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FULL PAPER

Nasopharingeal bacterial and fungal colonization in HIV-positive *versus* HIV-negative adults

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SUMMARY

Objectives: To compare mucosal flora in HIV-positive and HIV-negative subjects, to assess chemosusceptibility patterns of carriage isolates and to evaluate possible predisposing factors within the two groups.
Methods: We analyzed microbes isolated from nasopharyngeal swabs in virologically suppressed and immunologically stable HIV-positive adult outpatients (n=105) at baseline and after 12 months and in an age-matched cohort of HIV-negative outpatients (n=100) at baseline. Bacteria and Candida spp strains were isolated and identified through standard biochemical assays and chemosusceptibility tests were performed. Multi Locus Sequence Typing was also determined to characterize Staphylococcus aureus isolates from HIV-infected persistent carriers.
Results: In HIV-positive patients a significantly higher rate of colonization by *S. aureus* as compared to HIV-negative controls was observed (19% *vs* 8%, p=0.02), with a relevant percentage of penicillin resistant strains (15% *vs* 0, p=0.24). Methicillin resistant strains were recovered only from HIV-positive subjects. Overall HIV-positive status was the only predictor of *S. aureus* colonization (OR 2.77, 95% CI 1.03;7.41, p=0.04).
Conclusions: The nasopharyngeal bacterial flora differs between HIV-positive and HIV-negative subjects and appears relevant for possible development of staphylococcal infections in HIV-positive patients.

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INTRODUCTION

In HIV-infected patients, the colonization of nasopharyngeal mucosae is a critical event in the pathogenesis of invasive infections from both bacterial and fungal agents: after mucosal adherence, invasion can occur and bacteremia or other threatening invasive infections can develop (Lee *et al.*, 2016; Yehia *et al.*, 2011; Wu *et al.*, 2017; Jordano Q *et al.*, 2004; Menezes Rde *et al.*, 2015). Specifically, *Staphyloccocus aureus* is one of the most frequent bacterial agents colonizing the general population, but risk of nasal carriage is known to be higher in HIV-positive subjects. Individual nasopharingeal flora can influence carriage rate, acting, e.g., as a competitor with the main potential bacterial pathogens. Moreover, antibiotic pressure or health-care related expositions can affect carriage rates and increase the risk of acquiring multiresistant strains (Que and Moreillon, 2015).
As a consequence, characterization of the nasopharyngeal flora, with particular emphasis on the assessment of the chemosusceptibility pattern of these isolates, represents a pivotal step for developing prophylactic measures. This study analyzed nasopharyngeal colonizing microbes in a cohort of virologically suppressed and immunologically stable HIV-positive outpatients *versus* aged-matched HIV-negative adults and aims specifically to evaluate the factors associated with *S. aureus* colonization.

Key words:
HIV, colonization, microbial epidemiology, *Staphylococcus aureus*.

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MATERIALS AND METHODS

Patients included in the study were enrolled in an interventional study to evaluate immunological response in HIV patients immunized with different anti-pneumococcal vaccines (ClinicalTrials.gov identifier: NCT02123433; EudraCT number 2011-004518-40) (Lombardi *et al.*, 2016).
Briefly, study participants were ≥18 years-old, HIV-1 infected outpatients with CD4 cell counts ≥200 cells/μL. Main exclusion criteria were: age >65 years, non-HIV related immunosuppression, current immunomodulatory therapy, previous antipneumococcal vaccination, ongoing acute infectious disease, current or recent antimicrobial use (<1 weeks).
An age-matched group of HIV-negative outpatients with the same exclusion criteria (n=100) was also recruited in parallel as controls.
Previous antimicrobial therapy and/or prophylaxis with trimethoprim-sulfamethoxazole was also evaluated in the HIV-positive population.
A nasopharyngeal swab was obtained for each subject at baseline (T0) and for HIV-positive patients also after 48 weeks (T48) in order to detect persistent carriage states and a possible variation of chemosusceptibility patterns of isolates. Colonization was defined as a positive naso-

pharyngeal swab in the absence of signs and symptoms of bacterial or fungal infection. Swabs were serially streaked on selective media (Oxoid, Basingstoke, UK) and, after incubation, colonies were isolated and identified by MALDI-TOF spectrometry (Vitek MS, bioMérieux, Marcy l'Etoile, France). Susceptibility to antibiotics was tested according to Clinical and Laboratory Standards Institute (CLSI) guidelines. For *Staphylococcus aureus* cefoxitin

