

Comparison of aerobic conjunctival bacterial flora in pregnant, reproductive –aged and postmenopausal women

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Abstract

• **AIM:** To evaluate the effect of hormonal status on aerobic conjunctival flora in women.

• **METHODS:** One hundred fifty-eight women [reproductive-aged ($n=55$), pregnant ($n=51$), and postmenopausal ($n=52$)] who admitted to outpatient clinic of Obstetrics and Gynecology Department of Denizli State Hospital were enrolled. Age, body-mass index (BMI), obstetric history, cigarette smoking, drug usage, presence of systemic disease, and intraocular pressure (IOP) were recorded for each patient. The samples were taken from the lower fornix with two culture swabs and directly incubated in culture containing 5% sheep blood, eosin-methylene blue and chocolate agar. The other swab specimen was Gram stained. All growths and microscopic results were analyzed.

• **RESULTS:** The coagulase-negative *Staphylococcus* was the predominant organism isolated in the conjunctival samples in both three groups. The aerobic microorganism growth rate for all isolated aerobic organisms revealed no significant change in the three groups ($P>0.05$). The conjunctival culture positivity rates were similar in the three groups (49% in reproductive-aged, 57% in pregnant and 58% in postmenopausal women) ($P>0.05$). Age, IOP, BMI, gravidity, parity, cigarette smoking, drug usage, and presence of systemic diseases did not have an effect on culture positivity

in three groups.

• **CONCLUSION:** Results of this study showed that conjunctival aerobic flora and bacterial colonization did not differ between reproductive-aged, pregnant and postmenopausal women.

• **KEYWORDS:** aerobic conjunctival flora; coagulase-negative *Staphylococcus*; menopause; pregnancy; reproductive-aged
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INTRODUCTION

The normal flora of conjunctiva constitutes a reservoir for microorganisms. Various factors alter the conjunctival flora such as, seasonal variations, temperature, host age, environmental exposure, ocular trauma, surgical procedures, dry eye, contact lens usage, immunocompromising diseases, and general hygienic conditions^[1,2]. Periodontal microbial flora is known to be affected by hormonal status in pregnant women^[3,4]. Positive correlations between estradiol, progesterone levels and overgrowth of *Prevotella intermedia*, *Porphyromonas gingivalis*, *Tannerella forsythia* and *Campylobacter rectus* were reported^[3,7].

As pregnancy is accompanied by an increase in the production of estrogen and progesterone, changes in the composition of the subgingival microflora may be due to significantly higher estradiol concentrations in pregnant women compared to that of non-pregnant women^[8,9]. Nonetheless, to the best of our knowledge, there is no study in the literature investigating the aerobic conjunctival flora variation with hormonal or pregnancy status of women. Therefore, the aim of this study was to determine whether conjunctival bacterial flora differ between reproductive-aged, pregnant and postmenopausal women.

SUBJECTS AND METHODS

Subjects This single-blind cross-sectional study was carried out between January 2010 and July 2010. The study

Table 1 The demographic characteristics of patients in three groups including reproductive-aged, pregnant, and postmenopausal women

	Reproductive-aged women (n=55)	Pregnant women (n=51)	Postmenopausal women (n=52)	P
Age (a)	32.9±8.2 (18-46)	25.2±4.6 (17-38)	49.8±6.0 (39-68)	² 0.000 (Mena>Repr >Preg)
The intraocular pressure(mmHg)	13.1±2.6 (9-20)	11.8±2.6 (7-22)	13.3±2.7 (8-20)	² 0.01 (Preg<Repr=Mena)
BMI ¹ (kg/m ²)	24.3±4.3 (16.4-41.9)	25.2±3.8 (18.7-35.9)	29.7±4.8 (20.9-44.7)	² 0.000 (Mena> Repr =Preg)
Gestational week (weeks)	—	19.5±8.7 (5-40)	—	-
Duration of menopause (a)	—	—	3.4±2.9 (1-16)	-
Gravidity (n)	1.6±1.2 (0-4)	1.9±0.9 (1-4)	3.5±1.4 (1-8)	² 0.000 (Mena> Repr =Preg)
Parity (n)	1.3±0.9 (0-3)	0.8±0.7 (0-3)	2.8±1.0 (1-6)	² 0.000 (Mena>Repr >Preg)
Microorganism growth rate, [n(%)]	27(49.1)	29(56.9)	30(57.7)	³ 0.61

Values are presented as mean±SD (minimum-maximum range); ¹BMI = Body mass index; ²One-Way ANOVA and Tukey HSD; ³Chi-squared (χ^2) test; "Mena" means postmenopausal women; "Repr" means reproductive-aged women; "Preg" means pregnant women.

population consisted of 158 women [reproductive-aged ($n=55$), pregnant ($n=51$), and postmenopausal ($n=52$)] who were admitted to the outpatient clinic of the Obstetrics and Gynecology Department of Denizli State Hospital. The study protocol which adhered to tenets of the Declaration of Helsinki was approved by the local ethics committee and an informed consent was obtained from each participant.

