Diversity and stability of cultured vaginal lactobacilli in pregnant women from a multi-ethnic urban UK population

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Title

Diversity and stability of cultured vaginal lactobacilli in pregnant women from a multi-ethnic urban UK population

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Running headline

Vaginal lactobacilli

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Abstract

Aims

To determine the diversity and stability of cultured vaginal lactobacilli in a multi-ethnic population of pregnant women.

Methods and Results

A single centre, prospective, cohort study was performed in a tertiary perinatal centre in East London, UK. Self-collected vaginal swabs at 13 and 20 weeks gestation were obtained from women attending for routine antenatal care and cultured for lactobacilli. In women who provided both swabs, 37 of 203 (18%) had no lactobacilli cultured at either time. Only 53 (26%) had the same species at both times. Black women were less likely to have lactobacilli cultured at 13 weeks (p = 0.014) and Black and Asian women were less likely to have lactobacilli cultured at 20 weeks (p = 0.002) compared with those in the White and Other groups.

Conclusions

Significant differences exist between ethnic groups in the carriage and stability of vaginal lactobacilli.

Significance and Impact of Study

These differences have implications for the design of interventions aimed at normalising the vaginal microbiota in pregnant women.

Keywords

Lactobacilli, vaginal microbiota, pregnancy, preterm birth

Introduction

Preterm birth (PTB) makes a major contribution to infant mortality and long-term disability (Moser *et al.* 2007; Saigal and Doyle 2008). The mechanisms and causes of spontaneous PTB are poorly understood but known associations include ethnicity, low socio-economic status, a short interval between pregnancies, poor nutritional status, previous history of PTB, intrauterine infection and ethnicity (Goldenberg *et al.* 2008). In the US, the rate of PTB in Black women is 2-3 times that of white mothers (Adams *et al.* 2000; Collins *et al.* 2007; Kistka *et al.* 2007; Goldenberg *et al.* 2008). Similar but more complex patterns have been observed in Europe. The rate of PTB is higher in Black women but differences in PTB are seen between Black Caribbean and Black African groups, and within Black African subgroups. Studies in North Paris, East London, North West England, and England and Wales as a whole have found higher rates of PTB among women from the Caribbean and West Africa compared with women from Northern Africa (Zeitlin *et al.* 2004; Macfarlane *et al.* 2005; Balchin and Steer 2007; Datta-Nemdharry *et al.* 2012). A review of ethnic disparities in PTB pointed out that both social and biological factors are likely to play a part (Kramer and Hogue 2009).

Bacterial vaginosis (BV) is associated with PTB (Gibbs *et al.* 1992; Taylor *et al.* 1997). It is characterised by both the absence of lactobacilli and by the presence of large numbers of anaerobic species. Lactobacilli, principally the strains that produce higher levels of H_2O_2, appear to protect against vaginal colonisation by pathogenic species, particularly those causing BV (Klebanoff *et al.* 1991; Hawes *et al.* 1996). There is some evidence that vaginal colonisation with H_2O_2 producing lactobacilli reduces the risk of chorioamnionitis and PTB (Reid and Bocking 2003; Wilks *et al.* 2004; Mosbah and Mesbah 2009). In the US, BV is commoner in Black women (Antonio *et al.* 2009; Uscher-Pines and Hanlon 2009) and is significantly associated with PTB of a low birthweight baby in this ethnic group (Hittie *et al.* 2007). Despite substantial evidence linking bacterial vaginosis with PTB, the results of trials of antibiotic treatment of BV in pregnancy have not produced clear evidence of benefit (Nygren *et al.* 2008; Brocklehurst 2013).

Ethnic differences in the vaginal microbiota of sexually-active, non-pregnant women have been described in the US (Ravel *et al.* 2011). Previous cross-sectional (Wilks *et al.* 2004; Kiss *et al.* 2007; Mosbah and Mesbah 2009) and longitudinal (Verstraelen *et al.* 2007; Verstraelen *et al.* 2009) studies have reported on the presence and stability of vaginal lactobacilli in pregnant women who were predominantly White. Similar studies on pregnant women from multi-ethnic backgrounds have not reported before. The aims of this study were to determine the prevalent types and stability of vaginal lactobacilli in pregnant women from a multi-ethnic population in East London, UK using standard laboratory techniques.

