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Pathogenic Interplay Between *Chlamydia trachomatis* and *Neisseria gonorrhoeae* that Influences Management and Control Efforts—More Questions than Answers?

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Abstract

Purpose of Review To emphasize key gaps in knowledge impacting efforts to control single infection and co-infections with *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG), the most common bacterial sexually transmitted infections (STIs) worldwide.

Recent Findings Clinical and epidemiological studies describe gaps in understanding about female rectal CT infection, screening effectiveness, pelvic inflammatory disease, and influence of the microbiome. For NG, gaps in knowledge include factors increasing incidence in men who have sex with men, correlations between treatment and antibiotic resistance, the role of pharyngeal infection, and microbiome influence. CT/NG co-infections are poorly understood, and adequate models to explore pathophysiological consequences of co-infection urgently needed. The sole existing CT/NG co-infection mouse model showed that CT/NG interactions in vivo modulate host response and NG load/shedding—encouraging further consideration of this model and potential alternatives.

Summary We stress key challenges in controlling these important STIs. Appropriate, quality-assured animal models are essential to improve understanding of the pathogenic interplay in CT/NG co-infections.

Keywords *Chlamydia trachomatis* · *Neisseria gonorrhoeae* · Co-infection · Mouse model

This article is part of the Topical Collection on *Influences of Hormones and Other Microorganisms on Genital Tract Pathogens*

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Introduction

Chlamydia trachomatis (CT) and *Neisseria gonorrhoeae* (NG) cause the most commonly reported bacterial sexually transmitted infections (STIs) in humans worldwide, with an estimated 127 million new cases of chlamydia and 87 million of gonorrhea among adults in 2016, according to the World Health Organization (WHO) [1]. The primary site of infection for both bacteria is the columnar epithelium of the endocervix in women and the urethra in men; both also infect the rectum, pharynx, and conjunctiva and can be transmitted from an infected mother during pregnancy or delivery [2]. CT/NG co-infections are more frequent than would be expected by chance, and mathematical modeling studies suggest that biological interactions between the two increase susceptibility and/or transmissibility [3•]. In this review, we highlight some gaps in knowledge that challenge the effective management and control of these STIs. We focus on clinical or epidemiological studies and data from animal models published from 2014 to 2019, for both single infections and co-infections.

The Two Most Commonly Reported Bacterial STIs: Chlamydia and Gonorrhea

Chlamydia

The prevalence of CT, measured in surveys in the general population in high-income countries, is ~3–4% in young adults [4, 5]. Reported numbers of CT infections are increasing and/or high in many countries. In the United States of America (USA), more than 1.7 million cases of CT were reported to the Centers for Disease Control and Prevention (CDC) in 2017, nearly 7% more than in 2016 [6]. The highest rate of diagnoses is among women aged 15–24 years, reflecting recommendations for yearly testing in this group [6]. The majority of women and up to half of men infected with CT are asymptomatic, so infections often remain undiagnosed and unreported [2]. In women, cervical CT infection can ascend from the endocervix into the endometrium and fallopian tubes, resulting in pelvic inflammatory disease (PID), which can then cause tubal factor infertility (TFI) and ectopic pregnancy (EP). CT is associated with clinical PID in around 20% of cases; other STI agents, including NG, are also implicated [7]. In men, urethritis is the most frequent STI syndrome, with CT causing 15–40% of cases. Among MSM attending clinics in high-income countries, about 10% have CT detected in the rectum [8–11].

Doxycycline (a tetracycline) and azithromycin (a macrolide) are treatments of choice for CT infections [12–14]. Stable homotypic antibiotic resistance in CT in clinical specimens has not been reported to date [15], in stark contrast to multidrug-resistant NG strains circulating and increasing worldwide [16]. However, repeated detection of CT after treatment can be common, resulting from failure to cure the infection, poor treatment compliance, test of cure performed too early and/or detection of DNA/RNA from non-viable organisms, or re-infection from an untreated or new infected partner [17, 18].