Table 1 - *Characteristics of the study population (n=205).*

	HIV+ (n=105)	*HIV- (n=100)*	*p*
Male, n (%)	77 (73.3)	52 (52.0)	**0.002**
Age, mean (SD)	44.7 (9.7)	42.3 (13.2)	0.136
Caucasian, n (%)	97 (92.4)	93 (93.0)	0.865
Body mass index (Kg/m^2), mean (SD)	23.7 (3.9)	25.1 (5.4)	**0.043**
Cohabitation with children, n (%)	16 (15.2)	33 (33)	**0.003**
Age of cohabiting children, n (%)			0.121
<3 years	2 (12.5)	11 (33.3)	
4-16 years	14 (87.5)	22 (66.7)	
COPD, n (%)	2 (1.9)	0 (0)	0.165
Asthma, n (%)	1(0.9)	0(0)	0.328
Splenectomy, n (%)	0 (0)	1 (1)	0.304
CRF, n (%)	1(0.9)	0(0)	0.328
Diabetes, n (%)	3 (2.9)	1 (1)	0.337
Cirrhosis, n (%)	1(0.9)	1(1)	0.972
Cardiovascular disease , n (%)	8 (7.6)	13 (13)	0.204
Neoplasm, n (%)	1(0.9)	5 (5)	0.086
Hepatopathy, n (%)	16 (15.2)	39 (39)	**0.001**
Recent Hospitalization, n (%) (last 12 months)	6 (5.7)	8 (8)	0.517
Smokers*, n (%)	65 (61.9)	30 (30)	**<0.001**
Alcohol consumers**, n (%)	11 (10.5)	21 (21)	**0.038**
IDU attivo, n (%)	4 (3.8)	0 (0)	**0.048**
Years from HIV diagnosis, median (IQR)	11.0 (4.8-18.0)		
Years from HAART initiation, median (IQR)	7.5 (2.7-14.7)		
Risk factor, n (%)			
Heterosexual	37 (35.2)		
Homo/bisexual	43 (41.0)		
Injecting drug use	17 (16.2)		
Other /Unknown	8 (7.6)		
Nadir CD4 count cell/µL, mean (SD)	205 (164)		
CD4 count BL, mean (SD) cell/µL	614 (265)		
CD4 cell count category BL, n (%)			
200-350	14 (13.3)		
351-500	28 (26.7)		
>500	63 (60.0)		
Viral load ≤50 copies/µL BL, n (%)	93 (88.6)		
On HAART, n (%)	104 (99)		
Antiretroviral regimen, n (%)			
PI-based	57 (54.3)		
NNRTI-based	20 (19.0)		
INSTI	15 (14.3)		
HCV co-infection	17 (16.2)		
Past AIDS-defining events, n (%)	29 (27.6)		
CD4 cells count at T48, mean (SD) cell/µL	626 (271)		
HIVRNA ≤50 copies/µL at T48, n (%)	100 (95.2)		
CD4 cell count category T48, n (%)			
200-350	13 (12.4)		
351-500	22 (21.0)		
>500	70 (66.7)		

*>= 100 cigarettes/year
**>= 10 Alcoholic Unit/week (Alcoholic Unit: ethanol 12 gr)
Legend: COPD: Chronic o; CRF: chronic renal failure, IDU: Injection Drug Users; HAART: Highly Active Antiretroviral Therapybstructive Pulmonary Disease; PI: Protease Inhibitors; NNRTI: Non Nucleosidic Reverse Transcriptase Inhibitors; INSTI: Integrase strand transfer inhibitors

was used for detection of Methicillin-Resistant *Staphylococcus aureus* (MRSA). All agents were tested as suggested by CLSI guidelines with Kirby-Bauer disk diffusion test, D-test for highlighting inducible clindamycin resistance, or Minimum Inhibitory Concentration (MIC) tests when needed. In order to assess phylogenic relationship between *Staphylococcus aureus* isolates from T0 and T48 swabs, MLST was performed according to the MLST website protocol (http://saureus.mlst.net/, last access: 15 July 2014). PCR products were purified with High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany) and sequenced with prepaid plates through an external facility (Eurofins-MWG Operon, Martinsried, Germany). Patients were defined as persistent carriers if the same sequence type (ST) was obtained from isolates collected at T0 and at T48.