Material and Methods

This single centre, prospective, cohort study was performed with the approval of the Redbridge & Waltham Forest Local Research Ethics Committee which formed part of the UK National Research Ethics Service (REC reference number 08/H0701/26). The study population consisted of women attending the antenatal clinic at Homerton University Hospital NHS Foundation Trust (HUH), London between September 2008 and February 2009. Women referred to the antenatal clinic at HUH received an information leaflet about the study with the appointment letter for their first antenatal clinic visit. Participation involved permitting access to hospital obstetric and neonatal records, contact with the GP if required to enquire about prescribed medications, agreeing to self-collect vaginal swabs on two occasions, and permission to retain the specimens. Antibiotic usage during the period of pregnancy was determined by asking the participant. Ethnicity was self-defined by the participants and results were analysed by grouping ethnicity into the categories used in England, based on categories used in the 2001 population census: White (British, Irish and other White), Black (Caribbean, African, other Black and mixed Black and White), Asian (Indian, Pakistani, Bangladeshi, other Asian and mixed Asian and white) and Other (Chinese, other and not known).

The women in the study provided two self-collected swabs: the first at the time of the first antenatal clinic appointment at approximately 13 weeks gestation (swab A) and the second when the women attended for a routine ultrasound anomaly scan at approximately 20 weeks gestation (swab B). Women were provided with a sheet of written instructions and diagrams that described how to self-collect a vaginal swab. Briefly, women were asked to wash their hands, gently part their labia, remove the sterile swab from its plastic tube, insert the ‘cotton-bud’ end of the swab into their vagina to approximately half the swab length (about 6 cm), gently twist the swab about three times, part their labia, remove the swab and place it back into its plastic tube. The swab was then extracted into 3 mls brain heart infusion broth (BHI) containing 10% glycerol and 0.005% cysteine hydrochloride and stored at -70°C. After the women gave birth, maternal and neonatal hospital records were reviewed and data on maternal demographics, gestational age at birth, birth outcome, and birthweight collected.

Culture and identification of lactobacilli

Members of staff performing the microbiological assays were blinded to the clinical characteristics of the study population. Thawed vaginal secretions were vortexed for 10 secs, inoculated onto MRS agar (Unipath, Basingstoke, UK) and incubated for 48 h at 35 °C in an atmosphere of 10% CO_2, 10% H_2 and 80% N_2. Single colonies from recovered cultures were subcultured onto blood agar plates (5% horse blood, Oxoid, Basingstoke UK) and used for DNA extraction as described below and for determination of H_2O_2 production. H_2O_2 production was measured using a semi-quantitative assay (Merckoquant Peroxide Test, Merck, Leics, UK) as described previously.[17] Results from this test are expressed in bands of H_2O_2 production: negative, 1-3, 3-10,10-30 and 30-100 mg l^{-1}.

Following DNA extraction using a QIAamp DNA minikit (Qiagen, Manchester, UK), lactobacilli were identified to species level by 16S rDNA sequencing or matrix assisted laser desorption ionisation time of flight (MALDI-TOF) analysis. For 16S rDNA sequencing, a 1,350-bp fragment of 16S rRNA gene was amplified using oligonucleotide primers 5′-GAA CGC TGG CGG CGT GCC (Z1-forward) and 5′-TCC GCG ATT ACT AGC GAT TCC (Z2-reverse). During the course of the study, MALDI-TOF mass spectrometry was introduced into the laboratory and validated for the identification of lactobacilli using standard strains. For MALDI-TOF analysis, a single colony of a fresh culture was lysed with 70% ethanol, extracted with acetonitrile and formic acid, overlaid with hydroxy cinnamic acid matrix and analysed using a Bruker Microflex mass spectrometer running MALDI-TOF Biotyper 2.0 analysis software.

