Influence of TNF-α blockers on the oral prevalence of opportunistic microorganisms in ankylosing spondylitis patients

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Abstract

Objectives

To compare the oral prevalence and antimicrobial susceptibility of candida spp., staphylococci, enterobacteriaceae, and pseudomonas spp. from ankylosing spondylitis (AS) patients receiving conventional and anti-TNF-α therapy.

Methods

The study included 70 AS patients, diagnosed according to the modified New York criteria (1984). The volunteers were divided into 2 groups: a biological group (AS BioG) (n=35) (on anti-TNF-α therapy) and a conventional group (AS ConvG) (n=35). The control group (ContG) (n=70) was made up of healthy individuals matched for age, gender, and oral conditions. After clinical examination, oral rinse samples were collected and plated in specific culture media. The number of colony-forming units per milliliter (cfu/ml) was obtained, and isolates were identified using the API system. Antimicrobial susceptibility tests were performed according to the NCCLS guidelines. Prevalence and counts of microorganisms were statistically compared between the 3 groups, using the Mann-Whitney and Chi-square tests. Significance level was set at 5%.

Results

In both the AS BioG and the AS ConvG, staphylococci counts were higher than that in the ContG ($p<0.0001$). Candida albicans and staphylococcus epidermidis were the most commonly found species in all the groups. Serratia marcescens and klebsiella oxytoca were more prevalent in the AS BioG and the AS ConvG, respectively. Two candida isolates (2.8%) from the AS BioG and 5 (10.8%) from the AS ConvG were resistant to amphotericin B and 5-fluorocytosine. A low percentage of staphylococci isolates was resistant to amoxicillin, ciprofloxacin, and doxycycline.

Conclusion

Higher counts of staphylococci were observed in both AS groups, regardless of the current therapy, age, sex, and oral conditions. Anti-TNF-α therapy could not be correlated with increased counts of microorganisms.

Key words

ankylosing spondylitis, infection, super-infection, oral microbiology, TNF-α blockers, candida, staphylococcus

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Received on July 28, 2011; accepted in revised form on December 12, 2011.

Funding: this study was supported by grants provided by Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP (2008/57631-4 and 2008/56485-4).

Competing interests: none declared.

Introduction

Ankylosing spondylitis (AS) is a complex, disabling, chronic inflammatory disease that involves the enthesis of the joints and ligaments. Its etiology and pathophysiology are not yet fully understood, although it is known that there is a close relationship between AS and the presence of infectious triggers, especially Gram-negative pathogens from the intestinal tract (1-2). Moreover, men are 2–3 times more susceptible to AS than women.

Conventional therapy includes non-steroidal anti-inflammatory drugs (NSAIDs) and rehabilitation. Nowadays, the use of immunobiological agents, such as TNF-α blockers, is considered to be an excellent therapeutic option in some patients having an inadequate response to conventional treatment. Nevertheless, several studies reported a higher rate of infections following this treatment (3-4). The oral cavity represents a portal of entry for pathogens, especially in immunocompromised patients; therefore, reservoirs of oral opportunistic microorganisms, enterobacteria, staphylococci, and yeasts may cause systemic infections (5). These opportunistic microorganisms can be found in saliva, mucosa, supragingival plaques, and periodontal pockets (6-7).

Staphylococcus aureus is the most important pathogen related to severe human infections, such as pneumonia and sepsis, mainly after treatment with immunosuppressive agents (8). Under physiological conditions, *Candida* spp. is commensal in humans, but local or systemic changes may increase its pathogenicity (9). According to Chen *et al.* (10), septicemia by *Candida* spp. is associated with significant morbidity and mortality in oncologic, diabetic, and rheumatic patients. The use of TNF-α blockers in AS patients may play a relevant role in the prevalence of opportunistic microorganisms in the oral cavity, as well as in the perpetuation of these reservoirs, thus potentially increasing the risk of systemic infections. Notwithstanding this risk, there are no studies addressing this subject in the literature.

The aim of this study was to assess the oral prevalence, and the antimicrobial susceptibility, of *Candida* spp., staphylococci, enterobacteriaceae, and *Pseudomonas* spp. in AS patients treated with conventional and anti-TNF-α therapy, in comparison to healthy, control subjects.

