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Gene polymorphisms of Toll-like and related recognition receptors in relation to the vaginal carriage of *Gardnerella vaginalis* and *Atopobium vaginae*☆,☆☆

Hans Verstraelen[a,*,1], Rita Verhelst[b,1], Lieve Nuytinck[c], Kristien Roelens[a], Els De Meester[c], Daniel De Vos[c], Martine Van Thielen[c], Rudi Rossau[c], Wim Delva[d], Ellen De Backer[b], Mario Vaneechoutte[b], Marleen Temmerman[a,d]

[a] *Department of Obstetrics & Gynaecology, Faculty of Medicine & Health Sciences, Ghent University, De Pintelaan 185, B-9000 Ghent, Belgium*

[b] *Department of Clinical Chemistry, Microbiology, and Immunology, Faculty of Medicine & Health Sciences, Ghent University, Belgium*

[c] *Innogenetics n.v., Belgium*

[d] *International Centre for Reproductive Health, Faculty of Medicine & Health Sciences, Ghent University, Belgium*

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Abstract

Host genetic factors have previously been found to act as determinants of differential susceptibility to major infectious diseases. It is less clear whether such polymorphisms may also impose on pathogen recognition in mucosal overgrowth conditions such as bacterial vaginosis, an anaerobic overgrowth condition characterised by the presence of a vaginal biofilm consisting of the Gram-positive anaerobes *Gardnerella vaginalis* and *Atopobium vaginae*. We selected 34 single nucleotide polymorphisms pertaining to 9 genes involved with Toll-like receptor-mediated pathogen recognition and/or regulation (*LBP*, *CD14*, *TLR1*, *TLR2*, *TLR4*, *TLR6*, *MD2*, *CARD15* and *SIGIRR*) and assessed in a nested case–control study their putative association with bacterial vaginosis, as diagnosed by Gram staining, and with the vaginal carriage of *A. vaginae* and *G. vaginalis*, as determined by species-specific PCR, among 144 pregnant women. Carriage of *G. vaginalis* during early pregnancy was associated with the $-1155A>G$ substitution in the promoter region of the *MD2* gene ($p=0.041$). The presence of *A. vaginae* during the first half of the pregnancy was significantly associated with the *CD14* intron 2 $1342G>T$ ($p=0.039$), the *TLR1* exon 4 $743A>$G ($p=0.038$), and the CARD15 exon 4 $14772A>T$ ($p=0.012$) polymorphisms, and marginally significantly associated with the *LBP* exon13 $26842C>T$ ($p=0.056$), the CD14 promoter $-260C>T$ ($p=0.052$), and the TLR1 promoter $-7202A>G$ ($p=0.062$) polymorphisms. However, no association between gene polymorphisms and bacterial vaginosis as such could be documented. Our data suggest that some degree of genetic susceptibility involving pathogen recognition may occur with the key bacterial vaginosis organism, *A. vaginae*.

Keywords: Bacterial vaginosis; *Atopobium vaginae*; Single nucleotide polymorphisms; Toll-like receptor; Vaginal microflora

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* Corresponding author. Tel.: +32 9 240 37 96; fax: +32 9 240 38 31.
E-mail address: Hans.Verstraelen@UGent.be (H. Verstraelen).

[1] Contributed equally.

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1. Background

Bacterial vaginosis (BV), a condition characterised by massive anaerobic overgrowth of the vaginal mucosa, is a highly common infection among women of childbearing age and associated with a vast disease burden of adverse obstetric outcome, pelvic inflammatory disease, and the acquisition and secondary spread of HIV-1 and other sexually transmitted diseases (Sobel, 2000; Schwebke, 2003).

Although the pathogenesis of this enigmatic condition remains largely undetermined, it has generally been attributed to a limited number of risk factors, foremostly non-Caucasian ethnicity and behavioural factors including vaginal douching and sexual contact (Sobel, 2000). Little is known, however, about the putative role of intrinsic risk factors in BV. In particular, as host genetic factors have previously been found to act as determinants of differential susceptibility to major infectious diseases (Cooke and Hill, 2001; Segal and Hill, 2003), it is plausible that the human genetic make-up might also underlie mucosal overgrowth conditions such as BV. Genetic susceptibility to infectious disease most typically involves single nucleotide polymorphisms (SNPs) of genes coding for pathogen-associated molecular-pattern recognition receptors (PRRs) such as the Toll-like receptors (Schroder and Schumann, 2005).