Streptococcus pneumoniae penicillin susceptibility tests were interpreted in accordance with CLSI breakpoints (CLSI 2014). Culture for Gram negative strains was performed in order to highlight colonization by multidrug resistant strains, such as carbapenemase-producing *Klebsiella pneumoniae*. Carbapenemase-producing strains were identified by phenotypic methods as recommended by EUCAST using disks containing meropenem +/- boronic acid for class A carbapenemases, and meropenem +/- dipicolinic acid for class B carbapenemases (EUCAST 2013) Chemosusceptibility of *Candida spp.* strains was analyzed with a commercial broth microdilution test (SensiTitre Yeast One, Thermo Fischer Scientific, Waltham, MA, USA) and the results interpreted according to CLSI guidelines (CLSI 2014). Echinocandin susceptibilities were confirmed with standard MIC tests (Liofilchem, Roseto degli Abruzzi, Italy) on Roswell Park Memorial Institute medium agar, then interpreted according to CLSI indications.

Data were analyzed with IBM-SPSS Statistics version 22.0 (IBMCorp., Armonk, NY, USA). Categorical variables were compared using a chi-square test or a Fisher's exact test, as appropriate. Factors associated with colonization by S. aureus at baseline were investigated by logistic regression analysis. P-levels <0.05 were considered good evidence against the null hypothesis.

RESULTS

Between November 2011 and April 2012, a total of 205 nasopharyngeal swabs were obtained from HIV-positive patients (n=105) and HIV-negative patients (n=100) at baseline. Of those from the HIV-positive group, 102 subjects repeated the swab at T48.

Baseline main characteristics were homogeneous between HIV-positive and HIV-negative persons, except for gender, BMI, drug use, cohabitation with children, HBV-related liver disease, cigarette smoking status and alcohol intake (*Table 1*).

Table 2a - *Microbial strains isolated from enrolled HIV+ and HIV- subjects.*

Isolate	*Baseline (n; %)*		*p*	*T48 (n; %)*
	HIV+ (n=105)	*HIV- (n=100)*		*HIV+ (n=102)*
Gram +	23 (21.9)	15 (15)	0.274	22 (21.6)
S. aureus	20 (19.0)	8 (8.0)	**0.021**	20(19.6)
β-haemolytic Streptococci	1 (0.9)	3 (3.0)	0.289	1 (1.0)
S. pyogenes	0	0		1 (1.0)
Group C *Streptococcus*	0	1 (1.0)	0.304	0
Group G *Streptococcus*	1 (0.9)	0	0.328	0
Group F *Streptococcus*	0	1 (1.0)	0.304	0
Streptococcus intermedius	0	1 (1.0)	0.304	0
Streptococcus pneumoniae	1 (0.9)	1 (1.0)	0.972	0
Gram –	3 (2.8)	4 (4.0)	0.652	8 (7.8)
Klebsiella pneumoniae	1 (0.9)	1 (1.0)	0.972	1 (1.0)
Klebsiella oxytoca	0	0		1 (1.0)
Escherichia coli	0	0		1 (1.0)
Pseudomonas aeruginosa	0	0		1 (1.0)
Enterobacter cloacae	1 (0.9)	0	0.328	0
Enterobacter aerogenes	0	1 (1.0)	0.304	0
Serratia marcescens	0	1 (1.0)	0.304	0
Proteus mirabilis	0	1 (1.0)	0.304	0
Raoultella ornytholytica	0	0		1 (1.0)
Leclercia adecarboxylata	0	0		1 (1.0)
Citrobacter freundii	0	0		1 (1.0)
Morganella morganii	0	0		1 (1.0)
Acinetobacter spp.	1 (0.9)	0	0.328	0
Total bacterial isolates	26 (24.8)	19 (19)	0.408	30 (29.4)
Candida spp.	6 (5.7)	4 (4.0)	0.569	8 (7.8)
C. albicans	5 (4.7)	4 (4.0)	0.79	6 (5.8)
C. tropicalis	0	0		1 (1.0)
C. glabrata	1 (0.9)	0	0.328	1 (1.0)
Total bacterial and fungal isolates	**32 (30.5)**	**23 (23)**	**0.293**	**38 (37.25)**