Statistics

The data from the swabs and the clinical information were merged and checked for obvious errors. The analyses were performed using Stata 10. Log_e transformations of H_2O_2 were analysed by the Kruskal-Wallis test followed by Sidak's adjustment for multiple comparisons. Associations were tested using chi-squared or Fisher's exact tests for tables. Logistic regression was used to investigate associations with PTB, and any or specific lactobacilli carriage. For comparisons of White v Black, and White v Asian, a Bonferroni correction assuming 3 potential comparisons was made. The other group was not included because it is heterogeneous and small. This is a conservative correction. No adjustments were made for White v all others or Black v all others. All p-values are two sided and confidence intervals are 95%.

Results

The base line characteristics of the recruited women are shown in Table 1. Of the 293 women recruited to the study, gestational age and birth weights of live births were unavailable in 46 women (9 had a miscarriage or termination of pregnancy and 37 moved out of area). A second swab was not obtained from 90 women mainly because of researcher non-availability when these women attended for their routine ultrasound anomaly scan.

Overall, 75% of women were colonised with any lactobacillus at either of the sampling times (Table 2). The mean (SD) number of species of lactobacilli isolated from swab A was 1.15 (0.92) compared with 1.14 (0.87) from swab B (data not shown). The statistically significant effects of ethnic group on isolation of any lactobacillius in swab A and in Swab B among mothers with both swabs is associated with a significant reduction in carriage for Black compared to White mothers (p = 0.006 for both comparisons). Indian women had very similar reduced carriage for any lactobacilli in swab B as Black mothers, but the results are not significant because of smaller numbers (p = 0.105, chi-squared after Bonferroni correction). Compared with the White women in the study, the reductions in lactobacilli carriage appeared to be because fewer Black and Indian women were colonised with *L. crispatus* (p = 0.12 and p = 0.19, respectively), fewer Black women were colonised with *L. gasseri* (p = 0.32) and fewer Indian women with *L. jensenii* (p = 0.12) but none of these associations were significant (Fisher's exact test adjusted for 3 comparisons using Bonferroni's test for multiple comparisons).

Delivery of the fetus between 22^{+0} and 36^{+6} completed weeks of gestation occurred in 9 (5%) of 181 women who were lactobacillus positive at the first swab and 6 (9%) of 66 women who were negative (p = 0.23). Delivery during this range of gestational age was lower in White women (2.4%) compared to all others (9.9%) (p = 0.016, Fisher’s exact test). Excluding 4 multiple pregnancies which are themselves associated with PTB, non-White women were at increased risk with 9 PTBs (7.8%) from 144 births compared to White women with 2 PTBs(1.6%) from 122 births ($p = 0.030$, Fisher's exact test). The odds ratio for PTB for non-White women after adjustment for lactobacilli carriage at swab A, is 5.0 (CI 1.05 – 24, $p = 0.045$) while that for presence of any lactobacilli at swab A was not significant (OR = 0.7, CI .21 - 3.7, p=0.64 after adjustment for non-White ethnic group).

The amount of H_20_2 produced by *L. jensenii* was significantly higher than other common species (Table 3). A one-way analysis of variance comparing the $\log_e$ H_2O_2 produced showed that *L jensenii* was highly significantly different from *L. crispatus, gasserii* and *vaginalis*, ($p < 0.001$ after Sidak's adjustment for multiple comparisons). Similarly, regression analysis of the $\log_e$ H_2O_2 produced showed that *L jensenii* had nearly six times the level of H_2O_2 production *as L. crispatus, gasserii* and *vaginalis* (5.9, CI 4.3 to 8.1, $p < 0.001$).

Isolates of the same species were assumed to be the same strain of that species and the data were analysed to obtain basic information on the stability of lactobacillus carriage. The proportions of women who had specific strains at swab A and swab B were very similar but this masks a high turnover in species in individual women (Table 4). In women who provided both swab samples, 37 (18%) of 203 did not have lactobacilli isolated at either time, 53 (26%) had the same lactobacillus species isolated at both times, 71 (35%) gained a new species, and 68 (45%) of 150 who had a lactobacillus isolated at the first sampling time lost a species. In total, 90 of 203 (44%, CI 37 to 51%) had the same strains (or none) at both time points. Using multivariate analysis, Black women were less likely to gain a new species (OR 0.49, CI 0.25 to 0.98, $p = 0.043$) compared with all other ethnic groups combined. There were significant differences in the proportions of different ethnic groups losing either any species ($p = 0.008$) or all species ($p = 0.005$), with over 20% of Asian and Black women losing all species compared with only 4% of White women.