A complete ophthalmologic examination was performed by the same ophthalmologist in all patients in order to exclude clinical abnormality before sample collection. Patients wearing contact lens, currently taking topical or systemic antibiotics, with corneal or ocular anomalies such as dry eye, inflammation, untreated meibomianitis, and blepharitis or active ocular infection were excluded from the study. Age, body-mass index (BMI), obstetric history, cigarette smoking, drug usage, presence of systemic disease, and intraocular pressure (IOP) were recorded for each patient.

Methods Duplicate conjunctival swabs were taken without anesthesia by inserting the moistened swab into the lower conjunctival fornix at the nasal margin and rotating it along the fornix to the temporal margin. In reproductive-aged women, conjunctival swabs were taken at the follicular phase of their menstrual cycles. Care was taken to avoid the lashes. Samples were inoculated onto 5% sheep blood, eosin-methylene blue and chocolate agar plates. All culture media were incubated at 37°C in 5%-10% carbon dioxide for 24-72 hours and held for a week. The second swab specimen was Gram stained to examine not only the presence of polymorphonuclear leucocytes and gram-positive or gram-negative bacteria, but the correlation with growth of aerobic bacteria as well. All growths and

microscopic results were analyzed with standard microbiological methods at the Denizli State Hospital Microbiology Culture Laboratory.

Power calculation determined that 52 patients were required in the study to detect a 0.25 difference between the three study groups, assuming a positive culture rate of 0.80 or greater at baseline, power of 0.80 ($\beta=0.20$) and α -error of 0.05. Fifty five reproductive-aged, 51 pregnant, and 52 postmenopausal women were reached in this study.

Statistical Analysis Analysis was performed using the IBM SPSS version 19.0 software (SPSS Inc. Chicago, IL, USA). One-way ANOVA and Student's test were used for statistical analysis of patient's quantitative variables. Categorical variables were compared between groups using the Chi-squared (χ^2) test. The Fisher exact test was used when low expected frequencies were detected. A logistic regression model was formulated, and age, IOP, BMI, gravidity, parity, cigarette smoking, drug usage, and presence of systemic disease were included as co-variables in the model. $P \leq 0.05$ was considered statistically significant.

RESULTS

There were statistically significant differences between the three groups with respect to age, IOP, BMI, gravidity, and parity ($P < 0.05$). Age, BMI, gravidity, and parity were significantly higher in postmenopausal women while IOP was significantly lower in pregnant women than the other groups. There was no statistically significant difference in aerobic microorganism growth rate between the three groups ($P > 0.05$, Table 1).

The coagulase-negative *Staphylococcus* (CNS) was the most frequently isolated aerobic bacterium in reproductive-aged

Table 2 Species of bacteria isolated from the conjunctiva

	Reproductive-aged women (n=55)	Pregnant women (n=51)	Postmenopausal women (n=52)	¹ P
CNS	23 (41.8)	14 (27.5)	12 (23.1)	0.09
<i>Staphylococcus aureus</i>	3 (5.5)	3 (5.9)	7 (13.5)	0.24
<i>Corynebacterium</i> species	1(1.8)	6 (11.8)	4 (7.7)	0.13
α -Hemolytic streptococcus	—	2 (3.9)	3 (5.8)	0.22
Gram-negative basil	—	1 (2.0)	1 (1.9)	0.58
Gram-negative coccobasil	—	2 (3.9)	3 (5.8)	0.22
Gram-positive micrococcus	—	1 (2.0)	—	-
No bacteria isolated	28 (50.9)	22 (43.1)	22 (42.3)	0.38

¹Chi-squared (χ^2) test; CNS: coagulase-negative Staphylococcus; Values are given as number (percentage).