Table 2b - *Chemosusceptibility patterns of S. aureus strains.*

	Resistant strains			
Antibiotic	*Baseline (n, %)*		*p*	*T48 (n, %)*
	HIV+	HIV-		HIV+
Resistant isolates	20	8		20
Penicillin	14 (70.0)	6 (75.0)	0.791	15 (75.0)
Cefoxitin (MRSA)	3 (15.0)	0 (0)	0.246	2 (10.0)
Ciprofloxacin	3 (15.0)	0 (0)	0.246	0 (0)
SXT	0 (0)	0 (0)	/	1 (5.0)
Erythromycin	6 (30.0)	0 (0)	0.081	6 (30.0)
Clindamycin	4 (20.0)	0 (0)	0.171	2 (10.0)
Daptomycin	0 (0)	0 (0)	/	1 (5.0)
MDRSA	4 (20.0)	0 (0)		7(35.0)

Legend: SXT Trimethoprim/Sulfamethoxazole; MRSA Methicillin Resistant *Staphylococcus aureus*; MDRSA Multi Drug Resistant *Staphylococcus aureus*.

At baseline, 30.5% of HIV-positive patients (32/105) and 23% of HIV-negatives (23/100) were colonized by at least one microbial strain, without significant differences between the two groups (*Table 2a*).

The most frequently isolated microbial species was *Staphylococcus aureus*, found in 20 HIV-positive (19%) versus 8 HIV-negatives (8%) patients (p=0.021). *S. aureus* was also found in 20 HIV-positives (19.6%) at T48 (*Table 2b*).

All 20 *S. aureus* isolates from HIV-positive subjects at baseline were resistant to at least one antibiotic: 14 (70%) were Penicillin Resistant *Staphylococcus aureus* (PRSA), 3 (15%) MRSA, 6 (30%) were resistant to erythromycin, with a subset of 4 strains (20%) showing an inducible clindamycin resistance pattern. Four strains out of 20 (20%) were Multi-Drug Resistant (MDRSA), i.e., resistant to at least 3 classes of antibiotics. The 20 *S. aureus* isolates at T48 were resistant to at least one antibiotic: 15 (75%) were PRSA and 2 (10%) MRSA, 6 (30%) were erythromycin resistant and, among these, 2 showed a clindamycin-inducible phenotype. Seven (35%) T48 isolates were MDRSA. After adjustment for gender, smoking status, cohabitation with children, liver disease and alcohol consumption, HIV-positivity remained independently associated with *S. aureus* colonization (adjusted OR 2.77; 95% CI 1.03,7.41; p=0.043) (*Table 3a*).

Among HIV positive patients, at univariate analysis *S. aureus* colonization was associated with an AIDS-defining event in the past (OR 0.11; 95% CI 0.01, 0.84; p=0.034), co-trimoxazole previous use (OR 0.17; 95% CI 0.05, 0.63; p=0.008), age (per 1 year increase OR 0.94; 95% CI 0.89, 0.99; p=0.045) and nadir CD4 cells count (OR 1.004; 95% CI 1.001, 1.007; p=0.013) (*Table 3b*).

All eight *S. aureus* isolates from HIV-negative patients were also resistant to at least one drug. Of these, 6 (75%) were PRSA, while no MRSA or MDRSA were detected. No significant difference was observed between antibiotic susceptibility of *S. aureus* isolates from both groups. Complete data regarding *S. aureus* chemosusceptibility patterns are reported in *Table 2b*.

β-haemolytic *Streptococci* (BHS) were detected in relatively low percentages of HIV-positive patients, both at baseline (0.9%) and T48 (1%), and of HIV-negative controls (3%). *Streptococcus pyogenes* was found in 1 HIV-positive patient at T48, while all the other BHS were scattered among Group C, G and F, or *S. intermedius*, resulting in a completely susceptible bacterial population.

Streptococcus pneumoniae was found in one (0.9%) HIV-positive patient at baseline and in one (1%) HIV-negative, both were fully susceptible to antimicrobials (*Table 2a*).

