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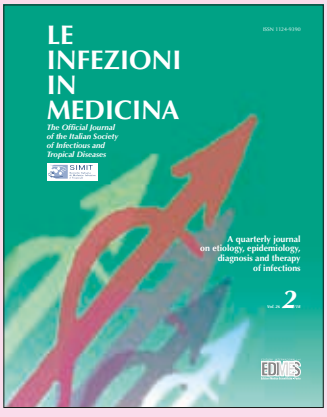


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# LE INFEZIONI IN MEDICINA

**THE OFFICIAL JOURNAL OF THE ITALIAN SOCIETY OF INFECTIOUS AND TROPICAL DISEASES**

**A quarterly journal covering the etiological, epidemiological, diagnostic, clinical and therapeutic aspects of infectious diseases**

# Microbiology and prognosis assessment of hospitalized patients with aspiration pneumonia: a single-center prospective cohort study

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## SUMMARY

Aspiration pneumonia has a high incidence in hospitalized patients with community-acquired pneumonia and results in high mortality rates. We aimed to evaluate microbiology and assess prognostic factors of aspiration pneumonia in the setting of a tertiary hospital pulmonology department. Community-acquired (CAAP) and healthcare-associated aspiration pneumonia (HCAAP) cases hospitalized over a period of a year were prospectively followed. Demographic, clinical, biological and radiological data were recorded at admission, while sputum, tracheal aspirates or bronchial washing samples were collected within 48 hours of admission. During hospital stay, therapeutic and supportive measures and resulting complications were recorded. Regression analysis was applied to find statistically significant prognostic factors. The sample consisted of 70 patients (67.1% men); 55.7% of them presented as HCAAP. 94.3% had positive culture of lower respiratory tract specimens with isolation of 115 pathogens, 47 of which were multidrug- or extensively drug-resistant. The most common pathogens were *Pseudomonas aeruginosa* (37.1%), *Klebsiella pneumoniae* (27.1%), *Staphylococcus aureus* (25.7%) and *Acinetobacter baumannii* (20%). Empiric antimicrobial therapy was combination therapy in 70% and included antipseudomonal and MRSA-targeted antibiotics in 61.4% and 11.4%, respectively. Patients in the HCAAP group had a higher rate of antibiotics usage in the previous trimester, more frequent isolation of resistant strains and were more likely to receive inadequate empiric treatment than those in the CAAP group. In-hospital mortality was 52.2%; no difference between groups was noted. Independent factors of increased mortality were older age (p=0.004), low serum albumin levels (p=0.039), increased radiological involvement (p=0.050) and ineffective initial therapy (p=0.001). We concluded that patients hospitalized for aspiration pneumonia have frequent contact with healthcare services and acquire multidrug-resistant Gram-negative bacteria. Empiric therapy should target these specific microorganisms as its success determines the prognosis.

*Keywords*: aspiration pneumonia, microbiology, empiric therapy, mortality.

## INTRODUCTION

Aspiration pneumonia is a distinct form of bacterial pneumonia resulting from macroaspiration of oropharyngeal or gastric content in patients with specific predisposing factors. Its incidence in hospitalized patients with community-acquired pneumonia ranges from 8.7% to as high as 60.1% in recent research, depending on the definition each study used [1-5]. In-hospital mortality rate also varies between studies, reaching up to 23% in patients treated at pulmonary or internal medicine departments and to 40% in patients admitted to intensive care units (ICU) [1, 3-9]. The microbiological etiology of aspiration pneumonia

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is reported to have significantly changed over the last 50 years, shifting from anaerobic-only isolates to mixed flora, with Gram-negative bacilli, often multi-drug resistant, dominating the picture [10]. The effectiveness of initial empiric antimicrobial therapy is considered a significant prognostic factor and the choice of drug or combination of drugs should be made according to knowledge of local epidemiological data [7, 9].

Previous research has examined microbiological data of hospitalized patients with aspiration pneumonia mostly in the context of ICU admission [7-9]. Since they do not represent the entire spectrum of aspiration pneumonia cases, it is possible that results cannot be generalized to guide treatment and determine prognosis in patients hospitalized in general wards. This study aimed to identify the bacteriological flora and resistance patterns of patients admitted for aspiration pneumonia in a pulmonology department over a year's period and assess prognostic factors related to mortality and length of hospital stay.

## PATIENTS AND METHODS

*Setting*

We conducted a prospective study of patients that were admitted with aspiration pneumonia within a year in a public tertiary hospital's pulmonology department in Greece. "Sismanoglio" General Hospital of Attica is a reference hospital for pulmonary diseases, serving the regional unit of North Athens, with a population of 591680 inhabitants, and the broader north-eastern part of Attica. All patients admitted in the 2nd Pulmonology department with a diagnosis of aspiration pneumonia between 1 December 2015 and 30 November 2016 were screened for enrolment and those included were followed during their hospitalization and up to three months from admission.

The study was conducted in accordance with the ethical standards of the institutional review board and the Helsinki Declaration of the World Medical Association and informed consent was obtained from participants or their health care proxy.

*Participants*

The presence of aspiration pneumonia required the following criteria assessed at initial presentation or within 48 hours of hospitalization:

a) acute onset of at least two symptoms or signs suggestive of lower respiratory tract infection (fever >38°C, cough or sputum production, dyspnoea or respiratory rate >20 breaths per minute, altered mental status, pleuritic chest pain, crackles or consolidation on physical examination);
b) evidence of a new gravity-dependent pulmonary infiltrate on thoracic imaging;
c) presence of aspiration risk factors (reduced level of consciousness, impaired swallowing reflex or abnormalities of the upper aerodigestive tract) or witnessed large aspiration [11]. A simple water-swallowing test was used to evaluate the adequacy of the swallowing reflex.

Patients were divided in two groups:

a) a healthcare-associated aspiration pneumonia (HCAAP) group, consisting of residents of nursing or long-term care facilities and patients receiving haemodialysis, intravenous chemotherapy, radiation therapy or specialized healthcare at home (intravenous therapy, wound care, urinary catheterization, etc.) in the last month before admission or being hospitalized for over two days in the previous trimester;
b) a community-acquired aspiration pneumonia (CAAP) group, including the rest [12].

Participants were evaluated continuously during their hospital stay until death or discharge and the latter also received a telephone call after three months to assess their clinical condition.

*Data sources*

Data collected upon admission included demographic information, type of residence, functional (ambulatory or bedridden) and nutritional (based on estimated body mass index with cut-off value 18.5 kg/m$^2$) assessment, comorbidities and calculation of Charlson index, history of hospitalizations, long-term oxygen therapy and protein pump inhibitors (PPIs), histamine-2 receptor antagonists ($H_2$ antagonists) and antibiotic usage in the previous trimester [13]. Within 48 hours after admission the following clinical, biological and radiological data were recorded: initial vital signs; presenting symptoms; mechanism of aspiration; arterial blood gases; biochemical and hematological tests (white blood cell count and type, platelet count, hematocrit, serum albumin, glucose, urea, creatinine, sodium and C-reactive

protein); and findings in chest radiograph (number of lung fields involved, bilateral involvement, presence of pleural effusion). Disease severity and prognosis assessment was made by calculation of the Pneumonia Severity Index (PSI) [14]. Septic shock was defined according to recently published criteria [15].

In all cases, a sputum, tracheal aspirate or bronchoscopy-directed bronchial washing sample was obtained within 48 hours following admission, while blood cultures were performed only in patients with fever. Standard suitability criteria of >25 leukocytes and <10 squamous epithelial cells per low-power field for bacterial cultures of lower respiratory tract specimens were applied, followed by qualitative analysis and antimicrobial susceptibility testing, according to EUCAST guidelines, in suitable samples, while cases with unacceptable specimens were excluded [16]. Urinary antigens for *Streptococcus pneumoniae* and *Legionella pneumophila* were not routinely ordered because of limited availability in our hospital throughout the study period. Therapeutic and supportive measures, such as initial antimicrobial therapy, use of systemic corticosteroids or inotropic drugs, blood transfusion, parenteral nutrition, placement of central venous catheters, nasogastric feeding tube or gastrostomy, invasive or noninvasive mechanical ventilation and therapeutic bronchoscopy were recorded in all patients. Endotracheal intubation was not performed in cases where a "do not resuscitate" order was applied. The choice of antibiotics was made by the treating physicians based on existing guidelines and local epidemiological factors. Patterns of antibiotic resistance were identified using expert proposed definitions [17]. Initial antimicrobial therapy was considered adequate if the isolated pathogens were susceptible to at least one of the used antibiotics and effective when a clinical response (decline of fever, leukocytosis and C-reactive protein, improvement of hypoxemia and shock) was observed in the first 72 hours of treatment. During the patients' hospitalization, the occurrence of pneumonia complications in the form of total lung atelectasis, hypoventilation and respiratory acidosis, bronchopulmonary hospital-acquired superinfections, secondary septic shock or multiple organ failure was also recorded. In-hospital mortality and mortality at the end of follow-up were the primary study endpoints. For patients discharged from hospital, length of stay (LOS) was also considered an outcome variable.

*Statistical analysis*

In descriptive statistical analysis, continuous variables were expressed as mean±standard deviation and categorical variables in the form of frequencies. Frequency differences between categorical variables were analysed by the chi-square test or Fisher's exact test, while the differences in means between continuous variables with Student's t test or Mann-Whitney test, depending on the normality of data. To examine prognostic factors of mortality and LOS we used multiple logistic and linear regression models with the backward stepwise method. In linear regression, high influential points were excluded when their Cook's distance value exceeded three times the sample mean value. All significance tests were two-sided, with p-value <0.05 being considered statistically significant. Analysis was conducted with the statistical software package IBM® SPSS® Statistics Version 20.

## RESULTS

*Sample demographics*

During the study period, 94 patients were admitted with diagnosis of aspiration pneumonia and were eligible for enrolment. Three patients did not consent and 21 were excluded for having unacceptable lower respiratory tract specimens, limiting the final sample to 70 patients. Forty-seven were males (67.1%) and 23 females (32.9%), while the mean age of the sample was 79.84±14.53 years. Reasons for aspiration were impaired swallowing reflex due to neurological conditions in 36, abnormalities of the upper aerodigestive tract in 13 (presence of feeding tubes=11, anatomical or functional disorders=2), reduced level of consciousness in 9 (cerebral infarct or tumour=3, encephalopathy=5, drug use=1) and witnessed large aspiration in 12 (vomiting=8, food aspiration=4) cases. Thirty-nine (55.7%) of the participants were assigned to the HCAAP group; of them 56.4% were nursing home residents, 5.1% received home care and 2.6% intravenous chemotherapy over the last month, 59% and 15.4% had one and two hospitalizations in the previous trimester respectively and 28.2% had been hospitalized for aspiration pneu-

**Table 1** - Demographic and clinical characteristics of study sample at admission.

| *Variable* | *CAAP* | *HCAAP* | *p* |
|---|---|---|---|
| *Sex* | | | |
| Male | 21 (67.7) | 26 (66.7) | 0.924 |
| Female | 10 (32.3) | 13 (33.3) | |
| Age (years) | 81.45±16.82 | 78.56±12.50 | 0.413 |
| *Function* | | | |
| Ambulatory | 4 (12.9) | 6 (15.4) | 1.000 |
| Bedridden | 27 (87.1) | 33 (84.6) | |
| *Nutrition* | | | |
| Adequate | 19 (61.3) | 25 (64.1) | 0.809 |
| Undernutrition | 12 (38.7) | 14 (35.9) | |
| *Comorbidities* | | | |
| Cardiovascular disease | 18 (58.1) | 19 (48.7) | 0.436 |
| Cerebrovascular disease | 9 (29.0) | 13 (33.3) | 0.700 |
| Dementia | 26 (83.9) | 33 (84.6) | 1.000 |
| Parkinson disease | 6 (19.4) | 4 (10.3) | 0.320 |
| Psychiatric disease | 2 (6.5) | 12 (30.8) | 0.012 |
| Chronic pulmonary disease | 2 (6.5) | 6 (15.4) | 0.287 |
| Diabetes mellitus | 7 (22.6) | 7 (17.9) | 0.630 |
| Malignancy | 3 (9.7) | 4 (10.3) | 1.000 |
| Charlson index | 2.48±1.90 | 2.67±1.90 | 0.740 |
| Median (IQR) | 2 (1, 3) | 2 (1, 4) | |
| *Drug usage* | | | |
| PPIs-$H_2$ antagonists | 7 (22.6) | 13 (33.3) | 0.323 |
| Antibiotics | 7 (22.6) | 31 (79.5) | 0.000 |
| Oxygen therapy | 2 (6.5) | 9 (23.1) | 0.096 |
| *Aspiration risk factors* | | | |
| Witnessed large aspiration | 7 (22.6) | 5 (12.8) | 0.236 |
| Reduced level of consciousness | 3 (9.7) | 6 (15.4) | |
| Impaired swallowing reflex | 18 (58.1) | 18 (46.2) | |
| Abnormalities of the upper aerodigestive tract | 3 (9.7) | 10 (25.6) | |
| *Presenting symptoms* | | | |
| Fever | 10 (32.3) | 12 (30.8) | 0.894 |
| Tachypnea | 13 (41.9) | 15 (38.5) | 0.768 |
| Altered mental status | 16 (51.6) | 13 (33.3) | 0.123 |
| *Biological data* | | | |
| Systolic blood pressure | 116.77±28.99 | 118.49±29.21 | 0.808 |
| Heart rate | 92.03±19.28 | 98.62±24.75 | 0.228 |
| $PaO_2/FIO_2$ | 182.32±66.03 | 191.13±81.51 | 0.628 |
| $PaCO_2$ (mmHg) | 45.39±19.53 | 41.56±17.16 | 0.378 |
| Median (IQR) | 39 (33, 52) | 38 (30, 45) | |
| pH | 7.38±0.13 | 7.41±0.11 | 0.254 |
| Lactate (mmol/L) | 1.59±1.08 | 1.71±1.15 | 0.558 |
| Median (IQR) | 1.3 (0.9, 2.3) | 1.5 (1.1, 2.1) | |
| White blood cell count (x103/μL) | 12.70±7.14 | 12.80±5.68 | 0.947 |
| % neutrophils (%) | 84.21±8.32 | 83.24±9.60 | 0.658 |
| Hematocrit (%) | 35.80±5.95 | 35.76±6.21 | 0.978 |
| Platelet count (x103/μL) | 265.03±114.59 | 243.38±93.57 | 0.387 |
| Glucose (mg/dL) | 145.94±66.72 | 142.26±63.79 | 0.929 |
| Median (IQR) | 128 (104, 177) | 129 (113, 155) | |
| Urea (mg/dL) | 76.29±41.50 | 66.54±53.10 | 0.099 |
| Median (IQR) | 67 (43, 109) | 40 (34, 88) | |
| Creatinine (mg/dL) | 1.17±0.59 | 1.06±0.64 | 0.190 |

| *Variable* | *CAAP* | *HCAAP* | *p* |
|---|---|---|---|
| Median (IQR) | 1.1 (0.8, 1.3) | 0.8 (0.6, 1.5) | |
| Albumin (g/dL) | 3.05±0.61 | 2.95±0.63 | 0.543 |
| Sodium | 136.68±11.77 | 135.69±11.22 | 0.722 |
| C-reactive protein (mg/L) | 151.51±122.21 | 167.52±139.24 | 0.616 |
| *Radiological data* | | | |
| Number of lung fields involved | 2.55±1.18 | 2.67±1.20 | 0.755 |
| Median (IQR) | 2 (2, 4) | 3 (2, 3) | |
| Bilateral involvement | 24 (77.4) | 28 (71.8) | 0.593 |
| Pleural effusion | 9 (29.0) | 5 (12.8) | 0.092 |
| Initial septic shock | 2 (6.5) | 5 (12.8) | 0.452 |
| PSI | 142.74±38.30 | 135.74±31.46 | 0.404 |
| Risk class I-III | 1 (3.2) | 2 (5.1) | 0.919 |
| Risk class IV | 12 (38.7) | 13 (33.3) | |
| Risk class V | 18 (58.1) | 24 (61.5) | |

CAAP: community-acquired aspiration pneumonia; HCAAP: healthcare-associated aspiration pneumonia; IQR: interquartile range; PPIs: proton pump inhibitors; $PaO_2$: partial pressure of arterial oxygen; $FIO_2$: fraction of inspired oxygen; $PaCO_2$: partial pressure of arterial carbon dioxide; PSI: Pneumonia Severity Index.
Categorical data are presented as frequency count (%) and analyzed with chi-square or Fisher's exact test.
Continuous data are presented as mean±standard deviation and analyzed with Student's t test, except from non-normal data presented also as median (IQR) and analyzed with Mann-Whitney test.

monia in the same period. The demographic and clinical characteristics of the study population at admission are presented in Table 1. The HCAAP group was more likely to present with a psychiatric comorbidity (p=0.012) and to have used antibiotics in the previous trimester (p<0.001) than the CAAP group.

*Microbiological data*

Blood cultures were obtained from 27 (38.6%) patients at admission and were positive only in one patient, consisting of *Staphylococcus hominis*. Thoracentesis was performed in six cases, revealing one transudate, four exudates and one empyema, with only the last having positive pleural fluid culture, consisting of *Streptococcus constellatus*. Positive cultures of lower respiratory tract specimens were found in 66 (94.3%) patients. Overall, 115 pathogens (47 in CAAP group and 68 in HCAAP group) were identified (Table 2); 40%, 40%, 12.9% and 1.4% of participants had one, two, three and four pathogens respectively. *Pseudomonas aeruginosa* was the most common pathogen isolated (37.1%), followed by *Klebsiella pneumoniae* (27.1%), *Staphylococcus aureus* (25.7%) and *Acinetobacter baumannii* (20%). *Corynebacterium* species were significantly more prevalent in the HCAAP group (p=0.015). Regarding antibiotic resistance, 24 multidrug-resistant (MDR) and 23 extensively drug-resistant (XDR) pathogens, including *Staphylococcus aureus*, *Enterobacteriaceae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, were isolated from 34 cases (7 cases in CAAP group and 27 in HCAAP group, p<0.001). HCAAP group was found to have significantly more *Klebsiella pneumoniae* (p=0.045) and *Escherichia coli* (p=0.036) MDR isolates than CAAP group. Mycobacterial cultures of lower respiratory tract specimens were performed in 17 patients and they were all negative. Urinary antigens for *Streptococcus pneumoniae* and *Legionella pneumophila* were tested in six patients and were negative in all of them.

*Follow-up data collection*

Therapeutic choices, complications and outcome data of the study population are reported in Table 3. Initial antimicrobial therapy was monotherapy in 30% and combination therapy in 70% of patients. The predominant single agent drug used was piperacillin-tazobactam (90.5%), while the most common combinations were ampicillin-sulbactam plus clindamycin or metronidazole (36.7%), piperacillin-tazobactam plus clindamycin (20.4%) and ceftriaxone plus clindamycin (8.2%). Meropenem-based regimens were initiated in 8 cases and amikacin was added in the empiric therapy in 4 cases. Only 5.7% of participants re-

**Table 2** - Microbiological data of study sample.

| *Pathogens* | *CAAP* | *HCAAP* | *p* |
|---|---|---|---|
| *Pseudomonas aeruginosa* | 13 (41.9) | 13 (33.3) | 0.459 |
| MDR | 0 (0.0) | 1 (7.7) | 1.000 |
| XDR | 2 (15.4) | 5 (38.5) | 0.378 |
| *Klebsiella pneumoniae* | 8 (25.8) | 11 (28.2) | 0.823 |
| MDR | 0 (0.0) | 5 (45.5) | 0.045 |
| XDR | 2 (25.0) | 1 (9.1) | 0.546 |
| *Staphylococcus aureus* | 8 (25.8) | 10 (25.6) | 0.987 |
| MDR | 2 (25.0) | 8 (80.0) | 0.054 |
| XDR | 0 (0.0) | 0 (0.0) | * |
| *Acinetobacter baumannii* | 5 (16.1) | 9 (23.1) | 0.470 |
| MDR | 0 (0.0) | 0 (0.0) | * |
| XDR | 4 (80.0) | 9 (100.0) | 0.357 |
| *Escherichia coli* | 2 (6.5) | 6 (15.4) | 0.287 |
| MDR | 0 (0.0) | 6 (100.0) | 0.036 |
| XDR | 0 (0.0) | 0 (0.0) | * |
| *Corynebacterium spp* | 0 (0.0) | 7 (17.9) | 0.015 |
| *Stenotrophomonas maltophilia* | 2 (6.5) | 5 (12.8) | 0.452 |
| *Enterobacter spp* | 3 (9.7) | 1 (2.6) | 0.315 |
| *Serratia marcescens* | 1 (3.2) | 3 (7.7) | 0.624 |
| *Haemophilus influenzae* | 3 (9.7) | 0 (0.0) | 0.082 |
| *Alcaligenes xylosoxidans* | 0 (0.0) | 1 (2.6) | 1.000 |
| *Moraxella catarrhalis* | 0 (0.0) | 1 (2.6) | 1.000 |
| *Proteus mirabilis* | 1 (3.2) | 0 (0.0) | 0.443 |
| *Streptococcus pneumoniae* | 1 (3.2) | 0 (0.0) | 0.443 |
| Polymicrobial | 15 (48.4) | 23 (59.0) | 0.377 |
| Number per patient | 1.52±0.89 | 1.74±0.79 | 0.231 |
| Median (IQR) | 1 (1, 2) | 2 (1, 2) | |
| MDR-XDR | 7 (22.6) | 27 (69.2) | 0.000 |
| Number per patient | 0.32±0.65 | 0.95±0.79 | 0.000 |
| Median (IQR) | 0 (0, 0) | 1 (0, 1) | |

CAAP: community-acquired aspiration pneumonia; HCAAP: healthcare-associated aspiration pneumonia; MDR: multidrug-resistant; XDR: extensively drug-resistant; IQR: interquartile range.
Categorical data are presented as frequency count (%) and analyzed with chi-square or Fisher's exact test.
Continuous data are presented as mean±standard deviation, median (IQR) and analyzed with Mann-Whitney test.

ceived adequate coverage for atypical pathogens. Patients in the HCAAP group were more likely to receive methicillin-resistant *Staphylococcus aureus* (MRSA)-targeted antibiotics as empiric therapy (p=0.007) and less likely to receive adequate initial therapy (p=0.013) than those in the CAAP group. Isolation of *Acinetobacter baumannii* and *Stenotrophomonas maltophilia* was related with significantly higher risk of being untreated during empiric therapy compared to other isolates (p=0.001 and p=0.014 respectively). Sixty-three patients were available for reevaluation after 72 hours; six cases died and one withdrew from the study before that time. Empiric therapy was judged ineffective in 25 (39.7%) patients, with 4 of them having adequate empiric antibiotic coverage. On the other hand, only 20 of the 38 effectively treated patients had adequate initial antimicrobial therapy. Patients with polymicrobial flora and drug-resistant pathogens were less likely to improve after 72 hours (p=0.020 and p=0.027 respectively), while those that received antipseudomonal or adequate

**Table 3** - Therapeutic choices, complications and outcome data of study sample.

| *Variable* | *CAAP* | *HCAAP* | *p* |
|---|---|---|---|
| *Empiric antimicrobial therapy* | | | |
| Combination | 20 (64.5) | 29 (74.4) | 0.372 |
| Anaerobic-specific | 19 (61.3) | 21 (53.8) | 0.532 |
| Antipseudomonal | 16 (51.6) | 27 (69.2) | 0.133 |
| MRSA-targeted | 0 (0.0) | 8 (20.5) | 0.007 |
| Atypical-targeted | 1 (3.2) | 3 (7.7) | 0.624 |
| Adequate | 17 (54.8) | 10 (25.6) | 0.013 |
| *Reevaluation after 72 hours* | | | |
| Effective | 18 (69.2) | 20 (54.1) | 0.225 |
| Antibiotic change | 9 (34.6) | 19 (51.4) | 0.188 |
| Change in $PaO_2/FIO_2$ | 59.50±73.49 | 31.86±99.49 | 0.234 |
| Change in WBC ($x10^3/\mu L$) | -1.96±4.43 | -2.01±4.74 | 0.968 |
| Change in CRP (mg/L) | -59.24±122.42 | -55.24±123.30 | 0.899 |
| Bronchoscopy | 1 (3.3) | 9 (23.1) | 0.035 |
| Mechanical ventilation | 1 (3.3) | 5 (12.8) | 0.223 |
| *Feeding tube placement* | | | |
| Nasogastric tube | 16 (53.3) | 18 (46.2) | 0.554 |
| Gastrostomy | 1 (3.3) | 2 (5.1) | 1.000 |
| Central venous catheter placement | 3 (10.0) | 10 (25.6) | 0.100 |
| Parenteral nutrition | 6 (20.0) | 18 (46.2) | 0.024 |
| Inotropic support | 5 (16.7) | 12 (30.8) | 0.178 |
| Corticosteroids | 6 (20.0) | 9 (23.1) | 0.759 |
| Blood transfusion | 7 (23.3) | 9 (23.1) | 0.980 |
| *Complications* | | | |
| Total lung atelectasis | 1 (3.3) | 9 (23.1) | 0.035 |
| Hypoventilation-respiratory acidosis | 2 (6.7) | 4 (10.3) | 0.690 |
| Hospital-acquired superinfections | 3 (10.0) | 8 (20.5) | 0.327 |
| Septic shock-multiple organ failure | 7 (23.3) | 11 (28.2) | 0.648 |
| *Outcome* | | | |
| LOS (days) | 13.33±8.82 | 18.67±17.51 | 0.334 |
| In-hospital mortality | 18 (60.0) | 18 (46.2) | 0.254 |
| 90-day mortality | 23 (76.7) | 27 (69.2) | 0.493 |

CAAP: community-acquired aspiration pneumonia; HCAAP: healthcare-associated aspiration pneumonia; MRSA: methicillin-resistant *Staphylococcus aureus*; $PaO_2$: partial pressure of arterial oxygen; $FIO_2$: fraction of inspired oxygen; WBC: white blood cell; CRP: C-reactive protein; LOS: length of stay.
Categorical data are presented as frequency count (%) and analyzed with chi-square or Fisher's exact test.
Continuous data are presented as mean±standard deviation and analyzed with Student's t test.

initial therapy were more likely to show clinical response at 72 hours (p=0.008 and p=0.003 respectively) (Table 4). In 39 cases, the initial antibiotic scheme was changed over the course of treatment, mainly consisting of replacement of a penicillin or a cephalosporin with a carbapenem in 20 cases and addition of colistin or a MRSA-targeted agent in 16 and 14 cases respectively.

Ten patients required therapeutic bronchoscopy for various degrees of atelectasis, four patients were intubated and transferred to the intensive care unit with mean LOS 22.5±17.41 days, three patients were treated with non-invasive ventilation for hypoventilation and emergency tracheostomy was performed in one patient after laryngeal edema. There was a significant difference in the number of patients that required bronchoscopy between the CAAP and HCAAP groups (p=0.035). Nasogastric tube and gastrostomy placement were performed in 49.3% and 4.3% of participants respectively, while central venous catheterization was performed in 18.8%. 34.8%

of patients received parenteral nutrition; significantly more in the HCAAP group (p=0.024). Inotropic support and corticosteroids were initiated in 24.6% and 21.7% of patients respectively and 23.2% were transfused during their hospital stay. There was no difference between groups in terms of observed complications, except from the fact that patients in the HCAAP group developed a total lung atelectasis more frequently than the CAAP group (p=0.035). During their hospitalization, 11 patients developed nosocomial pneumonia with alteration of their initial bronchial pathogens (*Klebsiella pneumoniae*=5, *Acinetobacter baumannii*=5, *Pseudomonas aeruginosa*=4, MRSA=2, *Stenotrophomonas maltophilia*=2), while three of them had positive blood cultures (*Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Candida parapsilosis*).