Chlamydia—Critical Gaps in Knowledge

Management and control of CT infection remain continuously challenging [4, 19]. Reasons are likely manifold, but there is prominent discussion about the potential importance of *rectal CT infection in women* in particular [20, 21]. Asymptomatic rectal CT has been hypothesized to be a reservoir of untreated infection, hindering chlamydia control [22]. Information is limited, however, about the clinical relevance of CT in anal canal swabs in females, when detected by a nucleic acid amplification test (NAAT) that do not distinguish between viable bacteria and CT DNA/RNA from non-viable bacteria. NAATs are often used as the sole diagnostic tool because of their high sensitivity [23], but CT-positive results could come from non-viable nucleic acid transferred from genital secretions on

fingers or toilet paper. A systematic review of studies of anal canal specimens analyzed by NAAT reported an average of 6% (95% CI 3–9%) CT-positive [23]. The finding that detection of CT in anal canal swabs is as common in women who report anal intercourse as those who do not [20, 23] is more consistent with detection of nucleic acid than with infection. In mouse models of CT infection and treatment, rectal CT is more refractory to antibiotic treatment particularly azithromycin than genital CT [24]. In a systematic review of observational studies in humans, doxycycline eradicated CT from anal canal specimens more often than azithromycin, but very few women were included in the studies [25]. Autoinfection from the female rectum to the cervix has also been suggested as a reason for presumed treatment failure, with infection arising through oral ingestion and persisting in the lower gastrointestinal tract [24]. A 2018 study of human gastrointestinal biopsy tissue found CT in the appendix of one female specimen, but this was compatible with sexually transmitted ascending infection [26], and there is no empirical evidence of autoinfection. A prospective cohort study in the Netherlands (FemCure), which will collect specimens for routine NAATs, PCR for detection of CT viability, culture, and genotyping, should help shed light on the potential role of rectal CT in women [27].

There are still *gaps in evidence about the effectiveness of widespread screening for chlamydia* to prevent reproductive tract complications and reduce infection prevalence [4]. Public health agencies in many high-income countries recommend screening all sexually active women or women and men under age 25–29 [6, 28, 29]. Definitive evidence about the effects of screening on CT transmission is absent because there were no randomized controlled trials (RCTs) before screening was first recommended in the 1980s and 1990s in Sweden and the USA, respectively [30]. Repeated estimates of CT prevalence in the general population have remained stable during the twenty-first century in adults in Great Britain [31] and the USA [32]. Two trials of strategies to increase general screening uptake in women and men aged 15–29 years did not find reductions in estimated CT prevalence [33, 34]. A study of CT screening uptake in young adults in Britain indicated that at least 25% of women and approximately 50% of men with CT risk factors had not been tested for CT in the last year [35]. Targeting women at highest risk for complications has therefore been suggested as a more effective strategy [19]. There is stronger evidence for an effect of screening for CT in the prevention of PID [4], but *identification of women at highest risk of progression to PID* is challenging [36]. Notably, a correlation between blood transcriptional profiles of CT- and/or NG-infected and uninfected women and clinical outcome was recently demonstrated [37], showing progress toward identifying those at risk for damaging long-term sequelae.

The interaction between STIs and the *vaginal microbiome* has gained attention as a potential factor for infection acquisition, transmission, and progression. Maintenance of a lactobacilli-dominant, inflammation-free environment could advance prevention of STIs and their adverse outcomes. A systematic review and meta-analysis, published in 2019, indicated that a vaginal microbiota dominated by *Lactobacillus crispatus*, *L. iners*, *L. gasseri*, and/or *L. jensenii* might have a role in protection against CT infection [38]. In line with these findings, a study that tested stored samples collected for vaginitis diagnosis found that those with bacterial vaginosis, either with or without concomitant *Candida* species infection, according to the BD MAX Vaginal Panel test, had an approximately 25% STI positivity rate, while those without bacterial vaginosis had an approximately 8% STI positivity rate [39]. This research is in its infancy and vaginal health is seldom routinely evaluated clinically. Thus, the impact of the vaginal microflora on CT (and NG) infection remains largely unclear [40]. However, microbiota-mediated defense against pathogenic intestinal bacterial infection has received considerably more attention (as reviewed in [41]) and might inform our future understanding of the potential protective role of the vaginal microbiome.