Patients and methods

Patients

This study was conducted in accordance with the principles laid down in the Declaration of Helsinki and was previously approved by the Local Human Ethical Committee (049/2008-PH/CEP). All volunteers gave their written informed consent prior to participation.

One hundred and fifty patients diagnosed with AS, according to the modified New York classification criteria (11), were evaluated. Among these patients, a total of 70 subjects were included in this study. Non-inclusion criteria included pregnancy, use of dentures and orthodontic appliances, smoking, occurrence of other systemic diseases (*e.g.* diabetes mellitus and cancer), patients with oral lesions or sicca symptoms, and those receiving treatment with drugs that could interfere with oral conditions (*e.g.* antibiotics, anti-depressants, anti-fungals, and mouthwashes) for the past 60 days. In addition, 70 healthy individuals, matched for gender, age, and oral conditions (assessed by the DMFT index: number of decayed, missing, and filled teeth), were included as controls, also adopting the non-inclusion criteria described above.

The present study comprises the following 3 groups:

a) biological group (AS BioG, n=35): patients taking TNF-α blockers for at least 90 days;
b) conventional group (AS ConvG, n=35): patients under conventional treatment, including non-steroidal anti-inflammatory drugs (NSAIDs), methotrexate (MTX), or sulfasalazine (SSZ); and
c) control group (ContG): 70 healthy individuals matched for age, gender, and oral conditions to the AS groups.

The AS patients received treatment at the Spondyloarthritis Section, Rheumatology Division, Universidade

Federal de São Paulo. Healthy, control group individuals were under treatment at São José dos Campos Dental School.

Anamnesis and clinical examination
Data on oral conditions and general health, associated diseases, and concomitant medication were obtained during anamnesis. The clinical examination, which evaluated the presence of oral lesions and the DMFT index, was conducted by a single examiner. Stimulated salivary flow rate was determined and classified according to Krasse (12). The volunteer received a graduated flask, in which stimulated saliva was deposited during a 5-minute period, and the results were expressed as ml/min. Salivary flow was classified as: normal (higher than 1.0 ml/min), hyposalivation (lower than 0.7 ml/min) or xerostomia (less than 0.1 ml/min). The determination of salivary flow rate was performed following oral rinse sampling.
Medical and joint examinations, including BASDAI (Bath ankylosing spondylitis disease activity index) assessment, were carried out by the rheumatologist (13).

Sample collection and processing
Oral rinse samples were obtained using 10 ml phosphate buffered saline (PBS 0.1 mol·l^{-1}, pH 7.2) for 1 min. Samples were kept on ice for a maximum of 3 h, before being processed.
Oral rinse samples were centrifuged at 8,000 × *g* for 10 min, and the supernatant was discarded. The pellet was resuspended in 2.5 ml PBS and vortexed for 30 s. From each sample, 0.1 ml was plated in duplicate on Sabouraud dextrose agar (Difco, Detroit, USA) supplemented with 0.1 mg/ml chloramphenicol (National Union Pharmaceutical Chemistry S.A.), for isolation of yeasts. The plates were incubated at 37°C for 48 h. In addition, 0.1 ml was plated in duplicate onto mannitol agar and McConkey agar, and incubated at 37°C for 48 h, for isolation of *Staphylococcus* spp., and *Enterobacter*/*Pseudomonas*, respectively.
After the incubation period, the colonies of yeasts and bacteria were counted. Each colony, with a different morphology, was confirmed microscopically by the Gram staining method. Five colonies of yeasts and bacteria were then isolated on Sabouraud dextrose (for fungi) or gelose agar (for bacteria). These samples were stored for further identification.

Isolate identification
Pure cultures were plated on Sabouraud dextrose agar for *Candida* spp., mannitol agar for *Staphylococcus* spp., and McConkey agar for *Enterobacter* spp. and *Pseudomonas*. These cultures were then incubated for 24 h at 37°C, and isolates were identified using API 20 C AUX, API Staphy, and API 20E systems (Bio-Meriéux, France).

