Gardnerella vaginalis diversity and ecology in relation to vaginal symptoms

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Abstract

Gardnerella vaginalis was first described in 1953, and subsequently identified as the causative agent of a cluster of vaginal symptoms currently known as vaginosis. Research has so far failed to confirm whether and by which mechanism *G. vaginalis* initiates vaginosis, with consequently poor diagnostics and treatment outcomes. Recent molecular analyses of protein-coding genes demonstrate that the taxon *G. vaginalis* consists of at least four distinct species. This development may represent a critical turning point in clarifying ecological interactions and virulence factors contributing to symptoms and/or sequelae of vaginosis.

Keywords:

Gardnerella vaginalis, phylogeny, virulence, microbial ecology, microbiome, bacterial vaginosis

Introduction

"*Gardnerella vaginalis*" was first described in the early 1950s, following a jump in the number of publications concerning sexually transmitted infections and vaginitis after the second world war (Fig. 1). Gardner was not the first to observe the Gram-variable vaginal bacillus eventually named after him (despite his disapproval), but he was the first to suggest it as the causative agent of what had previously been known as "non-specific vaginitis", in the seminal paper of the field [1]. The first paper to use the term "vaginosis", in 1964, was referring to cysts of non-microbiological origin (but coincidentally mentions Gardner by name) [2]. The term "vaginosis" did not re-appear until 1981 when it was used, with the qualifier "bacterial", to signify an overgrowth of *G. vaginalis* and other anaerobes, not characterized by typical inflammatory changes generally implied by the suffix '*–itis*' [3]. The utility of this clinical designation, also referred to as "cytolytic vaginosis", has recently been questioned and yet another qualifier has been suggested ("polymicrobial vaginosis") [4]. Clearly, the sizeable accumulation of clinical and microbiological observations, since Catlin's review [5], has yet to result in a coherent division between ubiquitous commensals of the genital tract and pathogens, resulting in either vaginal symptoms or in symptomless states that can nevertheless compromise sexual and reproductive health.

G. vaginalis is found in most women with vaginosis and in many or most women without vaginosis, especially in higher-resolution datasets [6]. These studies also confirm that *G. vaginalis* is present at higher concentrations and forms typically different ecological partnerships when women are experiencing vaginosis, or in women more likely to be affected by HIV, STI or pre-term birth. Several conceptual and technical advances have re-defined the modern understanding of *G. vaginalis* in relation to vaginosis, including: 1) massive expansion of readily

available molecular biology techniques and reagents, ranging from multi-target quantitative PCR to systems biology/omics via whole genome high-throughput sequencing and mass spectrometry techniques, 2) increasingly refined culture-based strategies to describe potentially virulent or protective properties of bacterial strains in vaginal secretions, 3) microscopic analysis of the arrangement of bacterial cells in mucosal strata including adherent polymicrobial biofilm; and, 4) increased characterization of mucosal innate and acquired immune effectors in response to specific virulence factors, microbes or microbial combinations. Freed from an exclusive reliance on culture, molecular microbiologists have discovered previously unrecognized microbial diversity within the vaginal microbiome and within *G. vaginalis*, suggesting potentially significant associations between *G. vaginalis* and other microbial species, as recently reviewed [6]. Despite this advance, enhanced culture techniques are still required in order to test hypotheses about microbial functions and interactions. Additionally, both culture and target-based molecular studies inherently under-emphasize the physical arrangement of cells of different species in vaginal mucosal layers, with subsequent analyses necessarily based on description of co-occurrence of *G. vaginalis* and other microbial species in proportional terms. In contrast, microscopic techniques ranging from wet mount and Gram stain to the most advanced confocal microscopy with phylogenetically-targeted fluorophores provide more or less detailed information about bacterial diversity, but are essential to understand physical arrangement of bacterial and human cells *in vivo*. Although *G. vaginalis* and/or polymicrobial biofilm has been recognized as a factor in vaginosis for decades as “clue cells”, microscopy has recently provided more insight into the phylogenetic diversity and physical structure of *G. vaginalis* biofilms intimately associated with the vaginal mucosa [7, 8], as well as of intracellular *G. vaginalis* [9].