Gonorrhea

Though the prevalence of NG in the general population in high-income countries (<0.5%) is much lower than that of CT [42], reports of NG diagnoses are increasing faster than those for CT. In the USA, in 2017, over 555,000 cases of NG were reported to CDC, a rate increase of almost 19% from the previous year and approximately 75% since 2009 [6]. Rates of reported NG were higher in men than in women, a finding attributable, in part, to the higher proportion of symptomatic infection (urethritis) in men and the high prevalence of NG in MSM [6]. In the European Union/European Economic Area (EU/EEA), the number of NG cases has increased by more than 200% since 2008, with the highest number of cases in the UK, France, the Netherlands, and Spain [43]. In this decade, substantial increases in NG cases occurred among MSM as well as among heterosexual men and women [43]. As with CT, untreated cervical NG infections may progress to the upper reproductive tract, contributing to PID, TFI, EP, and chronic pelvic pain [44].

In contrast to CT, NG antimicrobial resistance is widespread, developing rapidly with each successive treatment regimen [19]. Dual ceftriaxone/azithromycin therapy, a treatment regimen that should also eradicate concurrent CT infection, is the recommended regimen for empirical treatment in many countries [19]. However, ceftriaxone susceptibility is decreasing in European countries not yet reporting overt ceftriaxone resistance [16, 45] and high-level azithromycin-resistant strains have been spreading in some settings [46].

Alarming, with the emergence of confirmed ceftriaxone-resistant NG strains [47], untreatable NG might be anticipated in the near future [47–49].

Gonorrhea—Critical Gaps in Knowledge

Antiretroviral drugs, given as treatment for HIV infection and as pre-exposure prophylaxis (PrEP) to prevent acquisition of HIV, have been associated with increases in NG in MSM [50]. If drugs, rather than condoms, are the main method that MSM use to prevent HIV, *HIV and STI prevention have been decoupled*. The resultant reduced condom use and increases in other risky sexual practices will result in increased transmission of STIs, including NG, CT, syphilis, and *Mycoplasma genitalium* [19, 51, 52]. Sexual network analysis might be a useful tool in determining factors important in the spread and prevalence of STIs [53]. Frequent screening for STIs for MSM on PrEP is recommended, but a systematic review of observational studies, published in 2018, found no evidence of reductions in test positivity for either NG or CT [54], indicating that further study of STI screening efficacy to prevent STIs in MSM on PrEP is needed.

The emergence of highly antimicrobial-resistant NG is one of the most pressing public health concerns for STI control. Understanding the *factors influencing selection, spread, and fitness of antimicrobial resistance in NG is critical* to inform our continued efforts to curtail the emergence and global spread of multidrug-resistant, extensively drug-resistant, and potentially untreatable NG. Mathematical modeling indicates that the spread of antimicrobial-resistant NG in some settings may be explained by higher treatment rates, rather than differences in numbers of sexual partners, and is more common in MSM than in heterosexual men [55]. NG antimicrobial resistance, or decreased susceptibility, is also positively correlated with population-level antibiotic consumption rate [56], a finding that has been shown for other bacteria, such as *Staphylococcus* and enteric bacteria [57]. It has been postulated that “bystander selection” by exposure to antibiotics other than those specifically targeting NG infection may play a role in the emergence of antimicrobial-resistant NG, in addition to the “direct selection” of NG-therapeutic antibiotics. This has important implications for public health policy dependent on the degree to which both kinds of selection contribute to NG antimicrobial resistance [58]. Additionally, the prevalence of antimicrobial-resistant NG increases with age and is lower in some black minority ethnic groups than the white population in high-income groups, indicating that risk factors for antimicrobial-resistant NG infection are distinct from those for NG infection in general [59].

There is increasing interest in extra-genital NG infection sites, particularly *the pharynx, as an NG transmission source*. Pharyngeal NG is largely asymptomatic, often undetected and frequently exposed to anti-NG antibiotics at levels suboptimal

for clearance [60]. About 6% of MSM have pharyngeal NG [8, 61], even in the absence of NG positivity at the rectal and urogenital sites, so lack of pharyngeal NG testing will result in missed infections in MSM [62]. A 2019 study from a single STD clinic in the USA found NAAT-detected NG in about 3% of heterosexual women and men reporting extra-genital sexual activity, suggesting a potentially important role in heterosexual NG transmission [63]. In a study of more than 2200 patients with pharyngeal NG in the Netherlands, almost 90% of patients remained infected in the time (median 10 days) between original sampling for NG diagnosis and subsequent return for therapy [64]. Studies among MSM in Australia confirm the role of pharyngeal NG as a likely source of urethral NG [65] and suggest a role for tongue kissing/saliva in NG transmission from pharynx to pharynx [61, 62, 65]. Considering the correlation between pharyngeal carriage with NG and antimicrobial resistance [59], additional study of pharyngeal NG may be important to understand the development of NG antibiotic resistance [66].