Antifungal susceptibility testing
Isolates of *Candida* spp. were tested for *in vitro* susceptibility to amphotericin B, fluconazole, ketoconazole, and 5-fluorocytosine, according to the microdilution method (Clinical Laboratory Standards Institute – NCCLS [14]). Briefly, isolates of *Candida* spp. were grown in Sabouraud dextrose agar and incubated for 48 h at 37°C. Subsequently, 5 colonies, with a diameter exceeding 5 mm, were selected and suspended in sterile saline solution (NaCl 0.85%), to obtain an initial concentration of 1–5×10^6 cells/ml. Then, the suspension was diluted 1:2000 in synthetic medium: RPMI 1640 buffered to pH 7.0 with morpholinopropanesulphonic acid (MOPS), resulting in a final concentration of 0.5×10^3 to 2.5×10^3 cells/ml. The drugs amphotericin B and ketoconazole were dissolved in dimethyl sulfoxide (DMSO), while 5-fluorocytosine and fluconazole were dissolved in sterile water. The drugs were initially prepared at the following concentrations: 320 μg/ml for amphotericin B, 1000 μg/ml for 5-fluorocytosine, 1250 μg/ml for fluconazole, 640 μg/ml for ketoconazole (Sigma-Aldrich Co.), and were further diluted in synthetic medium to obtain final concentrations matching the following interval ranges, as mentioned in the literature (14). The ranges adopted were 4 to 0.02 μg/ml for amphotericin B, 32 to 0.02 μg/ml for ketoconazole and 5-fluorocytosine, and 64 to 0.02 μg/ml for fluconazole. Aliquots of the inoculum and the antifungal drug dilution were added to 96-well acrylic plates. Plates were incubated at 37°C, and readings were performed after 48 h. Plates with amphotericin B were covered with aluminum foil for light protection.
Each well was compared to a growth control. Minimum inhibitory concentration (MIC) values were taken to be the concentration at which 80% reduction in growth was observed for the fungistatic drugs. For amphotericin B, MIC values corresponded to the complete absence of growth (100%), whereas for 5-fluorocytosine and fluconazole, the isolates were classified as susceptible (S), intermediate (I), or resistant (R), according to the endpoints set by NCCLS (14). For amphotericin B (S<1/R>2) and ketoconazole (S<8/R>16), endpoint values were set by Sutton (15).

Antibiotic susceptibility testing
Antibiotic resistance tests were performed for *Enterobacteriaceae*, *Pseudomonas* spp., and *Staphylococcus* spp. isolates. The MIC of antimicrobials was determined using the Müeler-Hinton agar dilution method (16).
The following antibiotics were evaluated: amoxicillin, ampicillin, azithromycin, cephalexin, ciprofloxacin, clindamycin, doxycycline, erythromycin, metronidazole, norfloxacin, penicillin, and tetracycline (Sigma-Aldrich Co.). The antibiotics were diluted in water and filter-sterilised (cellulose filter disks, 0.22 μm pore size). A series of plates containing 0.25 to 256 μg/ml of each antimicrobial, in sequential dilutions, was prepared. The drugs were added to the medium at 50°C. Standardised bacterial suspensions (McFarlane 0.5) were obtained and inoculated with the aid of a Steers replicator, and plates were incubated at 37°C for 24 h. Readings were performed based on the presence or absence of colony growth on the agar surface. Classification of S, I, and R isolates was performed according to Clinical Laboratory Standards Institute (CLSI) endpoints (17).

Statistical analyses
Statistical analysis was performed by comparing the results obtained in the

2 AS patient groups, with those of the healthy control group. Data obtained for microbial counts were compared statistically using the Mann-Whitney U-test. The prevalence of microorganism species was compared among the groups using the Chi-square test. The significance level was set at 5%, using GraphPad Prism 5.0 for Windows.

Results

The demographic and clinical data, as well as the oral conditions, of all individuals who participated in this study, are shown in Table I. Sampling was performed on young, male patients, most of whom had long-standing disease (more than 10 years). Oral conditions (DMFT index) and salivary flow were similar among the 3 groups. On the other hand, the AS BioG had a lower BASDAI index than the AS ConvG, possibly due to better control of the disease achieved by the use of TNF-α blockers. Clinical examination showed a mean DMFT index of 8, for both the AS BioG and the AS ConvG. Salivary flow values were classified as normal in all groups studied, and no oral lesions were diagnosed.