The original case for fulfillment of Koch's postulates linking the cause of vaginosis with *G. vaginalis*, made by Gardner and Dukes (1955), continues to be defended and derided, even in current literature [10, 11], but its specific role in the natural history of specific vaginal symptoms and/or immune impairment leading to silent reproductive health risks remains elusive [12]. Since the clinical category "vaginosis" is poorly descriptive, with little agreement in the literature as to its etiology and natural course, and no cure in sight, our goal is to review the state of knowledge regarding the phylogenetic diversity, microbial associations and clinical significance of *Gardnerella vaginalis,* the Actinobacterium originally described as the cause of this enigmatic syndrome.

Phylogenetics of protein-coding genes reveals *G. vaginalis* diversity

Phenotypic heterogeneity within *G. vaginalis* has been recognized since the small, pleomorphic, rod-shaped organism was first identified and observed to give variable results in Gram staining. Based on current understanding of the cell wall structure and biochemical properties of *G. vaginalis* it is considered a Gram-positive bacterium [13]. Efforts to identify phenotypic traits shared universally by *G. vaginalis,* which would be clinically useful in order to distinguish it from other catalase negative coryneforms, resulted in a rather short list including beta-haemolysis on human blood agar, negative catalase reaction, hippurate hydrolysis, and lack of growth on nutrient agar or in the presence of 2% (w/v) sodium chloride [14, 15]. Proposals have been made for disambiguating *G. vaginalis* based on phenotypic properties ("biotyping") [16, 17], or targeted genotyping methods such as amplified ribosomal DNA restriction analysis (ARDRA) [18]. However, there has been little success in reconciling the genotypic and phenotypic characteristics with each other, or in identifying patterns of association of any

genotype or phenotype with demographic or clinical characteristics. Reports of correspondence between specific biotypes and clinical status are variable, with some authors reporting significant associations between particular biotypes and vaginosis symptoms [19-23]. Observations of ARDRA genotypes and their association with biotype or specific virulence factors are similarly variable [23-26]. While these approaches for classification are somewhat useful for examining cultured isolates, the requirement for culture means that they cannot be readily applied to addressing questions of the role of *G. vaginalis* in the context of the vaginal microbiome, which recent higher-resolution data indicates may normally contain a mixed community of *G. vaginalis* with strains that potentially vary in overall phenotype and virulence potential [27, 28]. Selective culture may bias observations of *G. vaginalis* diversity due to differential growth rates of strains in mixed samples, and choice of incubation atmosphere potentially affecting the recovery of obligate anaerobic strains [29]. For example, Schellenberg *et al.* [30] isolated 66 *G. vaginalis* strains representing all subgroups on *Brucella* medium with soluble starch and horse serum, in anaerobic conditions for 48 h. Two of these strains were later found to be strict anaerobes [31], indicating that they would not have been isolated under carbon dioxide enriched aerobic conditions, commonly used when isolating and culturing *G. vaginalis*.

Early efforts to exploit whole genome sequencing in describing and explaining diversity within *G. vaginalis* provided further evidence of disparities in virulence potential among isolates [32, 33]. Although the results of these comparative genomics studies revealed some clues regarding the distribution of genes responsible for virulence-associated traits such as adhesion [32] and degradation of mucus [33], conclusions were limited by the small number of strains studied and by the classification of strains in question as “pathogenic” or “commensal”, based solely on whether or not they had been clinically diagnosed with vaginosis. The latter issue is

119 particularly problematic given that most women in whom the four *G. vaginalis* subgroups have
120 been quantified were colonized with multiple strains of *G. vaginalis*. Numerous culture-based
121 studies have also highlighted the wide variety of phenotypes observed for *G. vaginalis* isolates in
122 terms of cytotoxicity, adhesion to epithelial cells, biofilm formation, sialidase production and
123 antibiotic susceptibility [32, 34-37, 23, 24, 38, 21].