**Table 4** - Association between effectiveness of empiric therapy and microbiology or antibiotic therapy used.

| *Variable* | *Effective* | *Non-effective* | *p* |
|---|---|---|---|
| *Microbiology* | | | |
| *Pseudomonas aeruginosa* | 14 (36.8) | 7 (28.0) | 0.466 |
| *Klebsiella pneumoniae* | 7 (18.4) | 9 (36.0) | 0.117 |
| *Staphylococcus aureus* | 9 (23.7) | 7 (28.0) | 0.700 |
| *Acinetobacter baumannii* | 7 (18.4) | 7 (28.0) | 0.371 |
| *Escherichia coli* | 5 (13.2) | 3 (12.0) | 1.000 |
| *Corynebacterium spp* | 2 (5.3) | 5 (20.0) | 0.103 |
| *Stenotrophomonas maltophilia* | 5 (13.2) | 1 (4.0) | 0.389 |
| *Enterobacter spp* | 2 (5.3) | 2 (8.0) | 1.000 |
| *Serratia marcescens* | 2 (5.3) | 1 (4.0) | 1.000 |
| *Haemophilus influenzae* | 2 (5.3) | 1 (4.0) | 1.000 |
| Polymicrobial | 16 (42.1) | 18 (72.0) | 0.020 |
| MDR-XDR | 15 (39.5) | 17 (68.0) | 0.027 |
| *Antibiotic therapy* | | | |
| Combination | 26 (68.4) | 20 (80.0) | 0.311 |
| Anaerobic-specific | 20 (52.6) | 18 (72.0) | 0.124 |
| Antipseudomonal | 28 (73.7) | 10 (40.0) | 0.008 |
| MRSA-targeted | 5 (13.2) | 2 (8.0) | 0.693 |
| Atypical-targeted | 3 (7.9) | 1 (4.0) | 1.000 |
| Adequate | 20 (52.6) | 4 (16.0) | 0.003 |

MDR: multidrug-resistant; XDR: extensively drug-resistant; MRSA: methicillin-resistant *Staphylococcus aureus*.
Data are presented as frequency count (%) and analyzed with chi-square or Fisher's exact test.

*Prognosis assessment*

In-hospital and 90-day mortality was 52.2% and 72.5% respectively; no difference between CAAP and HCAAP groups was found, neither in LOS among survivors. Median survival time at the end

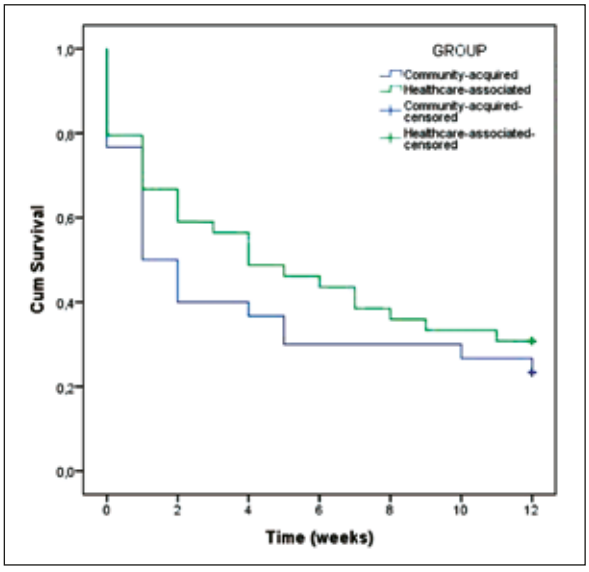


**Figure 1** - Kaplan-Meier graph of cumulative survival over time for CAAP and HCAAP groups (log-rank chi-square test: 0.834, p=0.361) during the three-month follow-up.

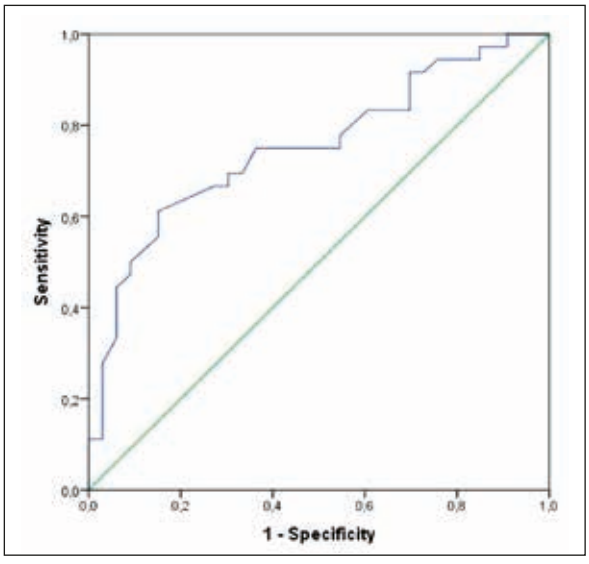


**Figure 2** - Receiver operator characteristic curve of PSI against in-hospital mortality (AUC: 0.752, 95%CI: 0.636, 0.867, p<0.001).

of follow-up for the whole sample was 3 weeks; no significant difference between CAAP (median: 1.5 weeks) and HCAAP (median: 4 weeks) groups was noted (p=0.322) (Figure 1). In-hospital mortality across PSI risk classes was 0%, 36% and 65.9% for classes I-III, IV and V respectively; patients in classes IV and V had significantly higher in-hospital mortality (p<0.001) compared to the original derivation cohort [14]. PSI significantly predicted overall in-hospital mortality, with an area under the receiver operator characteristic curve (AUROC) of 0.752 (95% CI: 0.636, 0.867, p<0.001) (Figure 2). PSI performed similarly for both groups (AUROC CAAP: 0.743, p=0.026, AUROC HCAAP: 0.771, p=0.004, AUROC difference: -0.028, p=0.818).

Regression analysis was performed to identify statistically significant predictors of in-hospital mortality and LOS among survivors, using different models for demographic, clinical, microbiological and therapeutic factors. For in-hospital mortality, increased age (p=0.002), use of oxygen therapy during the previous trimester (p=0.008) and impaired swallowing reflex as an aspiration risk factor compared to witnessed large aspiration (p=0.026) were significant demographic correlates; low serum albumin (p=0.032) and increased radiological involvement (p=0.027) were significant clinical correlates; ineffective initial therapy (p=0.015) was a significant therapeutic correlate, while none of the microbiological factors was found to be significant. Applying a unique model including all of the above significant predictors, four variables were found to be independently associated with in-hospital mortality: age (p=0.004), albumin (p=0.039), radiological involvement (p=0.050) and ineffective therapy (p=0.001). The model was statistically significant (p<0.001), explained 56.5% of the variance in aspiration pneumonia prognosis and correctly classified 81% of cases (AUROC: 0.892, 95% CI: 0.812, 0.973, p<0.001).

For LOS in patients discharged from hospital, lower age (p<0.001), being ambulatory (p=0.010), higher comorbidity burden (p=0.005), not using oxygen therapy (p=0.006), hospitalization in the previous trimester (p=0.015) and reduced level of consciousness as an aspiration risk factor compared to witnessed large aspiration (p=0.001) were significant demographic factors; high white blood cell (WBC) count (p=0.002), low serum creatinine (p=0.002) and albumin (p=0.005) and high serum sodium (p=0.001) were significant clinical factors; isolation of *Klebsiella pneumoniae* (p=0.029), *Corynebacterium* (p=0.050) or *Enterobacter* species (p=0.010) were significant microbiological factors; need for bronchoscopy (p<0.001) or change in antibiotic regimen (p<0.001) were significant therapeutic factors. Applying a unique model including all of the above significant predictors, the following variables were identified as independent correlates of increased LOS: age (p<0.001), comorbidities (p=0.018), oxygen therapy (p=0.005), WBC (p=0.002), serum creatinine (p=0.048), *Enterobacter* species isolation (p=0.005), bronchoscopy (p<0.001) and antibiotic change (p=0.001). The model was statistically significant (p<0.001) and explained 84.5% of the variance in LOS among patients discharged from hospital.

## DISCUSSION

The majority of patients that were hospitalized with diagnosis of aspiration pneumonia in our department over a year's period had increased contact with healthcare services. These cases (HCAAP) were more likely to have history of psychiatric disease, increased antibiotic usage and lower respiratory tract isolation of MDR-XDR pathogens. Although they were initially treated more frequently with MRSA-targeted agents, they were less likely to receive adequate empiric therapy than CAAP cases. They also developed more often total lung atelectasis requiring therapeutic bronchoscopy and were more likely to receive parenteral nutrition. Half the patients died during their hospital stay, but no difference in mortality rate was observed between the two groups. Effectiveness of empiric therapy was the most important prognostic factor, followed by older age, low serum albumin and increased radiological involvement.

Our study has several limitations. Even with the current definition of aspiration pneumonia, it could be difficult to distinguish aspiration pneumonia from aspiration pneumonitis and a degree of contamination may exist. The variety in the acquisition of lower respiratory tract specimens and the lack of anaerobic microbiological investigation was dictated by the daily routine of a large pulmonology department, since it was not

possible for all patients to undergo bronchoscopy or to maintain adequate sampling, transport and culture conditions respectively. The use of qualitative instead of quantitative cultures makes it less possible to clearly identify microbial etiology; however, a recent meta-analysis has shown that there is no clinical advantage in terms of mortality, length of mechanical ventilation or ICU stay and antibiotic change in patients with ventilator-associated pneumonia (VAP) [18].

Our microbiological data are in accordance with most recent research and confirm the predominance of Gram-negative bacilli in lower respiratory tract flora of patients with aspiration pneumonia and the growing challenge posed by antibiotic-resistant strains, especially in, but not limited to, healthcare-associated cases [8, 9]. Similar to Wei et al., we identified *Pseudomonas aeruginosa* as the leading pathogen and showed that isolation of *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Escherichia coli* has decreased since last decade's studies and that the emergence of XDR *Acinetobacter baumannii* is already becoming a major issue [6-9]. Our data also correspond to previous epidemiologic studies of hospital-acquired pneumonia (HAP), VAP and respiratory tract colonization in the ICU in Greece, where *Pseudomonas aeruginosa* - and especially resistant strains - is the most frequent isolate [19-21]. A novel finding of our study is the isolation of *Corynebacterium* species from nearly one fifth of the HCAAP cases. Recent reports have highlighted the pathogenic role of non-diphtheriae *Corynebacterium* species in lower respiratory tract infections, such as HAP or VAP and acute exacerbation of chronic obstructive pulmonary disease or bronchiectasis, occurring in immunocompromised or debilitated patients with pre-existing chronic pulmonary diseases, presence of medical devices, prolonged hospitalizations and use of broad-spectrum antibiotics [22, 23].

Our cohort exhibited higher in-hospital mortality than previous studies, while 90-day mortality exceeded 2/3 of the sample [1, 3-9]. The high proportion of old-aged, bedridden and demented patients may partially explain the observed worse prognosis. PSI modestly predicted in-hospital mortality, with an AUROC much lower than the summary of 0.81 from a recent meta-analysis of hospitalized patients with community-acquired pneumonia (CAP) [24]. Lanspa et al. also found that another prediction tool, CURB-65, performed significantly poorer in their retrospective cohort compared to a cohort of CAP patients from the same hospital [3]. Most of the prognostic factors for in-hospital mortality identified in this study were also evident in previous research, such as older age, chronic respiratory insufficiency, poor nutritional status, increased radiological involvement and ineffective initial therapy [7-9]. The finding that patients with impaired swallowing reflex had significantly higher mortality rate than patients with a witnessed large aspiration event can be possibly attributed to repeated silent aspiration episodes, accumulating a large bacterial load over time, in those with swallowing difficulties or misclassification of aspiration pneumonitis cases as aspiration pneumonia in those with macroaspiration.

We further tested statistically significant correlates of prolonged hospital stay in patients discharged from hospital, although, due to the small sample size, results must be interpreted with caution. Younger ambulatory patients had high LOS mostly because they were more likely to receive mechanical ventilation than older debilitated patients, who had more often a "do not resuscitate" order applied, while it is possible that patients not on long-term oxygen therapy had to stay longer in hospital to fully recover from hypoxemia. More comorbidities, previous hospitalization, malnutrition, high inflammatory markers and electrolyte disorders seem to complicate and prolong hospital stay, findings consistent with previous research in CAP [25, 26]. Patients that required bronchoscopic intervention for atelectasis and change in their antibiotic therapy because of clinical deterioration or hospital-acquired superinfections had also late discharge. In a study examining clinical impact of bronchoscopy in aspiration pneumonia patients admitted to ICU, those that underwent bronchoscopy within 24 hours after intubation had lower mortality and shorter duration of mechanical ventilation and ICU LOS than patients that had bronchoscopy later during their stay [27]. Finally, identified microbiological predictors of LOS are based on limited number of isolates to reach a safe conclusion.

Despite the unavailability of anaerobic cultures and the inability to distinguish between etiological pathogens and simple colonization in our study, we strongly believe that the microbiologi-

cal landscape of aspiration pneumonia has indeed changed over the last decades. Our findings correspond with those of recent research which has found that effective empiric antimicrobial therapy is the cornerstone of management of these patients. In light of the above, selection of antibiotics for empiric treatment should be guided by the need to cover for Gram-negative bacilli, especially *Pseudomonas aeruginosa*. Moreover, aspiration pneumonia patients who have increased contact with healthcare services or facilities are at risk of colonization with resistant strains of Gram positive bacteria, such as MRSA and *Corynebacterium* species, and should be evaluated for the necessity to receive glycopeptides or linezolid in the initial regimen. More studies are required to examine the impact of *Acinetobacter baumannii* isolation in HCAAP cases in order to determine the need for empiric coverage.

In conclusion, we have shown that Gram-negative bacilli are the main isolates from aspiration pneumonia cases and that they demonstrate multi-drug resistance patterns in healthcare-associated infections. Proper assessment of severity regarding demographic, clinical and radiobiological factors, along with administration of appropriate initial antibiotic therapy may lead to more favorable outcomes in clinical practice.

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**Conflict of interest**

None

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# Bismuth-based quadruple *Helicobacter pylori* eradication regimen alters the composition of gut microbiota

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## SUMMARY

Microbiota is a dynamic system showing individual differences in both the number and species of microorganisms. Dietary habits, lifestyle, age, genetic predisposition of the host and use of antibiotics are effective on microbiota. The aim of our research was to carry out a quantitative comparison of *Bifidobacterium spp*, *Bacteroides fragilis*, *Lactobacillus spp*, *Akkermansia mucinophilia* and *Faecalibacterium prausnitzii*, important bacterial microbiota species, before and after antibiotic therapy treated with tetracycline and metronidazole in patients who are diagnosed as positive for *Helicobacter pylori* (HP), and to determine the effects of antibiotic use on the microbiota. Eighteen HP-positive patients were enrolled in this study. A special extraction kit (QIAmp DNA Stool Mini Kit, QIAgen, Germany) was used for the DNA isolation procedure. Primers specific to the 16S rRNA region of the bacteria included in the study were used for the amplification of the target region. All the bacteria were subjected to real-time quantification procedure with PCR method on RotorGene® 20 device (Qiagen, Germany). According to quantification before and after antibiotic use in patients receiving HP treatment, statistically significant decreases were observed in *Bifidobacterium spp* (p=0.001), *B. fragilis* (p=0.001), *Lactobacillus spp* (p=0.001), *A. mucinophilia* (p=0.001) and *F. prausnitzii* (p=0.001). We were unable to identify *B. fragilis* in the microbiota of five patients after treatment. Based on the data obtained, it can be concluded that antibiotics used to treat HP can prepare the ground that could result in dysbiosis in microbiota.

*Keywords*: *Helicobacter pylori*, antibiotic treatment, bismuth, microbiota.

## INTRODUCTION

A considerably high fraction of the world population is infected chronically by *Helicobacter pylori* (HP). This bacterium has been known to be a cause of several diseases from a simple infection to stomach cancer. Thus, it warrants an immediate treatment but depending on the discrepancies at resistance rates in different countries, as well as due to incomplete implementations of the corresponding protocols, the success rates of the treatment protocols differ considerably among different countries. In the countries where bismuth procurement is possible and considering resistance rates in the country, bismuth plus two antibiotics and proton pump inhibitors (PPI), *i.e.*, a quadruple treatment protocol, is offered. On the other hand, in the developing countries where bismuth is not available, a combination of PPI plus three antibiotics is used as a varied foursome treatment protocol. In these treatment protocols, the antibiotics such as metronidazole and tetracycline are commonly used [1]. In our country, HP is as widespread as other countries in our region [2]. The primary use of antibiotics is the protection

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from a pathogenic agent and to ensure a therapy [3]. Antibiotics, besides their expected changes on target microorganisms, may also lead to undesirable changes in microbiota. Although found to be extremely safe medications so far, they have recently gained interest as their effects on microbiota and host. Antibiotics may deteriorate the stability of microbiota, which has been quite stable in a host. Regarding the antibiotic utilization, variations in terms of quantity used and the types have been observed. These conditions usually lead to dysbiosis which, in a long term, results in the changes in the functional properties of microbiota. The impact of antibiotics on microbiota has been studied since 1940s. However, at that time, only short-term effects on variations could be investigated by culture-based techniques [4]. The data obtained by these methods are not adequate to find out completely the whole range of antibiotic effects on the microbiota. At present, it is possible not only to put forward the short-term effects of antibiotics but also the long-term effects in terms of molecular techniques [5, 6]. The studies on antibiotic treatments have shown that the microbiota decreases by one-fourth to one-third of its initial quantity and more, resulting in considerable changes in the composition of microbiota [5]. In this context, the modern molecular methods have boosted both the sensitivity and specificity of the analysis done on microbiota [7].
In this study, we aimed to screen, by real-time polymerase chain reaction (qPCR), the changes in the gut microbiota of HP patients, on whom a treatment protocol involving PPI, tetracycline, metronidazole, and bismuth was applied.

## PATIENTS AND METHODS

*Ethics committee approval and informed consent*
An approval was obtained at the beginning of the study from the local ethics committee of the Zekai Tahir Burak Women's Health Training and Research Hospital. Written informed consent was obtained from all patients who participated in this study.

*Patients'selection*
For this study, 26 HP-positive patients admitted to Department of Gastroenterology, Gazi University, were enrolled after screening their baseline stool samples. Exclusion criteria were the history of antibiotic therapy right before up to one month of starting the study; the patients who were treated by corticosteroids, prebiotics or/and probiotics in last six months; and a medical history of kidney disorder, gut surgery, diabetes, obesity, inflammatory bowel disease, irritable bowel syndrome, mental disorder, neurological disorders or cardiopulmonary disease. The patients who were not able to complete the HP eradication treatment were not included.
Only 18 out 26 patients completed the study. The others were excluded due to medication side-effects (n=2), failure to comply with the treatment protocol (n=2), and protocol violation due to exclusion criteria [6].
The compliance to the treatment was checked by empty medication boxes returned after the treatment.
The HP eradication protocol was a quadruple bismuth-containing regimen (PPI bid, tetracycline 500 mg qid, metronidazole 500 mg tid, and bismuth subsalicylate 262 mg qid for 10 days). Among 18 patients, 15/18 (83%) were responders (HP eradication) and 3 were still positive for HP. These 18 patients had the full dosage of the treatment regimen and for this reason, the results were presented as per protocol analysis. These patients (completing the full protocol) were enrolled in the microbiome study.
Pre-treatment HP diagnosis was made by pathological evaluation of endoscopic biopsies (2 antrum, 2 corpus and 1 incisura angularis). Any positive result from these biopsies confirmed HP diagnosis. Post-treatment HP eradication was confirmed by $C^{13}$ urea breath test after 6 weeks.
Thirty-six stool samples were collected before and after six weeks of HP eradication therapy. All samples collected both before and after antibiotic medication were kept at -80 °C until the day of study.

## REAL TIME PCR CONDITIONS

*Preparation of Standards*
In this study, *Bifidobacterium breve* ATCC 15700, *Bacteroides fragilis* ATCC 25285, *Lactobacillus acidophilus* ATCC 4356, *Akkermansia muciniphila* ATCC BAA-835 and *Faecalibacterium prausnitzii* ATCC 27766 from the American type standard culture

**Table 1** - Primers belonging to standard strains, target region volume and PCR temperature.

| *Microorganism* | *Primer name* | *Primer (5'=>3')* | *Target Region Volume (bp*)* | *Annealing temperature (°C)* | *Reference* |
|---|---|---|---|---|---|
| *Bifidobacterium* spp. | g-Bifid-F | CTCCTGGAAACGGGTGG | 550 | 55 | [8] |
| | g-Bifid-R | GGTGTTCTTCCCGATATCTACA | | | |
| *Bacteroides fragilis group* | g-Bfra-F | ATAGCCTTTCGAAAGRAAGAT | 495 | 50 | [8] |
| | g-Bfra-R | CCAGTATCAACTGCAATTTTA | | | |
| *Lactobacillus* spp. | Lact-F | AGCAGTAGGGAATCTTCCA | 341 | 50 | [9] |
| | Lact-R | CACCGCTACACATGGAG | | | |
| *Akkermancia mucinophilia* | AM-1 | CAGCACGTGAAGGTGGGGAC | 327 | 60 | [10] |
| | AM-2 | CCTTGCGGTTGGCTTCAGAT | | | |
| *Faecalibacterium prausnitzii* | Fprau223F | GATGGCCTCGCGTCCGATTAG | 199 | 58 | [11] |
| | Fprau420R | CCGAAGACCTTCTTCCTCC | | | |

*bp: base pair.

collections (ATCC) were used (Table 1) [6-10]. The primary sequences specific to 16S rRNA of the bacteria were used and at least three standards were implemented to obtain the standard curve. The numbers of copies of the bacterium in the clinical samples were determined from the drawn standard curves.

*DNA extraction*
The DNA was isolated from the stool samples using an extraction kit designed for special stool samples (QIAamp DNA Stool Mini Kit, Qiagen, Hilden, Germany) following the extraction protocol prescribed for the kit (Protocol: Using Stool Tubes for Isolation of DNA from Stool for Human DNA Analysis).

*qPCR*
Amplification reaction for the quantitation was realized using the SYBR green ($RT^2$ SYBR Green qPCR, Qiagen, Germany) Rotor-Gene 6000 instrument. For each sample, an amplification mixture in a final volume of 25 µL was prepared. Melting curve analysis using the fluorescence by qPCR was drawn.

*Statistical analysis*
The data were fed into a Microsoft Excel sheet and the amount of bacterium in logarithmic scale was calculated. The evaluation of the data was realized by using Statistics package for Social Sciences (SPSS) 22 package program (Inc. USA). Average and percentile values were calculated and necessary comparisons were made. The average values of the data related to both pre- and post-antibiotic utilization were assessed by Mann-Whitney U test and $p<0.05$ was accepted as significant (SPSS version 22, Inc USA).

## RESULTS

The study population consisted of 7 males (39%) and 11 females (61%). The mean age value was 42±13 years. The quantitation of all bacterial species in the baseline stool samples revealed that the bacterial composition was similar at the baseline, regardless of the gender and the age of the patients. Seven patients were overweight according to their body mass index (BMI) (between 25 and 9.9 $kg/m^2$). However, we did not detect any significant difference in the gut microbiota composition of these patients (*Bifidobacterium* spp. p=0.508, *B. fragilis* p=0.456, *Lactobacillus* spp. p=0.204, *A. mucinophilia* p=0.714, *F. prausnitzii* p=0.714).
The quantification of bacteria in stool was represented under $\log_{10}/g$ logarithmic scale. In addition, the average values of the bacterium at $\log_{10}/g$ stool are also shown in Table 2.

*Bifidobacterium spp. qPCR results*
*Bifidobacterium breve* ATCC 15700 was used to determine the copy number in both pre and post-treatment samples. *Bifidobacterium* spp. were

**Table 2** - Bacteria qPCR results average and standard deviation.

| *Microorganism* | *Before antibiotic usage average±SD* (log10/g)* | *After antibiotic usage average±SD* (log10/g)* |
|---|---|---|
| *Bifidobacterium* spp | 7.81±1.12 | 6.11±2.74 |
| *B. fragilis* | 8.24±1.19 | 5.69±2.89 |
| *Lactobacillus* spp | 7.39±0.61 | 6.32±1.74 |
| *A. mucinophilia* | 8.08±0.60 | 6.10±0.86 |
| *F. prausnitzii* | 9.21±2.09 | 6.32±1.59 |

*SD: standard deviation.

significantly decreased (p=0.001) and were detected in four patients.

*B. fragilis qPCR results*
*Bacteroides fragilis* ATCC 252855 isolate was used to determine the copy number of *B. fragilis* before and after antibiotic medication. By comparing the samples taken before and after antibiotic medication, it was observed that *B. fragilis* was significantly decreased (p=0.001). This bacterium was not detected in five patients and all of them were of an age over 60 years.

*Lactobacillus spp. qPCR results*
*Lactobacillus acidophilus* ATCC 4356 was taken as a standard strain to determine the copy number of *Lactobacillus* spp. before and after antibiotic use. From the samples taken before and after the antibiotic medication, it was concluded that *Lactobacillus* spp. were significantly decreased (p=0.001).

*A. mucinophilia qPCR results*
Using the standard *Akkermansia muciniphila* ATCC BAA-835 isolate, indicated a decrease in the copy number of *A. mucinophilia* after antibiotic use at a statistically significant level (p= 0.001).

*F. prausnitzii qPCR results*
*Faecalibacterium prausnitzii* ATCC 27766 was used as a standard isolate, and copy numbers of *F. prausnitzii* were determined before and after the antibiotic use. We found that *F. prausnitzii* was significantly decreased after antibiotic treatment (p=0.001).

*Antibiotic effect on microbiota*
All bacterial groups were significantly decreased following HP eradication regimen. Mann-Whitney U test was carried out for each bacterial species and indicated that the differences were statistically significant (p=0.001). The degree of decrease in bacterial groups in patients who had eradicated or failed eradication was similar (p>0.05).

## DISCUSSION

In this study, we aimed to investigate the effects of antibiotics on microbiota, which are used for the treatment of HP patients in Turkey and other countries. For this purpose, volunteer patients, who were diagnosed with HP at the gastroenterology department, faculty of medicine at Gazi University, were enrolled in the study. A gastroenterology specialist implemented the standard treatment protocol to the volunteers. Regarding the protocol, a quadruple treatment protocol, involving PPI, tetracycline, metronidazole, and bismuth subsalicylate, was administered for ten days. Two stool samples were taken from the patients: before antibiotic medication and six weeks after the medication. We believe that 6 weeks is a short time for recovery of the gut microbiota. In the study, two different classes of antibiotics were used: tetracycline and metronidazole. The simultaneous medication of both tetracycline and metronidazole significantly affected *Bifidobacterium* spp., *B. fragilis*, *Lactobacillus* spp., *A. mucinophilia*, and *F. prausnitzii*, whose concentrations were found to be significantly lowered after antibiotic use as compared to the pre-medication condition. It has been reported that a couple of weeks after the treatment, the microbiota returns back to its pre-treatment state. However, in some other cases, this effect lasted for a long-term and even three months after the medication, the microbiota was not able to come back to its pre-medication state

completely [12-14]. Earlier analysis of gut microbiota in our study might yield a false result due to slow recovery in some patients.

In an experimental animal model, an antibiotic cocktail of ampicillin, gentamicin, metronidazole, neomycin, and vancomycin was implemented to animals for ten days. In the presence of a bacterial loading, anatomical, histological, and immunological variations were detected. After studying the microbiome by 16S rDNA and qPCR, it was noticed that the number of bacteria has decreased by ten times compared to that of the control group and this reduction was more prominent on *Firmicutes spp*. However, in the case of *Bacteroides* and *Akkermansia* spp, an increase was observed [15]. Similarly, in our study, the reduction of *Firmicutes* was more than ten-times but in contrast to the previous reports, we found a ten-fold decrease in *Bacteroides* and *Akkermansia* spp. Moreover, no *B. fragilis* were found in five patients.

The antibacterial activity of bismuth, involved in the treatment protocol, has been well established [16]. It can be speculated that the use of bismuth could have contributed to the decrease in the number of these bacteria. It was observed that in the HP patients on whom clarithromycin and metronidazole treatment protocol had been implemented, in culture-based studies, as a short-term effect of antibiotics, an absolute change at microbiota was noted [17-19].

A sharp and considerable decrease was noted soon after the treatment for the bacteria, *Bifidobacterium, Clostridium,* and *Bacteroides* spp, which can be cultured, and *Bifidobacterium* spp. and *Bacteroides* spp. bacteria showed a sustainable decrease lasting up to four weeks after the treatment [17]. A research group, considering the importance of individual evaluation of the effects of antibiotics, investigated both short and long term effects of antibiotics on intestine microbiota at three HP patients taking clarithromycin and metronidazole. The stool samples were evaluated at zero day, 8-13 day, first year, and fourth year. However, a decrease in bacteroides was observed in all patients. The discrepancies in the bacteria type were followed for all patients [20]. As a similarity to our study, a decrease in all groups of bacteria was noticed among the individuals along with their different decrease rates.