At baseline, 3 Gram-negative strains were isolated from HIV-positive patients (2.8%) and 4 (4%) from HIV-negatives, of which 1 *K. pneumoniae* from each group. At T48, among 8 (7.8%) Gram-negative strains found in HIV-positive, one *K. pneumoniae* was identified. All isolates were negative for carbapenemase production.

Candida spp. was isolated at baseline in 6 HIV-positive (5.7%) and 4 (4%) HIV-negative cases (p=0.569), and at T48 in 8 (7.8%) HIV-positives. Overall the most frequent species was *C. albicans* (15/18, 83.3%), followed by *C. glabrata* (2/18, 11.1%) *and C. tropicalis* (1/18, 5.5%). *C. albicans* and *C. tropicalis* strains resulted susceptible to all the tested antifungal agents. The two *C. glabrata* strains (from the same patient at baseline and T48) resulted resistant to fluconazole (MIC 128 μg/ml), voriconazole (4 μg/ml) and itraconazole (16 μg/ml), as expected.

A total of 9 distinct profiles were obtained after MLST analysis. In the HIV-positive group, 8 patients were colonized by *S. aureus* both at T0 and T48: 5 (62.5%) were colonized by the same ST at the two sampling times and thus were defined as persistent carriers. Eight out of nine

Table 3a - *Risk factors associated with Staphylococcus aureus colonization in the overall study population.*

Variable	*Unadjusted OR (95% CI)*	*p*	*Adjusted OR (95%CI)*	*p*
HIV Infection	2.706 (1.13, 6.47)	**0.025**	2.769 (1.034, 7.416)	**0.043**
Male gender	1.286 (0.550, 3.006)	0.562	1.161 (0.459, 2.936)	0.753
Caucasian ethnicity	1.030 (0.220, 4.831)	0.971		
Cohabitation with children	1.071 (0.426, 2.696)	0.884	1.437 (0.520, 3.975)	0.485
Cardiovascular disease	1.569 (0.487, 5.057)	0.451		
Neoplasm	1.274 (0.143, 11.328)	0.828		
Hepatopathy	0.656 (0.251, 1.710)	0.388	0.838 (0.307, 2.290)	0.731
Recent Hospitalization	2.783 (0.809, 9.579)	0.105		
Smokers	1.185 (0.534, 2.631)	0.676	0.885 (0.375, 2.091)	0.781
Alcohol consumers	0.612 (0.173, 2.163)	0.446	0.98 (0.214, 2.978)	0.737
Injecting drug use	2.148 (0.216, 21.409)	0.515		
Age (per 1 year increase)	0.992 (0.958, 1.027)	0.647		
BMI (per 1 unit increase)	0.942 (0.848, 1.046)	0.264		

Table 3b - *Risk factors associated with Staphylococcus aureus colonization at baseline in the HIV-positive population.*

Variable	*Unadjusted OR (95% CI)*	*p*	*Adjusted OR (95% CI)*	
Male gender	1.574 (0.477, 5.189)	0.456		
Race	1			
Caucasian ethnicity	0.684 (0.127, 3.668)	0.657		
Cohabitation with children	1.521 (0.434, 5.331)	0.512		
HCV co-infection	0.859 (0.231, 4.468)	0.872		
HIV 1-RNA>50 cps/mL	0.833 (0.168, 4.140)	0.824		
Recent Hospitalization	2.250 (0.382, 13.243)	0.37		
Past AIDS-defining events	0.107 (0.014, 0.842)	**0.034**		
Cardiovascular disease	1.463 (0.273, 7.851)	0.657		
Hepatopathy	0.761 (0.199, 2.914)	0.691		
Smokers	0.702 (0.262, 1.880)	0.481		
Alcohol consumers	0.938 (0.186, 4.722)	0.938		
Injecting drug use	1.439 (0.142, 14.603)	0.758		
Nadir CD4>200 cells/mmc	4.272 (1.570-14.232)	0.006		
Co-trimoxazole previous use	0.172 (0.047-0.632)	**0.008**	0.288(0.065-1.283)	0.102
Age (per 1 year increase)	0.944 (0.892, 0.999)	**0.045**	0.969 (0.914, 1.028)	0.297
BMI (per 1 unit increase)	0.979 (0.860, 1.116)	0.754		
Years from HIV diagnosis	0.962 (0.903, 1.025)	0.236		
Years from HAART initiation	0.960 (0.887, 1.038)	0.304		
Nadir CD4 (cells/µL)	1.004 (1.001, 1.007)	**0.013**	1.002 (0.998, 1.005)	0.347
Baseline CD4 (cells/µL)	1.001 (0.999, 1.003)	0.263		

The variable "Past AIDS-defining events", albeit statistically significant by univariate analysis, was not included in the multivariable analysis model as considered a mediator variable

ST (i.e., ST 5, ST 7, ST 8, ST 15, ST 22, ST 30, ST 72 and ST 398) were founders of their correspondent clonal complexes (CCs), while the remaining ST (ST 737) was a single locus variant of ST 22 (CC 22).