Higher counts of *staphylococci* were observed in the AS groups, when compared to healthy controls ($p<0.0001$), regardless of the therapy being used. No difference between counts in the AS BioG and the AS ConvG was detected. Despite this, no statistically significant differences between groups were detected for yeasts and *Enterobacter/Pseudomonas* spp. counts (Table II). The ConvG was additionally subdivided into DMARDs (n=13) and non-steroid anti-inflammatory drugs (NSAIDs) (n=22) groups, so as to elucidate the contribution of the different drug types on the prevalence of microorganisms in the oral cavity. There were significantly higher counts of *staphylococci* in the NSAIDs group, when compared to the MTX or SSZ groups ($p<0.0001$), but not when compared to the BioG. No difference was noted for *Candida* and *Enterobacteriaceae/Pseudomonas* spp. ($p=0.51$ and 0.35, respectively).

A total of 98 *sSaphylococcus* isolates were obtained in the AS BioG, 114 isolates in the AS ConvG, and 165 isolates in the ContG. The most prevalent species in all groups was *S. epidermidis*, followed by *S. aureus*. A total of 71 candida isolates were obtained in the AS BioG, 46 isolates in the AS ConvG, and 91 isolates in the ContG. *C. albicans* was the most prevalent species in all groups. For *Enterobacteriaceae* and *Pseudomonas*, a total of 41 isolates were obtained from AS BioG patients, the most frequently isolated species being *Serratia marcescens*. For the AS ConvG, 36 isolates were obtained, and the most frequently isolated species was *Klebsiella oxytoca*. For AS ConvG, 61 isolates were obtained, and the most frequently observed species was *Enterobacter cloacae* (Table III).

Antifungal susceptibility testing for *Candida* species isolates showed that most of the isolates classified as susceptible. All isolates were susceptible to ketoconazole and fluconazole, while 2 isolates from the AS BioG (2.8%)

Table I. Descriptive data obtained for AS patients and healthy controls.

	AS BioG (n=35)	AS ConvG (n=35)	ContG (n=70)	*p*-value
Age (years)	38±10	40± 12	39±11	0.60*
Time of disease (years)	13±5	13± 8	-0.66*	
Male (%)	71.4%	85.7%	78.6%	0.34**
DMFT	8±4	8±5	9±4	0.75*
Salivary flow (ml/min)	1.9±0.2	1.8± 0.2	1.8± 0.2	0.57*
Oral lesions	0	0	0	1*
BASDAI	1.8±1.6	2.4±1.9	-0.11*	

AS BioG: ankylosing spondylitis patients receiving biological treatment with TNF blockers; AS ConvG: ankylosing spondylitis patients receiving conventional therapy (NSAIDs, MTX, or SSZ); ContG: healthy control group; DMFT: decayed, missing, and filled teeth; BASDAI: Bath ankylosing spondylitis disease activity index; *Chi-square ($p<0.05$); **Mann-Whitney test for statistical analyses ($p<0.05$).

Table II. Counts of microorganisms (CFU/ml) in AS patients and healthy controls (mean±standard deviation).

	AS BioG (n=35)	AS ConvG (n=35)	ContG (n=70)	*p*-value*
Candida spp.	315±537	213±509	152±533	0.17
Staphylococcus spp.	2754±3260	3138±3596	868±890	<0.0001
Enterobacteria/Pseudomonas spp.	687±1032	596±1268	338±923	0.23

AS BioG: ankylosing spondylitis patients under biological treatment with TNF blockers; AS ConvG: ankylosing spondylitis S patients under conventional therapy (NSAIDs, MTX or SSZ); ContG: healthy control group; CFU: colony forming units; *ANOVA, Tukey's test.

Table III. Frequency of species isolated from oral cavities of AS patients and healthy controls.