Antibiotic usage occurred in the preceding month in 18 of 293 (6%) women who provided a swab A and 11 of 203 (5%) of those who provided a swab B. The oral antibiotics used were amoxicillin, cefalexin and co-amoxiclav. Of the 150 women who provided both swabs and had lactobacilli in swab A, 6 received antibiotics between swabs A and B and none of them lost any strains, while 65 of the other 144 who did not report antibiotic usage did lose a species. This difference is significant (p = 0.029, Fisher's exact test) and suggests that those receiving oral antibiotics were less likely to lose a species. The binomial exact one-sided confidence interval for the proportions losing a strain if they had received oral antibiotics is 0 - 46%. This suggests that of similar women given oral antibiotics fewer than half would be expected to lose a strain of Lactobacilli over the period.

Discussion

In this study, we found significant differences in cultured vaginal lactobacilli between ethnic groups at two time points during pregnancy. Black women were less likely to have vaginal lactobacilli at 13 and 20 weeks of gestation compared with White women. There was a high turnover of vaginal lactobacilli species in individual women.

To our knowledge, this is the first report to present longitudinal data on vaginal lactobacillus colonisation during pregnancy in an ethnically diverse population. Vaginal colonisation was determined using standard laboratory techniques only. We did this because interventions involving the administration of live lactobacilli (Vangelista *et al.* 2010; Yamamoto *et al.* 2013) require amongst other properties that the strains are easily culturable to allow manufacture of adequate quantities of the product and to allow the ready detection of the organism after administration not only to determine the success of colonisation but also for reasons of safety. Therefore, no attempt was made to identify strains such as *L iners* that are often difficult to recover in culture and require molecular methods of detection.

Our findings are in agreement with recent reports of ethnic variation in vaginal lactobacillus colonisation in non-pregnant women (Zhou *et al.* 2007). Three quarters of women in our study were found to be colonised with vaginal lactobacilli at both times of swabbing and this result is in agreement with previous cross-sectional reports (Bayó *et al.* 2002; Zhou *et al.* 2007). However, Black women at the time of both swabs A and B, and Asian women at the time of swab B, were less likely to have vaginal lactobacillus colonisation. The ethnic differences in vaginal microbiota found in this study and others may be due to a number of reasons including genetic influences on the immune system and differences in nutritional factors and cultural practices. The distribution pattern of the most common lactobacillus species varies between studies for reasons that are unclear. In earlier studies, the unreliablilty of biochemical identification methods made reliable speciation of lactobacilli unreliable (Wilks *et al.* 1984), but advances in the identification of lactobacilli by molecular methods such as 16S rDNA sequencing or MALDI-TOF suggests that reported differences in detected species are not due to technical factors.

In this study, mean gestational age of live births did not differ between the ethnic groups, although as expected the birth weight of Asian babies was lower than that of the other groups (Leon and Moser 2012). PTB occurred significantly more frequently in non-White women but not significantly more in the absence of lactobacilli in swab A. Reports in the literature suggest an association between preterm labour and reduced frequency of vaginal lactobacillus colonisation or BV (Hitti *et al.* 2007; Donders *et al.* 2009; Mosbah and Mesbah 2009). These findings have prompted trials both with antibiotics and probiotics designed to modify the vaginal microbiota with the objective of improving pregnancy outcome. Antibiotics administered to pregnant women can eradicate BV but are unable to reduce the risk of preterm labour and birth (Lams *et al.* 2008; Brocklehurst *et al.* 2013). Oral or vaginal administration with probiotic strains of lactobacilli has often been successful in establishing colonisation of the vagina by the probiotic strain but studies have not been sufficiently powered to determine an effect on preterm birth (Othman *et al.* 2007). If there are ethnic differences in the vaginal microbiota, any interventions designed to restore the normal microbiota must take this into account in addition to viability, dosage and strain/species of lactobacilli.