No decolonization strategy was performed, in particular in MRSA carriers, and no bacterial or fungal infection occurred in colonized subjects during a three-year clinical follow up.

DISCUSSION

The study involved a homogeneous HIV-positive group of patients, on efficient antiretroviral treatment and with an appropriate immune recovery, in comparison to HIV-negative healthy patients. The total number of bacterial and fungal isolates were similar in the two groups (30.5% in HIV-positive patients *vs.* 23% in HIV-negative, p=0.293); nevertheless, a significantly higher rate of HIV-positive patients (18%) carried *Staphylococcus aures* vs. HIV-negative (8%, p=0.03), in agreement with previously reported data (Melles *et al*., 2008; Padoveze et al., 2008; Nouwen *et al.*, 2005).

Such evidence could partially explain the greater risk in developing invasive staphylococcal infections (Wertheim et al., 2004). We recovered *S. aureus* isolates belonging to CCs 5, 8, 15, 30 and 45, that were previously described as being among the most prevalent clones causing invasive infections (Rieg, *et al.*, 2013; Rasmussen *et al.*, 2014), and those in CC22 that were linked to bloodstream infections in conjunction with osteomyelitis and septic arthritis (Rieg, *et al.*, 2013). ST7 has been reported as the predominant lineage of MSSA in Europe (Grundmann *et al.*, 2014), while ST 72 is a major representative of CA-MRSA in Korea (Jeon *et al.*, 2016) and ST 398, originally isolated in early 2000 from humans living in close contact with pigs, is able to produce invasive infections in human hosts with high prevalence.

Moreover, our results showed a significant strong association between HIV-positivity and *S. aureus* colonization, even after adjustment for gender, smoking, cohabitation with children, hepatopathy and alcohol consumption.

Among HIV-infected patients, previous exposure to co-trimoxazole for prophylaxis or therapy for opportunistic infections in more advanced patients, correlates with *S. aureus* colonization, suggesting the possible role of antibiotics in decolonization.

Interestingly, both MRSA (3% at baseline and 2% at T48) and MDRSA strains (4% at baseline and 7% at T48, p=0.04) were detected only in the HIV-positive group, in line with previous data (Cenizal *et al.*, 2008). These results could be explained at least in part by their frequent access to healthcare structures.

Regarding *S. pneumoniae*, *C. albicans* or other Gram positive/negative isolates, no significant differences between the two population or any correlation to CD4 cells count or other variables in the HIV-positive population were found.

The presence of *Candida non albicans* strains, especially among HIV-positive patients, could be hypothetically related to heavier exposition to antimycotics for both prophylactic and therapeutic strategies (Coleman *et al.*, 1998; Masia *et al*., 2000).

The results obtained indicate a potential higher risk of staphylococcal and *Candida non albicans* infection in HIV-positive subjects, with relevant clinical implication in empirical therapeutic management of infections: multiresistance could be a challenging issue and wide spectrum drugs should be chosen empirically.

Our study confirmed a strong association between HIV positivity and *S. aureus* colonization, but no invasive infection arose in our HIV-positive population of persistent carriers during observation. Constant and further observations of nasopharyngeal colonization type and rate in different HIV-positive cohorts could be useful in clinical management, in terms of prophylactic and therapeutic strategies of staphylococcal infections. Our population had a favorable viro-immunological profile: knowledge of the nasopharynx flora and further observations may contribute to understanding whether the increased risk of invasive disease in HIV-infected patients is a function of immunity impairment or a consequence of an increased risk of nasopharyngeal bacterial colonization. Chemosusceptibility analysis of bacterial colonizing strains in this type of patient could be of pivotal importance for prophylactic or eradicating strategies.

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