The role of Toll-like receptor signalling in BV has not been fully elucidated, although several observations are of particular interest here. Al-Harthi et al. documented that vaginal fluid from women with bacterial vaginosis strongly induces the nuclear transcription factor NF-κB in several types of cells (Al-Harthi et al., 1998), thereby regulating the genes responsible for the innate immune response, whereas indigenous *Lactobacillus* spp. seem to induce NF-κB activation at a very modest level (Klebanoff et al., 1999). Most recently, it has further been shown that NF-κB induction in bacterial vaginosis primarily occurs through a TNF-receptor-associated factor-6-dependent pathway (TRAF6), providing evidence that initiation of the immune response in bacterial vaginosis depends on Toll-like receptor signalling (St John et al., 2007a).

Vaginal epithelial cells strongly express the Toll-like receptors (TLRs) for Gram-positive recognition, TLR1, TLR2 and TLR6, though not the Gram-negative recognition molecule TLR4 (Fichorova et al., 2002; Fazeli et al., 2005). Neutrophils in turn express a wide array of Toll-like receptors (Hayashi et al., 2003) involved in both Gram-positive and Gram-negative recognition, but neutrophils are for some reason typically relatively absent in BV (Rein et al., 1996). Other immune cells in the vagina may play a role too, however, and recently, increasing attention has been paid to Toll-like receptor expression in monocytes in response to BV. From these experiments, the induction of TLR2 in monocytes and dendritic cells has emerged as a putatively important immune recognition mechanism with BV (Zariffard et al., 2005; Mares et al., 2008; St John et al., 2007b). The role of Gram-negative recognition with BV remains less clear, as in one study strong induction of TLR4 mRNA was reported in mononuclear cells in peripheral blood and in an immortalised monocytic cell line (Zariffard et al., 2005), whereas a similar study documented that vaginal fluid from women with BV failed to stimulate cells from a monocytic cell line expressing TLR4/MD2, although these cells do respond to purified lipopolysaccharide (LPS) (Mares et al., 2008).

We therefore hypothesised a link between defective Gram-positive recognition and overgrowth with the key species in BV, *Atopobium vaginae* and *Gardnerella vaginalis* (Verhelst et al., 2004; Ferris et al., 2004; Verstraelen et al., 2004; Menard et al., 2008), which were also found to be the main constituents of the epithelial biofilm in BV (Swidsinski et al., 2005). *G. vaginalis* as well as *A. vaginae* are frequently present with normal vaginal microflora; however, they display pronounced overgrowth in the case of BV (De Backer et al., 2007; Menard et al., 2008).

From this perspective, we selected 34 single nucleotide polymorphisms (SNPs) pertaining to 9 genes involved with Toll-like receptor-mediated and -related pathogen recognition and/or regulation (Table 1) and assessed in a nested case–control study their putative association with carriage of the primary constituents of the bacterial vaginosis biofilm, *A. vaginae* and *G. vaginalis*, as determined by species-specific PCR of 288 vaginal samples obtained from 144 women during the first- and second-trimesters of pregnancy respectively.

2. Methods and materials

2.1. Study population and design

As part of a prospective cohort study involving the study of the vaginal microflora during early pregnancy in relation to pregnancy outcome (Verstraelen et al., 2007), we conducted a nested case–control study comprising 144 unselected Caucasian pregnant women. In the original cohort, 221 women were consecutively enrolled on the occasion of their first antenatal visit, between January 2003 and May 2004 at the outpatient obstetric clinic of Ghent University Hospital. Women were scheduled to provide a first- and a second-trimester vaginal swab

Table 1
Basic pathogen-associated molecular-pattern recognition receptors for Gram-positive and Gram-negative recognition.