H_2O_2 production by vaginal lactobacilli is considered to be an important defence mechanism against vaginal colonisation by undesirable microorganisms. In a previous study we showed that the presence of H_2O_2 producing lactobacilli in the vagina of women who were at risk of PTB was associated with reduced risk of adverse birth outcomes (Wilks *et al.* 2004). The explanation for this finding is unclear because in vitro experiments have shown that the microbicidal activity of H_2O_2 is blocked by cervicovaginal fluid and semen (O'Hanlon *et al.* 2010). However, these findings may not be applicable in vivo where, for example, H_2O_2 producing lactobacilli may produce concentrations of H_2O_2 in their immediate vicinity that are sufficiently high to prevent adherence of a potential pathogen to the vaginal mucosa and thus prevent colonisation. In addition, it may be that H_2O_2 producing lactobacilli strains produce other microbicidal factors such as lactic acid or bacteriocins that prevent proliferation of pathogenic in the vagina.

In this study, approximately 5-6% of women received antibiotics in the month preceding either of the swab samples. Our figures are similar to that reported in a longitudinal study in the UK which also used self-reported data and showed that 8% of women reported antibiotic use in early pregnancy and 5% at 32 weeks gestation (Headley *et al.* 2004). By contrast, Petersen and colleagues used prescribing information recorded in a primary care database in South West London and found that 14% of women received at least one antibiotic in each trimester (Petersen *et al.* 2010). Taken together the data suggest that either the use of self-reporting underestimates the consumption of antibiotics during pregnancy or regional differences exist in the prescribing habits of GPs. In a study of non-pregnant women, use of antibiotics was associated with loss of vaginal lactobacillus strains (Vallor *et al.* 2001). However, we found that vaginal lactobacillus colonisation was relatively unperturbed by exposure to oral antibiotic administration even though lactobacilli show in vitro sensitivity to some of the antibiotics ingested by the women in this study (Hamilton-Miller and Shah 1994).

While this observational study was not powered to detect independent effects of ethnicity and lactobacillus colonisation on PTB, the combined results from this and previous studies warrant further research to investigate their effects on PTB. Two significant advances in recent years have made it more practical to undertake large studies in which multiple samples could be taken during pregnancy from different ethnic groups. Firstly, the validity of collecting self-taken swabs, enabling easier patient recruitment, is now well-established (Strauss *et al.* 2005; Srinivasan *et al.* 2010) and secondly the ready availability of molecular methods for the in-depth analysis of samples at relatively low cost. Further research along these lines will allow examination of the effects of ethnic, dietary and other factors on the vaginal microbiota and provide a more robust framework for interventions.

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Conflict of Interest

No conflict of interest declared.

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Table 1 Maternal ethnicity and age, gestational age at time of vaginal swabs A and B, and gestational age and birth weight of live births

	White	Black	Asian	Other	All
Number recruited to study (%)	158 (54)	89 (30)	32 (11)	14 (5)	293 (100)
Maternal age (years)	31.4 (5.8)	28.9 (6.1)	28.3 (4.3)	31.7 (7.0)	30.3 (5.9)
Gestational age swab A (w)	12.5 (2.0)	12.8 (2.0)	13.4 (2.3)	14.4 (2.1)	12.9 (2.2)
Gestational age swab B (w)	19.3 (2.6)	19.8 (3.2)	20.5 (0.8)	20.2 (0.4)	19.7 (2.7)
Gestational age of live births (w)	40.0 (1.9)	39.3 (2.6)	38.8 (2.6)	39.8 (2.8)	39.1 (4.0)
Birth weight of live births (kg)	3.47 (0.50)	3.39 (0.53)	3.05 (0.50)	3.17 (0.55)	3.34 (0.57)