Chlamydia and Gonorrhea Co-infection—Potentially Important Interactions

CT/NG co-infection is common, with around 10–40% of those with NG infection being concomitantly CT infected [67]. In most cases, the proportion of people with NG who have CT co-infection is higher than the proportion of people with CT who have NG [68], most likely reflecting higher CT prevalence. Mathematical modeling suggests that co-infection occurs too frequently to be random [3•], but the *impact of co-infection on CT and NG epidemiology and pathogenesis is poorly understood*. Pathogen-pathogen and pathogen-host interactions specific to co-infection may also affect interpretation of studies about transmission, re-infection, treatment failure, pathogenic immune response, and, importantly, vaccine development [69]—which may ultimately impact treatment and vaccine efficacy. There are substantial gaps in our knowledge of *factors associated with prevalence of co-infection and the role co-infection may play in acquisition, pathogenesis, bacterial load and transmission, and disease course and severity* for these two important bacterial STIs. Although many studies have examined different aspects of these issues, the reported findings are often contradictory.

Factors associated with CT/NG co-infection vary across studies/populations and include sex, age, sex work, and drug/alcohol use [70–72]. MSM have been reported as more likely to harbor co-infections than heterosexual males, with the co-infection rate increasing with age in MSM, but decreasing with age in heterosexual men [73], while others have found similar co-infection rates for MSM and heterosexual men [74]. However, MSM have higher overall infection rates

than heterosexual men, which may impact such analyses. Co-infection has, in most cases, been linked with increased risk of re-infections. Co-infection increases risk of (i) re-infection with CT or NG when retested between 6 weeks and 6 months [70], (ii) NG re-infection in a high NG prevalence population [71], and (iii) CT re-infection in women [75]. However, a study that collected daily samples for 28 days after antibiotic treatment, evaluating 23 patients with NG/CT co-infection for CT clearance, noted 100% clearance with no re-infections [17].

CT/NG co-infection might increase NG transmission compared with single infection, owing to increased bacterial load, but not contribute to increased symptom severity [76•]. In a study of women aged 14–17 who collected 12 weekly self-taken vaginal swabs twice per year, 16 women acquired NG infection, demonstrating variable bacterial load over the ensuing 11 weeks, co-infection (in 6 of 13 cases) associated with increased NG bacterial load, and largely asymptomatic infection [76•]. However, studies based on concordance of infection status in couples have suggested that, though NG was generally found to be more transmissible than CT, co-infection had no effect on NG transmission [77], or even reduced NG transmission [78]. Genital symptoms were more common in CT/NG co-infection (16%) than in single infections (NG 7.7%, CT 5.0%) among 568 high school students in the USA [68], but not among 382 STD clinic patients in the UK (CT/NG co-infection, 40% asymptomatic; NG alone, 33% asymptomatic) [79].

Associations between CT/NG co-infection and damaging long-term sequelae are also variable. A study of CT and NG as well as *Mycoplasma genitalium*, *Ureaplasma urealyticum/parvum*, *Gardnerella vaginalis*, *Trichomonas vaginalis*, and herpes simplex virus-1/2 found that CT and co-infections with two or more of the evaluated organisms were associated with similarly high risk of EP [80]. Similarly, risk of TFI was shown to be similar for single infection or CT/NG co-infection [81], while others showed an increased risk of endometrial infection associated with NG co-infection, but not *M. genitalium* or *T. vaginalis* co-infection or bacterial vaginosis, in a group of CT-infected women [82]. Most studies only considered CT/NG infections [68, 76•, 77–79, 81] and did not consider/exclude other STIs and/or bacterial vaginosis, so their findings might be affected by unknown co-infections.