Species	AS BioG		AS ConvG		ContG	
	n	%	n	%	n	%
Candida albicans	64	90.1	39	81.2	79	89.2
Staphylococcus epidermidis	57	58.2	55	48.2	89	56.8
Staphylococcus aureus	31	31.6	33	28.9	37	21.6
Serratia marcescens	17	41.5	5	13.9	6	9.8
Klebsiella oxytoca	9	21.9	16	44.4	8	13.1
Enterobacter cloacae	4	9.8	2	5.6	17	27.9

AS BioG: ankylosing spondylitis patients under biological treatment with TNF blockers; AS ConvG: ankylosing spondylitis S patients under conventional therapy (NSAIDs, MTX or SSZ); ContG: healthy control group; n: number of isolates.

were resistant to amphotericin B. Five isolates (10.8%) in the AS ConvG, and 3 isolates (6%) in the AS BioG and the ContG, were intermediate to 5-fluorocytosine.

Most of the staphylococci isolates were also susceptible to the tested antibiotics. Nevertheless, a lower number of resistant isolates was found in healthy controls, when compared to the AS groups. Similarly, susceptibility to the tested antibiotics for *Enterobacter/ Pseudomonas,* demonstrated that most of the isolates was susceptible to the quinolones.

Discussion

Our results revealed a high prevalence of microorganisms in the oral cavities of AS patients, in particular staphylococci. Moreover, although no statistically quantitative differences have been detected, there were also qualitative differences in *Enterobacter* species, among the groups.

To the best of our knowledge, there are no studies regarding the oral conditions and the presence of opportunistic microorganisms in patients with AS. According to our data, the mean DMFT for both the AS BioG and the AS ConvG was 8 (a value above 7 is considered to be a high index for caries [17]). This finding emphasises the need for preventive dental measures in this population, as well as multidisciplinary treatment strategies.

The prevalence of yeasts in the oral cavities of patients in both AS groups (45.7% for the AS BioG and 34.3% for the AS ConvG) is within the range reported previously for control subjects (25 to 65%) (18). These values, however, are lower than those observed in patients with other systemic diseases, such as malignancies (80%) (19), HIV-positive patients (60%–75%) (20), and cardiac transplant patients (88%) (21). On the other hand, higher oral levels of yeasts have been reported in patients treated with immunosuppressive drugs after kidney and liver transplantation (22) or undergoing multidrug therapy for Hansen's disease (65.8%) (23). The comparison with different systemic conditions is, admittedly, a limited tool; nonetheless, considering the lack of previous data in AS patients, this is the only method we have to compare our results, and we believe the results should be analysed carefully.

A high prevalence of *C. albicans* was observed in all groups; this result being similar to that reported by other authors, both in healthy subjects, as well as in those with systemic or oral diseases and candidiasis (23-24). The prevalence of non-*C. albicans* species (10% of the isolates in the AS BioG group and 15% in the AS ConvG) is relevant, because reports of infections caused by non-*C. albicans* species have increased significantly in recent years (25). Moreover, candidemia caused by C. albicans has a better prognosis than that related to non-*C. albicans* species (25-27). The higher variability of *candida* spp. has been suggested to be related to an imbalance of the oral microflora (21, 28). In our study, the ConvG showed the greatest diversity in species of *Candida*.

Interestingly, significant differences between staphylococci counts in AS groups (both anti-TNF-α and conventional therapy groups) and healthy controls were detected. Considering that the therapeutic effects of NSAIDs and DMARDs are different, the ConvG was further subdivided in NSAIDs or MTX/SSZ patient groups, for the evaluation of higher susceptibility to infection. Both for the ConvG as a whole, and for the subdivided groups, only staphylococci counts were higher, in relation to controls. Higher prevalence of *S. epidermidis*, followed by *S. aureus*, was observed, both in AS patients and control groups. Besides, a higher prevalence of *S. aureus* nasal or oral carrier state had already been demonstrated in rheumatoid arthritis patients taking TNF-α inhibitors and MTX (29). A previous report has described the increased susceptibility of individuals receiving anti-TNF-α therapy to ocular *S. aureus* infections (30); however, in this study, no higher oral prevalence of this species was detected. The reason for this increased incidence remains unclear. The counts of other opportunistic microorganisms were similar to those found in controls – this observation suggests the need for additional investigation; in particular, the molecular effects of both the disease and the therapy on the immunity of the host.