PAMP recognition receptor	Gene locus	Pathogen-associated molecular pattern/function	
		Gram-positive	Gram-negative
Lipopolysaccharide-binding protein (LBP)	20q11.23-q.12	Lipoteichoic acid (LTA) and other Gram-positive cell wall components	Lipopolysaccharide (LPS)
Monocyte differentiation antigen CD14 (CD14)	5q23-q31	LTA/other components-LBP complex	LBP–Lipopolysaccharide (LPS) complex
MD2 protein (MD2) (*LY96*)	8q21.11		TLR4 accessory protein, confers LPS responsiveness onto TLR4; enhances TLR4-mediated activation of NF-κB
Caspase recruitment domain family, member 15 (CARD15) (*NOD2*)	16q21	Confers responsiveness to peptidoglycan (PGN) leading to NF-κB activation	Confers responsiveness to intracellular LPS and PGN, involved in intracellular signalling, leading to NF-κB activation
Toll-like receptor 1 (TLR1)	4p14	Recognition of peptidoglycan (PGN) and lipoproteins (LP), NF-κB activation	
Toll-like receptor 2 (TLR2)	4q32	Recognition of peptidoglycan (PGN) and lipoproteins (LP), NF-κB activation	
Toll-like receptor 4 (TLR4)	9q32-q33		Recognition of CD14–LBP–LPS, NF-κB activation
Toll-like receptor 6 (TLR6)	4p14	Recognition of peptidoglycan (PGN) and lipoproteins (LP), NF-κB activation	
Single immunoglobulin and Toll-interleukin 1 receptor (TIR) domain (SIGIRR) (*TIR8*)	11p15.5	Down-regulator of the Toll-like receptor signalling pathways	Down-regulator of the Toll-like receptor signalling pathways

for bacterial 16S rDNA extraction and a blood sample for maternal DNA extraction. Of those, only a single swab was obtained from 61 women, while a blood sample was not available for another 14 women, and hence these subjects could not be included in the analyses. The Ghent University Hospital Ethical Board approved the study protocol (IRB protocol no. 2002/340) and all study subjects agreed to participate through written informed consent.

2.2. Sampling procedures

Vaginal samples were collected for the purpose of Gram staining and bacterial DNA extraction by inserting two sterile cotton-tipped wooden swabs into the vagina, after placement of a non-lubricated speculum. The swabs were rolled through 360° against the vaginal wall at the midportion of the vault and carefully withdrawn to prevent vulvar microflora contamination. The first swab was rolled out on a plain glass slide to allow for Gram staining followed by microscopy, and the second swab was placed in a sterile tube for transport.

A venous blood sample was drawn upon study enrolment and collected in an EDTA tube and stored at −80 °C until further processing.

2.3. Sample processing

Gram-stained vaginal smears were assigned a modified Nugent sore (Nugent et al., 1991) according to Verhelst et al. (2005).

For DNA extraction from the vaginal swabs, the QIAamp DNA mini kit (Qiagen, Hilden, Germany) was used according to the manufacturer's recommendations, with minor modifications, as described previously (Verhelst et al., 2004). The DNA concentrations of the vaginal samples ranged from 4.20 to 76.29 ng/μL with a mean concentration of 17.8 ng/μL. DNA extracts were stored at −20 °C until further processing for the purpose of detection of *A. vaginae* and *G. vaginalis* through species-specific PCR.

A primer set that allowed amplification of the 16S rRNA gene of *A. vaginae* and that lacked homology with non-target bacteria was used as described previously

(Verhelst et al., 2004). Briefly, a 10-μL PCR mixture contained 0.2 μM each of the primers ato167f (5′ GCGAATATGGGAAAGCTCCG) and ato587r (5′ GAGCGGATAGGGGTTGAGC), 5 μl of Promega master mix (Promega, Madison, WI, USA), 1 μL of Qiagen DNA extract of the samples and distilled water. Thermal cycling consisted of initial denaturation of 5 min at 94 °C, followed by three cycles of 1 min at 94 °C, 2 min at 58 °C and 1 min at 72 °C, followed by 35 cycles of 20 s at 94 °C, 1 min at 58 °C and 1 min 72 °C, with a final extension of 10 min at 72 °C. Five microlitres of the amplified product was visualised on 2% agarose gel to verify the presence of an *A. vaginae* DNA fragment of 420 base pairs.