The potential for unknown co-infection variables to impact progress in vaccine development, perhaps the most pressing need to effectively control CT and NG, is becoming clear. A study published in 2017 provided the first evidence that a vaccine might protect against NG infection. A commercial vaccine for *Neisseria meningitidis*, used in a campaign in New Zealand in the early 2000s, had an estimated anti-NG vaccine efficacy (VE) of 31% [83]. VE was reduced in the case of CT/NG co-infection [83], emphasizing the need for greater understanding of CT and NG biology. Basic science

research plays an important role in informing clinical study design and helping to explain how one pathogen might affect the immune response to another. A study published in 2018 found that CT can prevent human neutrophils from producing neutrophil extracellular traps (NETs) in response to NG [84], providing interesting insights into the potential for CT/NG interaction.

In Vivo Infection and Co-infection: Animal Models of CT, NG, and CT/NG

Animal models of CT and NG infection have been pivotal in understanding information we have gleaned from clinical and epidemiological studies. Clinical and mathematical modeling data demonstrate that human genital and rectal NG/CT co-infections are frequent and suggest active interplay between these two pathogens [3•, 85]. As most published experimental animal studies have focused on each pathogen individually, CT/NG co-infection models in which the pathophysiological consequences of co-infection can be explored are needed. There are several *Chlamydia* in vivo genital infection models, including (i) *C. muridarum* (CM), CT, and *C. suis* (CS) in mice; (ii) *C. caviae* (CC) in guinea pigs; (iii) *C. suis* in swine; and (iv) CT in several non-human primate species [86•, 87, 88, 89••]. In contrast, the most well-established animal NG genital infection models are in mice and chimpanzees [90]. The most widely used NG mouse model, introduced almost 20 years ago and still currently in use, has been valuable in examining NG colonization kinetics, fitness, vaccine candidates, host immune response, influence of host estrus state on infection, and pharmacokinetics of gonorrhea therapeutic antimicrobials [69, 90–92]. To our knowledge, *only one CT/NG co-infection animal model*, a modification of the above-described NG single-infection model, has been published to date. Vonck et al. vaginally CM-infected BALB/c mice, prior to subsequent 17- β -estradiol treatment and vaginal NG infection. Vancomycin (VAN) and streptomycin (STP) injections were used to suppress normal microflora and promote NG colonization. Viable NG and CM were recovered in vaginal swabs for 8–10 days, and significantly more gonococci were observed in co-infected compared with NG singly infected mice [93••]. Vaginal neutrophil numbers and leukocyte chemokine concentrations also increased in co-infected mice compared with either CM or NG singly infected mice, which may explain the more severe symptoms observed in co-infected women [94].

The murine model established by Vonck et al. has revealed interesting facets of co-infection, but the authors suggested two main limitations of the model, both due to specific requirements for robust NG infection. First, estradiol treatment is immunomodulatory, reducing vaginal cytokine expression in CM-infected mice [93••]. Second, VAN/STP treatment is

required to suppress growth of vaginal microflora [93••]. Therefore, animal models that require neither exogenous hormone nor antibiotic administration would be advantageous. Transgenic mice expressing human carcinoembryonic antigen-related cell adhesion molecule 1 (hCEACAM1), a receptor for gonococcal Opa proteins, support vaginal NG colonization in the absence of estradiol treatment for at least 15 days [95]. As in the Vonck et al. study, VAN/STP treatment was used to suppress vaginal microflora. However, the authors did not report attempting NG infection without antibiotics [95], raising the possibility that VAN/STP treatment may not be required in hCEACAM1-expressing mice. Nonetheless, a NG mouse model that does not require estradiol treatment offers advantages, particularly for studying host immune responses to co-infection, even if VAN/STP treatment is required. Interestingly, a human NG clinical isolate has been shown to colonize, grow, and form biofilms on explanted pig vaginal tissue under aerobic conditions [96]. Given that epithelial cells within the NG-infected tissue remain viable despite extensive NG biofilm formation [96] and that CS readily infects explanted pig cervical and uterine luminal and glandular epithelial cells [97], an NG/CS co-infection model using explanted vaginal tissue seems feasible. These data also suggest that establishing genital co-infections in a female pig model is feasible because (i) a model of *C. suis* genital infection already exists and (ii) the porcine genital immune system and estrus cycle are more similar to those in humans than to those in the mouse [88, 89••].