124 The advent of culture-independent methods for determining the composition of the
125 vaginal microbiome has provided an unprecedented opportunity to investigate *G. vaginalis*
126 diversity. In an early study of the vaginal microbiome based on PCR amplification and
127 sequencing of the "universal target" (UT) region of the gene encoding the 60 kDa chaperonin
128 (*cpn*60), Hill *et al.* [39] described four clusters of *G. vaginalis*-like sequences detected in the
129 microbiomes of Canadian women. The same four groups were observed in a much larger study
130 of vaginal microbiomes of African women [30]. Hummelen *et al.* [40] subsequently reported
131 four *G. vaginalis*-like phylotypes based on single nucleotide differences in the V6 variable
132 region of the 16S rRNA gene. Although it is impossible to directly reconcile these categories
133 with other molecular categories using existing data, it has been clearly shown that intra-subgroup
134 variability in 16S rRNA sequence overlaps with inter-subgroup variability [26], and so is
135 unreliable as a subgroup-specific target. Confirmation that *cpn*60-based subdivisions of *G.*
136 *vaginalis* was not the result of PCR artifact was provided by phylogenetic analysis of cultured
137 isolates based on *cpn*60 UT sequences [25, 26] and whole genome sequences [41].
138 Reconciliation of the *cpn*60 based subgroups described by Jayaprakash *et al.* [25] and whole
139 genome sequence based "clades" proposed by Ahmed *et al.* [41] was achieved in a recent study
140 by Schellenberg *et al.* [26] where *cpn*60 subgroups A, B, C and D [25] were shown to
141 correspond to clades 4, 2, 1 and 3 [41], respectively. These observations underline the general

142 superiority of protein-coding sequences to differentiate *G. vaginalis* subgroups, and point
143 tantalizingly to a near future of cheap and abundant whole genome and metagenomics-based data
144 providing information about every known protein-coding gene.

145 Based on a phylogenomic species definition [42] there are at least four species within *G.*
146 *vaginalis*, since whole genome average nucleotide identity values between *cpn*60-defined
147 subgroups are less than 95% [26] (Fig. 2). Establishment of phenotypic properties that
148 differentiate the four subgroups is so far limited to the observation that all subgroup B isolates
149 (and only some subgroup C isolates) are sialidase activity positive [26, 43], and lipase activity
150 may characterize subgroup A [25]. Studies of many more isolates will be required to confirm this
151 relationship and identify other differentiating traits. Sub-speciation within *G. vaginalis* is not a
152 recent evolutionary event, since the same four subgroups have been detected among isolates
153 from women in North America, Europe and Africa [26]. Albert *et al.* [27] demonstrated in a
154 *cpn*60-based microbiome profiling study of healthy, non-pregnant Canadian women, that the
155 previously defined vaginal "community state type" (CST IVA, [44], which is dominated by *G.*
156 *vaginalis*, could be subdivided based on the relative abundance of different *cpn*60-based
157 subgroups. Out of 310 microbiome profiles, 33 were found where *G. vaginalis* formed at least
158 50% of the microbiome, and all but two contained at least two subgroups. Similarly, using multi-
159 target quantitative PCR with subgroup-specific primers designed by Balashov *et al.* [28],
160 multiple subgroups were detected in 70% of the 60 vaginal samples examined. The limited
161 evidence to date suggests that many if not most women are colonized with multiple *G. vaginalis*
162 subgroups, and that *G. vaginalis* subgroups may express different virulence determinants.
163

164 **Microbial ecology at the mucosal interface**

Observations of bacterial cell types in vaginal smears have included "normal", "abnormal" and "intermediate" profiles since as early as 1921 [45], suggesting ecological succession and numerical fluctuations in vaginal microbial communities. More or less detailed schemes for enumerating bacterial cell types have been described since [46, 47]. Similarly, cross-sectional and longitudinal culture-based and molecular monitoring of vaginal microbes reveal a dynamic microbiota transitioning between *Lactobacillus* dominance, *G. vaginalis* dominance, and/or *G. vaginalis* co-dominance with other anaerobes. Physical associations of micro-organisms and human cells at the mucosal surface have been observed for decades as a defining feature of vaginosis (clue cells). Although vaginal biofilm is addressed elsewhere in this issue [48], it is discussed here as the specific milieu in which *G. vaginalis* may create conditions leading to acquisition or overgrowth of normally sub-dominant bacteria such as *Atopobium*, *Dialister*, *Escherichia, Megasphaera*, *Mobiluncus*, *Prevotella*, *Pseudomonas* or *Sneathia*. Resistance conferred by the biofilm structure is generally understood as an explanation for difficulties in eradicating vaginosis using conventional antibiotic treatment [49, 12, 50] Physical association of *G. vaginalis* with different types of mucus (for example membrane-bound MUC4, secreted gel-forming MUC5AC and MUC7 [51]) may determine pathogenic characteristics and extent of biofilm. Although microscopy using phylogenetically-specific probes can only so far reveal the broad phylogenetic outlines of the actual participants in mucosal biofilm, thereby proving co-localization rather than functional associations, a combination of microscopy, phylogenetic census and functional analysis is on the horizon [52]. Whether structured *G. vaginalis* biofilm can assemble spontaneously from co-existing strains in the right combination or under certain conditions, or is a co-evolved structure that must be transmitted as biofilm via transfer of colonized epithelial cells [7], remains speculative. Re-infection by *G. vaginalis,* or by