Species-specific primers for *G. vaginalis*, designed by Zariffard et al., were used (Zariffard et al., 2002). Briefly, a 20-μL PCR mixture contained 0.05 μM primers, 10 μL of Promega master mix (Promega, Madison, WI, USA), 2 μL of Qiagen DNA extract of the samples and distilled water. Thermal cycling with the primers consisted of initial denaturation of 10 min at 94 °C, followed by 50 cycles of 5 s at 94 °C, 45 s at 55 °C and 45 s at 72 °C, and a final extension of 10 min at 72 °C. Five microlitres of the amplified product was visualised on 2% agarose gel.

Using Qiagen extracted DNA from pure cultures, the detection limit for *A. vaginae* was 1.3 pg/mL and for *G. vaginalis* it was 43.2 pg/mL. Species-specific PCR is estimated to pick up these species beyond a detection limit of 3.5×10^6 cells/mL for *A. vaginae* and 4.5×10^6 cells/mL for *G. vaginalis*, respectively.

For assessment of single nucleotide polymorphisms (SNPs), DNA was extracted from 1 mL of maternal blood using QIAamp Blood Midi Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. SNPs in the following genes were analysed, in coding as well as in non-coding regions, based on their known or postulated association with infectious disease: lipoprotein-binding protein (LBP), the monocyte differentiation antigen CD14, the lymphocyte antigen 96 LY96 (MD2), the nucleotide-binding oligomerisation domain protein NOD-2 (CARD15), Toll-like receptor 1 (TLR1), Toll-like receptor 2 (TLR2), Toll-like receptor 4 (TLR4), Toll-like receptor 6 (TLR6), and the single immunoglobulin domain-containing interleukin-1-related protein (SIGIRR; Table 1). Primers were developed for selective amplification of the relevant genomic DNA sequences. Genotyping was carried out by a multi-parameter assay allowing simultaneous detection of the relevant DNA variants in these genes using reverse hybridisation technology (Stuyver et al., 1993). Briefly, specific probes designed to hybridise with their complementary sequences amplified from the target DNA were coated as dots on nitrocellulose strips. The hybridised probes were visualised as coloured dots and were interpreted visually.

2.4. Statistics

Genotype distributions between groups (cases versus controls) were compared through ordinary χ^2 tests or, when appropriate, Fisher's exact tests. On account of the sample size, we calculated *exact* rather than ordinary *asymptotic* p values as fixed exact p values for 2×2 contingency tables and as estimated exact p values through Monte Carlo simulations based on 10,000 reiterations for 2×3 contingency tables. When Monte Carlo simulations were applied, the exact p value was presented as a point estimate with its 95% confidence interval to the p value. As the study objective was exploratory, no adjustment was made for multiplicity (Ohashi et al., 2001). Correlations between genotype distributions across the various loci were explored under the non-parametric assumption. The Hardy–Weinberg equilibrium (HWE) for each polymorphism was calculated as "$A^2 + 2Aa + a^2 = 1$" with "A" being the wild type allele and "a" the variant allele. p values for the HWE denote the significance of the departure from the expected allele distribution. All analyses were performed with use of the statistical software SPSS v 15.0 (Chicago, IL, USA).

3. Results

Based on Gram staining, 116 women (80.6%) had normal/grade I either grade I-like microflora throughout the first- and second-pregnancy trimesters, whereas 28 parturients (19.4%) presented on at least one test occasion with either intermediate microflora or overt bacterial vaginosis.

Based on the DNA analysis of the 288 vaginal samples obtained during the first- and second-trimesters, 45 women could be categorised as having had at least 1 positive PCR test for *A. vaginae* during the first- and/or second-trimester, whereas 79 women had at least 1 positive PCR test for *G. vaginalis* (Table 2). Hence, comparisons according to vaginal microflora status could be made with a 1–2.2 and a 1–0.8 case–control ratio for *A. vaginae* and *G. vaginalis*, respectively.