Regarding our data, it can be speculated that the statistically significant decline of all the bacteria may be brought by the high sensitivity of the anaerobic bacteria to metronidazole. In fact, in our country, the rate of metronidazole resistance of *B. fragilis* is under 1% [21].

A continuous and long-term medication using antibiotics leads to the occurrence of dysbiosis in the intestine, which actually triggers the factors leading to several diseases. In a study carried on 718 volunteers with a four-year follow-up, a positive correlation between antibiotic use of the patients and the evolution of intestine inflammatory disease was observed. Moreover, it was expressed that the intensity of the disease had increased with the increasing rate of antibiotic use [22].

There are studies investigating the role of antibiotic-induced dysbiosis and carcinogenesis [23]. In a similar study involving 4029 patients, antibiotic-induced dysbiosis was related with colon cancer risk. Moreover, an antibiotic prescription for five or more times in a year significantly increased cancer risk [24].

There are many weak points in our study. We had chosen only 5 bacterial species which represents the major species that might change after antibiotic therapy. Another reason is that these bacteria were associated with many conditions in previous reports. This approach (may be not complete) might facilitate the translation of this complex and expensive microbiome analysis procedure into clinical practice.

Probiotics have been studied for their protective role in antibiotic-induced dysbiosis. Some researchers claimed that probiotics are effective in reducing side effects of HP eradication and no probiotic is superior to the other [25]. On this aim, in a study, a group of patients took anti-HP treatment and another group had HP treatment plus a probiotic. Consequently, in both groups, *Firmicutes filum* was decreased statistically but the change was mild in the group treated with the probiotic. It has been proved that probiotics are effective in both protecting microbiota balance connected to antibiotic use and keeping the changes caused by antibiotics at minimum levels [26]. Probiotics also increase patient adherence to HP eradication regimens by attenuating antibiotic-related side effects [27, 28]. Recent Maastricht Guideline recommended probiotic supplementation in HP eradication [29].

Our study is the first attempt in the literature investigating the effect of bismuth-based quadru-

ple therapy on gut microbiota. Bismuth is widely used in areas where clarithromycin resistance has increased. However, bismuth salt is a strong topical antibiotic with widespread effects on local gut community. This study, which assesses the short-term effect of HP treatment on microbiota, is a preliminary study. We need of long term studies to assess whether dysbiosis in HP-treated patients is a risk factor for disease.

**Conflict of interest**

There is no conflict of interest regarding the publication of this article.

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# Antibiotic prophylaxis in children undergoing abdominal surgery for neoplastic diseases

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## SUMMARY

Little is known about the effectiveness of antibiotic prophylaxis for prevention of surgical site infections (SSIs) in paediatric abdominopelvic surgical oncology. A retrospective analysis was performed upon the incidence of SSIs in children receiving a 24-hour antibiotic prophylaxis with cefazolin for abdominopelvic oncological surgery.
In all, 145 patients (57% females) with a median age of 4 years underwent surgical procedures for abdominopelvic tumours. No SSIs were detected, despite the various risk factors known to be associated with their occurrence (such as pre- and post- surgical chemotherapy, long hospitalization, intensive care unit admission and drain placement). Cefazolin prophylaxis seems to be safe and effective in preventing SSIs in children undergoing abdominopelvic surgery for oncological diseases.

*Keywords*: surgical site infections, antibiotic prophylaxis, paediatric surgical oncology.

## INTRODUCTION

Surgical site infections (SSIs) are among the most common health care associated infections in children, with rates ranging from 2.5% to 5.4%. They are connected to significant postoperative morbidity (such as delayed wound healing, increased antibiotics administration with their related adverse effects and possible systemic spread) and mortality [1-6].
Oncological patients could be considered at particular risk of SSIs because of the neoplastic process itself and the immunosuppression induced by the chemotherapy [7].

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Antibiotic prophylaxis is a well-known strategy for preventing SSIs also in the field of surgical oncology [8]. Unfortunately, there are no data on its effectiveness of in children.
The aim of this study is to investigate the incidence of SSIs in children undergoing surgery for abdominopelvic neoplastic diseases receiving a 24 hours prophylaxis with cefazolin.

## PATIENTS AND METHODS

The Istituto Giannina Gaslini (IGG) in Genoa-Italy is a tertiary care center for children.
Clinical data of children undergoing abdominopelvic surgery for neoplastic diseases from January 2008 to December 2016 were retrospectively analyzed. The incidence of SSIs in the 30 days following the surgical procedure was investigat-

ed. All patients received antibiotic prophylaxis with cefazolin 25 mg/kg (maximum 2000 mg) 30 minutes before skin incision and two more times within the first 24 hours after surgery (total: 3 doses). Adherence to prophylactic protocol was monitored through a checklist routinely completed before skin incision. Povidone-iodine was used as skin preparation agent in all cases.

For each patient the following data were collected: demographics, type of malignancy, the American Society of Anesthesiologists status (ASA), type of surgical procedure, wound classification [12], insertion of a central venous catheters (CVC), requirement of blood transfusion, placement of urinary catheter, suction or open drain placement, postoperative intensive care unit admission, pre- and post- operative length of hospital stay, previous hospitalization within 30 days, pre- and post-surgical chemotherapy.

SSIs were diagnosed when the infectious process affected either the incision or the deep tissue at the operation site [9]. Wound cultures and medical imaging were performed in case of sign of SSIs as erythema, tenderness, induration or purulence at the site of incision, associated or not with systemic signs. Blood cultures were performed in case of clinical signs of systemic infections (*i.e.*, fever, chills, and/or hypotension). Central venous catheter related infections were diagnosed according to our previous definitions [10]. Urine cultures were performed in case of fever in absence of signs of SSIs or in case of suspected urinary tract infections.

In consideration of the descriptive nature of the study and in the absence of any prospective randomization or historical control comparison, no statistical analysis was performed.

## RESULTS

During the study period a total of 146 patients underwent surgery for abdominopelvic tumors. Table 1 reports tumor diagnosis in prevalence order, while Table 2 summarizes demographic data. Particularly, median age at surgery was 4 years (1 month -28 years), with 4 patients (3%) younger than 30 days of life. Median weight was 15 kg (4 kg - 76 kg). In 38% of cases chemotherapy was administered before surgery. The majority of patients (96%) was hospitalized more than 24 hours before surgery with 56% of them at least 30 days before it. The ASA score was I-II in ¾ of patients, with only 1 patient that was ASA score IV. The median duration of the surgical procedure was 180 minutes (25 minutes - 845 minutes), but in ¼ of cases the procedure was longer than 5 hours. Laparoscopic surgery was performed in near 1/5 of cases. After surgery a drain was placed in 57% of patients. Admission in intensive care unit was necessary in 23% of patients. Twenty-six percent of cases required blood transfusions. Finally, a CVC (80% tunneled) was inserted concomitantly with the abdominal surgery in near a half of patients, while urinary catheter was positioned in 66%. Median duration of hospitalization was 10 days, in 8% of cases it was longer than 30 days.

Surgical wound was classified as clean (class I) in 135 (92%) cases, as clean/contaminated (class II) in 7 (5%) and as class III (dirty) in 4 (3%) be-

**Table 1** - Histological types categorized in relation to tumor primary site.

| *Diagnosis* | *n (%)* |
|---|---|
| *Adrenal tumor* | 82 (56%) |
| Peripheral neuroblastic tumor | 78 |
| Mesothelial cyst | 1 |
| Adrenaline producing tumor | 3 |
| *Ovarian tumor* | 26 (18%) |
| Teratoma | 14 |
| Cistoadenoma | 8 |
| Leydig Sertoli cell tumor | 1 |
| Fibrothecoma | 1 |
| Embrional carcinoma | 1 |
| Dysgerminoma | 1 |
| *Renal tumor* | 25 (17%) |
| Wilms tumor | 21 |
| Sarcomatoid cancer | 1 |
| Rhabdoid tumor | 1 |
| Carcinoma | 1 |
| Cystic nephroma | 1 |
| *Pelvic tumor* | 8 (6%) |
| Sacrococcygeal teratoma | 3 |
| Pelvic rhabdomyosarcoma | 3 |
| Pelvic york sac tumor | 1 |
| Pelvic schwannoma | 1 |
| *Gastrointestinal tumor* | 3 (2%) |
| Gastric teratoma | 1 |
| Hepatoblastoma | 1 |
| Anaplastic tumor of the colon | 1 |
| *Abdominal wall tumor* | 2 (1%) |
| Sarcoma | 2 |

**Table 2** - Patient demographics, surgery-related data and admission-status features.

| | *n (%)* |
|---|---|
| *Patient demographics* | |
| Sex<br>- Male<br>- Female | <br>63 (43%)<br>83 (57%) |
| Age, median, range | 4 years (1 month -28 years) |
| Weight (kg), median, range | 15 (4-76) |
| *ASA physical status*<br>- I - II<br>- III - IV | <br>107 (73%)<br>39 (27%) |
| *Surgery-related data* | |
| Wound class<br>- I<br>- II<br>- III | <br>135 (92%)<br>7 (5%)<br>4 (3%) |
| Surgical time (minutes), median, range | 180 (25-845) |
| *Type of procedure*<br>- Open<br>- Laparoscopic | <br>115 (79%)<br>31 (21%) |
| Procedures requiring blood transfusion | 38 (26%) |
| Central venous catheter placement concomitantly with surgery<br>- Partially implanted central venous catheters (Broviac)<br>- Totally implanted central venous catheters (port)<br>- Non-tunneled central venous catheters<br>- Peripherally inserted central catheters | 71 (49%)<br><br>57<br><br>10<br><br>3<br><br>1 |
| Urinary catheter placement | 97 (66%) |
| Drain placement<br>- Suction<br>- Open<br>- Both | 82 (57%)<br>76<br>6<br>1 |
| *Admission-related data* | |
| Preoperative chemotherapy | 56 (38%) |
| Previous hospitalization within 30 days | 82 (56%) |
| Being inpatients | 140 (96%) |
| Postoperative admission<br>- Intensive care unit<br>- Surgery ward | <br>34 (23%)<br>112 (77%) |
| Postoperative chemotherapy | 63 (43%) |
| Length of hospital stay (days), median, range | 10 (2-384) |

cause of gross contamination or spillage of the operative field. After surgery, fever occurred in 18 (12%) patients. No case of SSIs was detected. In 5 cases (28% of febrile episodes) an infection of the CVC-insertion site that required its removal occurred. In 3 cases (3% of patients with urinary catheter) urinary tract infections were diagnosed, in 2 cases due to *Escherichia coli*, in a single case due to *Candida albicans*.

Chemotherapy was administered to 43% of patients within 1 month after surgery, but no case of SSIs was observed, even in presence of granulocytopenia.

No adverse event related with cefazolin administration was observed.

## DISCUSSION

In the present study we evaluated the incidence of SSIs in children undergoing surgery for abdominopelvic neoplastic diseases who received 24 hours antibiotic prophylaxis with cefazolin that still represents the recommended prophylaxis in many surgical procedures [11-16]. Although oncological surgery is usually clean (as in the 92% of our patients), considering the lack of specific recommendations, we decided to administer an antibiotic prophylaxis because of the immunocompromission induced by the neoplastic disease itself and by the chemotherapy administered. Even if chemotherapy-induced granulocytopenia is a well-known risk factor for infections in oncological patients, data from adults indicate that surgical and ICU-related factors are more critical [7].

Our patients presented many risk factors generally associated with SSIs [2, 8, 17-20]. As a matter of fact, 3% percent were neonates, 25% of the surgical procedures lasted more than 5 hours, 57% required drains, 49% CVCs and 66% urinary catheters, while surgical wound contamination was infrequent (3% only). Moreover, 96% were inpatient for more than 24 hours at time of surgery, 23% required ICU admission and postoperative length of hospital stay was longer than 30 days in 12%. In spite of all these risk factors, no SSIs was observed.

Few studies have been published in the last 10 years on the rate of infectious complications in children undergoing surgery for abdominal solid tumors [2, 21-24]. Qureshi et al. reported a 4% of in-

cidence of SSIs in 106 neuroblastoma patients treated from 2006 to 2011, while Ritchey et al. rated a 2% of SSIs in 534 children required surgery for Wilms' tumor from 1986 to 1994 [23, 24]. However no data on antibiotic prophylaxis were documented.
In conclusion, in the absence of a randomized clinical trial, our results demonstrate that antibiotic prophylaxis with cefazolin is safe and effective in preventing SSIs in children undergoing abdominopelvic surgery for tumors.

**Conflict of interest**
No conflict of interest has to be declared.

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# Day-On, Day-Off emtricitabine, tenofovir disoproxil fumarate and efavirenz single tablet regimen (DODO) as maintenance therapy in HIV-infected patients

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## SUMMARY

Reduced dose schedules may be feasible options to simplify antiretroviral therapy (ART) in selected HIV-1-infected individuals. Efficacy and safety of a Day-on, Day-off (DODO) schedule of tenofovir disoproxil fumarate, emtricitabine and efavirenz (FTC/TDF/EFV) single tablet regimen (STR) was assessed. Twenty-seven patients were prescribed the DODO schedule and were monitored for 48 weeks. Switching criteria were: no previous ART failure, no AIDS-defining illnesses, T CD4 cell nadir >200/mm$^3$, and HIV-RNA below detection limit (40 copies/mL) for at least six months. Clinical and laboratory data, including plasma HIV-RNA levels, T CD4 and CD8 counts, liver and kidney function, lipid levels and ultrasensitive C-reactive protein (us-CRP) were assessed at baseline, week 4, 12, 24, and 48. Statistical analysis was performed by paired Student's T-test for comparison between baseline and each time point, and Chi square test for CD4/CD8 ratio comparison. In all, 26 out of 27 patients maintained plasma HIV-RNA levels below the detection limit through the entire follow-up. One patient experienced low level plasma HIV-RNA rebound at week 36 (47 copies/ml) and immediately reverted to the conventional dose schedule of FTC/TDF/EFV; plasma HIV-RNA was undetectable after four weeks. No major changes on liver and kidney function tests, lipid levels and us-CRP were observed. Although no profound modifications of T CD4 count were observed during follow-up, the CD4/CD8 ratio increased significantly at week 48 compared to the baseline ($p<0.05$). In conclusion, 48-week DODO administration of the fixed dose FTC/TDF/EFV STR combination was safe and effective in maintaining HIV viral replication below the detection limit in a selected group of HIV-1-infected individuals.

*Keywords*: HIV-1, antiretroviral therapy, simplification/dose reduction, emtricitabine/tenofovir DF/efavirenz, single tablet regimen.

## INTRODUCTION

Antiretroviral therapy (ART) has dramatically improved the quality of life of people living with HIV; despite its remarkable effectiveness and tolerability, ART is currently a lifelong treatment that can be associated with long-term side effects on liver, kidney, bone and lipid metabolism, according to type of drug regimen used [1]. Pill burden and fatigue are also common issues of lifelong treatments and both are major causes of poor adherence and treatment failure [2]. Furthermore, the cost of a lifelong ART is a major concern for healthcare systems worldwide [3]. To date, several strategies have been attempted to reduce pill burden and/or long-term side effects. Single-tablet regimens (STR) are the

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therapeutic options with the lowest pill burden, intended for use in both naïve and experienced patients. Available combinations include two nucleoside reverse transcriptase inhibitors (NRTIs) plus a third drug (either non-nucleoside reverse transcriptase inhibitors, NNRTI or integrase inhibitors, INSTI) in a single tablet, administered once daily. However, STR long-term side effects are comparable with non-STR regimens, given that the number of drugs used is unchanged. The so-called "less drug regimens" (LDR) represent an alternative therapeutic approach, widely evaluated in recent years, either constituted by a single antiretroviral drug (mono therapy, usually boosted protease inhibitors) or a combination of two drugs (dual therapy) [4, 5]. Mono/dual therapy are feasible options in selected groups of patients as maintenance regimens, allowing to reduce the number of drugs for long-term treatment and sometimes to reduce the overall pill burden [6, 7]. Such treatment is, however, not recommended for patients naïve to ART, with history of AIDS-defining illnesses or ART-experienced with multiple drug resistance [8]. Besides, data on the long-term benefit of such reduction on overall liver, kidney, bone and lipid metabolism are currently unavailable. A third ART simplification strategy, namely reduced schedule of administrations, has been recently proposed and this option may potentially reduce the burden of long-term side effects and poor adherence due to pill fatigue. The FOTO and BREATHER trials have shown that reducing the number of administrations (*e.g.* five days on/two days off) is a potentially safe and effective option, as long as drugs with prolonged half-lives (*i.e.* efavirenz, EFV) are included in the regimen [9, 10]. To date, there is little evidence on EFV-based STR regimens with alternative administration schedule in clinical practice. We report on effectiveness and safety of an alternate-day administration schedule of emtricitabine (FTC)/tenofovir disoproxil fumarate (TDF)/efavirenz (EFV)-based STR performed in a real-life setting.

## PATIENTS AND METHODS

Between July 2014 and August 2015, twenty-seven HIV-1-infected patients undergoing once daily FTC/TDF/EFV STR were switched to a Day-On Day-Off (DODO) schedule, as simplification strategy in the context of routine clinical practice. The switch was proposed to patients meeting the following criteria: no previous failure to ART; no previous AIDS-defining illnesses; T CD4 cells nadir >200/mm$^3$; HIV RNA below detection limit (undetectable or <40 copies/mL) for at least 6 months prior study entry. Extensive information was provided to the patients and a written informed consent was obtained before switching to DODO schedule. To reduce the risk of missed doses due to forgetfulness, patients were instructed to take their medication every odd day of the month. Clinical and laboratory data were collected at baseline, week 4, 12, 24, 36, and 48 of treatment; an additional time point (week 52) was settled to further confirm end of follow-up plasma HIV viral load levels. In case of viral rebound (defined as a single detection of plasma HIV-RNA equal to or above 40 cp/ml) patients were instructed to switch to the conventional administration schedule and viral load was reassessed after 4 weeks. The main endpoint was to assess the proportion of subjects who maintained plasma HIV RNA below the detection limit (<40 copies/mL or undetectable); secondary endpoint was to define the impact of DODO regimen on renal and liver function tests, lipid profile, mean T CD4 and T CD8 cell count, CD4/CD8 ratio and ultrasensitive C-reactive protein (us-CRP). T cell subsets were assessed by flow cytometry (FacsCanto, Becton Dickinson, NJ); HIV plasma viral load was assessed by Real-Time HIV-1 assay (Abbott, IL). Statistical analysis was performed by coupled paired Student's T-test to compare baseline data to those obtained at the subsequent time-points, and Chi square test for CD4/CD8 ratio comparison; differences were considered statistically significant if $p<0.05$.

## RESULTS

Twenty-seven patients were included in the analysis; no patient was lost to follow-up during the 48-week monitoring. Four subjects (14.8%) were females; mean age at enrollment was 48.5±9.5 years. Mean nadir of T CD4 cell count was 346/mm$^3$ (range 204-736) and mean T CD4 cell count at enrollment was 735/mm$^3$ ±350. Mean number of previous ART regimens was 2.26 (range 1-8) and mean number of years from initial HIV diagnosis

was 13.9 (range 5-30). Duration of suppression of HIV replication before the initiation of the DODO schedule was 65 months on average (range 6-204). Twenty-six out of 27 subjects (96%) maintained plasma HIV viral load below the detection limit throughout the 48-week follow-up. One patient experienced low-level viral rebound at week 36 (plasma HIV-RNA: 47 cp/ml) and was instructed to revert immediately to the conventional once daily FTC/TDF/EFV schedule; HIV viral load was again undetectable 4 weeks after interruption of the DODO schedule and in subsequent assessments. No increased prevalence of HIV-RNA values "<40 copies/ml" over "undetectable" was observed among the other 26 patients during the follow-up: 22 of them were "undetectable" and 4 were "<40 copies/ml" both at baseline and at week 48. In addition, only 1 patient out of the 26 had plasma HIV-RNA <40 copies/ml at the additional time point of week 52, while in the others

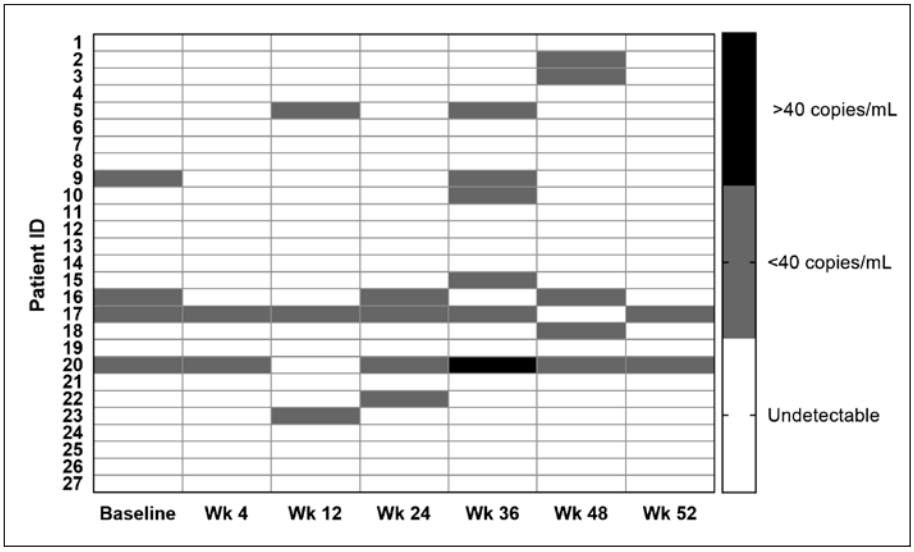


**Figure 1** - HIV-RNA plasma viral load trend during the day-on, day-off FTC/TDF/EFZ dose reduction schedule follow-up.

Note: Each row represents a single patient. Data from the patient who experienced a viral load rebound (patient 20) are showed during the day-on day-off schedule and after the once daily administration was resumed (week 36).

**Table 1** - Laboratory test results during the day-on, day-off FTC/TDF/EFZ dose reduction schedule.

| *Test* | *NR* | *Baseline* | *Wk 4* | *Wk 12* | *Wk 24* | *Wk 36* | *Wk 48* |
|---|---|---|---|---|---|---|---|
| Creatinine (mg/dl) | 0.60-1.40 | 0.89 (±0.16) | 0.86 (±0.13) | 0.85 (±0.16) | 0.82 (±0.15) | 0.85 (±0.14) | 0.91 (±0.15) |
| eGFR (ml/min) | >90 | 112.8 (±32.35) | 117.9 (±31.65) | 117.9 (±36.25) | 120.8 (±33.01) | 115.8 (±29. 06) | 108.2 (±31.42) |
| AST (U/l) | 0-40 | 20.3 (±5.98) | 18.3 (±5.97) | 18.28 (±5.07) | 18.68 (±6.92) | 20.4 (±7.63) | 20.5 (±9.53) |
| ALT (U/l) | 0-40 | 33.6 (±12.95) | 33.2 (±14.47) | 29.8 (±11.9) | 32.0 (±19.4) | 34.0 (±14.4) | 33.9 (±16.3) |
| Cholesterol (mg/dl) | <200 | 201.8 (±40.05) | 183.8 (±56.7) | 186.4 (±55.95) | 197.5 (±53.55) | 190.1 (±54.8) | 191.5 (±44.5) |
| Triglycerides (mg/dl) | <170 | 149.6 (±96.6) | 168.9 (±56.7) | 160.7 (±103.61) | 163.8 (±121.0) | 187.7 (±162.6) | 150.3 (±78.0) |
| us-CRP (mg/l) | <3 | 1.06 (±0.94) | 1.10 (±1.18) | 2.20 (±3.86) | 2.40 (±4.77) | 1.23 (±1.23) | 1.59 (±1.26) |

Mean and standard deviation (in brackets) are showed for each test at different time points. Abbreviations: NR, normal range; Wk, week; eGFR, estimated glomerular filtration rate (Cockroft-Gault formula); AST, aspartate aminotransferase: ALT, alanine aminotrasferase; us-CRP, ultrasensitive C-reactive protein.

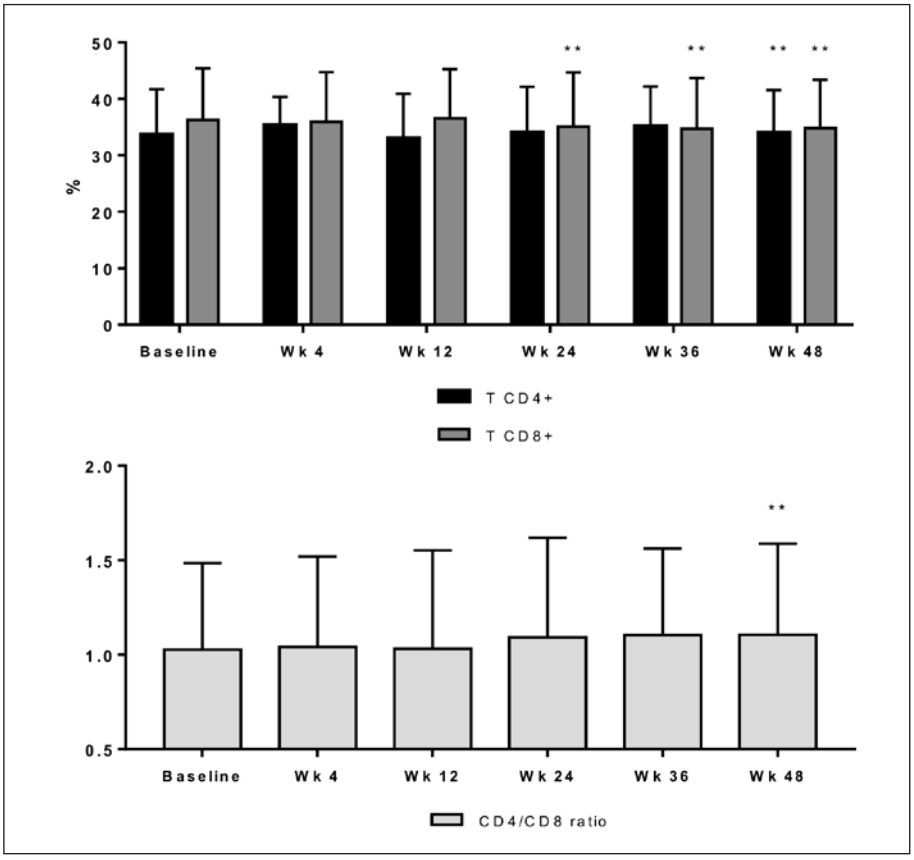


**Figure 2** - Immunological trend during the day-on, day-off FTC/TDF/EFZ dose reduction schedule.

Note: Timepoint percentages of T CD4 and T CD8-positive cells (Panel A), and CD4/CD8 ratio (Panel B) are represented. Mean values and standard deviations are shown; **p value <0.05 compared to baseline.

it was undetectable (Figure 1). Self-reported compliance to the Day-On, Day-Off schedule was excellent. No major changes of liver function tests, total cholesterol, triglycerides and us-CRP were observed during follow-up (Table 1); a slight increase in estimated glomerular filtration rate was observed at week 24 ($p<0.05$), but reverted to baseline values by week 36. A small but significant increase in T CD4 cell count ($p<0.05$) was observed at week 48 compared to baseline (Figure 2 panel A), while circulating T CD8 cell counts decreased significantly by week 24 ($p<0.05$); CD4/CD8 ratio increased by week 48 compared to baseline values ($p<0.05$, Figure 2 panel B).

## DISCUSSION

The present study was conducted with the aim to report our clinical experience obtained by prescribing an FTC/TDF/EFZ dose reduction schedule in the context of our clinical routine. Forty-eight weeks Day-On, Day-Off administration of the fixed dose combination FTC/TDF/EFV STR was safe and effective in maintaining HIV viral replication below the detection limit in a selected group of HIV-1-infected individuals. To our knowledge, this is the first report suggesting this peculiar reduced dose administration schedule as a potential simplification strategy for patients with sustained undetectable HIV replication, stable immunological profile and no previous ART failures.

Robust evidence of the feasibility of an EFZ dose reduction schedule comes from the double-blind, placebo-controlled ENCORE1 study. Week 48 and week 96 analysis demonstrated durable virological non-inferiority of EFZ 400 mg to the standard 600 mg dose when given in combination with TDF and FTC to a large population of HIV-infected antiretroviral-naïve patients, regardless of baseline HIV viral load levels [11, 12].