Another approach to modeling co-infection is to use *Neisseria* or *Neisseriaceae* species that naturally colonize the genital tract of animal species that can also be *Chlamydia* infected. *Uruburuella suis*, a novel species in the family *Neisseriaceae*, was isolated from pigs with pneumonia and pericarditis in 2005 [89••, 98]. A new commensal *Neisseria* species (*N. musculi*) has also been recently identified in the wild house mouse [99, 100]. *N. musculi* can be cultured and transformed to produce knockout and gene-complemented strains and colonizes the gut and oral mucosa of C57/B6 mice without producing overt disease. *N. musculi* colonization in mice requires neither estradiol or antibiotic treatment nor expression of human-specific receptors. The *N. musculi* genome also contains predicted homologs for several NG and *N. meningitidis* virulence factor-encoding genes [100]. Because CM establishes gastrointestinal infection in mice via the oral route [101, 102], co-infecting mice orally with CM and *N. musculi* should be relatively straightforward. A significant drawback to developing a *Chlamydia/U. suis* or *N. musculi* co-infection system is that neither *Neisseriaceae* species has been shown to colonize the genital tract of their host species. More importantly, each lacks key virulence factors present in NG—though select NG genes could be expressed in *N. musculi*. However, it seems likely that interactions

between these pathogens might be radically different than those of NG and CT in the human genital tract.

A 2016 study demonstrated that the host nectin-1 protein is required for efficient murine genital CM infection but not for rectal infection [103], suggesting that host factors required for CM infection may vary at different anatomical sites. Thus, observations from in vivo studies of genital single infection or co-infection may not be entirely applicable to co-infections of the rectal mucosa. Interestingly, CM-infected mice exhibited attenuated tubal fibrosis in the absence of gastrointestinal CM carriage [104], suggesting that gastrointestinal CM infection influences distal genital tract pathology, at least in the context of the murine model. Rectal NG/CT co-infections occur in humans, and thus, it is desirable to develop experimental animal models of rectal co-infection. Though there is no established murine NG rectal infection model, the CM rectal infection model [103] provides a platform with which to attempt rectal CM/NG co-infection in either male or female mice. Intestinal hCEACAM1 expression is observed in the hCEACAM1-transgenic mice described above [95], suggesting that these mice may be susceptible to rectal NG infection and could be used to establish a relevant co-infection model. Because significant physiologic differences are observed in female, versus male, animal models, it is also important to evaluate sex as a biologic variable during co-infection. Male mice and guinea pigs can be genitally infected with CM and CC, respectively, and both models have the additional advantage that natural male to female transmission can be studied [105, 106]. Though much needed, there is no established animal model for NG genital infection in males and, thus, no characterized system in which to attempt male genital co-infection. However, a rectal co-infection model in male mice, such as that discussed above, would inform whether there are host sex-specific differences in the progression or outcome of *Chlamydia*/NG co-infection.

Conclusions

CT and NG are common bacterial sexually transmitted pathogens and CT/NG co-infections are also common. Both primarily infect the female endocervix and male urethra and share many clinical characteristics. Differences in the biology and infection dynamics of CT and NG, however, contribute to differences in their epidemiology and to challenges for management and control in high-income countries. For CT, high prevalence in the general population of young heterosexual adults makes screening of asymptomatic people an attractive intervention. However, prevalence has not, thus far, decreased in high-income countries that recommend screening. Rectal CT infection in women is hypothesized as a reason for sustained prevalence, but its clinical relevance has yet to be shown convincingly. NG is uncommon in the general heterosexual population, but incidence is increasing among

MSM. The spread of antimicrobial resistance in NG is a serious threat to control, with verified exceedingly difficult-to-treat cases and untreatable NG cases likely in the near future. Pharyngeal NG is common in MSM and may be a niche where resistance development is promoted. There is surprisingly little epidemiological research about CT/NG co-infection. Mathematical modeling studies suggest that each infection could increase susceptibility and/or transmissibility of the other, and bacterial load might be higher in CT/NG co-infection than in NG alone. The vaginal microbiome probably influences the risks of CT and NG and hence CT/NG co-infection as well. With increasing interest in the development of new treatments and vaccines against NG and CT, it will be important to investigate their effectiveness in the context of co-infections. Finally, future advances will require studies in animal models. There are substantial challenges, however, because both CT and NG are human-specific pathogens. Appropriate, quality-assured animal models are essential to improve understanding of the pathogenic interplay in CT/NG co-infections.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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- Of major importance

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