polymicrobial biofilms containing *G. vaginalis,* between sex partners may also be an important contributor to relapse, with increasing evidence of colonization of "vaginal" organisms and clue cells in the male reproductive tract [53, 54].

Most literature regarding physiological interactions between *G. vaginalis* and other microbes concerns inhibitory activities of *Lactobacillus* known to produce anti-*G. vaginalis* effectors such as hydrogen peroxide, lactic acid and bacteriocins [55-58]. More recently, it has also been suggested that lactobacilli and *G. vaginalis* compete for access to the mucosal surface [59, 37, 60], and that the biofilm phenotype helps *G. vaginalis* tolerate acid and hydrogen peroxide exposure [61]. Co-dominance of *G. vaginalis* with primarily *Bacteroides*, *Porphyromonas* and *Prevotella* species has been described, particularly in some higher resolution molecular datasets of vaginal samples [27, 30, 62]. Potential nutritional interactions between *G. vaginalis* and *Prevotella* were first proposed based on co-culture of vaginal isolates [63], with amino acids produced by *G. vaginalis* consumed by *Prevotella*, which in turn produces ammonia taken up by *G. vaginalis*. Consistent with this hypothesis, increased *G. vaginalis* biofilm mass has been shown when co-cultured with *Prevotella bivia* [64]. A similar pattern was observed with *Atopobium vaginae*, *Fusobacterium nucleatum* and *Mobiluncus mulieris* in this study [64], as well as in studies of *A. vaginae* [65, 66], and another study concerning *Enterococcus* and *Escherichia* [67]. Although no specific physiological interactions were proven, besides the creation and maintenance of an *Atopobium*-promoting anaerobic environment by *G. vaginalis,* these observations indicate that *G. vaginalis* may initiate biofilm formation (early colonizer), and create favourable conditions for other micro-organisms (late colonizers). Since isolates of *G. vaginalis* and *Prevotella* grow to higher concentrations in culture when pH is as high as 9 [63], factors resulting in an elevated pH may provide an

advantage for these assemblages. Ovulatory mucus, semen deposition, menstrual flow, and disappearance of *Lactobacillus* populations for any reason, are cyclical or punctual alkalization events in the vagina, perhaps setting the stage for increases in *G. vaginalis* populations.

Ecological relationships within complex microbial communities of the vagina have yet to be fully defined, although preliminary studies indicate several potential competitive or cooperative nutritional interactions possibly defining whether or not *G. vaginalis* populations rise or fall in response to shifts in the vaginal environment (succession). Whether *G. vaginalis* creates physiological conditions that reduce *Lactobacillus*, prior to becoming numerically dominant, or if *G. vaginalis* is simply an opportunist that moves in when *Lactobacillus* levels drop for other reasons, cannot be fully established in the absence of extensive longitudinal data and a more fundamental understanding of typical community compositions and shifts within an individual over time. Balashov *et al.* [28] found that subgroups C and D (clades 1 and 3) were associated with high Nugent scores, and subgroup B (clade 3) was associated with intermediate scores, but no association between subgroup A (clade 4) and vaginosis defined by either Amsel's criteria or Nugent scores was observed. The association of sialidase activity positive subgroup B with intermediate Nugent scores suggests that this subgroup may play a role in microbial succession, either enabling the establishment of a milieu consistent with vaginosis, the resolution of vaginosis and the re-establishment of a *Lactobacillus*-dominated milieu, or transition to yeast infection or aerobic vaginitis, also shown to involve high sialidase levels [68].