The week 96 intention-to-treat analysis showed that 90% of patients in the reduced 400 mg EFZ group versus 90.6% in the standard dose 600 mg group had plasma HIV viral load of less than 200 copies/mL (primary endpoint). In addition, non-inferiority was also demonstrated for viral load threshold of 50 copies/mL. Compared to the standard 600 mg, lower 400 mg dose was associated with fewer EFZ-related adverse events and reduced number of drug discontinuations over the 96 weeks follow-up period [12]. Of note, study population was heterogeneous in terms of ethnic origin, geographical areas, baseline T CD4 count and plasma HIV-RNA levels, making the results of the study broadly applicable. Based on these results, the authors advocated the redefinition of the efavirenz dose for first line treatment among HIV-infected adults. In the FOTO study, 48-week Five days-On, Two days-Off Lamivudine (3TC), TDF and EFV regimen was effective in maintaining HIV-1 replication suppressed in a small group of adult patients [9]. More recently, encouraging results were obtained by the open-label non-inferiority trial BREATHER, that compared continuous FTC/TDF/EFV with short cycle treatment, consisting in two days off therapy per week, in 199 HIV-infected patients aged 8-24 years who had been virologically suppressed for at least 12 months before enrollment [10]. Efavirenz, tenofovir DF and emtricitabine are drugs characterized by long half-lives whose pharmacokinetics and wash-out timing have been elucidated; the potency of this combination, in the absence of HIV resistance mutations, has also been demonstrated [13-15]. Finally, a study presented at the 2016 HIV Drug Therapy Conference held in Glasgow, reported that 96% of patients undergoing a four days-on three-days off schedule with different drug regimens maintained undetectable viral load throughout a 48-week period, despite low or undetectable plasma concentrations after three days of treatment interruption [16]. Economic savings are among the potential advantages of dose reduction schedules; indeed, the burden of cost of HIV treatment represents an important issue, both in the prospect of a progressive scaling up of antiretroviral treatment worldwide and at single country level [3]. Recently, Costa and coll. conducted a local budget impact analysis of reducing EFZ dose from 600 mg to 400 mg daily, obtaining a cost saving of about 55,000 Euro over two-year period [17]. Of note, none of the 39 HIV-infected patients undergoing reduced 400 mg EFZ dose experienced viral rebound over the follow-up period. Although these results cannot be generalized, they underline the potential economic benefits coming from reduction of antiretroviral drugs dosage. In our experience, 96% of the patients that switched to the DODO schedule maintained plasma HIV-RNA levels below the detection limit throughout the 52-week monitoring. Collectively, these data suggest that a reduced administration schedule of an EFV-based antiretroviral drug regimen is a feasible option for selected HIV-infected patients with uncomplicated disease; this strategy may also be evaluated with the most recent once daily antiretroviral drug combinations (either STR or not) such as those including the INSTI elvitegravir/cobicistat and dolutegravir, although no supporting data are available at the moment. Of note, the only patient who experienced an HIV viral rebound had a previous history of small vessel cutaneous vasculitis and showed increased us-CRP levels throughout the entire observation period (range: 5.5 - 22.9 mg/L, upper laboratory limit 3 mg/L). Transient low level viral replication in the context of a well functioning antiretroviral therapy has been associated with increased risk of viral rebound and treatment failure over time [18-20]. Therefore, to minimize the risk of drug failure and development of resistance, the patient resumed immediately the once daily FTC/TDF/EFV administration; plasma HIV-RNA level decreased below the detection limit 4 weeks later and in further assessments. The immune reconstitution process was not impaired during the follow-up; although no robust changes of T CD4 cell number were recorded, week 48 mean CD4/CD8 ratio increased significantly compared to baseline. Data from literature point out that interruption of TDF administration is associated with an increase of total cholesterol levels, due to a possible statin-like effect of TDF; in our study, no significant modification of total cholesterol levels, LDL, HDL, triglycerides, and liver and renal function tests (with the exception of transient increase in mean glomerular filtration rates) was observed compared to baseline [21-23]. Of note, us-CRP values also remained stable, suggesting the absence of a consistent increase in inflammation due to potential - although not detectable by clinical routine methods - outbreak of

low level viral replication following FTC/TDF/EFV dose reduction, that could potentially lead to loss of viral replication control in the future. No missed doses or lack of adherence to the alternate administration schedule were recorded; self-reported-adherence to the alternate day regimen was excellent, throughout the follow-up, further supporting the feasibility of the DODO schedule. Additional follow-up may be helpful to better establish the long-term effectiveness of this regimen. Limitations of the study were the single-arm design, the small number of patients enrolled and the short duration of the follow-up. However, the persistence of high rates of HIV viral suppression during the observation period suggests that the Day-On, Day-Off FTC/TDF/EFV dose reduction schedule is an effective, safe and cost-saving choice as a potential maintenance regimen in selected HIV-infected patients. Moreover, our findings suggest that dose reduction schedules may also be conceived with other once daily antiretroviral drug combinations, including those containing the INSTI elvitegravir/cobicistat and dolutegravir, given that the prolonged half-lives of these drugs may allow a reduced schedule of administrations. Further studies are needed to confirm these findings.

**Conflict of interest**

None of the authors has any conflict of interest to declare.

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# Risk factors for recurrences in patients with hepatitis C virus after achieving a sustained virological response: a multicentre study from Turkey

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## SUMMARY

In this study, we aimed to determine the late relapse rate in hepatitis C patients with sustained virological response after interferon-based regimens, and evaluated the predictors of late relapse while comparing the real-life data of our country with that of others. A multicenter retrospective study was performed to investigate the data of patients infected with HCV who obtained sustained virological response after classical or pegylated interferon alpha (PegIFNα) and ribavirin (RBV) for 48 weeks. Sustained virological response was based on negative HCV RNA level by PCR at the end of six months after the therapy. The information of patients enrolled in the study was retrieved from the hospital computer operating system and outpatient follow-up archives. We evaluated the age, gender, HCV RNA levels, HCV genotype, six-month and further follow-up of patients with sustained virologic response, presence of cirrhosis, steatosis and relapse. In all, 606 out of 629 chronic hepatitis C patients (mean age was 53±12 years; 57.6 % of them were female) with sustained virological response were evaluated. We excluded 23 patients who relapsed within six months after the end of treatment (EOT). The mean follow-up period of the patients was 71 months (range: 6-136) after therapy. Late relapse rate was 1.8% (n=11) in all patients. Univariate Cox proportional hazard regression models identified that cirrhosis and steatosis were associated with the late relapse [(p=0.027; Hazard Ratio (HR) 2.328; 95% confidence interval (CI): 1.309-80.418), (p=0.021; HR 1.446; 95% CI: 1.243-14.510, respectively]. In multivariable Cox regression analysis, steatosis was the only independent risk factor for late relapse (p=0.03; HR 3.953; 95% CI: 1.146-13.635). Although the late relapse rate was approximately 2% in our study, clinicians should consider that pretreatment steatosis may be an important risk factor for late relapse.

*Keywords*: hepatitis C, HCV, relapse, steatosis.

## INTRODUCTION

A great deal of progress and success have been achieved in treatment of chronic hepatitis C (CHC) infection with newly introduced direct-acting antiviral agents [1]. Besides, on a global perspective, all these favorable achievements still remain as a distant hope for patients living in countries with limited resources [2]. The rate of long-term relapse (developing after 24 weeks) in patients with CHC infection has been reported to be 0-17% [3, 7]. We could not find any study including quite a number of cases genotype 1 patients and focused their late relapse in medical literature. In another aspect, we are of the opinion that this late relapse rates may reflect the new therapies long term results.
In this study, we aimed to determine the late relapse rate in a total of 629 patients with sustained virological response and the risk factors of late relapse.

## PATIENTS AND METHODS

*Study patients and design*
In this study, the records of adult CHC patients who were admitted to 15 center of chronic hepatitis outpatient clinics of university and training and research hospitals after presenting sustained virological response (SVR) following therapy with recombinant or pegylated interferon alpha (PegIFNα) + ribavirin (RIB) and were followed up between 2000-2015 were evaluated retrospectively. SVR was based on negative HCV RNA level by PCR at the end of six months after EOT. Late relapse was defined HCV RNA detectability after have achieving an SVR.
Patients who were not treated adequately in terms of therapy duration (12 months for genotype 1 or 4 patients, and 6 months for genotype 2 or 3 patients) or due to drug-related side effects and those who were lost to follow-up were excluded. The patients with HBV/HCV, HIV/HCV co-infection, drug abuse, malignant disease, or autoimmune hepatitis and those who were pregnant were also excluded. Patients who relapsed within six months after EOT were not included in the study.
The data of the patients in terms of age, gender, serum HCV RNA level by PCR, HCV genotype, treatment regimens, presence of cirrhosis and Child Pugh scores, presence of steatosis were obtained from patients' data files.

*Treatment regimens*
Patients who received interferon-alpha 2a or 2b or pegylated INF alpha 2b 1.5 mg/kg/w or 2a 180 mg/kg/w; subcutaneously + RIB (1200 mg/day or 1000 mg/day for those with body weight of >75 kg and <75 kg, respectively; per oral) combination were included. The treatment regimen was continued for 12 months in patients with HCV genotype 1 or 4 and 6 months in those with genotype 2 or 3.

*HCV RNA and HCV genotype assays*
HCV RNA was assessed using bDNA (branched DNA) signal amplifier (Versant HCV RNA 3.0 Assay, Bayer Corporation Diagnostics, USA [detection range 615-7690000 U/ml]) or RT-PCR (real time polymerase chain reaction, Cobas TaqMan HCV test v 2.0 [detection range 25-391000000 U/ml]) in 70% of patients. The patients were grouped according to their pre-treatment HCV RNA levels. That is, one group comprised the patients with pre-treatment HCV RNA levels of ≥600000 IU/L and the other group included patients with pre-treatment RNA levels of <600000 IU/mL.
HCV genotype was assessed by using Innolipa HCV II commercial kit (Bayer Diagnostics, USA). The patients were grouped according to their HCV genotypes. That is, the patients having genotype 1 and those with genotype 2, 3 or 4 were analyzed as separate groups.

*Evaluation of cirrhosis and liver steatosis*
Liver biopsy findings were evaluated using the modified Knodell (Ishak) scores. A fibrosis score of 5/6 was accepted as cirrhosis. Child Pugh score was calculated if a patient had been diagnosed as cirrhosis. Cirrhosis was also accepted according to radiological (ultrasonography, magnetic resonance imaging) appearances (liver contour lobulation, caudate/left lobe hypertrophy splenomegaly and thrombocytopenia). All patients were evaluated with ultrasonography for the diagnosis of hepatic steatosis.

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*Statistical analysis*

The data were expressed as mean ± SD or %. Late relapsed and non-relapsed HCV patients were presented by age, genders, quantitative HCV RNA, follow up time and the proportion of cirrhosis and steatosis in Table 1.

The Chi-square test or Fisher's exact test (when Chi-square test assumptions do not hold due to low expected cell counts), where appropriate, was used to compare proportions in late relapsed and non-relapsed patient groups. A *p*-value lower than 0.05 was considered as statistically significant.

Associations between possible risk factors and late relapse were evaluated using the Multivariable Cox proportional hazard regression models based on variables with a *p*-value of ≤0.05. The following variables were considered in the univariate analysis for late relapse: age (<60 years, ≥60 years), gender, HCV RNA level (<6x10$^5$ IU/mL, ≥6x10$^5$ IU/mL), HCV genotype, treatment regimens (standard IFN or pegylated IFN), presence of cirrhosis and steatosis. Variables that were found significant in univariate analysis were evaluated with multivariable analysis. All *p*-values were based on two-sided hypothesis tests, and α were set at 0.05. Analysis was performed with SPSS Statistics version 16.0 (IBM, Chicago, IL, USA).

## RESULTS

Investigation of the hospital records of a total of 606 patients with sustained virological response after treatment with pegylated INF α2b + RIB (n=298), pegylated INF α2a + RIB (n=285), INF α2b + RIB (n=32), or INF α2a + RIB (n=14) combinations revealed that they completed the therapy period and showed virological response at the end of therapy. The mean follow-up period of the patients was 71 months (range, 6-136) after the therapy.

The mean age of the patients was 53±12 and 58% of them were female. Genotype 1 was detected in 98% of the patients. Late relapse was recorded in

**Table 1** - Clinical and laboratory features of study patients.

| | *Non-relapsed patients (n = 595)* | *Late relapsed patients (n =11)* | *P value* |
|---|---|---|---|
| *Variables* | | | |
| Age | 53±12 years | 58±8.5 years | 0.133 |
| Gender (Female) | 349/595 (58.6) | 5/11 (45.5 ) | 1 |
| HCV RNA | 850000 (IQR, 284946- 2835000 IU/ml) | 2107950 (IQR, 594500- 3130000 IU/ml) | 0.699 |
| Cirrhosis | 9/585 (1.5%) | 1/11 (9.1 %) | 0.169 |
| Steatosis | 164/589 ( 27%) | 7/11 (63.6 ) | 0.016 |
| Follow up duration | 43±27 months | 42±30 months | 0.0001 |
| Relapse duration | - | 17±5.6 months | - |

**Table 2** - Results of univariate and multivariate Cox regressions model analysis for the development of late relapse.

| *Variable* | *N (%)* | *Hazard Ratio* | *(%95 Confidence Interval)* | *P value (univariate)* | *P value (multivariate)* |
|---|---|---|---|---|---|
| Gender (Female) | 349 (58.6) | 1.134 | 0.346-3.716 | 0.835 | |
| Age >60 years | 379 (62.7) | 2.342 | | 0.276 | |
| Liver steatosis | 171 (28.2) | 1.446<br>3.953 | 1.243-14.510<br>1.146-13.635 | 0.021 | 0.03 |
| Cirrhosis | 10 (1.7) | 2.328 | 1.309-80.418 | 0.027 | 0.063 |
| Pre-treatment HCV RNA level (>2x10$^6$IU/mL) | 361 (59.7) | 1.945 | 0.516-7.73 | 0.326 | |
| Standard INF+RIB | 48 (8.1) | 0.966 | 0.124-7.553 | 0.974 | |

11 patients (1.8%). The mean late relapse time was 16.9±5.3 months. Ten patients were diagnosed as cirrhosis before the treatment. All cirrhotic patients had a Child Pugh classification of A. The clinical and laboratory features of late relapsed vs. non-relapsed patients are summarized in Table 1. Gender, age, genotypes, HCV RNA load at the beginning of therapy, classical IFN- based treatment were not found as predictive factors to predict late relapse rate. Univariable Cox proportional hazard regression models identified that cirrhosis and steatosis were independently associated with the late relapse [$p$=0.027; Odds Ratio (OR) 2.328; 95% confidence interval (CI): 1.309-80.418, and $p$=0.021; OR 1.446; 95% CI: 1.243-14.510, respectively]. Multivariable Cox proportional hazard regression models identified that steatosis is only independent factor for late relapse ($p$=0.03; HR 3.953; 95% CI: 1.146-13.635) (Table 2).

## DISCUSSION

Chronic hepatitis C virus (HCV) infection, which is a global health problem, affects approximately 170 million people all over the world [2]. Additionally, long-term carriage may lead to the development of cirrhosis, liver decompensation, hepatocellular carcinoma and even death [6]. Although the treatment regimens for hepatitis C virus (HCV) infection has moved beyond interferons and toward direct-acting antiviral agents (DAAs) such as protease inhibitors and NS5A and NS4B inhibitors, payers and the governments even in high income countries have been limiting the coverage of these therapies because of their high costs. This situation makes it difficult to access DAAs in some regions of the world. For this reason, in some countries, interferon based regimens are still in use.

It is well-known that SVR is a good marker of response to CHC treatment [8-10]. Moreover, late HCV recurrence (relapse or re-infection) still remains as a problem. In high-risk group patients (such as HIV-positive subjects, patients on hemodialysis, intravenous drug users), increased recurrence rates support the higher possibility of re-infection [11]. In this study, particularly patients who have low risk of re-infection were investigated and compared with medical literature in Medline (Table 3).

The phenomenon of late relapse and re-infection

**Table 3** - Chronic HCV infection studies with late relapse rates in patiens with low risk for reinfection.

| *Study/publicationyear* | *Total number of patients with SVR* | *Follow Up Time* | *Late Relapse, n (%)* | *Risk factors for relapse* |
|---|---|---|---|---|
| Veldt et al, 2004 | 286 | 5 years | 12 (4.7) | None |
| E. Formann, 2006 | 187 | 29 months | (0) | NA |
| Desmond et al, 2006 | 147 | Mean 2.3 years (range 0.3-10.3) | (0) | NA |
| daCostaFerreira et al, 2010 | 174 | Median duration 47 months (12-156) | (0) | NA |
| Swain et al, 2010 | 1077 | Annually (for 5 years) | (0) | NA |
| Sood et al, 2010 | 100 | 6 months to 8 years | 8 (8) | Cirrhosis |
| Giannini et al, 2010 | 231 | Median duration 41 months weeks | 2 (1) | NA |
| Li et al, 2012 | 146 | 33.45 +/- 16.41 months (range: 12-85) | 8 (8.9) | Older Age |
| Rutter et al, 2013 | 103 | 21 months (range: 7-64) | 2 (1) | One patient was cirrhotic, both carried the genotype 1b |
| Uyanikoglu et al, 2013 | 196 | 33.5 months (range, 6 to 112) | 2 (0) | NA |
| Papastergiou et al, 2013 | 145 | 68.8 ± 35 months | 2 (1) | NA |
| M. P. Manns 2013 | 1002 | 5 years | (0) | NA |
| Giordanino, Chiara, 2014 | 115 | 9.2 years | (0) | NA |

in patients with CHC infection may be confused with each other. Even though the term "late relapse" is used, it shouldn't be overlooked that some of the patients with high relapse prevalence constitute those with high risk of re-infection [7, 12]. In a meta-analysis, the rate of late relapse in low-risk patients for re-infection has been reported as less than 2% [11]. In a study conducted with 196 CHC patients in our country, relapse after SVR has been detected in 1.02% in a period of 33 months in average and those cases had low levels of HCV viremia [13].

Most of the studies stated/emphasized the predictive factors for relapse developing after SVR as age, HCV genotype, IL-28B genotype/polymorphism, presence of steatosis, cirrhosis, viruses in peripheral blood mononuclear cells, ribavirin concentration at the end of treatment [5, 14, 20].

In addition to difficult achievement of SVR in cases with advanced liver injury (especially in patients infected with genotype 1 HCV), there are some studies which reveal that risk of development of early relapse is higher in those patients [21, 23]. There are few studies investigating the relationship between late relapse and advanced liver injury (except for transplanted patients) other than this study [24, 25]. Late relapse was detected in five out of 28 cirrhotic patients and this result was statistically significant compared to non-cirrhotic patients in the study conducted by Sood et al. [24, 25]. Rahman et al reported significantly higher rates of late relapse in treatment experienced cirrhotic patients (three out of six) who achieved SVR compared to non-cirrhotic patients during a 5-year prospective study [26].

Hepatosteatosis develops as a result of deposition of fat droplets in hepatocytes [27]. HCV (especially genotype 3) is a risk factor for hepatosteatosis in addition to environmental factors triggering it [27]. It is known that presence of steatosis causes progressive fibrosis and decreases SVR rates [28]. Furthermore, it is under debate whether hepatosteatosis is an independent risk factor for recurrence in patients with SVR or not.

In a study evaluating patients infected with Genotype 2 and 3, it has been stated that presence of steatosis had positive correlation with early relapse and the relapse rate was around 36.4% in patients with steatosis and high viral load [15]. This situation may be interpreted as being irrelevant to genotype 3. In studies focusing on genotype 1 HCV patients similar to our study, it has been reported that hepatosteatosis had a negative effect on SVR [34]. In another study evaluating liver transplant patients, the sensitivity of presence of steatosis in forecasting of virologic relapses in post-transplant liver biopsies was around 89% [30]. To the best of our knowledge, we could not come across any studies focusing on relation between late relapse and steatosis in non-transplanted HCV patients in medical literature. We believe that our study is unique in this aspect.

Major limitations of our study are its retrospective design, inability to perform genetic studies in patients with relapse, and detection of steatosis only by ultrasound. However, inclusion of large number of patients and long-term follow-up are its strong aspects.

In conclusion, the rate of late relapse after SVR in patients with CHC infection in our study is consistent with previous medical literature. Although SVR has been achieved in patients with cirrhosis and steatosis, the possibility of late relapse should be kept in mind.

**Conflict of interest**

There is no conflict of interest.

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# HPV and *Chlamydia trachomatis* coinfection in women with Pap smear abnormality: baseline data of the HPV Pathogen ISS study

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## SUMMARY

*Chlamydia trachomatis* (Ct) and human papillomavirus (HPV) are the most common sexually transmitted pathogens. Whereas it is well known that infection with oncogenic HPV genotypes increases the risk of cervical cancer (CC), the implication of Ct in the pathogenesis of CC is still controversial. Hence, to investigate the possible implication of Ct infection alone, or with concomitant HPV infection, in the severity of cervical lesions, we conducted a study in 164 Caucasian HIV-negative women with abnormal Pap. Genomic HPV and Ct DNA were detected in 97 (59%) and 16 (10%) women respectively, and 15 (9%) of women were infected by both. Of the HPV positive samples, 89 (79%) were HR-HPV types or probable HR types and HPV16 was the most represented genotype. Interestingly, it was observed that co-infection was more frequent than HPV infection alone in women with high grade lesions.

*Keywords*: *Chlamydia trachomatis*, HPV, genotyping, Pap test.

## INTRODUCTION

*Chlamydia trachomatis* (Ct) is the most common sexually transmitted bacterial pathogen, frequently asymptomatic and consequently unrecognized and untreated. The infection can become persistent and may promote a pathogenic process leading to chronic diseases such as pelvic inflammatory disease and infertility [1]. Serious Ct sequelae including cervical hypertrophy and induction of squamous metaplasia, suggest a possible relationship with human papillomavirus (HPV) infection [1-4]. In Italy, Ct infection is not subject to mandatory reporting, and a sentinel surveillance study recognized by a network of Italian clinical centers of sexually transmitted infections showed an overall prevalence of 3.2% [5]. High-risk oncogenic HPV genotypes (HR-HPVs) are the most important etiological agents of cervical cancer (CC). A systematic review of studies conducted in Italy revealed a HR-HPV infection prevalence of 8% in the general population with no difference among the northern, central and southern regions of Italy. However, this percentage is higher in women aged 18-29 participating in CC screening programs [6, 7].

The involvement of Ct in the pathogenesis of CC is controversial and its specific role in this neoplasia has not yet been completely clarified. Chlamydial infection may damage the mucosal barrier thus increasing the susceptibility to HPV co-infection. Alternatively, the bacterium may interfere with the host immune response by decreasing the clearance of a concomitant HPV infection, thereby increasing the persistence of the

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virus and the possibility of progression to cancer [3-4]. The aim of the present study was to investigate a possible implication of Ct infection alone or with a concomitant HPV infection, in the severity of cervical lesions.

## PATIENTS AND METHODS

The survey was carried out in DNA samples from 164 Caucasian HIV-negative women with abnormal Pap test, randomly selected among those participating in the Multi-center Cohort Study, known as the HPV Pathogen ISS study. This study was designed to determine risk factors for the progression of cervical lesions to cancer in HIV-positive and -negative women. All patients were enrolled from March 2002 to November 2003 [8-10]. The women, who were referred for colposcopic examination to the gynecological departments of five Italian hospitals in Milan, Bologna and Rome, were submitted to an epidemiological interview, and sampling for molecular and serological assays.

**Table 1** - Patient's characteristics, Pap test and histology data.

| | *Frequency, no. (%)* | |
|---|---|---|
| *Age group (years)* | | |
| < 20 | 5 | (3.05) |
| 21-30 | 51 | (31.10) |
| 31-40 | 60 | (36.59) |
| 41-50 | 30 | (18.29) |
| > 50 | 18 | (10.98) |
| *Smoking* | | |
| No smoker | 93 | (56.71) |
| Current smoker | 64 | (39.02) |
| Ex - smoker | 7 | (4.27) |
| *Age of earlier sexual activity (years)* | | |
| < 15 | 16 | (9.76) |
| 15-20 | 128 | (78.05) |
| 21-25 | 20 | (12.20) |
| *Sexual partners during life* | | |
| 1-2 | 78 | (47.56) |
| 3-5 | 74 | (45.12) |
| > 5 | 12 | (7.32) |
| *Pregnancies* | | |
| None | 142 | (86.59) |
| 1-2 | 21 | (12.80) |
| 3-4 | 1 | (0.61) |
| *Contraceptive method* | | |
| None | 60 | (36.59) |
| Oral contraceptive | 82 | (50.00) |
| Intrauterine device | 5 | (3.05) |
| Condom | 8 | (4.88) |
| Natural method | 9 | (5.49) |

| | *Frequency, no. (%)* | |
|---|---|---|
| *Previous genital infection reported* | | |
| No | 73 | (44.51) |
| HSV | 2 | (1.22) |
| Candida | 88 | (53.66) |
| Chlamydia | 1 | (0.61) |
| *Previous HPV infection reported* | | |
| No | 129 | (78.66) |
| Yes | 35 | (21.34) |
| *Frequency of Pap test* | | |
| Regular (every 1-3 years) | 59 | (35.98) |
| Irregular | 98 | (59.76) |
| Never | 7 | (4.27) |
| *Pap test* | | |
| ASC-US | 41 | (25.00) |
| LSIL | 97 | (59.15) |
| HSIL | 26 | (15.85) |
| *Histology* | | |
| Normal | 15 | (9.15) |
| CIN1 | 36 | (21.95) |
| CIN2 | 12 | (7.32) |
| CIN3 | 35 | (21.34) |
| HPV-NCIN | 21 | (12.80) |
| ND | 45 | (27.44) |

ASC-US: atypical squamous cells of undermined significance; LSIL: low-grade squamous intraepithelial lesions; HSIL: high-grade squamous intraepithelial lesions; CIN: cervical intraepithelial neoplasia; HPV-NCIN: HPV lesions without evidence of CIN; ND= not determined.

Informed consent was obtained from all participants, and the study was approved by the institutional Ethical Committees of all the hospitals that contributed to the HPV Pathogen ISS study. Our work used the baseline data and cervical samples collected for the HPV Pathogen ISS study. For the molecular analyses, DNA was extracted from cells of cervical smears by QIAamp DNA mini kit (QIAGEN). The HPV genotyping was performed by sequencing of GP5/GP6 PCR amplicons, as previously described [9]. The Ct DNA was detected with primer set CTR 70/71 targeting 16S-23S spacer rRNA, while Ct genotyping was performed by MS-1F and MS-1R primers, spanning an approximately 936-bp region of the *omp*A gene [11, 12]. PCR amplicons were purified, sequenced and analyzed with BLAST program (http://www.ncbi.nlm.nih.gov/BLAST).
Cytology and histology data were jointly modeled by ordered logit regression in STATA13 (StataCorp. 2013) within a structural equation modeling (SEM) framework.

## RESULTS

The characteristics of the patients are listed in Table 1. All women presented an abnormal Pap test diagnosed as: atypical squamous cells of undermined significance (ASC-US) in 25% of cases, high-grade squamous intraepithelial lesions (HSIL) in 59.15% and low-grade squamous intraepithelial lesions (LSIL) in 15.85%. A total of 149 patients (90.85%) revealed moderate or severe intra-epithelial lesions.
After colposcopy examination and biopsy of lesional tissues, histological results highlighted Cervical Intraepithelial Neoplasia (CIN) 1, CIN 2 and CIN 3 in 21.95%, 7.32%, and 21.34% of the women, respectively. Biopsies without evidence of CIN (HPV-NCIN) were detected in 12.8 % of patients (Table 1).
Genomic HPV DNA was detected in 97 (59%) and Ct DNA in 16 (10%) women, while 15 women (9%) had both infections. Out of the 112 HPV positive samples, 89 (79%) were considered HR-HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59) or probable HR type (53, 66, 81). The most represented genotype was HPV16, found alone in 44% of the samples. Six different Ct serovars were identified: G (25%), H (25%), F (19%), D (12%), E (12%) and K (7%). In the HPV-Ct co-infected samples, the HPV 16 genotype was the most prevalent, being detected in 8 samples (53.33%), while the HPV 6, 31, 33, 45, 52,5 3, and 66 were detected in similar percentages (6.67%). Excluding HPV 6, all the genotypes found are considered at high risk or probable high risk for CC.
The association of patients' demographic and clinical characteristics, including the presence of HPV infection, with cytological and histological diagnoses has been described for the whole cohort in a previous publication [8]. In our study the univariate ordinal logit models for Pap test results (categorized as ASCUS, LSIL, HSIL) showed that women infected with HPV, alone or concurrently with Ct had a twofold (2.71; 95%CI: 1.38; 5.32) and fourfold (4.18; 95%CI: 1.31; 13.35) higher risk, respectively, of presenting a cervical intraepithelial neoplasia than their uninfected counterparts. Multivariate ordinal logit models for Pap test results, adjusted for factors significantly associated with the outcome in the univariate analysis (smoking and Pap test frequency), showed that women infected by HPV alone or in conjunction with Ct had proportional odds ratio (OR) of 2.17 (95%CI: 1.08; 4.39) and 3.87 (95%CI: 1.19; 12.56) with respect to their uninfected counterparts, for any cytological level (Table 2). Univariate ordinal logit models for histological diagnosis (categorized as HPV-NCIN, CIN 1, CIN 2 and CIN 3) found that the proportional OR of histological diagnosis was 2.83 (95%CI: 1.16; 6.89) for women infected by HPV alone, and 2.89 (95%CI: 0.82; 10.16) for those infected by HPV and Ct, compared to the not infected. The multivariate ordinal logit model for histological diagnoses adjusted for age at first sexual intercourse, which was significantly associated with the outcome at the univariate analysis, showed that women infected with HPV alone (3.08; 95%CI: 1.26; 7.53) or with HPV and Ct (2.95; 95%CI: 0.83; 10.46), had a threefold risk of presenting a cervical intraepithelial neoplasia with respect to their uninfected counterparts (Table 2).