Table 3 Comparison of conjunctival culture results in each group with respect to cigarette smoking, presence of systemic disease and drug usage

Women	Reproductive-aged (n=55,%)		P	Pregnant (n=51,%)		P	Postmenopausal (n=52,%)		P	Total (n=158,%)		P
	Conjunctival culture			Conjunctival culture			Conjunctival culture			Conjunctival culture		
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative				
Cigarette smoking	9 (64.3)	5 (35.7)	² 0.23	1 (50.0)	1 (50.0)	² 1.00	6 (54.5)	5 (45.5)	¹ 0.81	16 (59.3)	11 (40.7)	¹ 0.58
Systemic disease	3 (27.3)	8 (72.7)	¹ 0.18	5 (83.3)	1 (16.7)	² 0.22	13 (59.1)	9 (40.9)	¹ 1.00	21 (53.8)	18 (46.2)	¹ 0.93
Drug usage	3 (30.0)	7 (70.0)	² 0.29	3 (75.0)	1 (25.0)	² 0.62	14 (60.9)	9 (39.1)	¹ 0.68	20 (54.1)	17 (45.9)	¹ 0.96

¹Chi-squared (χ^2) test; ²Fisher exact test; Values are given as number (percentage).

(42%), pregnant (28%) and postmenopausal women (23%). Although it was higher in reproductive-aged women compared to the other groups, there was no statistically significant difference between the three groups. *Staphylococcus aureus* was the second most commonly isolated bacterial species in reproductive-aged (6%), and postmenopausal women (14%), whereas *Corynebacterium* species was the second most commonly isolated bacterial species in pregnant women (12%). Among all aerobic bacterial isolates, no statistically difference in bacterial colonization was detected between the three groups ($P > 0.05$). Furthermore the percentage of sterile eyes (with no growth of aerobic bacteria from the conjunctiva) was similar in three groups (51% in reproductive-aged, 43% in pregnant and 42% in postmenopausal women) ($P > 0.05$, Table 2). There was no significant difference in conjunctival culture positivity between the three groups subjects who smoked cigarettes, used drugs, and with systemic disease, and those not smoking cigarettes or using drugs, and without systemic disease ($P > 0.05$, Table 3).

Age, IOP, BMI, gravidity, parity, cigarette smoking, drug usage, and presence of systemic disease were found to have no effect on culture positivity in the three groups when evaluated by the logistic regression model.

DISCUSSION

Conjunctival bacterial flora develops after birth and varies throughout life [10,11]. In the literature, the relations between the microbial flora of the conjunctiva and eyelids and postoperative infection have been revealed [12-14]. In light of

this study, a better understanding of the changes in conjunctival bacterial flora will enrich our methods of approach to eye infections and will be useful in interpreting culture results from suspected cases of bacterial eye infections to determine more efficient diagnosis, prophylaxis and treatment strategies [15-18].

As reported in various studies regarding normal conjunctival flora, coagulase-negative *Staphylococcus*, *Staphylococcus aureus*, and *Corynebacterium* species are the most commonly isolated ocular flora microorganisms in the conjunctiva [2,19-23]. α -hemolytic *Streptococcus* and other gram positive rods are also isolated from normal conjunctival flora frequently [24-26]. Other organisms, such as anaerobes and fungi, can be isolated in small numbers [27,28]. *Staphylococcus epidermidis* has usually been considered to contribute to normal skin flora [29]. Furthermore an important source of conjunctival flora is thought to be the skin and upper respiratory tract with the majority being gram-positive bacteria [30-33]. Concordant to the studies above, in this study, the most common aerobic microorganisms isolated was CNS in all groups.

The second most commonly isolated bacterial species was *Staphylococcus aureus* in reproductive-aged and postmenopausal women, and *Corynebacterium* in pregnant women, however. A plausible explanation for this, *Corynebacterium* species is usually labile [19], and thus may be more sensitive to the immune system which may limit the number of positive cultures in the reproductive-aged and postmenopausal women. During pregnancy, maternal

Table 4 The demographic characteristics of patients and microorganisms commonly isolated from conjunctival flora (a summary of previous literature)