Of the 34 loci under study, 3 did not show any allelic variability in this cohort, while in the remaining variants, allele frequency ranged from 0 to 52%, with a median frequency of 7.5% (interquartile range 2.0–29.0%; Table 3). Overall, genotype distribution proved consistent with the Hardy–Weinberg equilibrium for all polymorphisms

Table 2
Correlation between vaginal microflora status on Gram staining and the presence of *Gardnerella vaginalis* and *Atopobium vaginae* as determined by species-specific PCR during the first two trimesters of pregnancy ($N = 144$).

	Normal microflora and grade I-like microflora ($n = 116$)	Intermediate microflora and bacterial vaginosis ($n = 28$)
At least one positive PCR for *G. vaginalis*	61 (52.6%)	21 (75.0%)
No positive PCR for *G. vaginalis*	55 (47.4%)	7 (25.0%)
At least one positive PCR for *A. vaginae*	30 (25.8%)	15 (53.6%)
No positive PCR for *A. vaginae*	86 (74.2%)	13 (46.4%)

except for the *TLR6* 1083C > G SNP genotype distribution (Table 3).

Correlation analysis revealed several significantly positive and negative associations between related loci. A strong linkage disequilibrium (Spearman's rho >0.5) was, however, only observed between the *LBP* $-205A > G$ and *26842T* > *C* polymorphisms ($r = 0.703$, $p < 0.001$), between the *CD14* $-1461G > T$ and $-260C > T$ substitutions and the CD14 *2 1342G* > *T* polymorphism ($r = -0.589$ and -0.571, $p < 0.001$, respectively), and between the *TLR1* $-7202A > G$ and *743 A* > *G* SNPs ($r = 0.971$, $p < 0.001$).

Women who carried *G. vaginalis* during the first- and/or second-trimester showed a significantly higher rate of the homozygous $-1155A > G$ mutation in the promoter region of the *MD2* gene ($p = 0.041$; results not displayed). Women who harboured *A. vaginae* during the first- and/or second-trimester (Table 2) were significantly more likely to be heterozygous for the *CD14* intron 2 *1342G* > *T* mutation ($p = 0.039$), to be heterozygous for the *TLR1* exon 4 *743A* > G mutation ($p = 0.038$), while conversely significantly more likely to have the wild type genotype at the CARD15 exon 4 *14772A* > *T* locus ($p = 0.012$). In addition, the presence of *A. vaginae* was marginally significantly associated with the *LBP* gene exon13 *26842C* > *T* polymorphism ($p = 0.056$), with the CD14 promoter $-260C > T$ polymorphism ($p = 0.052$), and with the TLR1 promoter $-7202A > G$ polymorphism ($p = 0.062$; Table 2). In contrast, BV, as defined by Gram staining, did not show any significant correlation with the SNPs under study (results not displayed).

4. Discussion

Of the 34 SNPs pertaining to 9 selected candidate genes (*LBP*, *CD14*, *TLR1*, *TLR2*, *TLR4*, *TLR6*, *MD2*, *CARD15* and *SIGIRR*, respectively), none was associated with BV. A single SNP of the *MD2* gene was significantly associated with the presence of *G. vaginalis*. Carriage of *A. vaginae* was in turn significantly associated with three polymorphisms located on the *CD14*, *TLR1* and *CARD15* genes, respectively and marginally significantly associated with another three polymorphisms belonging to the *LBP*, *CD14* and *TLR1* genes. Our data, therefore, may point to some degree of genetic susceptibility to the key organisms in BV and *A. vaginae* in particular, although this was not apparent from the correlation analysis with the Gram staining scores, possibly as a result of the limited sample size.