## DISCUSSION

In agreement with previously published studies, our results showed that women with a high

**Table 2** - Univariate and multivariate analyses of HPV and Ct infections in cytological and histological diagnoses.

| | *Cytological diagnosis* | | | | | | *Histological diagnosis* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Univariate analysis* | | | *Multivariate analysis** | | | *Univariate analysis* | | | *Multivariate analysis*** | | |
| | OR | (95%CI) | p | OR | (95%CI) | p | OR | (95%CI) | p | OR | (95%CI) | p |
| Age group | | | 0.351 | NI | | | | | 0.795 | NI | | |
| 18-30 (n = 56) | 1 | | | | | | 1 | | | | | |
| > 30 (n = 108) | 1.35 | (0.72; 2.53) | | | | | 0.92 | (0.47; 1.78) | | | | |
| Smoking | | | 0.058 | | | | | | 0.624 | NI | | |
| No (n = 93) | 1 | | | 1 | | | 1 | | | | | |
| Yes (n = 71) | 1.82 | (0.98; 3.38) | | 1.58 | (0.84; 2.98) | 0.155 | 1.18 | (0.61; 2.26) | | | | |
| Age of earlier sexual activity | | | 0.646 | | | | | | 0.132 | 1 | | |
| 13-20 (n = 144) | 1 | | | | | | 1 | | | 0.36 | (012; 1.13) | 0.079 |
| >20 (n = 20) | 0.80 | (0.32; 2.03) | | | | | 0.42 | (0.14; 1.30) | | | | |
| Sexual partners during life | | | 0.350 | NI | | | | | 0.265 | NI | | |
| 1-2 (n = 78) | 1 | | | | | | 1 | | | | | |
| >2 (n = 86) | 1.33 | (0.73; 2.45) | | | | | 1.44 | (0.76; 2.76) | | | | |
| Pregnancies | | | 0.767 | NI | | | | | 0.542 | NI | | |
| None (n = 142) | 1 | | | | | | 1 | | | | | |
| 1-2 (n = 22) | 1.15 | (0.45; 2.93) | | | | | 1.38 | (0.49; 3.86) | | | | |
| Contraceptive method | | | 0.662 | NI | | | | | 0.673 | NI | | |
| None (n = 60) | 1 | | | | | | 1 | | | | | |
| Oral (n = 82) | 1.31 | (0.68; 2.53) | | | | | 1.13 | (0.56; 2.28) | | | | |
| Others (n = 22) | 0.97 | (0.37; 2.59) | | | | | 1.14 | (0.43; 3.07) | | | | |
| Previous genital infection | | | 0.733 | NI | | | | | 0.441 | NI | | |
| No (n = 73) | 1 | | | | | | 1 | | | | | |
| Yes (n = 91) | 0.90 | (0.49; 1.66) | | | | | 0.77 | (0.39; 1.51) | | | | |
| Frequency of Pap test | | | 0.018 | | | | | | 0.242 | NI | | |
| Regular (n = 59) | 1 | | | 1 | | | 1 | | | | | |
| Irregular/never (n = 105) | 0.45 | (0.24; 0.87) | | 0.56 | (0.28; 1.10) | 0.091 | 0.68 | (0.35; 1.30) | | | | |
| Previous HPV infection | | | 0.739 | NI | | | | | 0.888 | NI | | |
| No (n = 129) | 1 | | | | | | 1 | | | | | |
| Yes (n = 35) | 1.14 | (0.54; 2.40) | | | | | 1.05 | (0.52; 2.15) | | | | |
| Microbial agent | | | 0.005 | | | | | | 0.003 | | | |
| Negative (n = 51) | 1 | | | 1 | | | 1 | | | 1 | | |
| HPV (n = 97) | 2.71 | (1.38; 5.32) | | 2.17 | (1.08; 4.39) | 0.031 | 2.83 | (1.16; 6.89) | | 3.08 | (1.26; 7.53) | 0.014 |
| HPV + Ct (n = 15) | 4.18 | (1.31; 13.35) | | 3.87 | (1.19; 12.56) | 0.025 | 2.89 | (0.82; 10.16) | | 2.95 | (0.83; 0.46) | 0.095 |

OR: Odds Ratio; 95%CI: 95% confidence interval; NI: not included in the multivariate analyses. The association of demographic and clinical characteristics of patients, including HPV infection, with cytological and histological diagnoses was already described in a previous publication not reported in the text [8-9]. *Adjusted for smoking and Pap test frequency. **Adjusted for age at first sexual intercourse.

percentage (15/16; 94%) of Ct-HPV co-infections have more frequent high grade cervical lesions than those infected only with HPV [13-16]. Several epidemiological studies corroborate our findings evidencing that Ct may enhance the susceptibility to HPV infection and promote the viral persistence [4, 13, 14]. Ct infection is involved in cell proliferation, inhibits cell apoptosis and induces chronic inflammation.

Specifically, Ct may increase the risk of HPV infection through a plausible involvement in the epithelial disruption associated to the inflammatory response [15]. Furthermore, Ct may interfere with the immune response by decreasing the number of antigen presenting cells, and reduce the cell-mediated immunity thus allowing the persistence of HPV [16].

The main limitations of our study are represented by the small group of patients examined, the low number of Ct infections found and the lack of follow-up data. Therefore, we were unable to verify whether previous Ct infection can promote the infection with HPV first and then, possibly, the formation of cytological lesions and the tumor progression of these.

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**Conflict of interest**

None

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# *In vitro* culture of *Toxoplasma gondii* in HeLa, Vero, RBK and A549 cell lines

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## SUMMARY

*Toxoplasma gondii* is a protozoan parasite which can be grown *in vivo* and *in vitro*. Various cell lines are used for *T. gondii* culture in vitro. In this study, four cell lines of HeLa, Vero, RBK and A549 were compared with each other for *T. gondii* tachyzoites culture. The four cell lines were cultured and infected with $5 \times 10^6$ tachyzoites, respectively. The number of tachyzoites and viable host cells and pH of the media were assessed in each culture. The highest tachyzoite yield was seen in HeLa cell culture. The lowest number of viable host cells and the lowest pH were seen in HeLa cell line culture. The lowest tachyzoite yield, the highest viable cell and the highest pH were observed in Vero cell line culture. HeLa and Vero cell lines are thus appropriate for rapid and long-term propagations of *T. gondii* tachyzoites, respectively.

*Keywords*: *Toxoplasma gondii*, cell culture, cell line.

## INTRODUCTION

*Toxoplasma gondii*, a protozoan parasite with world-wide distribution, is the agent of toxoplasmosis in humans [1]. *In vivo* and *in vitro* production of *T. gondii* tachyzoites is critical for *Toxoplasma* researches [2]. Due to ethical issues and infra structural deficiencies in *in vivo* culture models such as animal models, these models have been replaced by cell culture systems [3]. *Toxoplasma gondii* tachyzoites can be cultured and maintained in various culture systems and cell lines [4]. These tachyzoites are used in fundamental studies and diagnostic assays [5-9]. Furthermore, cell culture systems can be used in *T. gondii* cyst formation [10]. The aim of this study included comparison of the four cell lines of HeLa (human cervix carcinoma), Vero (African green monkey kidney carcinoma), RBK (Razi bovine kidney) and A549 (human lung carcinoma) for culture of *T. gondii* tachyzoites *in vitro*.

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## MATERIALS AND METHODS

*Preparation of T. gondii tachyzoite*

Tachyzoites of *T. gondii* (RH strain) were inoculated intraperitoneally into BALB/c mice. After 3-4 days, tachyzoites were harvested using intraperitoneal wash with phosphate buffered saline (PBS; pH 7.3) and then were counted. Adherent cell lines of HeLa, Vero, RBK and A549 were grown in 10 mL of Dulbecco's Modified Eagle culture media (DMEM; KB cell, Iran), supplemented with 10% of inactivated fetal calf serum (FCS; Bovogen, Australia), 10 mμ of hepes and 1% of penicillin-streptomycin (Biowest, France) using 25 $cm^2$ flasks (Nunc, Denmark) and then were incubated in 5% $CO_2$.

After formation of confluent monolayers, the media was replaced by the maintenance media (DMEM/hepes with 5% FCS).

**Table 1** - Yield of tachyzoites, viable host cells and pH of each infected culture with 5×10⁶ tachyzoites of *T. gondii*, RH strain, on Days 3 and 5 post infection.

| | *HeLa* | | *Vero* | | *RBK* | | *A549* | |
|---|---|---|---|---|---|---|---|---|
| *Cell line* | *Day 3* | *Day 5* | *Day 3* | *Day 5* | *Day 3* | *Day 5* | *Day 3* | *Day 5* |
| Yield of tachyzoites (×10⁶/ml ) | 60.2 | 107.7 | 53 | 90.1 | 59.5 | 95.6 | 54.3 | 93.1 |
| Viable host cells (×10⁶/ml) | 2.1 | 1.05 | 2.4 | 1.91 | 1.9 | 1.1 | 2 | 1.75 |
| pH of media | 7.7 | 7.53 | 7.82 | 7.62 | 7.91 | 7.69 | 7.93 | 7.71 |

*In vitro cell culture of T. gondii*

Cell lines were infected 1:1 by tachyzoites of *T. gondii*, RH strain. Therefore, each flask was infected with 50×10⁶ tachyzoites. The culture media was replaced after 4 and 24 h by DMEM without FCS. Flasks were investigated on Days 3 and 5 post infection. Number of tachyzoites, viability of cell lines and pH of media were calculated on Days 3 and 5 post infection.

## RESULTS

Number of tachyzoites of *T. gondii*, RH strain, in the four cell lines (HeLa, Vero, RBK and A549), number of the host cells and pH of each flask were shown in Table 1. On Days 3 and 5 post infection, number of derived tachyzoites from the four cell lines were in the following order of HeLa > RBK > A549 > Vero. The highest and the lowest numbers of tachyzotes of *T. gondii*, RH strain, on Days 3 and 5 post infection were seen in HeLa and Vero cell lines, respectively. On Days 3 and 5 post infection, pH of the culture media for these four cell lines was as follows: A549 > RBK > Vero > HeLa. The pH of culture medium is an indicator of tachyzoite reproduction, so the lowest pH was seen in HeLa cell line in comparison with the others. The order of viable host cells in these four cell lines on Days 3 and 5 post infection was as follows: Vero > A549 > RBK > HeLa. The lowest number of viable cells was seen in HeLa cell line, indicating multiplication of the tachyzoites and hence rupture of the host cells in this cell line. Comparison of the tachyzoite yields in these four cell lines with tachyzoites of *T. gondii*, RH strain, on Days 3 and 5 post infection is shown in Figure 1.

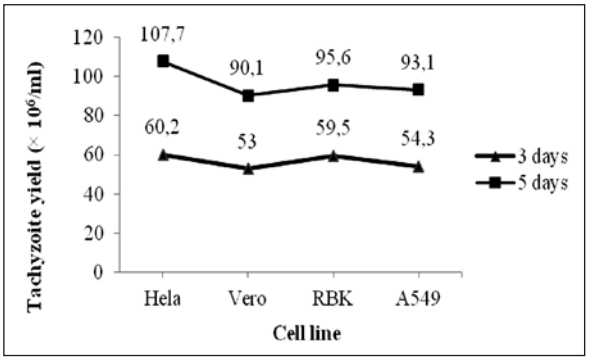


**Figure 1** - Comparison of tachyzoite yields in HeLa, Vero, RBK and A549 cell lines on Days 3 and 5 post infection with *T. gondii*, RH strain.

## DISCUSSION

Propagation of *Toxoplasma* tachyzoites in cell culture systems includes multiple advantages compared to that in animal models. These advantages include ethical value, low cost and easy management [11]. Various cell lines have been used to produce *T. gondii* tachyzoites *in vitro* [12-14]. Unlike passage in mice, passage of tachyzoites in cell cultures is not known to alter the virulence of the microorganism. Tachyzoites multiply in almost all mammalian cell lines. Yield of tachyzoites varies based on the cell line and strain of *T. gondii*. Mean generation time of the tachyzoites of *T. gondii*, RH strain, is reported nearly 5 h (15). In the present study, four cell lines were compared with each other for *T. gondii*, RH strain, cell culture. At the same condition, HeLa cell line was shown more appropriate than Vero, RBK and A549. Low levels of the host cell contamination and high levels of tachyzoite yield were seen in HeLa cells on Days 3 and 5 post infection. In Vero cell line, the highest contamination and the lowest yield rates of tachyzoites were observed. Furthermore, pH of the culture media was more acidified in HeLa cell culture on Days 3 and 5 post infection than that of the other cell lines, indicating high reproduc-

tion rate of the parasite. Hughes et al. compared four cell lines of HEP2, Vero, MRC5 and AGMPK for *in vitro T. gondii* culture. They found that optimal growth conditions occurred in AGMRK cell line [16]. Evans et al. used three cell lines of HeLa, LLC and Vero for continuous production of *T. gondii* tachyzoites and investigated that HeLa cell line included a higher tachyzoite yield than that LLC and Vero cells did [17]. In the present study, RBK and A549 cell lines were ranked second and third based on the tachyzoite yield, host cell contamination and pH of media. Therefore, RBK cell line can be substituted by HeLa cell line to have a large number of tachyzoites in cell culture. The Vero cell line seems to be appropriate for the long-time production of *T. gondii* tachyzoites in cell cultures.

## CONCLUSION

Of the assessed four cell lines of HeLa, Vero, RBK and A549 for *T. gondii* cell culture, HeLa is the best and Vero is the worst due to the number of derived tachyzoites. However, the Vero cell line is appropriate for the long-time culture of the parasite.

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**Conflict of interest**
The authors declare no conflict of interest.

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# Species diversity and molecular analysis of Staphylococcus in confectioneries of a developing country, Iran

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## SUMMARY

Confectionery is one of the potential sources of contamination and transmission of gastrointestinal infections to humans. Staphylococcus species, and particularly the coagulase-positive ones, have the remarkable capability to produce high amounts of enterotoxin in food. In the present study, the frequency and diversity of Staphylococcus in confectioneries in Iran were assessed by using a combination of conventional and molecular methods. A total of 55 confection samples were collected from 30 confectioneries of Isfahan. They were analyzed for the presence of Staphylococcus using standard protocols for isolation and characterization of the isolates. The conventional tests were used for primary identification and the sequence analysis of 16S rRNA was used for the species identification. A total of 47 out of 55 samples were gram-positive cocci (85.45%). They belonged to 39 Staphylococcus spp., 7 Macrococcus spp., and one Micrococcus spp. The most prevalent 11 various Staphylococcus species were S. aureus 30.8%, S. warneri 20.5% and S. succinus 17.9. Identification and characterization of Staphylococcus species can be important for epidemiological investigations and assessment of virulence factors such as enterotoxin production and development of specific management practices to prevent staphylococcal food poisoning.

*Keywords*: Staphylococcus, confectioneries, DNA sequence analysis.

## INTRODUCTION

Foodborne diseases are considered a global public health challenge. According to the World Health Organization (WHO) estimate, 600 million cases of illness were induced by over 30 foodborne pathogens in 2010. Over 200 diseases are caused by contamination of foods with harmful bacteria, parasites, viruses and chemical materials [1]. Among bacteria, the genus *Staphylococcus* is one of the most prominent food poisoning agents [2]. Staphylococcal food poisoning (SFP) often occurs following ingestion of at least 1.0 µg of staphylococcal enterotoxin in food [3]. *Staphylococcus* spp. are considered as skin and mucosal normal flora of humans and several animal species such as cows and sheep [4]. They can also be found in environmental sources and a wide range of foodstuffs [5]. Additionally, they are used as the starter in some fermented foods such as cheeses in order to produce favorite flavor and aroma [6]. Confectioneries often contain various amounts of different dairy products and can be produced by food handlers; thus they can act as sources of contamination and transmission of enterotoxigenic staphylococci. Currently, more than 80 species and subspecies of the genus Staphylococcus have been characterized [7]. They are commonly divided into 2 groups: coagulase-pos-

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itive staphylococci (CPS) and coagulase-negative staphylococci (CNS) [5]. *Staphylococcus aureus*, the prototype CPS, is enterotoxigenic and responsible for the majority of SFP cases. However, several studies reported that CNS isolated from foods can also produce enterotoxins and may be involved in food poisoning [3, 6, 8-12]. Accurate identification of staphylococci to species level is necessary to follow up toxigenic species and for epidemiological investigations. Manual and automated phenotypic identification methods are not entirely reliable to accurately identify all staphylococcal species [13, 14]. Most of these methods have been designed for detection of frequently involved species in human clinical samples and cannot identify rare species and atypical strains that may exist in food stuffs [13]. In addition to routine phenotypic methods, several DNA-based methods for *Staphylococcus* species identification have been described. Some studies have demonstrated that molecular methods are preferable to phenotypic methods [15, 16]. One applicable molecular method is the evaluation of polymorphism in housekeeping genes such as 16S rRNA gene by Polymerase Chain Reaction (PCR) and sequencing.

In Iran, national standards are focused only on detection of *S. aureus* in sweets [17]. As previously mentioned, other *Staphylococcus* species can also be the causative agent of SFPs. Since pastries are popular and are widely consumed in many countries, isolation and identification of contaminating *Staphylococcus* species can have important health ramifications [18]. The diversity of *Staphylococcus* species isolated from confectioneries has not been studied in our region. In this study, we aimed to isolate staphylococci from confectioneries in Isfahan using routine phenotypic methods and identify bacterial species by partial 16S rRNA sequencing.

## MATERIALS AND METHODS

*Sampling, isolation and conventional identification*

In a descriptive study conducted during September to December 2015, 55 confectionery samples including sweet pastries, cakes, and similar baked goods were collected from 30 confectionery stores in various sections of Isfahan, Iran. The samples were processed within 12 h of the collection in microbiology laboratory of Isfahan Infectious Diseases and Tropical Medicine Research Center. To isolate staphylococci, 10 grams of the collected samples were suspended in 90 ml sterile phosphate buffered saline, pH 7.4 (PBS) for 10 min. Ten mL of the suspension was added to 10 mL of Giolitti - Cantoni broth (HiMedia, India). The tubes were incubated at 37°C for 18-24 hours. Then, 10 µL of the enriched cultures were streaked on Baird Parker Agar containing egg yolk tellurite emulsion (HiMedia, India). The plates were incubated at 37°C for 18-24 h. After that, a single colony was streaked onto blood agar plates with 5% sheep blood (HiMedia, India) and further incubated at 37°C for 12-18 h. Suspected Staphylococcus colonies were identified using the standard microbiology tests including Gram staining, catalase reaction, resistance to bacitracin (0.04 units), modified oxidase test, tube coagulase reactions, DNase test and growth on mannitol salt agar [3].

## MOLECULAR IDENTIFICATION

*DNA extraction*

Chromosomal DNA was extracted using a simple boiling method [18]. In brief, a few colonies of each isolate was added to 100 µL of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.8) and boiled for 10 minutes at 100°C. After centrifugation of bacterial suspensions at 9,000 × g for 30 second at 4°C, the supernatant was stored at -20°C for future PCR analysis.

*Molecular identification of staphylococci isolates*

Oligonucleotide primers were synthesized at Bioneer (Daejeon, Republic of Korea) based on universal bacterial primers 27F (5'-AGA GTT TGA TYM TGG CTC AG-3') and 515R (5'-TTA CCG CGG CKG CTG GCA C-3') [18]. Fifty µl PCR reactions were set up each containing 5 µl of 10X reaction buffer, 2 µl of 50 mM $MgCl_2$, 1 µl of 2.5 mM dNTPs, 2 µl of each 20 pmol/µL primer, 0.4 µl Taq DNA polymerase 5 U/µl (SinaClone, Tehran, Iran) and 35.6 µL distilled water. Each PCR reaction was run in a Master cycler (Eppendorf, Germany) with the following conditions: 94°C for 3 min; 35 cycles of 94°C for 1 min, 58°C for 45 seconds, and 72°C for 1 min; 72°C for 10 min and 12°C. PCR products were visualized in 1.5% agarose gels stained with ethidium bromide.

For DNA sequencing, amplified products of 16S rRNA gene underwent bidirectional Sanger sequencing using the ABI 3730 XL DNA analyzer (Applied Biosystems, USA). The primers used for sequencing the 16S rRNA gene were same as those used for PCR amplification. The sequences were blasted against the Staphylococcaceae nucleotide database and 16S ribosomal RNA sequences of the National Center for Biotechnology Information (NCBI) available at http://www.ncbi.nlm.nih.gov/BLAST.
The staphylococcal sequences were aligned using the Clustal W v2.0 software and Phylogenetic tree was constructed by MEGA Version 6.0 [19] and UPGMA method [20].

*Nucleotide sequence accession numbers*
Partial 16S rRNA sequences from the 39 isolates, representing the 12 species and subspecies identified in the present study, were registered in GenBank under accession numbers KY411652 to KY411690.

## RESULTS

Forty-seven Gram-positive cocci with a positive catalase reaction were isolated from 37 (67.3%) of the 55 confectionery samples. From these isolates, one *Micrococcus* spp. and seven *Macrococcus* spp. were detected using phenotypic methods [resistance to bacitracin (0.04 units) and Modified oxidase test]. The other Gram-positive cocci (39 isolates) belonged to the genus Staphylococcus. According to tube coagulase reaction, twenty CPS isolates and 19 CNS isolates were detected. Results of DNase test and growth on mannitol salt agar were shown in Table 1.
After performing phenotypic tests, the 39 Staphylococcus isolates recovered from confectionery samples were subjected to partial 16S rRNA gene sequencing. The alignment of sequences to those available in GenBank (nucleotide database) was discriminative enough to differentiate 26 Staphylococcus strains at the species and subspecies level with similarities of 100%. However, 13 strains

**Table 1** - The results of phenotypic methods for detection of *Staphylococcus* spp.

| | | *CPSc (nb=20)* | *CNSd (n=19)* | *Total* |
|---|---|---|---|---|
| *Growth on MSA*[a] | Positive | 16 | 15 | 31 |
| | Negative | 4 | 4 | 8 |
| *DNase Test* | Positive | 11 | 2 | 13 |
| | Negative | 9 | 17 | 26 |

[a]MSA = Mannitol Salt Agar medium.
[b]n = Number of isolates.
[c]CPS = Coagulase-positive *Staphylococcus*.
[d]CNS = Coagulase-negative *Staphylococcus*.

**Table 2** - Phenotypic and molecular features of Staphylococcus confectioneries isolates.

| *Isolates* | *Phenotypic features* | | | | *16S rRNA analysis* | |
|---|---|---|---|---|---|---|
| *Designation* | *Catalase* | *Coagulase* | *DNase* | *MSA*[a] | *Similarity*[b] *(%)* | *Identification* |
| LH (8, 17, 47, 49, 52, 56, 57/2, 60/1, 65, 67, 69, 75) | + | + | + | + | 100 | *S. aureus* |
| LH (4, 6, 43, 48, 68, 76) | + | + | - | + | 100 | *S. warneri* |
| LH (3/2, 58) | + | - | - | + | 100 | *S. vitulinus* |
| LH (5,50) | + | + | - | + | 100 | *S. pasteuri* |
| LH 7 | + | - | - | + | 100 | *S. succinus* |
| LH (11, 16/1, 60/2, 71, 77, 79) | + | + | - | + | 100 | *S. succinus subsp. casei* |
| LH (9, 10) | + | - | - | + | 100 | *S. sciuri subsp. sciuri* |
| LH 13 | + | - | - | - | 100 | *S. lugdunesis* |
| LH 15/1 | + | - | - | + | 100 | *S. saprophyticus subsp. bovis* |
| LH (46, 61/2) | + | - | - | - | 100 | *S. epidermidis* |
| LH 54 | + | - | - | - | 100 | *S. gallinarum* |
| LH (78, 80, 81) | + | - | - | - | 100 | *S. carnosus* |

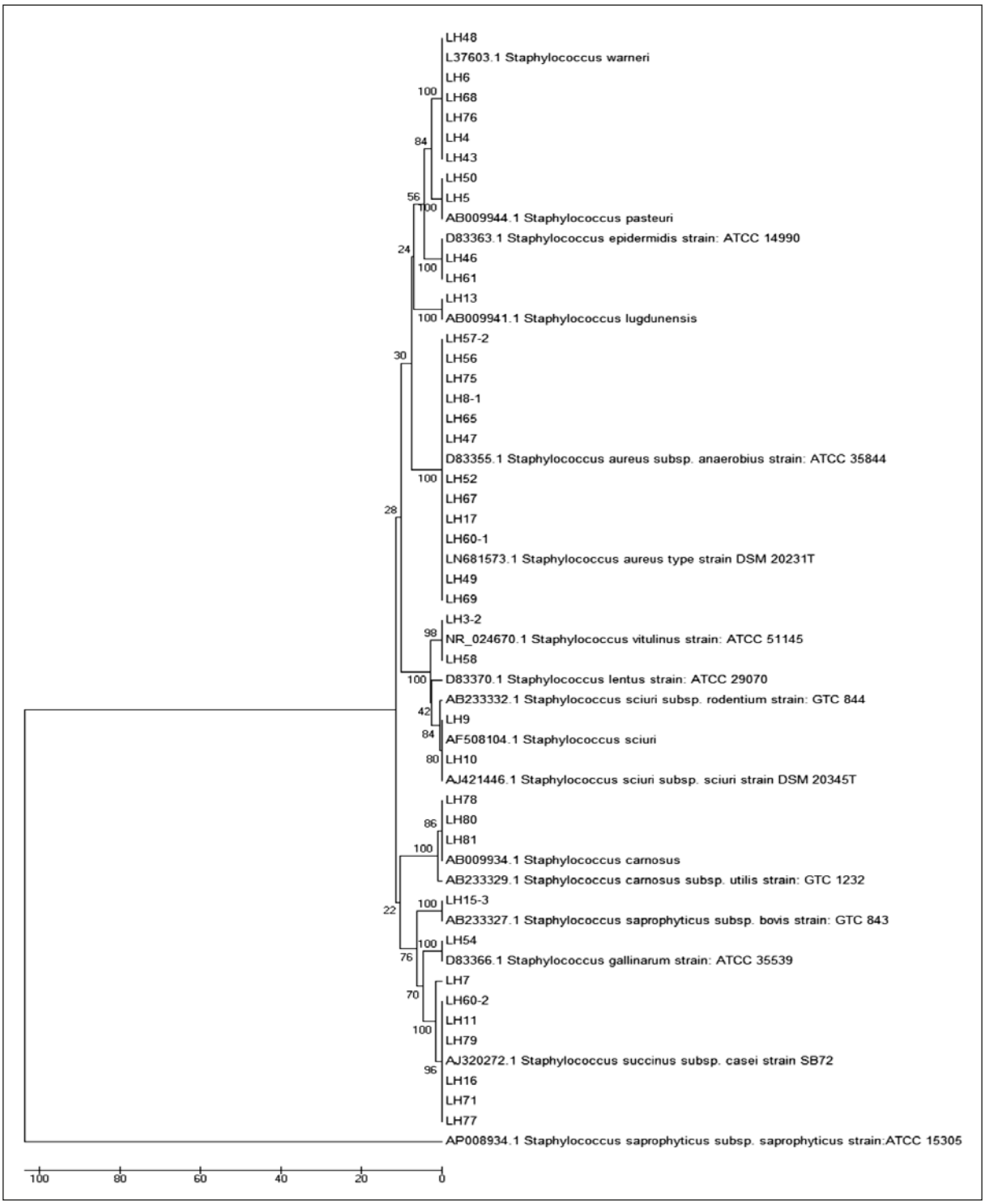


**Figure 1** - Phylogenetic trees generated from the multiple alignments of 16S rDNA sequences from the 39 Staphylococcus strain isolates found in confectionery samples and nearest validated species of Staphylococcus species. The Clustal W v2.0 software using Mega 6.0 and the unweighted pair group method (UPGMA) were used.

were not distinguishable between the two or three species (Table 2). All strains were distinguished at the species and subspecies level with similarities of ≥99% using the 16S ribosomal RNA sequences database of NCBI (Table 2).