Physical and chemical fluctuations in the *G. vaginalis* niche contributing to ecological succession patterns can be divided into five sets of factors: 1) Chemical/structural aspects of the mucous membrane, including mucus layers and flow, epithelial secretion of immune factors and nutrient-rich substrates, and changing access to the mucosal surface for attachment and biofilm

formation; 2) Consequences of episodic sexual intercourse, possibly including vaginal lubrication (endogenous or applied), physical disturbance, homogenization and oxygenation of the mucosal layer, introduction of non-vaginal organisms to the vaginal environment and the deposition of a rich source of bacterial nutrients that raises the pH of the vaginal lumen to neutral, facilitating conception; 3) Cyclical menstruation, including predictable fluctuations in estrogen and progesterone, changes in mucus consistency during ovulation, presence of blood and tissue in menstrual flow, as well as intentional dysregulation of these pathways through different forms of birth control, and physiological consequences of hormonal attenuation in menopause;4) Parity and childbirth, including physical changes in the reproductive tract, suspension of monthly cycles, lactation, oxytocin production, and different routes of transfer of maternal/parental microbiota to the infant; 5) Broader environmental factors affecting women in population and public health terms, including social customs, diet and coping with stress. We are currently pursuing multidisciplinary studies in order to establish the magnitude of the effects of these factors on succession of microbial assemblages *in vivo*.

Virulence factors and modulation of host immune responses

Vaginolysin, sialidase and prolidase are frequently described virulence factors of *G. vaginalis*, with a range of hypothetical or predicted effects on biofilm formation (addressed elsewhere in this issue [48]) and modulation of immune responses in vaginosis. *G. vaginalis* haemolysin (Gvh), a cholesterol-dependent cytolysin, was initially discovered in *G. vaginalis* culture medium and found to have cytolytic activity against human erythrocytes [69]. Studies with the purified native protein suggested functional similarities to *Clostridium perfringens* theta-toxin and *Escherichia coli* haemolysin [69]. Additionally, IgA specific for the 59 kDa pore-

forming cytolysin was detected in 60% of women with symptoms and a Nugent score indicative of vaginosis [70]. Although purification of the native protein allowed initial characterization of its activity, complete characterization was delayed until the whole genome sequence of the *G. vaginalis* type strain (ATCC 14018) became available, facilitating the identification of the gene encoding the toxin [71]. Gelber *et al.* proposed the name "vaginolysin" for this cholesterol-dependent pore-forming protein toxin, and confirmed its specificity for human erythrocytes [71]. Vaginolysin activity was found to depend on the complement regulatory molecule CD59, and expression of human CD59 in hamster cells resulted in increased susceptibility to cytolysis by vaginolysin. Further evidence of a specific interaction between vaginolysin and target cells was provided by experiments showing that single-chain antibodies against vaginolysin inhibit cytolytic activity [72]. Vaginolysin expression levels have been associated with level of cytotoxicity in cell culture models [23, 36] but no link between expression level and *G. vaginalis* genotype or biotype has been established [23].

Vaginosis-associated bacteria, including *G. vaginalis,* have been associated with a pro-inflammatory cytokine response in vaginal fluid, although vaginosis may not be associated with clinical signs of inflammation such as leukocyte infiltration, pain, redness or swelling. A recent review by Mitchell and Marrazzo [73] summarizes the contradictory reports of relative levels of cytokines and anti-microbial peptides in vaginal secretions from women with or without BV, emphasizing the complexity of relationships between the microbiome and the cervico-vaginal immune system. Even in the absence of symptoms or only mild symptoms, which may be due to potential abrogation of inflammatory changes by bacterial effectors, subclinical effects of abundant *G. vaginalis*, biofilm and/or proliferation of other anaerobes such as *Prevotella*, in terms of increased risk of negative reproductive health outcomes must be considered. A specific

IgA response to Gvh has been described, and found to correlate with IL-8 expression in vaginal secretions [74]. Stimulation of pro-inflammatory cytokine expression by *G. vaginalis* has also been documented *in vitro* [75]. Coincident with eliciting a pro-inflammatory response, hydrolytic enzymes produced by *G. vaginalis,* including sialidase and prolidase, play important roles in abrogation of inflammation.