Several limitations owing to our study design must be accounted for and therefore our results should be interpreted with caution. In particular, it must be acknowledged that although we have demonstrated several associations between the genotypes for selected Toll-like receptor and related pattern recognition and immune regulation genes and the presence of *G. vaginalis* and *A. vaginae* on the vaginal epithelium, these associations do not necessarily equate to a cause–effect relationship. We did not correlate evidence of gene expression or gene product at the level of the vaginal mucosa with the presence of genotype differences or with the presence of the microorganisms studied. In addition, other genes not included in this analysis may have played a more important role or alter the findings because of interactions for which we were unable to control. Similarly, the associations demonstrated in this study may also be the result of closely linked loci. Also, even though we aimed to explore putative associations between genotype and the two index species, it cannot be ruled out that the associations observed can be explained by the occurrence of synergistic organisms, although it may also be noticed that the associations were explored regardless of vaginal microflora status. Finally, we handled the presence of the *A. vaginae* and *G. vaginalis* as a binary variable, whereas a quantitative measure of bacterial concentration might have been more informative, as documented by Genc et al. (2004). It may be added that detection errors due to multiple comparison may also have generated spurious associations.

Only two studies thus far have been aimed at establishing an association between bacterial vaginosis and

Table 3
Single nucleotide polymorphisms of the LBP, CD14, TLR1, TLR2, TLR4, TLR6, MD2, CARD15 and SIGIRR genes in relation to vaginal colonisation with *A. vaginae*.

SNP			Genotype	Variant allele frequency	HWE (*p* value)	Genotype frequency (%)	Genotype frequency (%)	Monte Carlo/exact significance
Gene region, rs nomenclature	Contiguous position	Amino acid change				*A. vaginae* negative (*n* = 99)	*A. vaginae* positive (*n* = 45)	
LBP gene								
Promoter, rs2232578	−205 A > G	–	AA	0.22	*0.81*	0.657	0.556	0.488
			AG			0.293	0.378	0.478–0.498
			GG			0.051	0.067	
Exon 4, rs5744204	7892 G > A	V166M	GG	0.01	*0.99*	0.990	1.000	1.0
			GA			0.010	0.000	(exact)
			AA			0.000	0.000	
Exon 8, rs2232607	18414 A > G	D283G	AA	0.02	*0.97*	0.960	0.956	1.0
			AG			0.040	0.044	(exact)
			GG			0.000	0.000	
Intron 9, rs1780627	22713C > T	–	CC	0.42	*1.0*	0.364	0.289	0.287
			CT			0.495	0.467	0.278–0.296
			TT			0.141	0.244	
Exon 10, rs2232613	22736 C > T	P333L	CC	0.06	*0.73*	0.838	0.956	**0.058**
			CT			0.162	0.044	(exact)
			TT			0.000	0.000	
Exon 13, rs2232618	26842 T > C	F436L	TT	0.12	*0.72*	0.818	0.667	**0.056**
			CT			0.172	0.333	0.051–0.060
			CC			0.010	0.000	
CD14 gene								
Promoter, rs3138078	−1461G > T	–	GG	0.25	*0.17*	0.535	0.511	0.456
			GT			0.444	0.422	0.446–0.466
			TT			0.020	0.067	
Promoter, rs2569190	260C > T	–	CC	0.45	*0.12*	0.394	0.244	**0.052**
			CT			0.343	0.556	0.048–0.057
			TT			0.263	0.200	
Exon 2, rs2228049	698A > G	*N204D*	AA	0.0	*NA*	1.000	1.000	–
			AG			0.000	0.000	
			GG			0.000	0.000	
Intron 2, rs2563298	1342G > T	–	GG	0.27	*0.98*	0.525	0.556	**0.039**
			GT			0.364	0.444	0.035–0.043
			TT			0.111	0.000	