Twelve coagulase-positive isolates were identified as *S. aureus* by partial 16S rRNA sequencing. Of these, 11 and 10 isolates were DNase and MSA-positive respectively.

The phylogenetic tree constructed based on the 16S ribosomal RNA sequences showed a high bootstrap value between the validated *Staphylococcus* species sequences from German Collection of Microorganisms and Cell Cultures (DSMZ) and our isolates sequences (Figure 1).

Partial 16S rRNA sequences from the 39 isolates, representing the 12 species and subspecies identified in the present study, were registered in GenBank under accession numbers KY411652 to KY411690.

## DISCUSSION

Staphylococcal food poisoning may not cause high mortality but can cause a lot of morbidities and economic loss. Confectioneries, particularly cream-filled pastries, and cakes, are foods that have been frequently considered as SFP agents [21]. In this study, among the 55 confectionery samples, we detected 6 different species and subspecies of staphylococcus based on nucleotide database and *Staphylococcus aureus* contamination rate was 21.8%. This rate in different parts of the country varies from 19% to 48.7% [22-25]. According to Institute of Standards and Industrial Research of Iran report number 2395, colony count of *Staphylococcus aureus* in pastry and confectionaries should be negative [17]. Since after cooking the confectioneries are in contact with surfaces and worker's hands, therefore the present data imply pastries might be contaminated by food handlers and environments. While the isolated *Staphylococcus* spp., carry the enterotoxin genes and the environmental conditions such as temperature and acidity are suitable for the toxin production, epidemic SFP is likely to occur with the consumption of these products. Unfortunately, in this study, testing of humans and surfaces that came into contact with the study products were not evaluated and therefore we suggest that further investigation should be done in this case.

In the present study, with respect to phenotypic identification tests, 10 *S. aureus* isolates were coagulase/DNase/MSA positive; one isolate was coagulase/DNase positive and one isolate just coagulase positive. According to previous studies, these two isolates with atypical characteristics may be methicillin-resistant *Staphylococcus aureus* (MRSA) [26, 27]. In current study, the survey of antimicrobial susceptibility pattern of isolates was not considered. However, laboratories should be considered such atypical strains.

Identification of *Staphylococcus* spp., other than *S. aureus* is not routinely conducted in food safety reference laboratories. However, the identification of these species might be important for epidemiological investigations, the assessment of virulence factors such as enterotoxin production and the development of specific management practices to prevent SFPs caused by CNSs [28]. Not only the identification of CNS is important, but also this process is difficult and relatively costly. Since in our country, limited phenotypic tests were used for *Staphylococcus* species identification in food laboratories, most of the isolates were not completely identified or misdiagnosed. Unfortunately, we could not properly detect the CNSs using phenotypic tests; however, 78.2% of identified *Staphylococcus* species were CNSs by partial 16S rRNA sequencing method. With comparing the partial sequences of 16S rDNA to the sequences available at GenBank database, 14 (52%) strains from 27 CNSs were identified at the level of species and subspecies while, 13 (48%) strains were confirmed as belonging to two or three species (similarity 100%). Unlike to our results, Casaes Nunes et al. identified 42 strains at the level of species and subspecies from 45 *Staphylococcus* strains isolated from Minas Frescal cheeses that were submitted to the sequencing of the V5 region of the 16S rDNA and only three strains were confirmed as belonging to *Staphylococcus* genera [28]. Chakravorty et al. determined that V1 region is the best region to differentiate among *S. aureus* and CNSs [29]. In the present study, we also identified 12 *Staphylococcus aureus* strains by partial 16S rRNA sequencing with 100% similarities. We also used 16s ribosomal RNA sequences database and all of the CNSs were identified at the level of species and subspecies. Even though 16S ribosomal sequences are widely used for the

bacterial species identification and perform taxonomic classification studies, some inadequacy can be seen in currently available databases and this subject was confirmed in our study and by other researchers [15, 30, 31].

## CONCLUSION

In the current study, we isolated a considerable number of staphylococcus strains from confectionaries that almost one-third of them were *S. aureus* by partial 16s RNA sequencing method and rest of them belonged to different *Staphylococcus* species. According to our findings, using this method for detection of *S. aureus* could be useful but not suitable for identification of other species of *Staphylococcus* genera.

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**Conflicts of interest**
There are no conflicts of interest.

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# Three cases of non-Hodgkin's lymphoma in HIV-infected Bulgarian patients

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## SUMMARY

HIV-associated lymphoma was first classified as an AIDS-defining disease by the American Center for Disease Control and Prevention (CDC) in 1985. Non-Hodgkin's lymphomas (NHLs) are frequent malignancies in AIDS patients. The risk of NHL in the case of an underlying HIV infection is estimated to be 100 times greater than in the general population, and it increases with the progression of the retrovirus-related immunosuppression. Cases of HIV-related non-Hodgkin's lymphoma are widely documented in the literature. In this article we present three cases of NHL and HIV hospitalized over a period of three years (2013-2016) at our specialized department for AIDS patients. Two of them were initially diagnosed with NHL and then with HIV infection. In one patient, NHL developed despite the patient's taking background antiretroviral therapy. The first case was a 38-year-old male diagnosed previously with HIV, who developed a palpable mass in the left zygomatic bone. The second case was a 52-year-old male who was first diagnosed with a cutaneous lymphoma, and subsequently with HIV infection. The third patient was a 63-year-old male who presented with two palpable masses: one in the left part of the mandible, and the other in the right inguinal region, the latter subsequently diagnosed as lymphoma. Following the latter diagnosis, the patient tested positive for HIV. The histological findings of the three lymphomas were as follows: an NHL plasmoblastic lymphoma, a cutaneous large B-cell anaplastic lymphoma, and a diffuse large B-cell lymphoma. The first patient received antiretroviral therapy (ART) and EPOCH (etoposide, pharmarubicin, vincristin, endoxane, uromitexan) plus radiotherapy, while the second received ART and CHOEP (endoxan, epirubicin, vincristin, etoposide, prednisolone). The third patient died a few days after beginning antiretroviral therapy.

*Keywords*: AIDS, HIV, non-Hodgkin's lymphoma, antiretroviral therapy.

## INTRODUCTION

HIV-associated lymphoma was first classified as an AIDS-defining disease by the American Center for Disease Control and Prevention (CDC) in 1985 [1]. Prior to the introduction of antiretroviral therapy, the incidence of HIV-associated lymphoma was 3 to 4% of all AIDS - determining diseases [2-4]. It has been found that without effective antiretroviral therapy 5-10% of all HIV-infected patients will develop lymphoma as an initial or a subsequent AIDS-defining condition [4, 5]. Non-Hodgkin's lymphomas (NHL) are frequent malignancies in AIDS patients. More than 90% of HIV-associated NHL are derived from B-cells and the majority are of a high grade [6]. Study data show that over time, before the era of

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highly active antiretroviral therapy, the incidence of NHL has been between 60 and 200 times higher in HIV-infected adults compared to the general population [7, 8].

The following have been considered to be the most important prognostic factors for the development of HIV-associated NHLs: patients naïve to antiretroviral therapy, men who have sex with men (MSM), age of 35 years, and, most significantly, a low CD4 cell count [8, 9].

In this article we report all cases of HIV-positive patients who have developed NHL at our Department over the past three years (2013-2016). The patients have been monitored and treated for HIV-infection at the Department for AIDS of the Specialized Hospital for Infectious and Parasitic Diseases in Sofia. The NHL diagnosis was made, and accordingly chemotherapy was administered at the National Specialized Hospital for Active Treatment of Hematological Diseases in the same city.

## CASE REPORTS

*Case 1*

A 38-year-old male with proven HIV infection since 1998. Until May 2014, the patient did not appear to be registered and monitored at our Department. The patient's complaints started in May 2014 with general malaise, chills, shortness of breath, fever, cough, and expectoration. In May 2014, he was treated for bilateral interstitial pneumonia at the Pulmonology Department. After that, he was hospitalized at our Department, and was treated for pneumocystosis and candidiasis. The patient started antiretroviral therapy with the 3TC/ABC + ATZ/r combination. One week later, a solid formation with an increasing size occurred in the area of the left zygomatic bone. Following a biopsy and a histological examination of the formation at the National Specialized Hospital for Active Treatment of Hematological Diseases, the diagnosis was confirmed as plasmablastic non-Hodgkin's lymphoma. A whole-body CT scan was performed, and the lymphoma was graded stage 4. An infiltrate engaging the left maxillary sinus was found. The soft tissues of the left cheekbone were also infiltrated. The upper border of the formation penetrated into the left orbit (72/63/44 mm). A soft-tissue lesion in the right anterior diaphragmatic sinus with a pore-size of 30 mm was also established. There was no evidence of enlarged lymph nodes. The trephine biopsy did not reveal involvement of the bone marrow. Chemotherapy with 6 courses of an EPOCH regimen (etoposide, pharmarubicin, vincristin, endoxane, uromitexan), and radiotherapy were conducted. The patient is currently being followed-up for progression of his lymphoma. So far, it has shown remission.

Since the onset of the antiretroviral therapy (ART), an optimal immunological response has been observed: CD4 T+ cell count at baseline being at 79 cells/μL, and currently standing at 685 cells/μL. The initial CD4/CD8 ratio was 0.41, whereas at present it is 1.29. The viral load before ART initiation was 25,000 copies/μL, but following ART commencement, an optimal viral suppression has been achieved.

*Case 2*

A 52-year-old man was diagnosed with HIV infection in July 2016. The patient's complaints dated back to February 2016, when a cutaneous red plaque about 5 cm in size appeared in the right temporal area. Subsequently, a "nodular" formation emerged in the right temporal area, approximately 5 cm in size. Smaller nodes of similar appearance developed also on the neck and the trunk. A weight reduction of about 6 kg was also observed. The diagnosis was established after a biopsy and a histological examination of the formation of the calvaria (Figure 1): anaplastic large-cell lymphoma, which required the ruling out of the following differential diagnosis:

a) primary cutaneous anaplastic large cell lymphoma;
b) systemic anaplastic large-cell lymphoma with skin involvement.

The CT scan of the chest, abdomen and the lesser pelvis revealed no pathological findings or enlarged lymph nodes. The CT scan of the head showed a subcutaneous soft-tissue formation in the right parietal region, 37/17 mm in size, without destruction of the underlying bone, and a second similar lesion, 5/12 mm, in the right occipital region. The trephine biopsy showed no involvement of the bone marrow. The patient commenced ART in July 2016 with TDF/FTC + RAL at a CD4 count of 177 cells/μL, a CD4/CD8 ratio of 0.77, and a viral load of 265,493 copies/μL. A month

later, the patient started chemotherapy with the CHOEP regimen (endoxan, epirubicin, vincristin, etoposide, prednisolone), and received 8 courses of it. He is currently being followed-up for his lymphoma. At present, a remission has been achieved. The current CD4 count is 252 cells/μL, the CD4/CD8 ratio is 0.77, and the HIV viral load is undetectable (<40 μL).

*Case 3*

A 63-year-old patient was diagnosed as HIV-positive in August 2016 during his hospitalization at the Department of Hematologic Diseases, where he was found with diffuse B-cellular NHL. In this patient the disease debuted in February 2016 with the onset of tumor formations on the left side below the mandible, and in the right inguinal region (Figure 2). The patient's lymphoma was graded stage 4. The CT-scan showed a tumor formation in the left half of the facial skull: part of the formation was tracked to penetrate into the intrapharyngeal space; another lesion was found in the left mandible. It involved the regional lymph nodes: a mesh-like lymphadenomegaly at the level of the crura of the diaphragm in the posterior mediastinum, 70/26 mm in size. A tumor formation in the right inguinal fold of 12/8 cm was also established.

This patient was admitted to our Department for initializing antiretroviral therapy. He started with 3TC/ABC + MVC at a CD4 count of 221 cells/μL, a CD4/CD8 ratio of 0.38, and a viral load of 1,274,788 copies/μL. On the 15$^{th}$ day of ART, a lethal outcome due to cardiac arrest was observed. The third patient's lethal outcome was probably related to his advanced age and the large tumor mass, and the respective aggressive course of the NHL.

## DISCUSSION

Patients infected with HIV are at an increased risk of developing NHL. AIDS-related NHL (AIDS-NHLs) are mostly, but not all, of high-grade and B-phenotype, as in the presented cases [8, 9]. The risk of developing lymphoma in patients with symptomatic HIV infection appears to be approximately 1.6% per annum [9]. The most common sites of involvement are the CNS (26%), the bone marrow (22%), the gastrointestinal tract (17~25%), and the liver (12%) [3]. The exact pathogenesis of AIDS-related lymphomas is not fully understood. One likely factor is the immune suppression itself. Chronic antigenic stimulation of the B lymphocytes by antigens, mitogens, or viruses, including the Epstein-Barr virus (EBV) and HIV, may play a role, but the continued HIV viral burden on B cells in association with the EBV and Herpes simplex virus 8 (HHV-8) is also believed to play a part [10, 11]. All patients presented in this article had IgG antibodies against EBV and CMV. PCR and testing for HHV- 8 have not been carried out for financial reasons.

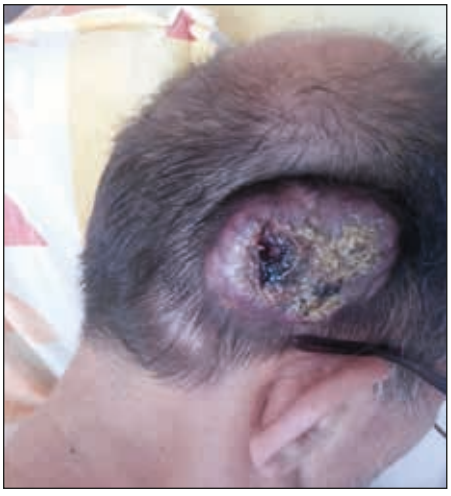

**Figure 1** - The tumor formation on the head.

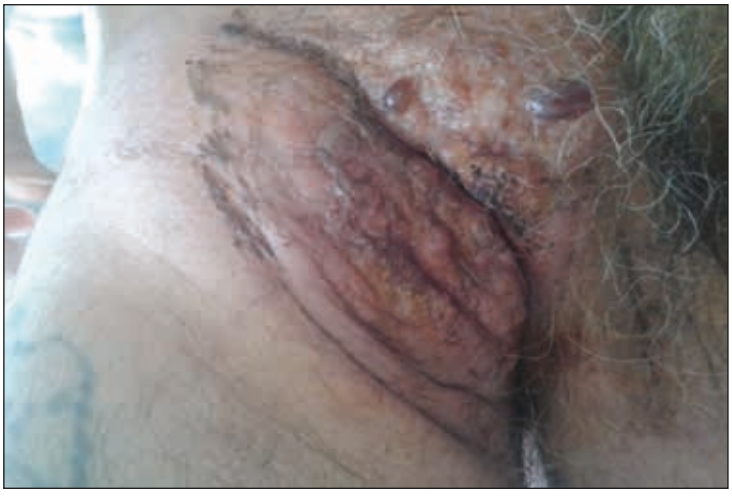

**Figure 2** - The tumor formation in the right inguinal region.

According to other authors, HCV infection is also associated with low-grade lymphoproliferative disorders that can progress to NHL. Bacterial infections have also been associated with NHL [16]. All the patients presented in this article were negative for HCV. The second patient was positive for HBV and had serological evidence of a past infection of syphilis. But in all patients, there were no concomitant bacterial infections.

Cutaneous lymphomas are characterized by initial accumulation of mononuclear cells, mostly lymphocytic, in the skin [12]. Primary cutaneous lymphomas represent 5% to 10% of the total extranodal NHLs, and rank second in frequency after the lymphomas occurring in the gastrointestinal tract [12]. The clinical presentation of cutaneous NHL includes single or multiple subcutaneous nodes, papules, and ulcerative and infiltrative lesions. Patients with NHL show cutaneous involvement in 15% to 20% of cases, and in 5% to 10% of them, skin lesions are the first manifestation of the disease. The number of lesions is a prognostic factor in primary cutaneous B-cell lymphomas. Fine Needle Cytology (FNC) combined with ancillary techniques can also provide the correct diagnosis in most cases. In patients suffering from NHL, FNC also plays an important role in the differential diagnosis between a relapse of a primary disease and reactive lymph nodes enlargement [16].

In the pre-antiretroviral therapy era, the incidence of AIDS-related lymphoma remained constant at about 6~7 cases per 1000 persons per annum [2, 4]. The declining incidence following the introduction of HAART is promising; there have been significant improvements due to the improved immunity status in the cases of boosted patients, and this has been highlighted by the increase in CD4 T - cells counts [13]. In the early years of treatment, the recommended optimal first choice of therapy for AIDS-related lymphoma was a combination chemotherapy that consisted of high doses of cytosine arabinoside, methotrexate, and cyclophosphamide [14]. However, the combination chemotherapy regimens that are commonly used for the treatment of intermediate or high-grade lymphomas are so toxic that the occurrence of hematologic complications and opportunistic infections has generally resulted in a poor outcome for patients, especially for those with AIDS-related lymphomas. A recent study using 6 courses of a dose-adjusted EPOCH regimen has shown a complete response rate of 74% [4, 14, 15]. The first two patients in our case, treated with EPOCH and CHOEP, respectively, responded well to chemotherapy. The first one received also radiotherapy. The third patient died before starting the chemotherapy. The response to the chemotherapy and the patient survival were related to the existing comorbidities and the patients' overall state. The CD4 count has been considered one of the most important predictors in AIDS-related lymphoma patients. The invasion of the tumor elsewhere than in the lymph node, the performance status, the histological subtype, and the clinical stage could also be useful as predictors [14, 15]. The adverse prognostic factors for AIDS-related lymphomas include a CD4 count of $<100/mm^3$, an age of >35 years, stage III or IV disease, an elevated LDH-level, and a history of drug injection abuse (11). AIDS-related lymphoma patients exhibit various degrees of immune suppression. At the time of the lymphoma diagnosis, the CD4 counts of our patients were $79/mm^3$, $177/mm^3$, and $221/mm^3$, respectively. The CD4/CD8 ratios were 0.41, 0.77, and 0.31, respectively. All three patients were diagnosed with advanced HIV/AIDS disease with severe immunosuppression and low CD4 T - cell counts at the time of the neoplasm diagnosis, age >35 years, and stage IV disease. The 1-year survival rate of AIDS-related lymphoma patients is 30%, which is lower than in the general lymphoma population. The survival period for our two patients to date is three years, and one year, respectively.

## CONCLUSION

We present three cases of NHL that developed in HIV-infected patients. This is a brief report on these HIV-infected lymphoma patients; the lymphomas had a different anatomical location, and distinctive clinical signs at the time of presentation. In two of the cases reviewed in this study, combined antiretroviral therapy and chemotherapy was administered, and these treatments proved to be effective for improving the prognosis of these patients. The third patient died before starting the chemotherapy. The tumor invasion, together with the performance status and the age were the most important factors for the poor outcome in the third patient.

**Disclosure of conflict of interest**
No conflict of interests.

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# Antiviral activity of maraviroc plus mirtazapine in a low-risk HIV-negative patient with progressive multifocal leukoencephalopathy

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## SUMMARY

A case of progressive multifocal leukoencephalopathy (PML) is described in an HIV-negative patient with mixed connective-tissue disease (MCTD) on a minimally immunosuppressive treatment with hydroxychloroquine. The patient presented with right-sided weakness, episodes of disorientation and loss of short-term memory and of vision in her right eye. PML was diagnosed by JCV DNA on cerebrospinal fluid and radiological criteria. She was treated with off-label maraviroc and mirtazapine but died two months after hospital admission, despite a surprising decrease in the viral load of cerebrospinal fluid three weeks after starting therapy. Prompt diagnosis and antiviral treatment of PML even in low-risk patients are warranted. Future studies are required to define the therapeutic role of maraviroc (MVC) and mirtazapine in this setting.

*Keywords:* Progressive multifocal leukoencephalopathy, maraviroc, mirtazapine, hydroxychloroquine, Sjögren's syndrome, IRIS, JCV.

## INTRODUCTION

Progressive multifocal leukoencephalopathy (PML) is a rare, deadly demyelinating disease of the central nervous system (CNS), caused by the John Cunningham virus (JCV) and classically observed in advanced stages of human immunodeficiency virus (HIV) infection [1, 2]. However, in the last decades, PML has been increasingly reported among patients with haematological and solid malignancies, organ transplant recipients on immunosuppressive drugs and most recently among patients receiving immunomodulating drugs, like natalizumab [1, 2]. Surprisingly, PML may also develop occasionally in individuals with minimal immunosuppression, such as in those affected by sarcoidosis [1, 2]. In HIV-negative patients, the main therapeutic strategy is to decrease the immunosuppression, whenever possible; serotonin reuptake inhibitors (by blocking 5-HT2a, used by JCV for cell-to-cell spread) and the CCR5-antagonist MVC (for its immune-modulating properties) have shown promising results in limited case series [3-7].

Here we describe a case of PML in an HIV-negative patient with mixed connective-tissue disease (MCTD) on a minimally immunosuppres-

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sive treatment with hydroxychloroquine. To our knowledge this is one of the fewest cases treated with off-label maraviroc and mirtazapine, resulting in a divergent clinical, radiological and virological outcome.

## CASE REPORT

A 50-year-old Caucasian woman with autoimmune thyroiditis and MCTD was admitted to our clinic for right-sided weakness, episodes of disorientation and loss of short-term memory and right eye's vision. In the previous months she reported significant difficulty in driving, weakness and visual disturbances. MCTD was diagnosed 5 years before with a clinical and serologic pattern overlapping Sjögren syndrome. She started hydroxychloroquine (200 mg) and low-dose corticosteroids (withdrawn after 3 years). At the admission, she was on oxycodone, hydroxychloroquine and levothyroxine. Neurological examination revealed right hemi-paresis, bilateral ophthalmoplegia and right hemi-spatial neglect. Blood tests were unremarkable, but total lymphocyte count of 236 cells/µL with 217 CD4+ T-cell (68,5%). HIV test was negative. Brain magnetic resonance (MRI) showed extensive T2/FLAIR non-enhancing signal abnormalities involving subcortical white-matter of both parietal-occipital lobes, corpus callosum and the rear frontal lobe (Figure 1). Cerebrospinal fluid (CSF) analysis were within the normality range (proteins, glucose, cells and CSF-serum albumin ratio). CSF opportunistic infections were ruled out (*Cryptococcus* spp, *Toxoplasma*, herpetic viruses and *Mycobacterium tuberculosis*), but JCV-DNA was 119370 copies/mL. After informed consent and the approval by the off-label committee of the hospital, maraviroc (300 mg twice daily) and mirtazapine (15 mg once-daily; then twice-daily) were started, while hydroxychloroquine was concurrently withdrawn. Nevertheless, her clinical conditions worsened: after treatment was started, her neurological status continued to deteriorate with a new onset of dysarthria, temporo spatial disorientation and progressive development of tetraplegia. After three weeks of treatment, a brain MRI revealed slightly increased white-matter lesions (Figure 1), while CSF JCV-DNA was reduced to 459 copies/mL. Plasma and CSF maraviroc concentrations were 85 ng/mL and <1 ng/mL, respectively (ultra high performance liquid chromatography coupled to tandem mass spectrometry methods SE, Waters, Milan, Italy). Eventually, the patient went into a coma and passed-out two months after hospital admission.

## DISCUSSION

Nowadays, an increasing number of HIV-negative individuals are at risk for developing PML,

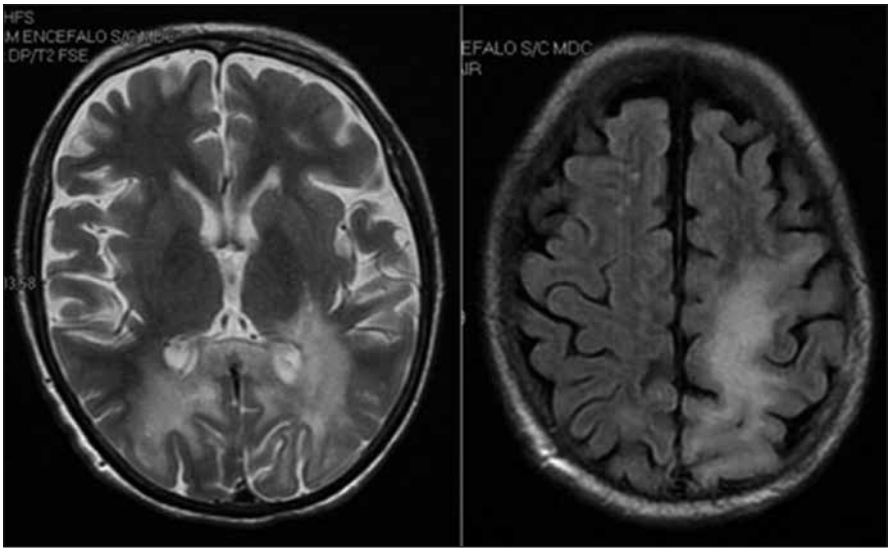


**Figure 1** - Brain Magnetic Resonance Imaging. Axial T2-weighted sequence (left) and axial fluid-attenuated inversion recovery (FLAIR, right). Typical progressive multifocal leukoencephalopathy-associated lesions are seen in parietal-occipital lobes and corpus callosum. The lesions were hyper intense on T2/FLAIR sequences and did not enhance with contrast.

due to the broader use of immunosuppressive/immunomodulatory treatment in different clinical settings [1, 2]. In these cases, the new onset of subacute neurologic signs or symptoms should raise the suspicion for PML, even in minimally immunosuppressed patients suffering from hepatic cirrhosis, chronic renal failure, pregnancy, dementia or idiopathic CD4+/CD8+ lymphocytopenia [1, 2]. While for HIV-positive patients highly-active antiretroviral therapy may add adjunctive help, in HIV-negative patients the prognosis remains poor, with a median survival of 3 months [3]. For the latter, the only strategy is the reduction of immunosuppressants' doses, whenever possible, or the removal of biological agents through plasmapheresis [4, 5].

Several medications have been used to treat PML with anecdotal success (e.g., cytarabine, cidofovir, mefloquine), although these drugs have failed to show efficacy in randomized trials or prospective studies [8, 9]. On the contrary, no randomized trials or prospective studies on large cohort have been carried on about MVC and mirtazapine use in PML [10-12].

We observed a significant decrease in CSF JCV-DNA (2.4 $\log_{10}$ copies/ml within three weeks) using MVC plus mirtazapine. *In vitro* studies have showed that JCV may entry into target cells through the serotonin receptor 5-HT2a, suggesting a potential role for mirtazapine, a serotonin reuptake inhibitor licensed for major depression [6]. Some evidence claimed beneficial effects of mirtazapine for PML treatment, showing concurrent clinical improvement and CSF virological clearance due to the restriction of viral cells spread [6, 7, 11]. On the other side, MVC is a CCR5 receptor antagonist, approved for R5-tropic HIV infection. Evidence regarding the usefulness of MVC in PML is limited and conflicting. It has been shown that CCR5+ lymphocytes may play a pivotal role in the pathogenesis of a subgroup of PML cases, associated with immune reconstitution inflammatory syndrome (IRIS), an overwhelming and life-threatening inflammatory reaction occurring during immune recovery [13, 14]. High percentages of CCR5 + cells, predominantly CD8+ cytotoxic T-cells, has been found in natalizumab-associated PML-IRIS, as well as in other cases of inflammatory PML lesions, suggesting a role in tissue damaging for this T-cell subpopulation [15, 16]. In an inflammatory milieu, MVC may directly hamper the exaggerated inflammatory reaction by decreasing the influx of lymphocytes and macrophages into the infection site and modulating immune activation [16]. Recently, 3 HIV-negative patients diagnosed with PML were treated with MVC and mirtazapine, showing a reduction up to undetectable CSF JCV-DNA value, as well as long-term clinical and radiological improvements [17]. However, compared to our patient, these cases presented lower CSF viral loads at the diagnosis, which has been associated with a better prognosis [18]. In addition, the absence of inflammatory components in our patient, as evidenced by the lack of enhancing lesions at the MRI, may have made MVC vain. Scarce data are available regarding CCR5 inhibitors usefulness or risks in PML cases without IRIS [19, 20]. In this setting, MVC may potentially aggravate immune suppression, leading to the inhibition of innate and adaptive responses; additionally, the appropriate dose is uncertain [21, 22]. Considering the conflicting evidence from scant reports and the observed discrepancy between virological and clinical outcome, further studies are needed to assess the real efficacy of MVC and mirtazapine and their eventual association for different PML subtypes.