Schmidt-Westhausen *et al.* (31) described *Enterobacteriaceae/Pseudomonas* as transient microorganisms in the oral cavity and that, in general, their presence reflects an imbalance in the oral microflora. In this study, there was no quantitative difference between the groups, although there were differences in the detected species. The large variability of species in the AS ConvG (standard treatment with NSAIDs, MTX, or SSZ) may suggest an imbalance in oral microflora as compared to the AS BioG group (TNF-α antagonists). AS patients had greater counts of *S. marcescens* and *K. oxytoca* than did healthy controls. These data may reinforce the role of infectious agents, particularly Gram-negative bacteria, in triggering pathogenetic development and the perpetuation of inflammation in patients with AS (2, 32-34).

This is the first study revealing the presence of these microrganisms in the oral cavity of AS patients, as opposed to the intestinal or genitourinary tracts (36). Furthermore, previous studies reported a higher frequency of anti-*Klebsiella* antibodies in the sera of AS patients (34-36). Further investigations of *Klebsiella* spp. and *Serratia* spp. may, therefore, provide interesting results for the better understanding of these diseases.

Few studies have evaluated the susceptibility of microbes to antifungals and antibiotics in oral samples, and there is no such previous study in AS patients. The finding of low prevalence of oral isolates resistant to antifungal agents is similar to findings of previously published literature (23, 37).

Several authors have stressed the importance of the presence of antimicrobial-resistant microorganisms in the mouth, including methicillin-resistant *S. aureus* (38). In the present study, a low rate of antibiotic-resistant staphylococci isolates was observed (up to 6%) in both groups, including those resistant to quinolones, beta-lactams, and macrolides. One striking finding, in patients with AS taking TNF-α blockers,

was the high number of isolates resistant to ampicillin (almost 30%), implying that this antibiotic should not be the therapeutic option for oral infections in these individuals. De Carvalho *et al*. (38) reported that 100% of staphylococci isolates from saliva of health professionals were resistant to penicillin and oxacillin, and almost 60% of the isolates were resistant to clindamycin. These values were much higher than those observed in AS patients (BioG=11%; ConvG=3.5%). Similarly, we observed a low rate of resistance to the quinolones in *Enterobacter* and *Pseudomonas* spp. isolates. Future studies evaluating third or fourth generation cephalosporins, as well as carbapenems, are needed.

The lack of antibiotic susceptibility testing for anaerobic agents that may also be involved in systemic infections in patients with chronic inflammatory diseases can be considered a limitation of this study. Another relevant point is the lack of follow-up of these patients, due to cross-sectional design. More longitudinal research is needed to determine whether AS patients, positive to pathogenic bacteria in the oral cavity, have a higher risk of infections. This would then enable us to establish the relationship between cause and effect.

To the best of our knowledge, this is the first comprehensive study about oral microorganisms in AS patients, including the prevalence and antimicrobial susceptibility, as well as the patients' oral health status and salivary flow. This research revealed that AS patients have higher counts of staphylococci, independently of the therapy they are on (conventional *vs*. TNF-α blockers). While these aspects should be emphasised when selecting antimicrobial therapy for AS patients, especially during an infectious process, prospective studies are needed in order to understand whether these findings can be related to a higher risk of infections in AS patients taking TNF-α blockers.

Conclusion

In summary, the present study showed that high counts of staphylococci in AS patients, regardless of the therapy they are receiving, as compared to healthy, control individuals, might increase their risk of opportunistic infections. Although potentially hazardous and pathogenic, these microorganisms have a low rate of antibiotic resistance in clinical practice, including resistance to β-lactams and quinolones. Similarly, in AS patients, microorganism susceptibility to antibiotics used routinely to treat infections caused by enterobacteriaceae/pseudomonadaceae and yeasts or quinolones in particular, showed a low rate of resistance, regardless of treatment with TNF-α blockers.

Thus, we can speculate that in AS patients suffering from community infections caused by fungi, staphylococci, or enterobacteria, it is not necessary to use broad-spectrum antibiotics, such as fourth-generation cephalosporins or carbapenems, regardless of treatment with TNF-α blockers. The use of quinolones or third-generation cephalosporins should be enough.

Acknowledgements

The authors would like to thank Prof. Ivan Balducci for his suggestions and statistical analysis, and for the grants provided by Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP (2008/57631-4 and 2008/56485-4).

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