Sialidase enzymes cleave the terminal sialic acid residues of sialoglycans in the vaginal environment and play critical roles in providing nutrition for vaginal bacteria through sialic acid catabolism, in revealing attachment sites for bacterial adhesion to the epithelium, contributing to biofilm formation and in modulation of the immune response [43, 76]. In addition to mucin, immune system targets for sialidase include IgA, and cell surface receptors for chemokines and immunoglobulins, and toll-like receptors [76].

Prolidases are expressed by a variety of vaginosis-associated bacteria, including *G. vaginalis*. These proteolytic enzymes can degrade extracellular matrix components including mucin, and prolidase activity is strongly associated with vaginosis. In a study of vaginal secretions of women with vaginosis, prolidase activity was inversely correlated with innate and *G. vaginalis* antigen-specific IgA responses [77]. Additional enzymatic activities implicated in the pathogenesis of vaginosis continue to be identified [78] and the increasing availability of complete genome sequences will no doubt facilitate determination of the specific contribution of *G. vaginalis* to the complex cocktail of hydrolytic enzymes produced in vaginosis.

Future perspectives

There remains a great deal of work to be done in elucidating the basic biology and metabolism of *G. vaginalis* subgroups, determining mechanistic aspects of adhesion, biofilm

303 formation, immunomodulatory and antimicrobial activities. At the time of writing, there are over
304 40 published *G. vaginalis* whole genome sequences at various stages of assembly and
305 annotation. These data offer a rich resource for studies of *G. vaginalis* species population
306 structure and phenotypic potential, and provide reference data for transcriptomic and proteomic
307 studies. The lack of a good animal model for the human vaginal microbiome remains a
308 significant obstacle to investigating interactions of *G. vaginalis* with the vaginal epithelium.

309 Taken together, the evidence suggests that "*Gardnerella vaginalis*" may not be a
310 particularly useful operational designation for this diverse collection of organisms: at least four
311 new species are likely to be established soon. The roles of various *G. vaginalis* subgroups in the
312 vaginal microbiome and their individual or collective contribution to vaginosis and its sequelae
313 may be elucidated with a combination of omics and deep sequencing methods that examine *G.*
314 *vaginalis* in the context of the entire microbiome. The four-subgroup division of *G. vaginalis* that
315 has been developed based on sequencing of protein-coding genes offers a rational framework for
316 future studies since it is consistent with the phylogenomic species definition, and the subgroups
317 can be easily detected in *cpn*60 sequence-based microbiome profiles and quantified in vaginal
318 samples.