**Conflict of interest**

Giovanni Di Perri has received honoraria from Abbvie, BMS, Gilead, Janssen-Cilag, MSD and ViiV. Andrea Calcagno has received honoraria from Abbvie, BMS, Gilead, Janssen-Cilag, MSD and ViiV, and he is currently receiving research grants from BMS, Gilead and ViiV. For the remaining authors none were declared.

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# A fatal case of cytomegalovirus disease in an immunocompetent young woman: a case report

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## SUMMARY

Cytomegalovirus can cause severe disease with adverse outcome in immunocompromised patients. Severe cytomegalovirus infection in previously healthy individuals is rare. Here we present an unusual case of cytomegalovirus infection with neurological and pulmonary involvement in a previously healthy young woman with no history of immuno-suppression. Unfortunately, the disease followed a malignant course and despite the efforts of the medical staff the patient died. CMV infection should be considered in the diagnostic work-up of immunocompetent patients with fever and unexplained neurological or pulmonary manifestations. Although uncertainty exists regarding the optimal treatment of CMV in healthy individuals, early recognition and administration of ganciclovir may prevent a fatal outcome.

*Keywords*: Cytomegalovirus, pneumonitis, neurological impairment, immunocompetent

## INTRODUCTION

Cytomegalovirus (CMV) is a double stranded DNA virus that belongs to the family of Herpesviridae. In immunocompromised patients can cause serious disease through primary CMV infection or re-activation secondary to immune dysfunction [1]. In healthy individuals CMV infection follows an initially asymptomatic benign course with a flu like illness and mild symptoms [1].
It has been reported that CMV seroprevalence in the United States varies widely, ranging from 21% to 95% of the population [2]. Additionally, CMV is considered the most common cause of congenital infection in the developed countries, affecting 0.1-2% of live born infants [3, 4]. In the vast majority of immunocompetent hosts CMV remains latent for years [1]. Here we present an unusual case of fatal CMV disease in a previous healthy young woman.

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## CASE PRESENTATION

A 40-year-old previous healthy woman was referred by his general practitioner to the Saint George General Hospital of Chania, Crete, Greece reporting sudden weakness of the lower limbs with sensory alterations. A history of lower respiratory tract infection, with high fever was reported 1 week before admission. Her vital signs at admission were as follows: blood pressure, 140/60 mmHg; heart rate 90 min; body temperature, 36.2 C; respiratory rate 12 min; oxygen saturation 97% on ambient air. Abdomen was soft in palpation and electrocardiogram revealed sinus

rhythm. Neurological examination showed diminished muscular strength in the lower limbs (grade 3/5) and reduced pain sensations bellow L1. Cranial nerves examination was normal.
Blood tests revealed a normal white blood cell count (WBC) of $7.1\times10^9$/L (4-9.5). Liver functions tests disclosed a 2-fold elevation of hepatic transaminases, with alanine aminotransferase 122 U/l (normal range: 0-45) and γ glutamyl transferase 133 U/l (normal range: 0-55). Due to the neurological deficit a lumbar punction test was consequently performed.
Polymerase chain reaction assay in cerebrospinal fluid (CSF) for CMV was positive in two consecutive tests. Further analysis for human immunodeficiency virus, varicella zoster virus, measles, mumps and mycoplasma pneumonia was negative. The patient was treated with ganciclovir (5 mg/kg twice daily for two weeks) and methylprednisolone due to the neurological deficit of the lower limbs (500 mg once daily for 7 days). A progressive clinical improvement of the neuromuscular deficit was recorded and the patient was discharged home 4 weeks later. Serum tests including antinuclear antibody (ANA), antiJo-1 antibody, anti-DNA antibody, anti-CCP, anti-RNP antibody, rheumatoid factor, and thyroid tests were all negative.
After two months, she was readmitted to the Pulmonology department of our hospital with acute respiratory distress. Her oxygen saturation was 88% on ambient air. Arterial blood gases showed pH 7.5, $pCO_2$ 31 mmHg and $pO_2$ 76 mmHg. The chest X ray showed butterfly pulmonary opacities bilaterally (Figure 1). C-reactive protein was 5 mg/dl with normal white blood cells count. Polymerase chain reaction serum test was positive for CMV DNA with high viral load. A diagnosis of CMV pneumonitis was made. Since the respiratory status continued to deteriorate the patient was transferred to the Intensive Care Unit of our Hospital and was intubated. Treatment with ganciclovir 500 mg twice daily was initiated. Response to antibiotic therapy was poor and the patient died 4 days later.

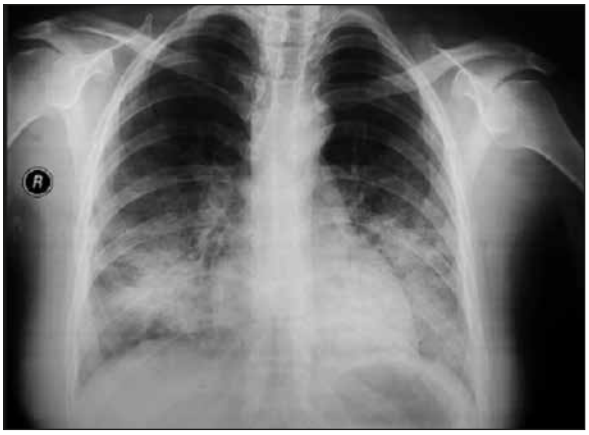

**Figure 1** - Chest X-ray showing pulmonary opacities in both lungs.

## DISCUSSION

Although CMV infections in immunocompromised patients is well documented, those observed in immunocompetent individuals have not been well described [1]. A literature review by Rafailides et al., reported that CMV infections in healthy patients may not be so unusual as previously believed [1]. It can be manifested as a wide range of conditions, from asymptomatic to invasive disease [5].
Almost every anatomical region can be involved [1]. Gastrointestinal tract is the location most frequently affected, following by the central nervous system, blood cells, eye, liver and last by pulmonary and vascular system [1, 6].
More specifically, gastrointestinal manifestations include gastroenteritis, colitis, duodenitis and proctitis [1]. Fever, abdominal pain in the lower abdomen, anorexia, nausea and vomiting are the main symptoms patients admitted while abdominal and rebound tenderness are the principal signs during palpation [1].
Central nervous system is the second prevalent site of CMV infection in immunocompetent individuals [1]. It is often presented with myelitis, encephalitis, and meningitis [1]. Fever, myalgia, muscular weakness, paraplegia are the principal symptoms while numbness, hypoesthesia, disorientation, confusion, visual loss are the main signs recorded during neurological examination. Advanced age is an aggravating factor for a favorable outcome [7]. Two types of meningo-encephalitis have been described in immunocompetent patients: the paroxysmal type with benign outcome and the monophasic type characterized by seizures and an adverse prognosis [7].
Remarkably in a retrospective study of 116 im-

munocompetent adults (range 19-68 years old), only two presented with interstitial pneumonitis and encephalitis [8]. Similarly in a cohort study by Wreghitt et al. among 124 immunocompetent patients with a diagnosis of acute CMV infection, aged from 16 to 86 years old, almost 3 out of 10 suffered from respiratory symptoms, about 1 out of 4 had jaundice and 3% were reported to be confused [9].

The traditional method for the diagnosis of CMV infection is by virus isolation from culture of the human specimen [10]. Similarly, serological tests are useful for determining whether a patient has had CMV infection in the past, by the presence or absence of CMV IgG [8]. Remarkably, polymerase chain reaction (PCR) is considered a fast method for CMV detection characterized with a high sensitivity [10].

We presented an unusual case of CMV multi-organ infection in an apparently immunocompetent patient. Our case is unusual since it represents concurrent manifestation of severe CMV disease with neurological and pulmonary involvement in a healthy young female. We presume that the resistance of the virus to ganciclovir was responsible for the unfavorable outcome.

Furthermore, compelling evidence suggests that unknown genetic parameters may influence the clinical expression of the disease [11]. It has been reported that natural killer (NK) cells play a crucial role in host defense against CMV infection and few studies demonstrated the KIR and HLA repertoire may influence the risk of developing symptomatic or asymptomatic disease after primary CMV infection in the immunocompetent host [12].

## CONCLUSION

Despite the rarity of CMV infections with neurological and pulmonary involvement, clinicians involved should have high index of suspicion in order to make an early diagnosis of the disease. Timely and appropriate antiviral therapy may improve prognosis and prevent lethal eventualities.

**ACKNOWLEDGMENTS**

Part of this case information has been presented to the 5th Southeast European Conference on Chemotherapy and Infection, Bled Slovenia, 16-19/10/2014.

**Informed consent**

Written informed consent was given from the next of kin of this patient for the publication of this case report.

**Conflict of interest declaration**

The authors declare no conflict of interest

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# Madura foot: an imported case of a non-common diagnosis

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## SUMMARY

Mycetoma (or "madura foot") is characterized by deformation, cutaneous lesions, infection of tissues extending from the cutaneous layer to the underlying fascia, and an indolent course. A number of fungal or bacterial agents that are introduced through traumatic inoculation can be responsible for the disease, but *Actinomadura madurae* is among the most common agents of mycetoma occurring worldwide. We report a case of madura foot caused by *A. madurae* in an immunocompetent young Somali man who was admitted with a diagnosis of skin and soft tissue infection of the left foot with osteomyelitis. The present report emphasizes the importance of the knowledge of this infection, which is sporadic but problematic to treat and, above all, difficult to diagnose. Moreover, a multidisciplinary approach with involvement of an infectious diseases specialist with experience in tropical diseases and a microbiology unit performing rapid molecular diagnostic tests is required for early diagnosis and an optimal antibiotic therapy.

*Keywords*: mycetoma, Madura foot, *Actinomadura madurae*, skin and soft tissue infection.

## INTRODUCTION

Mycetoma or Madura foot is a chronic granulomatous infection involving cutaneous and subcutaneous tissue, with possible and gradual extension to fascia and bone, becoming even a large and mutilating lesion [1].

The development of the disease seems to be correlated also to the patients' immune system and, in particular, to some single nucleotide polymorphisms and Th2-response, that predisposes to the evolution of the infection. It is caused by bacteria (Actinomycetoma) or fungi (Eumycetoma), both found in the soil, which enter the body by a lesion of the skin caused by thorn pricks, splinters, stone cuts or insect bites. The principal species involved is *Actinomadura* spp a bacterium that belongs to the genera of actinobacteria [1, 2].

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Actinomycetoma and eumycetoma have a similar clinical presentation: the affected part of the body appears increased in volume, deformed and firm, characterised by the presence of nodules and multiple sinus, with a seropurulent discharge containing grains. The lesion is typical painless. The foot and the hand are the parts of the body more involved (more than 80% of cases), followed by the other parts of the leg and arm, and back [3]. Nevertheless, unlike eumycetoma, actinomycetoma has a more aggressive clinical course with early bone involvement and frequent lymphatic spread.

In order to establish the correct treatment, it is mandatory the identification of the causal microorganism, since actinomycetoma and eumycetoma are treated differently.

The diagnostic tools should be employed on deep surgical biopsy material. Direct examination and histopathology aim at identifying the filaments and grains characteristics using special stains: in actinomycetoma, the filaments can be identified with Gram stain and are weekly stained by hae-

matoxylin and eosin; in eumycetoma, the filaments stain with periodic acid-Schiff and, strongly, with haematoxylin and eosin [4]. Nevertheless, it is hard to distinguish the species because of their similar appearance. Even the culture of grains, carried out using recommended media (Columbia agar, Brain Heart Infusion, Lowenstein-Jensen and Sabouraud glucose agar) for at least 10-15 days, is difficult and time-consuming [4].
Molecular methods applied on biopsy specimens, based on PCR and sequencing, even if more expensive, result more reliable in species identification and more rapid [5].
Once the microorganism is identified, the main challenges of the therapy are the long duration, the possibility of collateral effects and the poor availability of alternative effective drugs.

## CASE REPORT

A 24-year-old immunocompetent Somali man was admitted to the Infectious Diseases Unit of the University Hospital of Palermo (Sicily, Italy) in May 2017 because of a skin and soft tissues infection of the left foot with osteomyelitis. He had arrived in Lampedusa one week before, after a migratory route that lasted seven years, crossing Sudan, Kenya and Libya. He reported the first lesion in the left sole at the age of 13, with a slowly progressive deformation of the foot. On admission, the foot was swollen, warm and tender with limitation of the IV and V toes movement and multiples, poorly draining sinus on the dorsal face (Figure 1). The rest of the physical examination showed no anomalies. He had no pain. No fever or any other systemic symptom appeared.
Laboratory tests showed a modest leukopenia (WBC 3010/mmc, N 63% and L 29%) and an increased C- reactive protein (76 mg/L).
A plain radiography and CT scanning of the foot revealed marked soft tissue swelling of the metatarsal and between III-V toes, with bone sclerosis and cavities of the metatarsal and V toe.
The blood cultures were sterile. Quantiferon TB Gold Plus test was positive, but an active tubercular infection was excluded. The patient signed an informed-consent form before biopsy analysis.
We performed a surgical biopsy of the sole: pathology showed chronic, granulomatous inflammation with PAS positive grains; the specimen ZN coloration and cultures for common aerobic and anaerobic bacteria were negative.
The biopsy, before and after decontamination by N-acetyl L-cysteine (NALC) sodium hydroxide (NaOH) method, was inoculated on Löwestein-Jensen (L-J) and Blood Agar and incubated at 37°C for 8 weeks.
On the basis of epidemiology, clinical aspect and pathology result, under the hypothesis of eumycetoma, waiting for the microbiological results, we started a therapy with itraconazole.

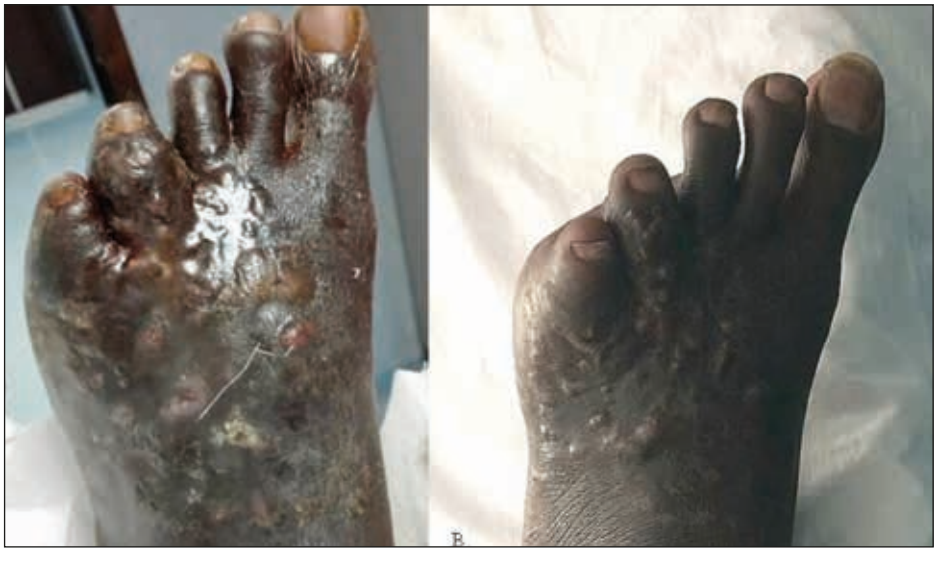


**Figure 1** - A. Madura foot on admission and B. after one month of treatment.

**Figure 2** - *A. madurae* on Löwenstein-Jensen Agar.

Two weeks later, the colonies grown (Figure 2), were identified as *Actinomadura madurae* by PCR and sequencing of 16S rRNA and hsp65 genes.
In June, we switched therapy to trimethoprim-sulfamethoxazole (TMP/SMX) 240/1200mg BID and 1g of amikacin for the first month. No renal dysfunction or ototoxicity occurred, but a worsening of leukopenia led us to using folic acid permanently and filgrastim twice, with stabilisation of the WBC count. At the discharge, the patient was given custody by a community one hour far from Palermo. In order to avoid the lack of adherence due to the daily intramuscular administration of amikacin, we decided to continue the therapy with TMP/SMX in association with rifampicin and isoniazid, in consideration of the latent tuberculosis infection. Nevertheless, two days later the patient had vomit and abdominal pain probably rifampicin-correlated, so rifampicin was stopped, and treatment with TMP/SMX, in association with folic acid and isoniazid was continued monitoring WBC count twice week.
After four months of treatment, the swelling subsided and the foot decreased in dimension. The patient carries on the follow-up and the treatment is currently on going.

## DISCUSSION

Mycetoma is a rare neglected tropical disease. According to the first and last biggest worldwide meta-analysis on the global burden of mycetoma, carried on in 2013 by van de Sande and reporting 8673 cases since 1944, the most endemic countries are Sudan, Senegal and Togo in Africa, India in Asia and Mexico in North America [6].
Nevertheless, lacking established surveillance programs, the real global incidence and prevalence are not known.
In Europe, thirty autochthonous cases were described in immunocompetent hosts, coming from Bulgaria, Albany, Italy, Greece, and Turkey [7, 8]. The only species found on all continents in equal amount is *A. madurae* [6].
In consideration of the increasing number of refugees from endemic areas observed in Europe since 2014, we underline the need to improve the awareness on this disabling disease [8, 9].
In Italy, only few cases are reported: among them, three cases are autochthonous and all of them by *A. madurae*. In Italy, was reported also an imported case of mycetoma due to *Actinomadura pelletieri* [8-10].
It is not known the entity of the national and European burden of imported cases that is likely to be increased due to the growing number of migrants.
Actinomycetoma is well responsive to antibiotic treatment, even if continued for a long period. The recommended first-line regimen is based on trimethoprim/sulfamethoxazole 240/1200 mg BID in cycles of 5 weeks, with the possible association of amikacin 15 mg/kg per day divided in 12 hours im or ev for 3 weeks, that seemed to increase the efficacy of the treatment [6]. Amoxicillin-clavulanate, rifampicin and carbapenems could be used in case of allergy to co-trimoxazole or amikacin or in refractory cases [11-13]. Amoxicillin-clavulanate is generally not effective against *A. madurae* [14]. The duration of the therapy is not well established: among all the studies, none lasts less than five months, until a maximum of one year.
Eumycetoma requires surgical management in association with a long period of antifungals.
In our case, despite a prompt diagnostic suspicion, we delayed the correct treatment because of initial misunderstanding of the causal agent waiting for the slow growth of microorganism.
In conclusion, international and national surveillance programs are necessary to establish the real burden of mycetoma. It is important to improve the awareness on mycetoma of physicians, pathologists and microbiologists, above all in those centres more involved in the increasing of migratory routes. All of these figures should have to work in strict collaboration to reach rapidly the correct diagnosis and promptly start a long and difficult treatment.

**Conflicts of interest**
None

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# Potential sexual transmission of *Giardia* in an endemic region: a case series

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## SUMMARY

We present four cases in which probable sexual transmission of *Giardia lamblia* was suspected.
Diagnosing this mode of transmission in endemic areas is often difficult and should be considered only as possible, because exposure to poor sanitation and a potentially contaminated environment are always latent. However, as patients reported, there was no history of drinking tap water, exposure to recreational water, eating contaminated food, or other potential sources of infection but anilingus with an infected partner. We consider that in endemic countries, even when other more frequent modes of transmission could be playing the main role, the possibility of (re) infection due to sexual transmission should not be forgotten.
Talking openly with patients, strengthening patient-specific preventive measures and counselling appear to be needed to reduce risks of *Giardia* infection transmission due to this often neglected route.

*Keywords*: *Giardia*, giardiasis, sexual transmission, HIV, diarrhoea, combination therapy.

## INTRODUCTION

*Giardia lamblia*, the aetiological agent of human giardiasis, is a protozoan widespread throughout the world and it is estimated that infects approximately 5-10% of the world´s population, especially in low- and middle-income countries where the prevalence rates may range from 4-43% [1, 2]. *Giardia* transmission mainly occurs when faecal excretion of cysts by ill persons or healthy carriers is followed by oral ingestion of contaminated water or food by a susceptible host.

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In the same way, faecal-oral transmission can occur within households, daycare centres and custodial institutions, and in those people who include and practice anilingus in their sexual repertoire, especially in men who have sex with men (MSM) [3-6]. *Giardia* is often an asymptomatic -or self-limited- infection of the upper small intestine. When symptomatic, this infection presents with non-specific manifestations like diarrhoea, abdominal pain, anorexia, nausea, vomiting, weight loss, and increased flatulence [7]. Were these reasons not enough, the value of the recent *Giardia* research is increased by the recognition that this protozoan has been linked to irritable bowel syndrome and chronic fatigue [8].
Relatively non-specific clinical features of this disease lead to diagnostic difficulties. High indexes of

suspicion by clinicians, as well as thorough evaluation using sensitive techniques, are essential to diagnose this infection. Treatment of giardiasis is based on 5-nitroimidazole compounds (mainly metronidazole and tinidazole), nitazoxanide and albendazole (ABZ). When treatment fails to cure, a number of factors must be considered, including re-infections, medication non-compliance, resistance, etc. [7].

While in endemic areas the main sources of *Giardia* infection seem to be water- or food-borne, other modes of infection including sexual transmission could have a role in transmission. Herein we report our experience with 4 cases, living in an endemic region in which the sexual transmission was considered as possible.

## CASE REPORTS

**Case 1:** A 35-year-old healthy woman started with diarrhoea, hives, abdominal pain of several days'duration; mild nausea and a decreased appetite. On physical exam her abdomen was diffusely tender to palpation. There was no obvious rash seen. Up to three faecal specimens were requested and *Giardia* cysts were observed. She was treated with secnidazole (SCZ) with resolution of symptoms and three negatives faecal specimens in follow up consultation. Four weeks later, she started with similar symptoms and *Giardia* cysts were found in faecal specimens again. On repeated questioning, exposure to persons with diarrhoeal illness or to contaminated food and water were ruled out. However, she stated that had heard that *Giardia* could be transmitted sexually -by receptive vaginal intercourse-, and someone told her that if she had giardiasis, maybe her husband should receive treatment at the same time. We denied this route of transmission, but on continued questioning, she stated that she enjoyed actively performing anilingus during the sexual intercourse with her husband. Her husband was invited to attend to the consultation. He was asymptomatic but when he was parasitologically examined, *Giardia* infection was confirmed, too. Other family members, who lived in the same house, were parasitologically examined and were negative for *Giardia* cysts in faecal specimens. The patient and her husband were given giardiasis treatment at the same time with a single dose of SCZ, which resulted in parasitological cure. She had completely recovered, with resolution of her symptoms and both, she and her husband remained well.

**Case 2.** A 24-year-old healthy MSM noticed a change in bowel habits, with increased frequency and decreased consistency of faeces. Yellowish foul watery diarrhoea, 3-4 times a day, abdominal pain and a decreased appetite were the main clinical manifestations. These started around 10 days after a sexual encounter with an adult male with whom he sporadically had sexual encounters. He was very concerned about the possibility of *Giardia* infection because his occasional partner had had giardiasis no less than 4 times before. He denied both receptive and insertive anal intercourse; however, they practiced fellatio and anilingus without protection, considering these less risky practices in relation with HIV infection. Exposure to contaminated food and water were ruled out. The findings of a physical examination revealed moderated periumbilicar tenderness. Faecal specimens for ova and parasites, and modified acid-fast staining were requested. HIV testing was also offered. HIV tests were negative and modified acid-fast staining was negative for intestinal coccidia. After microscopic detection of *Giardia* cysts in faecal specimens, metronidazole (MTZ) was prescribed for 5 days to the patient and he received the recommendation of abstaining anilingus without protection. In the follow up, his faecal examination were negative for ova and parasites. He improved clinically with resolution of diarrhoea, and weight gain. Despite history of recurrent giardiasis, his sporadic sexual partner had been asymptomatic throughout the patient's evaluation and management. He was invited to attend to the doctor office and to be examined for intestinal parasites. He was also confirmed to be infected with *Giardia* and successfully treated with a 7-day course of MTZ.

**Case 3:** A 56-year-old, HIV-infected MSM (11 years diagnosed, receiving treatment with HAART the last 5 years with apparent adherence) with a past medical history of hypertension and asthma, started complaining with abdominal pain, diarrhoea, flatulence and weight loss. The findings of a physical examination were unremarkable, only diffuse abdominal tenderness. At the faecal examination it was found

*Giardia* and *Entamoeba coli* cysts. Modified acid-fast staining of faecal specimens was negative for intestinal coccidia. A thorough history revealed no potential source of infection other than sexual. He protected himself avoiding drinking unboiled water or eating food out of his house in order to prevent enteric parasitic infections that may complicate his HIV seropositive status. He practiced protected anal intercourse (both receptive and insertive). He participated in group sex, used sex toys and also practiced anilingus without protection. He had been previously diagnosed with intestinal amoebiasis, enterobiasis and hepatitis A. He was asked about the health of his most recent partner and he stated he used to have recurrent *Giardia* infection. The patient was initially and successfully treated with MTZ for 5 days, parasitologically confirmed by faecal tests for ova and parasites, three weeks later, on the day of their follow-up visit. A month later, symptoms reappeared, he reported being with 3 nights history of severe pruritus ani; enterobiasis was suspected; however, it was not confirmed. The patient reported a sexual encounter with the same last partner and having had active anilingus with him, again. Three additional faecal specimens were requested. Once again exposure to contaminated food and water were ruled out. *Giardia* cysts were found again and he was successfully treated with MTZ and ABZ, both for 5 days and repeating 200 milligrams of ABZ after 15 days, according to the guidelines for the treatment of *Enterobius vermicularis* infections. Additionally, he was recommended abstaining anilingus without protection. Apart from mild nausea and bitter taste he tolerated the treatment well. Within several days after therapy, he improved clinically with resolution of diarrhoea, and weight gain. He achieved a complete parasitological cure. His occasional partner was invited to attend to the consultation but he never came.

**Case 4:** A 21-year-old healthy MSM attended with his 28-year-old male partner to the doctor´s office. Both were complaining with recurrent abdominal pain with moderate intensity, increased flatulence, diarrhoea and weight loss. They had visited a camping in the countryside and drank unboiled water a month before. They stated anilingus without protection as a common sexual practice. The findings of their physical examination were normal. HIV testing was offered to both, and was negative. Up to three faecal specimens were requested and *Giardia* cysts were observed. Once etiological diagnosis was established, they were counseled about *Giardia* and its mode of transmission. Also, it was prescribed MTZ for 7 days for the patient and his partner, and the recommendation of drinking boiled water and abstaining anilingus until three negative faecal specimens after completion of a 7 day course of MTZ were obtained. Only the 21-year-old patient attended to follow up and the three faecal specimens requested revealed the complete parasitological cure. However, three weeks later, he re-attended with symptoms again. The findings of his faecal examination revealed *Giardia* cysts again. On repeated questioning, it appeared that the 28-year-old male partner had only taken MTZ for three days, time in which resolution of abdominal pain and diarrhoea occurred and he stopped taking the drug. So, they recommenced their sexual activities including mutual anilingus without protection. A repeat course of the same therapy led to a complete parasitological cure in both men. They improved clinically with resolution of diarrhoea, and weight gain. The complete parasitological cure was achieved.

## DISCUSSION

*Giardia* is a common and globally distributed intestinal protozoan, although this infection is mainly observed in developing countries. It is the commonest intestinal parasitic protozoan infection in Cuba, where the highest prevalence has been found in children [9]. According to Cuban studies, despite the high proportion of the population who lives with improved water supplies, water seems to play a major role in the transmission of this protozoan [10]. In the present case series, the mode of transmission in each instance was thought to be probably via faecal oral contact during anilingus, a common risk factor found in the cases reported.

