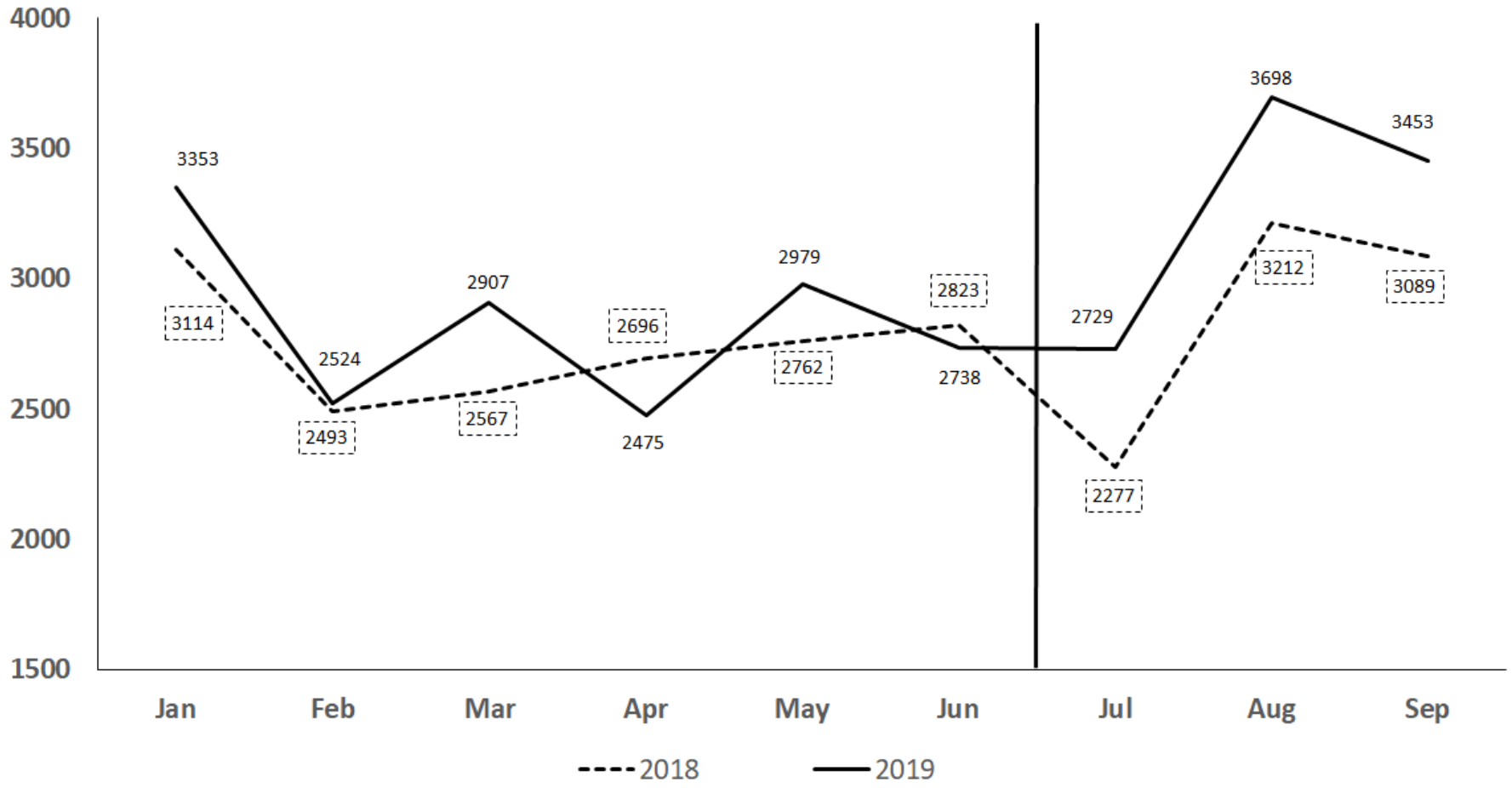

Figure 2