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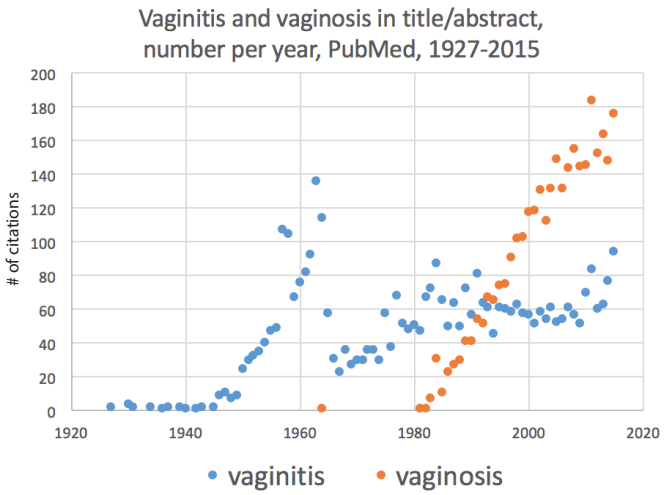
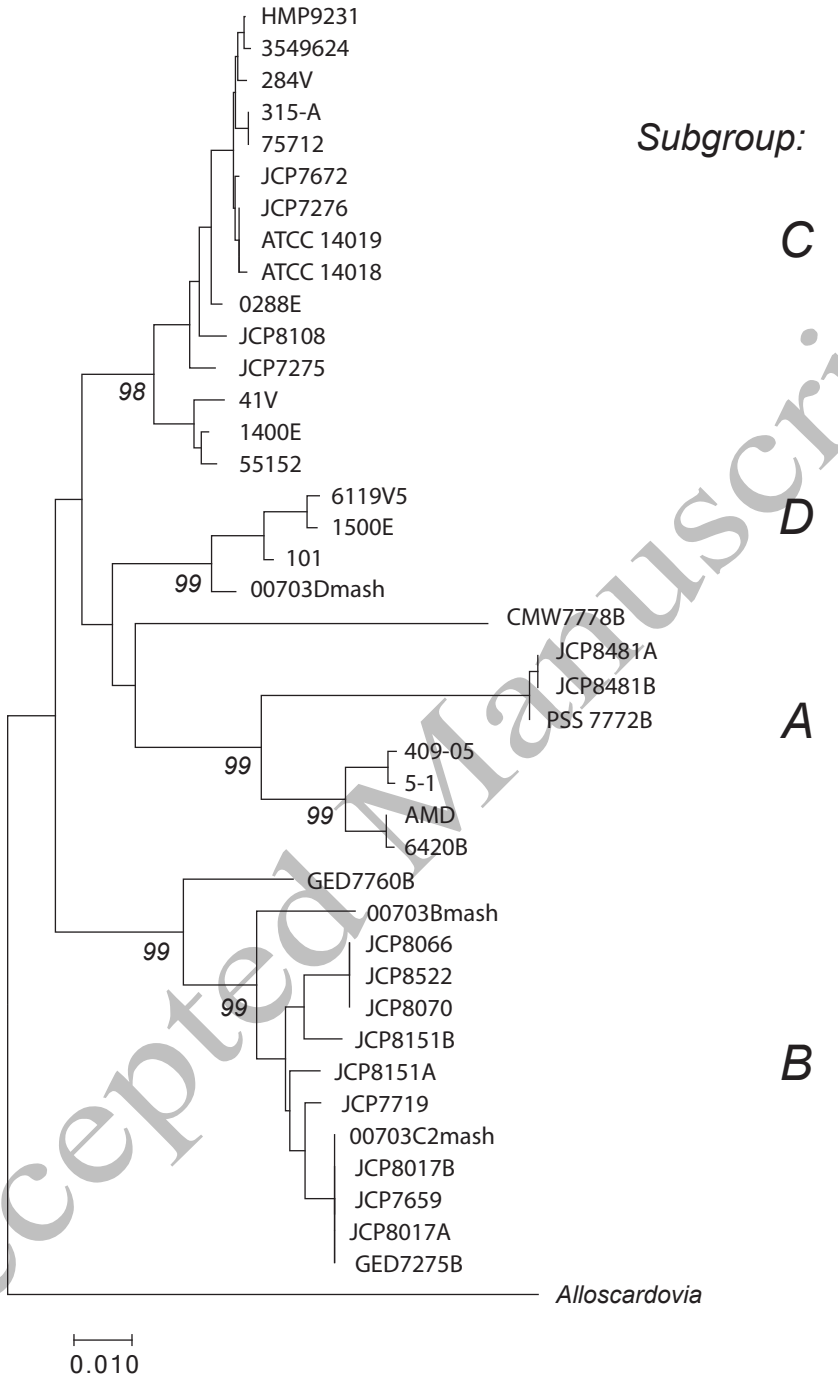


Fig. 1. Left: Use of the terms "vaginitis" and "vaginosis" in PubMed articles since 1927, indicating year of publication for articles by Gardner & Dukes, linking "*G. vaginalis*" to "vaginosis". Note that the first use of vaginosis (1964) does not concern vaginal microbiology. Right: H.L. Gardner at the First International Conference on Vaginosis – Nonspecific Vaginitis, Kristiansand, Norway, April 16-17, 1982. He provided the introduction to the proceedings [79] and, unfortunately, was also the subject of the leading obituary.



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544 **Fig. 2.** Phylogeny of *cpn*60 universal target sequences from published *G. vaginalis* genomes,
545 rooted with *Alloscardovia omnicolens* as the outgroup, inferred using the Neighbor-Joining
546 method with selected bootstrap values shown (500 replicates). The tree is drawn to scale, with
547 evolutionary distances computed using the Maximum Composite Likelihood method in base
548 substitutions per site, using MEGA7.