MD2 gene (LY96)								
Promoter, rs1809440	−1155 A > G	–	AA	0.52	*0.89*	0.232	0.244	0.944
			AG			0.475	0.489	0.939–0.948
			GG			0.293	0.267	
Exon 2, rs6472812	13407G > A	*G56R*	AG	0.05	*0.80*	0.111	0.089	0.777
			GG			0.889	0.911	(exact)
			AA			0.000	0.000	
Exon 5, rs11466004	37598C > T	*P157S*	CC	0.02	*0.70*	0.960	0.956	1.0
			CT			0.040	0.044	(exact)
			TT			0.000	0.000	
CARD 15 gene								
Exon 4, rs2066844	14772C > T	*R702W*	CC	0.06	*0.82*	0.848	0.956	**0.012**
			CT			0.152	0.022	0.010–0.014
			TT			0.000	0.022	
Exon 8, rs2066845	253855G > C	*G908R*	GG	0.01	*0.99*	0.980	0.956	0.589
			GC			0.020	0.044	(exact)
			CC			0.000	0.000	
Exon 11, rs2066847	32624-/C	*3020 (1006) insC*	-/-	0.02	*0.96*	0.949	0.956	1.0
			-/C			0.051	0.044	(exact)
SIGIRR (TIR8)								
Intron, rs7482596	9293 A > C	–	AA	0.0	*NA*	1.000	1.000	–
			AC			0.000	0.000	
			CC			0.000	0.000	
Exon 9, rs3210908	3392G > A	*R312Q*	GG	0.24	*0.10*	0.616	0.600	0.732
			AG			0.283	0.333	0.723–0.741
			AA			0.101	0.067	
Exon 9, rs3087588	3402G > T	*P315P*	GG	0.24	*0.10*	0.616	0.600	0.731
			GT			0.283	0.333	0.723–0.740
			TT			0.101	0.067	
TLR 1 gene								
Promoter, rs5743551	−7202A > G	–	AA	0.28	*0.90*	0.576	0.378	**0.062**
			AG			0.374	0.511	0.057–0.067
			GG			0.051	0.111	
Exon 4, rs5743611	239G > C	*R80T*	GG	0.07	*0.67*	0.848	0.889	0.610
			GC			0.152	0.111	(exact)
			CC			0.000	0.000	

Table 3 (*Continued*)

SNP			Genotype	Variant allele frequency	HWE (p value)	Genotype frequency (%)	Genotype frequency (%)	Monte Carlo/exact significance
Gene region, rs nomenclature	Contiguous position	Amino acid change				*A. vaginae* negative ($n = 99$)	*A. vaginae* positive ($n = 45$)	
Exon 4, rs4833095	743A > G	*N248S*	TT	0.27	*0.97*	0.596	0.378	**0.038**
			TC			0.354	0.511	0.034–0.042
			CC			0.051	0.111	
Exon 4, rs3923647	914A > T	*H305L*	AA	0.05	*0.83*	0.889	0.933	0.549
			AT			0.111	0.067	(exact)
			TT			0.000	0.000	
TLR 2 gene								
Promoter, rs1898830	−15607A > G	–	AA	0.35	*0.99*	0.444	0.378	0.305
			AG			0.465	0.444	0.296–0.314
			GG			0.091	0.178	
Exon 2, rs3804100	1350T > C	*S450S*	TT	0.08	*0.39*	0.838	0.911	0.557
			CT			0.141	0.089	0.547–0.567
			CC			0.020	0.000	
Exon 2, rs5743708	2258G > A	*R753Q*	GG	0.05	*0.85*	0.919	0.889	0.545
			AG			0.081	0.111	(exact)
			AA			0.000	0.000	
Exon 2, rs	2029C > T	*R677W*	CC	0.0	*NA*	1.000	1.000	–
			CT			0.000	0.000	
			TT			0.000	0.000	
TLR 4 gene								
Promoter, rs1927914	−2026A > G	–	AA	0.32	*0.31*	0.434	0.422	0.963
			AG			0.485	0.511	0.960–0.967
			GG			0.081	0.067	
Exon 4, rs4986790	4795A > G	*D299G*	AA	0.06	*0.73*	0.879	0.867	0.793
			AG			0.121	0.133	(exact)
			GG			0.000	0.000	
Exon 4, rs4986791	5095C > T	*T399I*	CC	0.07	*0.67*	0.869	0.844	0.796
			CT			0.131	0.156	(exact)
			TT			0.000	0.000	
TLR 6 gene								
Promoter, rs1039559	−502T > C	–	CC	0.42	*0.92*	0.333	0.356	0.554
			CT			0.455	0.511	0.544–0.563
			TT			0.212	0.133	