Data are shown as mean (SD) unless otherwise indicated

Table 2 Lactobacilli in women who provided swabs A and B

	White	Black	Asian	Other	Total	p-value *
Women with swab A	158	89	32	14	293	
Any lactobacillus in swab A	128 (81)	56 (63)	24 (75)	12 (86)	220 (75)	0.014
Women with swabs A and B	108	64	21	10	203	
Any lactobacillus in swab A	84 (78)	41 (64)	16 (76)	9 (90)	150 (74)	0.165
Any lactobacillus in swab B	89 (82)	39 (61)	13 (62)	10 (100)	151 (74)	0.002
L. jensenii						
Sample A	41 (49)	21 (51)	3 (19)	2 (22)	67 (45)	0.058
Sample B	36 (40)	18 (46)	1 (8)	2 (20)	57 (38)	0.051
L. crispatus						
Sample A	37 (44)	10 (24)	3 (19)	5 (56)	55 (37)	0.042
Sample B	40 (45)	10 (26)	2 (15)	3 (30)	55 (36)	0.062
L. gasseri						
Sample A	32 (38)	8 (20)	8 (50)	3 (33)	51 (34)	0.098
Sample B	34 (38)	9 (23)	7 (54)	3 (30)	53 (35)	0.174
L. vaginalis						
Sample A	14 (17)	9 (22)	3 (19)	0 (0)	26 (17)	0.514
Sample B	19 (21)	8 (21)	1 (8)	1 (10)	29 (19)	0.717
Other lactobacilli						
Sample A	14 (17)	9 (22)	6 (38)	1 (11)	30 (20)	0.307
Sample B	17 (19)	10 (26)	5 (38)	3 (30)	35 (23)	0.559

Data are shown as number (%). * chi-square test for types of lactobacilli; Fisher's exact test for individual 4 (ethnicity) x 2 (yes/no) tables for each row.

Table 3 H_2O_2 production by Lactobacilli isolated from swab A

Lactobacillus species (isolates tested)	H_2O_2 production * (mg l^{-1})	
	Median	Interquartile range
L. jensenii (175)	10 - 30	3 – 10 to 30 – 100
L. crispatus (177)	1 - 3	0 – 1 to 3 – 10
L. gasseri (177)	1 - 3	1 – 3 to 3 – 10
L. vaginalis (68)	1 - 3	1 – 3 to 3 – 10
Other strain (115)	0 - 1	0 – 1 to 1 – 3

* There was significant interspecies variation in H_2O_2 production ($p = 0.0001$, Kruskall-Wallis test).

Table 4 Gain and loss of lactobacilli between swabs A and B

Ethnicity	White	Black	Asian	Other	Total	
Total number of women	**108**	**64**	**21**	**10**	**203**	
Gain of lactobacilli						**p-value ***
Any lactobacillus species	45 (41.7)	15 (23.4)	7 (33.3)	4 (40.0)	71 (35.0)	0.111
L. jensenii	7 (10.5)	1 (2.3)	0 (0.0)	1 (12.5)	9 (6.6)	0.204
L. crispatus	11 (15.5)	2 (3.7)	0 (0.0)	0 (0.0)	13 (8.8)	0.071
L. gasseri	12 (15.8)	4 (7.1)	2 (15.4)	0 (0.0)	18 (11.8)	0.336
L. vaginalis	12 (12.8)	3 (5.5)	1 (5.6)	1 (10.0)	17 (9.6)	0.473
Any other lactobacillus species	12 (11.1)	5 (7.8)	4 (19.1)	2 (20)	23 (11.3)	0.70
Loss of lactobacilli						
Any lactobacillus species	38 (45.2)	14 (34.2)	13 (81.3)	3 (33.3)	68 (45.3)	0.012
L. jensenii	12 (29.3)	4 (19.1)	2 (66.7)	1 (50.0)	19 (28.4)	0.316
L. crispatus	8 (21.6)	2 (20.0)	1 (33.3)	2 (40.0)	13 (23.6)	0.788
L. gasseri	10 (31.3)	3 (37.5)	3 (37.5)	0 (0.0)	16 (31.4)	0.648
L. vaginalis	7 (50.0)	4 (44.4)	3 (100.0)	0 (0.0)	14 (53.9)	0.226
Any other lactobacillus species	10 (71.4)	4 (44.4)	5 (83.3)	0 (0.0)	19 (63.3)	0.209

Data are shown as number of women (%).* Fisher's exact test for individual 4 (ethnicity) x 2 (yes/no) tables for each row.