	de Kaspar <i>et al</i> ^[19]	Martins <i>et al</i> ^[20]	Gunduz <i>et al</i> ^[21]	Kusbeci <i>et al</i> ^[22]	Sen <i>et al</i> ^[23]	Current Study
Patient number-related disease	81-with glaucoma 258- with cataracts	103-with diabetes mellitus 60-control	50-with behçet's disease 50-normal	106-with parkinson's disease 102-control	81-with glaucoma 67-control	55-reproductive-aged 51-pregnant 52-postmenopausal 33- ² 25- ⁵ 50
Mean age (years)	n/a	¹ 267	¹ 236	¹ 63- ² 61	¹ 57- ² 55	³ 33- ⁴ 25- ⁵ 50
Female/Male Ratio	n/a	¹ 55/45	¹ 32/18- ² 31/19 ^b	¹ 56/50- ² 50/52	¹ 27/54- ² 21/46	n/a
Aerobic/Anaerobic bacteria	Aerobic/Anaerobic	Aerobic	Aerobic	Aerobic/Anaerobic	Aerobic	Aerobic
Fungus	n/a	n/a	+	+	n/a	n/a
Culture media	Sheep blood, chocolate, blood culture broth	Sheep blood, BHI	Sheep blood, EMB, chocolate, BHI, Sabaroud dextrose	Sheep blood, chocolate, BHI, Sabaroud dextrose	Sheep blood, EMB, chocolate	Sheep blood, EMB, chocolate
Swab type	Moistened	Moistened	Moistened	Dry	Moistened	Moistened
Most commonly isolated bacteria (%)	CNS ¹ 52- ² 65	CNS ¹ 84- ² 62	CNS ¹ 64- ² 48	CNS ¹ 42- ² 48	CNS ¹ 30- ² 30	CNS ³ 42- ⁴ 28- ⁵ 23
Second commonly isolated bacteria (%)	<i>Propionibacterium acnes</i> ¹ 33- ² 22	<i>Corynebacterium</i> spp ¹ 26- ² 38	<i>S. aureus</i> ¹ 24- ² 4	<i>S. aureus</i> ¹ 30- ² 10	<i>S. aureus</i> ¹ 7- ² 3	<i>S. aureus</i> ³ 6- ⁴ 6- ⁵ 14
Third commonly isolated bacteria (%)	<i>Corynebacterium</i> spp ¹ 14- ² 36	<i>S. aureus</i> ¹ 15- ² 12	<i>Moraxella</i> spp ¹ 16- ² 4	<i>Corynebacterium</i> spp ¹ 24- ² 15	<i>Corynebacterium</i> spp ¹ 3- ² 5	<i>Corynebacterium</i> spp ³ 2- ⁴ 12- ⁵ 8
Fourth commonly isolated bacteria (%)	<i>S. aureus</i> ¹ 7- ² 5	<i>Streptococcus</i> ¹ 2- ² 0	<i>Streptococcus</i> spp ¹ 16- ² 2	α -Hemolytic <i>Streptococcus</i> ¹ 15- ² 11	Gram-negative rods ¹ 4- ² 0	α -Hemolytic <i>Streptococcus</i> ³ 0- ⁴ 4- ⁵ 6
Fungus	n/a	n/a	<i>Candida</i> spp ¹ 6- ² 2	n/a	n/a	n/a
Bacteria growth rate (%)	¹ 77- ² 83	¹ 94- ² 73	¹ 92- ² 58	¹ 81- ² 72	¹ 46- ² 37	³ 49- ⁴ 57- ⁵ 58

n/a: not available; DM: diabetes mellitus; ¹: indicated as study group; ²: indicated as controls; ³: indicated as reproductive-aged group; ⁴: indicated as pregnant group; and ⁵: indicated as postmenopausal group; CNS: Coagulase-negative Staphylococcus; BHI: brain heart infusion agar; EMB: eosin-methylene blue agar.

immune reaction reduces on the whole since increased levels of progesterone induces a blocking factor that either deviates the T-helper cell response towards the production of T-helper type 2 (TH2) cytokines or up-regulates a number of other immunologically active molecules ^[34].

In the case of postmenopausal women, a decrease in reproductive hormones level is known to reduce Goblet cell number and tear production in the conjunctiva ^[35]. Goblet cell number is also reported to affect by menstrual cycle, especially around the time of ovulation ^[36]. A decreased density of Goblet cells in conjunctiva was, quite predictably, associated with an increase in bacterial microflora ^[37]. It was also linked to increased susceptibility to dry eye syndrome among post-menopausal women ^[35,37]. On the other hand, there was no statistical difference in the percentage of aerobic culture-positive eyes of reproductive-aged (49.1%), pregnant (56.9%) and postmenopausal women (57.7%) in this study. The prevalence of positive aerobic cultures is in agreement with other conjunctival flora studies in patients with diabetes (94.1%) ^[20,38], Behçet's disease (92%) ^[21], Parkinson's disease (81.1%) ^[22], cataract (83%) ^[19], glaucoma (45.7%) ^[23], patients undergoing intravitreal injections (48%) ^[39], and patients before lens wear (34.5%) ^[40]. Moreover, the percentage of positive aerobic cultures may decrease to 30%^[41-43]. The demographic characteristics of patients including age, gender, and pathology, aerobic or anaerobic bacteria and culture media are also different in the previous literature as summarized in Table 4.