Exon 1, rs5743810	745C>T	*P249S*	CC	0.39	*0.91*	0.343	0.467	0.332	
			CT			0.475	0.422	0.322–0.341	
			TT			0.182	0.111		
Exon 1, rs3821985	1083C>G	*T361T*	CC	0.34	*0.04*	0.465	0.533	0.301	
			CG			0.343	0.378	0.292–0.310	
			GG			0.192	0.089		
Exon 1, rs5743815	1280T>C	*V427A*	TT	0.01	*0.99*	0.990	1.000	1.000	
			TC			0.010	0.000	(exact)	
			CC			0.000	0.000		

polymorphisms of the *TLR* genes, and *TLR4* in particular. In a large cohort of predominantly African–American women, Goepfert et al. found no association between vaginosis and the TLR4 4795A>G polymorphism, whereas the TLR4 5095C>T polymorphism appeared to be protective, although the latter difference lost statistical significance after controlling for ethnicity (Goepfert et al., 2005). In a smaller cohort of white, Hispanic, and African–American pregnant women, Genc et al. also failed to document a correlation between the *TLR4* 4795A>G polymorphism and bacterial vaginosis, although the polymorphism was significantly associated with vaginal fluid *G. vaginalis* concentrations (Genc et al., 2004). In accordance with these studies, neither the aforementioned co-segregating *TLR4* 4795A>G and 5095C>T polymorphisms nor a third polymorphism of the *TLR4* promoter region (−2026A>G) were found to correlate with *A. vaginae* or *G. vaginalis*.

Since the very same *TLR4* polymorphisms have previously been associated with numerous other infectious conditions (Schroder and Schumann, 2005), the apparent lack of such associations with bacterial vaginosis in three subsequent studies may indicate that Gram-negative infection or at least LPS recognition may not be a key mechanism here. This is not so unexpected, given the fact that the two most abundant species in bacterial vaginosis, *G. vaginalis* and *A. vaginae*, actually both have a Gram-positive cell wall. Accordingly, for the associations between the *TLR4* 4795A>G polymorphism and *G. vaginalis* concentrations (Genc et al., 2004) and between *G. vaginalis* and the *−1155 A>G* polymorphism of the *MD2* gene found here, *G. vaginalis* is likely to act as a proxy variable for one or more synergistic Gram-negative bacterial species, e.g. *Prevotella bivia* (Pybus and Onderdonk, 1999), unless it could be documented that TLR4 could recognise *G. vaginalis*.

Although similar confounding with *A. vaginae* cannot be ruled out, it is of interest that the associated SNPs involving the *LBP*, *CD14*, *TLR1* and *CARD15* genes are at least potentially consistent with Gram-positive recognition, as would be expected. Indeed, the LBP and CD14 proteins, historically considered Gram-negative (LPS) signal receivers, have recently been shown to recognise several Gram-positive bacterial surface components as lipoteichoic acid (Zweigner et al., 2006). Similarly, the CARD15 or NOD2 protein is now recognised as an intracellular sensor of bacterial cell wall peptidoglycan, present in both Gram-positive and -negative bacteria (Kufer et al., 2006).

It may be concluded that polymorphisms of the Toll-like receptor and related pattern recognition and regulation genes may be involved in the presence of the

primary constituents of the BV biofilm, *G. vaginalis* and *A. vaginae*, and that this seems particularly apparent for *A. vaginae*. On the other hand, no such association could be established with BV. Even if the associations proved a genuine biological phenomenon as postulated, it should be acknowledged that, overall, the attributable risk of these polymorphisms appears to be of limited magnitude. Future studies in this area may benefit from a more dynamic assessment of innate immune gene functioning at the gene expression or translational level, with particular interest in genes involved in the modulation of the Toll-like receptor-related immune response, including the expression of the *SIGIRR*, *MyD88* and *TOLLIP* genes among others.

Conflict of interest

None.

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