In our previous study, it was determined that the duration of antiglaucomatous medication, and number of medications used, age, gender, presence of diabetes and asthma did not affect culture positivity in patients with glaucoma and healthy controls ^[23]; similarly, age, IOP, BMI, gravidity, parity, cigarette smoking, drug usage, and the presence of

systemic disease did not have any effect on culture positivity in reproductive-aged, pregnant and postmenopausal women in our present study.

Conjunctival flora's association with systemic diseases varies from one study to another. Kusbeci *et al* ^[22] demonstrated that there is no statistically significant difference in the percentage of culture-positive eyes among patients with Parkinson's disease (PD) and healthy subjects. They also reported that, eye blink rate reduced, but did not affect the conjunctival flora of the patients with PD ^[22]. On the other hand, Martins *et al* ^[20] showed that the frequency of positive conjunctival cultures in patients with diabetes was significantly higher than non-diabetic subjects. The high prevalence of organisms in the conjunctival flora of diabetic patients was associated with the immunologic alterations described in this population ^[20]. Although no statistical evaluation was stated, Gunduz *et al* ^[21] detected that bacterial growth percentage was 92% and 58% in the Behçet group and in the age- and sex-matched healthy subjects, respectively. Furthermore, it was determined that conjunctival cultures were not changed by using topical glaucoma medications or undergoing glaucoma surgery ^[19,23]. It should be noted that all these studies included different amount of patients and subjects with different diseases.

The ocular surface microbiota has commonly been established through conventional culture techniques as in the present study. On the other hand, a much greater diversity of bacteria could be detected by using molecular fingerprinting methods and sequence analysis of cloned microbial small subunit ribosomal RNA genes [16S ribosomal DNA (rDNA)] in parallel samples ^[37,44-47]. So that, several additional atypical ocular bacteria including *Rhodococcus erythropolis*, *Klebsiella oxytoca*, and *Erwinia* sp, and many others were commonly identified on both inflamed and on the normal

conjunctiva^[37,44]. In addition, the medium used in culture based methods is important for the detection of bacteria. In a study evaluating microbial contamination during cataract surgery, of the 58 positive cultures, 50 were obtained using enrichment culture techniques and enrichment media have been reported to be valuable with a much higher sensitivity to identify bacteria^[48]. In another study, evaluating of ocular bacterial flora with two different culture media including brain hearth infusion broth and blood agar plate, both types of media have been found to raise the chance of bacterial recovery²⁰. Despite the possibility of increasing relatively low culture positive rate (approximately 50%) in our results, we did not prefer the use of liquid medium due to the possibility of contamination during the inoculation process^[49] and being impractical in outpatient. We also used the standard culture methods as a result of a lack of technical equipment.

There are several limitations in the current study that should be noted. First, this study does not normalize variables including IOP, BMI, gravidity and parity among the groups. Because our study groups are not in the same period, normalization of these groups is very difficult. As expected, BMI, gravidity, and parity increases during pregnancy and with increasing age and IOP reduces during pregnancy as a result of the effect of progesterone. In addition, not evaluating certain bacterial prevalence by using culture-independent molecular methods, neglecting to isolate or identify Chlamydia, viruses or anaerobic organisms, and failing to measure the sexual hormones levels in tear fluids to compare them with conjunctival culture results due to the absence of necessary equipment are the other potential limitations of the current study. Furthermore, it is particularly true in the light of the latest findings by Human Microbiome Project that a major proportion of normal human microflora is uncultivable in standard media conditions because of their dependence on unique microenvironment^[45-47,50]. The use of culture-positivity criteria instead of certain bacterial prevalence is also perplexing, since the latter criteria is typically correlated with environmental changes, while culture-positivity merely reflects relative abundance of bacterial groups capable of growing on limited number of media utilized in this study. So culture-positivity of conjunctival swabs is rather vague parameter for comparative analysis, particularly for the study where normal subjects with different hormonal levels are being compared. This parameter is more appropriate in healthy *vs.* diseased type of comparisons. Finally, an inadequate number of organisms isolated other than CNS, *Staphylococcus aureus* and *Corynebacterium* species has prevented us to reach clinically significant conclusions about these microorganisms growth rate in all three groups.

In conclusion, results of this study show as the first time in the literature that the bacterium most frequently found in the conjunctival flora of the reproductive-aged, pregnant and postmenopausal women was CNS. Moreover, no statistically significant difference was detected between the three groups regarding all aerobic bacterial colonization. Further studies, in which culture-based methods, such as microscopy/direct counting of labeled bacterial cells in swab content are combined with PCR/sequencing-based detection and identification with culture-independent approaches, are needed in order to increase the sensitivity and scientific value of studies on the conjunctival microbiota.

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