Direct transmission from person to person is an established mode of transmission for some enteric pathogens including *Shigella*, *Entamoeba*, *Enterobius*, and *Giardia* when there is an oral contact with the perianal area, previously contaminated with faeces [6, 11-13]. As our cases live in endemic areas,

diagnosing this mode of infection is often difficult and should be considered only as possible; however, the sexual route appears to have provided the necessary link for transmission in each one of our 4 cases, according to the each case history.

In endemic areas, sexual transmission of *Giardia* infection may be underappreciated, due to the continuous exposure to a potentially contaminated environment because of poor hygienic conditions. That is why in these regions, the sexual transmission of *Giardia* infection is hard to be distinguished from other routes of transmission. However, it might be more common than is currently recognized, especially if the sexual repertoire of couples is taken into consideration. In addition, although this route has been mainly reported in homosexual males, anilingus may be a practice carried out independent of sexual orientation, as in our first case.

It is important to highlight that our 4 cases were highly motivated and we were able to form a close doctor-patient relationship throughout their period of diagnosis, treatment and follow up to talk in an open manner. We were therefore almost confident of excluding other potential sources of reinfection but anilingus. They denied history of exposure to drinking tap water, eating contaminated food or contact with diaper-age children, neither exposure to recreational contact with fresh water.

HIV/AIDS awareness may have a side-effect on the transmission of *Giardia* and other enteric parasitic infections, due to the HIV transmission through the oral route is considered uncommon [14]. In this way, because of the perceived "relative safety" of oral sex, in comparison to other types of sexual behaviour, oral sexual practices have been prevalent among many high-risk groups and this could increase the possibility of sexual transmission of this protozoan, mainly if it is considered the high number of asymptomatic cysts passers, the high cyst excretion rate and the long-term faecal shedding from infected human host, the immediate infectivity of cysts released in the faeces, and the low infectious dose necessary to initiate an infection (10 organisms) [7, 15].

From a public health perspective, the major challenge is how best to avoid acute infections in at-risk populations, and for those already infected, how to prevent consequent morbidity and transmission to other members in the community. A combination of both prevention and treatment is required to minimize the ongoing transmission of *Giardia* in the general population. For giardiasis, primary prevention is difficult, because a human vaccine is not available nor is one likely to be available in the near future. It seems necessary that providers have open and non-judgmental conversations with patients about the varying levels of risk for *Giardia* infection also based on sexual activity. Providers can intervene with education about harm reduction techniques; for instance, messages that include information that individuals with giardiasis are infectious during the cyst shedding. These patients should be counselled to avoid oral-anal contact during this time, and it should be explained that being asymptomatic, improvement of abdominal pain or the achievement of diarrhoea resolution do not unequivocally mean parasite clearance neither cessation of infectivity.

The present case series highlights some important points; firstly, the importance of considering the possibility of sexual transmission of *Giardia* infection in endemic countries, at least in those with recurrent *Giardia* infection or when other causes of treatment failures were ruled out, even among heterosexual couples: in this way the patient can be appropriately investigated and promptly - and properly - treated. Secondly, it is an important reminder that the human factor of compliance to therapy and preventive measures are at least as relevant, if not more so, than the drugs we choose for a regimen after failure of first-line therapy. Thirdly, the importance of tailoring messages according to the route and mode of transmission; patients could be receiving counselling messages sufficiently tailored to the epidemiological reality of the local endemicity regarding *Giardia* transmission that emphasize drinking unboiled water, eating contaminated food or swimming in contaminated pools, etc., forgetting other important modes of transmission that could be implicated.

While the importance of sexual transmission of *Giardia* infection in an endemic region need not to be overemphasized, its inadequate assessment makes re-infections possible. So, it is necessary to find an easy route into discussion about sexual transmission of this protozoan, avoiding moral judgments. Additionally, patients and their couples should be warned to use protection during this kind of sexual practice or abstain from it until a negative ova and parasites control after comple-

tion of a course of antigiardial drug was obtained. In the field of sexually transmitted infections (STIs), the effective management of these infections depends on appropriate testing, treatment, partner management, complete and timely reporting of positive the sexually transmitted disease tests and the implementation of preventive measures. However, in the case of *Giardia* infection, it is non-notifiable disease in many countries and it is not considered in the STIs setting, so the health department takes no action to notify partners. If sexual transmission is suspected, the responsibility mostly lies with the patient and most likely most physicians rely on the patient to notify his/her partner(s), which could persuade him/her to look for diagnosis and care, but probably not.

## CONCLUSIONS

Although *Giardia* is mainly transmitted through contaminated water or food, and sexual transmission is not the primary route of transmission in endemic countries, the potential of sexual transmission of this protozoan should be ruled out, mainly in patients who actively report anilingus or those who report after questioning. Increased public awareness is essential for the treatment and control of this disease in different settings. People, in general, should be counselled accordingly with clear-designed messages about the relative contributions of the main modes of transmission of *Giardia*, so that they can make informed choices about the preventive measures they should take. As no immune protection can be expected from previous *Giardia* infections, infections may repeatedly occur, as long as high-risk practices or exposure to an ongoing source continue. Thus, considering *Giardia* in the STI setting, sexual partners should be screened for this protozoan infection and treated if necessary.

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**Conflicts of interest**
None declared.

**Ethical approval**
Not required; all patients were assessed, investigated and treated in accordance with standard clinical procedures in Cuba.

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# Acute enterocolitis causing an appendicitis like symdrome

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Dear Editor,

A 24-year-old man came to our outpatient clinic due to epigastric abdominal pain which migrated to the right lower quadrant during the next few days. The patient also presented persistent watery and non-bloody diarrhea. On physical examination, the patient was afebrile (body temperature, 36.5°C). He had right lower abdominal tenderness with rebound pain and guarding. Physical signs suggestive of acute appendicitis, such as McBurney's or Lanz' point tenderness, Rovsing's and obturator signs, were all positive. However, the patient was afebrile and the laboratory data indicated the absence of inflammatory findings (peripheral white blood cell count, 6,600/$\mu$l; C-reactive protein level, 0.08 mg/dL). Plain abdominal computed tomography (CT) scanning demonstrated a normal appendix, but a severely swollen ascending colon with prominent thickening of the bowel walls (Figure 1, arrow heads). It appeared to totally occlude the luminal space, showing the appearance of a pseudoabscess-like formation with stratified structures inside. Since there was no diverticulum in the colon, acute diverticulitis was not likely the diagnosis. Additionally, an abdominal X-ray revealed the absence of mechanical bowel obstruction (Figure 2), which suggested the unlikeliness of carcinoma or intussusception. The lesion extended continuously in the right lower abdominal area (Figure 1A to B), which strongly supported a diagnosis of acute enterocolitis in the ascending colon. Oral rehydration was initiated with the administration of empiric antibiotics together with probiotics. Intravenous administration of ceftriaxone (1 g/day) was initially started, followed by oral administration of cefcapene pivoxil (300 mg/day) for seven days. The patient remained afebrile, but his symptoms and signs, including watery diarrhea and right lower abdominal pain, subsided several days after the initiation of the treatment.

Abdominal imaging is not usually warranted in patients with acute diarrhea. However, in patients who have significant peritoneal signs, CT scanning is a useful diagnostic approach to evaluate undifferentiated abdominal pain. The typical CT findings of acute enterocolitis include thickening of the bowel walls due to intramural edema and pericolic fat stranding, sometimes showing the appearance of a pseudoabscess-like formation (Figure 1) [1, 2]. There are many causes of infectious acute enterocolitis, such as bacterial, fungal, viral, parasitic causes and sometimes by amebiasis and tuberculosis [1, 3]. In our case, however, the affected portion of the ascending colon suggested infection with specific organisms, such as *Shigella*, *Salmonella*, *Campylobacter* or *Yersinia enterocolitica* [4]. Since initial stool culture results for routine organisms, such as *Shigella*, *Salmonella* and *Campylobacter*, revealed negative, *Yersinia enterocolitica*, which usually grows more slowly on a separated culture media, was most likely to be the causative pathogen.

In young adult males, the differential diagnoses of right lower quadrant abdominal pain include, appendicitis, diverticulitis, mesenteric lymphadenitis, inguinal hernia, testicular torsion or nephrolithiasis [5]. In our case, except for the persistent watery diarrhea and the absence of inflammatory findings, the patient's symptoms and signs mimicked those of acute appendicitis. As we previously reported in a patient with Fitz-Hugh-Curtis syndrome complicated by appendicitis, the

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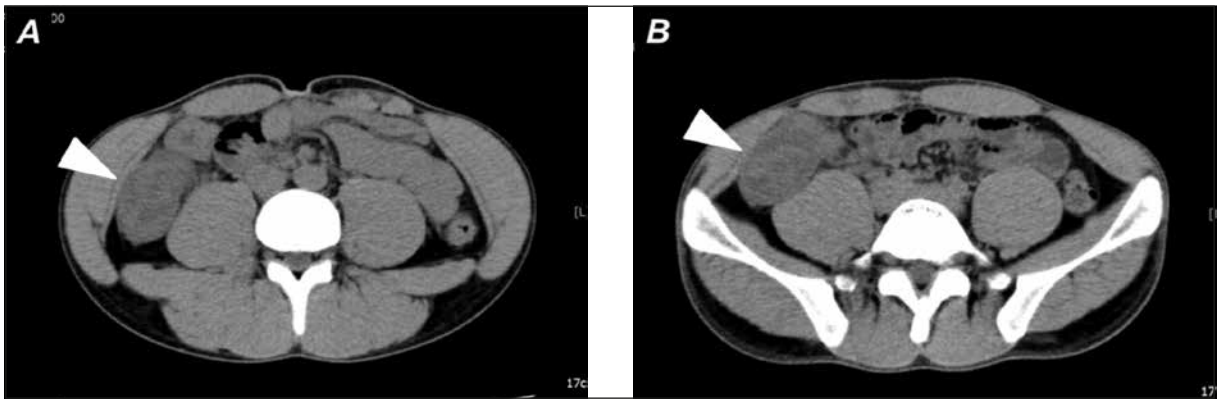


**Figure 1** - Plain abdominal computed tomography (CT) scanning at the umbilical (A) and pelvic (B) levels. It demonstrated a severely swollen ascending colon with prominent thickening of the bowel walls (arrow heads), showing the appearance of a pseudoabscess-like formation with stratified structures inside. The lesion extended continuously in the right lower abdominal area (A to B). There was no diverticulum in the colon and the appendix was normal.

extension of pericolic inflammation into the right lower quadrant was likely to be responsible for the parietal pain stimuli [6]. In our case, an abdominal X-ray revealed the absence of mechanical bowel obstruction (Figure 2), which suggested the unlikeliness of carcinoma or intussusception. However, it does not totally exclude the possibility of the malignancy in the right colon, although it is unlikely from the patient's history and age. In this regard, further investigations, such as colonoscopy and repeated CT scan, should be necessary in the future, once the enterocolitis has been resolved.

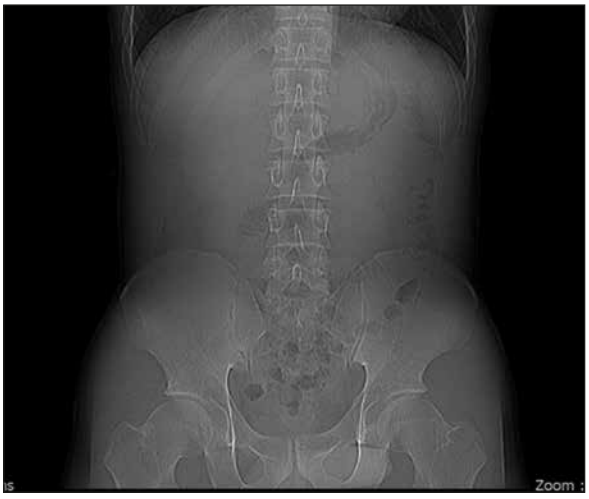

**Figure 2** - An abdominal X-ray film revealed the absence of a mechanical bowel obstruction.

**Acknowledgements**
We thank the staffs at Iwakiri Hospital for their assistance. This work was supported by MEXT KAKENHI Grant, No. 16K08484, the Salt Science Research Foundation, No.1725 and the Intelligent Cosmos Scientific Foundation Grant to IK.

**Conflict of interest**
None

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# Mapping Zika in the 125 municipalities of Antioquia department of Colombia using Geographic Information System (GIS) during 2015-2016 outbreak

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Dear Editor,

Zika epidemics have significantly impacted in the Americas region [1]. Countries such as Brazil and Colombia had regions with high incidence [2, 3]. Nevertheless, there is still a lack of epidemiological studies showing the spatial pattern of distribution, with their implications for public health and infectious diseases practitioners [2-4]. Also, travelers to those endemic areas should be aware about the risk of infective biting exposure when visiting for different purposes these areas [3]. In order to help in the advice to travelers and public health, epidemiological information is of utmost importance, including the availability of detailed maps in order to assess the risk when visiting specific destinations [2, 5, 6]. For these reasons, we have developed and published epidemiological maps for Zika in Colombia using geographical information systems (GIS) for different regions, in this case at one of the largest departments, Antioquia, constituted by 125 municipalities [2-6].

Use of GIS for development of epidemiological maps in Zika and other emerging arboviral diseases has not been used enough in Colombia and Latin America [2, 3, 6].

Surveillance cases data (2015-2016) (official reported by the National Institute of Health of Colombia) were used to estimate cumulative incidence rates using reference population data, on Zika RT-PCR and clinically suspected cases (both estimated as cases/100,000 pop.) to develop the first maps of Zika in the department of Antioquia (constituted by 125 municipalities). GIS used was Kosmo® 3.1. Four thematic maps were developed according municipalities. Determination of ZIKV infection includes either laboratory and syndromic surveillance (clinical definition of fever, rash, conjunctivitis and arthralgias in a municipality with previously ZIKV circulation, at least one case confirmed by RT-PCR). The clinical definition has been recommended by World Health Organization, Pan American Health Organization as well the US Centers for Disease Control and Prevention.

Total number of cases also included those in which clinical diagnostic criteria, *i.e.*, the case definition was met, but which were reported in a municipality without RT-PCR confirmation. After one case is confirmed by RT-PCR in a municipality, patients not classified as risk groups (pregnant women, children <1 y-old, people >60 y-old and patients with comorbidity), can be diagnosed by clinical definition.

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Since September 1, 2015 up to April 23, 2016, 1,965 cases were reported in Antioquia, Colombia (Figure 1), corresponding to: 139 RT-PCR confirmed and 1,826 clinically suspected (1,479 from municipalities with RT-PCR confirmed cases and 347 from other municipalities, without previous confirmed cases) (Figure 1), for a cumulative rate for the department of 30.43 cases/100,000 pop. Highest incidence rate was estimated in Chigorodó (208.58), followed by Carepa (188.75), Apartadó (186.16), Zaragoza (173.47) and Mutata (161.31) (Figure 1). Seventy-nine (out of 125) municipalities reported cases. Medellin (the capital city) reported 323 cases (16.4%) for a rate of 12.99 cases/100,000 pop. Eleven municipalities (high incidence at maps), reported >100 cases/100,000 pop. (Figure 1). The disease is concentrated in northwest municipalities (the whole central Urabá) of the department (neighbor municipalities) (Figure 1).

Data derived from these maps can be used to guide decisions for prevention and control of emerging health problems. Undoubtedly, Zika represents a significant issue in the region and the country, particularly in pregnant women and newborns [2, 3, 7]. And these maps should be used for counseling of travelers and pregnant patients who should be aware about the risk of infective mosquito biting. But also about the possibility of asymptomatic disease and the risk of disease transmission through sexual intercourse that should lead to the use of contraceptive barrier methods even weeks after visiting these areas, such as Antioquia [2, 3, 7]. A previous study assessed the basic reproduction

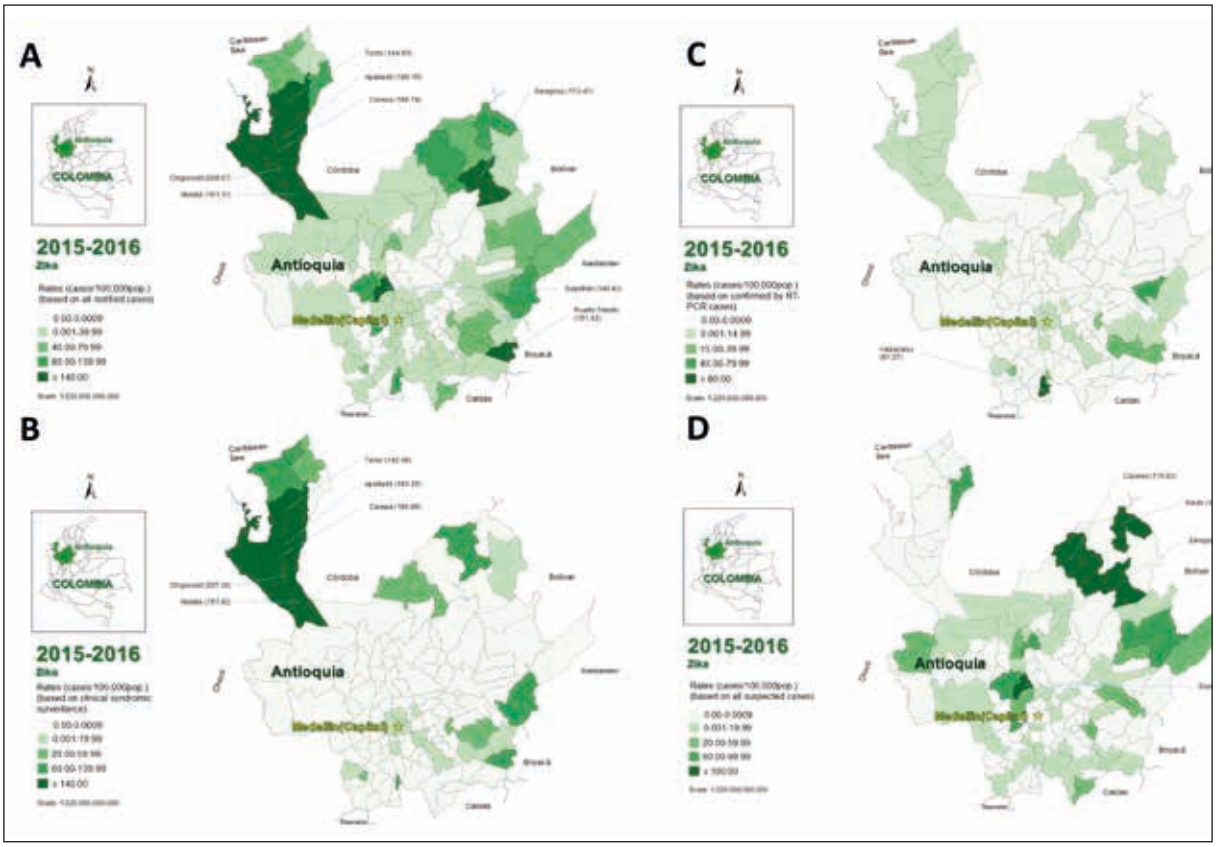


**Figure 1** - Geographic distribution of Zika incidence rates (cases/100,000pop) in Antioquia department, Colombia, 2015-2016. A. Based on all notified cases. B. Based on clinical syndromic surveillance (clinical definition of fever, rash, conjunctivitis and arthralgias in a municipality with previously ZIKV circulation, at least one case confirmed by RT-PCR). C. Based on RT-PCR-confirmed cases. D. Suspected cases (clinical definition of fever, rash, conjunctivitis and arthralgias in a municipality without previously confirmed ZIKV circulation).

number ($R_0$) in Antioquia for this epidemic period, which was found as 1.12, but did not develop incidence maps [8]. For Colombia, a previous report indicated a $R_0$ ranging from 3.0 to 6.6, that study found that the Urabá had a $R_0$ between 1.1 to 5.0, consistent to the high incidence we found of >140 cases/100,000 pop [8] (Figure 1).

Colombia officially reported during 2015-2016, a total of 106,659 cases; 2.4% were from Antioquia department. Given the ecoepidemiological conditions of the department and particularly of the northwestern municipalities, these are becoming now endemic for Zika. Other factors, including environment and climate, as have been studied in another *Aedes*-borne disease, such as Dengue, are important in future studies [3, 9]. Public health policies and strategies, considering these conditions, for an integral control of Zika in people living, but also in travelers, in these areas, should be developed and urgently implemented [3, 5, 7, 8].

Use of GIS-based epidemiological maps allows to integrate preventive and control strategies, as well as public health policies, for joint control of this vector-borne disease in this area of the country [2, 6]. As Zika is transmitted primarily by *A. aegypti*, the Dengue and Chikungunya virus vector, maps of both infections as well for coinfections will be also needed [10, 11]. Finally, the availability of relevant information, to assess the risk of travelers with specific destinations, in highly transmission areas, is highly important for prevention advice. Even more, because they play also an important role in the virus spread, as occurred in Colombia and the Antioquia department during 2015-2016 [2, 3, 6, 10].

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# Ancient treatment for lice: a source of suggestions for carriers of other infectious diseases?

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## SUMMARY

Louse infestation is one of the oldest contagious pestilential diseases of humankind, which has recently re-emerged in several developing countries as well as in homeless individuals and migrants. The present work provides the first phase of an historical excursus of louse remedies based on the classics of pharmaceutical literature, codes, pharmacopoeia and treatises. The second phase involves a literature search, based on the principal medical databases (SciFinder, Pubmed, Google Scholar, ISI-Web of Science and Scopus), to match ancient raw materials and active principles for the treatment of pediculosis and their possible applications, with other current infectious pathologies transmitted by different carriers. In this regard, *Rhododendron tomentosum* has revealed repellent insect activity, particularly against *Aedes aegypti*, responsible for Dengue fever, Chikungunya, Zika fever, Mayaro, yellow fever and other infectious diseases. *Petroselinum crispum* is an insecticide employed for resistant strains of *A. aegypti*. In the case of *Delphinium staphisagria*, the phytochemical profile was further investigated with the identification of further molecules in addition to delphinine. The latter shows interesting activities against *Trypanosoma cruzi* and *Leishmania*. *Anthemis pyrethrum*, now renamed as *Anacyclus pyrethrum*, although not containing pyrethrins present in several plants of the genus Chrysanthemum, revealed pediculicidal activity but did not produce satisfactory results in antiprotozoal activity.

*Keywords*: louse, remedies, typhus, mosquito-borne diseases, trypanosomiasis, Chagas disease, leishmaniasis.

## INTRODUCTION

Louse infestation, called pediculosis, one of the oldest pestilential diseases of humankind, is very contagious and easily transmitted by close body-to-body contact or contact with infested linen, brushes, or clothes, according to the species of louse. *Pediculosis capitis*, caused by head lice, is the most common louse infestation; it particularly affects school-children 3-11 years of age and its clinical hallmark is scalp pruritus [1]. *Pediculosis corporis*, caused by body lice, represents a major public health concern. It is strongly associated with close body-to-body contact, and occurs only when clothes are not changed or washed regularly. These conditions are more prevalent in individuals living in crowded and unhygienic environments, such as refugee camps or shelters for the homeless and migrants.

Clinical manifestations include generalized pruritus associated with scratching and lesions are typically localized to the neck, thorax, waist, and ankles. Diagnosis is based on a finding of adult lice and, more importantly, eggs in clothing seams.

Three types of louse can affect humans, but only body lice act as vector for human pathogens such as *Rickettsia prowazekii*, *Borrelia recurrentis* and *Bartonella quintana*, which are known to cause epidemic typhus and louse born relapsing fever

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(LBRF) which must be considered in any febrile refugee regardless the country of origin, and trench fever, respectively.
These infections particularly affect populations living in poor-hygiene conditions where body louse infestations are prevalent. Whereas epidemic typhus and epidemic relapsing fever are particularly prevalent in vulnerable populations in developing countries, trench fever is common worldwide, especially in homeless individuals.
Louse-borne diseases are associated with a high prevalence of body louse infestation, and have recently reemerged in jails and refugee camps in central and eastern Africa, Peruvian Andes and in rural louse-infested populations in Russia [2, 3]. LBRF, a neglected and forgotten disease by western physicians has recently reemerged among East African migrants seeking asylum in Europe [4]. Migrants from endemic areas can carry the vector with them; healthcare providers should be aware of this condition to implement adequate diagnostic, therapeutic, and public health measures.
The historical path that has led to today's remedies against pediculosis has been long and laborious, although it is worth recalling that the insecticide action on the common louse can now be exploited against the carriers of severe infectious diseases now rapidly spreading from a continent to another.
In 1939, the dichlorodiphenyltrichloroethane (DDT) came into pratical use revolutionizing the cornerstones of disinfestation although it was banned in 1978 for its adverse health effects. After DDT benefit, treatment of pediculosis has been continued since the late 1970s by the employment of synthetic pyrethroids developed from natural molecules drawn from *Chrysanthemum cinerariaefolium*. Among them, permethrin has shown to be the most effective product. Other synthetic pyrethroids are phenotrine, deltamethrin and sumitrin. A second choice treatment consists of malathion, an organophosphorus pesticide.
The present work provides a first part on an historical excursus of pediculosis remedies based on the classics of pharmaceutical literature, codes, pharmacopoeia and treatises (Figure 1).
A second phase part will involve a literature search, based on the principal medical databases (*i.e.*, through SciFinder, Pubmed, Google Scholar, ISI-Web of Science and Scopus), to match ancient raw materials and active principles for the treatment of pediculosis and their possible applications, with other current infectious pathologies transmitted by different carriers.

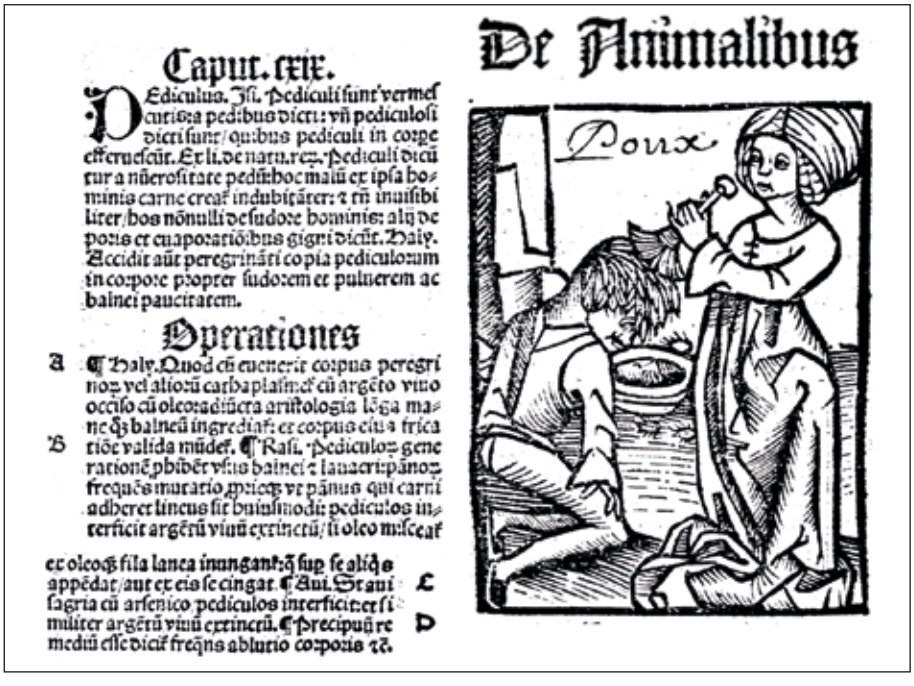



**Figure 1** - *De Animalibus: Pediculus*, Ortus sanitatis, 1517.

*Historical notes*

The role of *Pediculus* as a vehicle of epidemic typhus infection was established in 1909 by the Nobel Prize in Medicine Charles Nicolle who discovered that epidemic typhus is transmitted by body lice (*Pediculus humanis corporis*) [5]. The epidemiology of head louse and body louse infestations, and of louse-borne epidemic typhus, indicates that the head lice are potential vectors of *R. prowazekii* in the field [6].

Epidemics occurred during wars and famine and epidemic typhus has been one of the earliest pestilential diseases that have strongly influenced humanity [7].

Paleomicrobiology enabled the identification of the first outbreak of epidemic typhus in the 18th century in the context of a pan-European great war in the city of Douai, France, and supported the hypothesis that typhus was imported into Europe by Spanish soldiers returning from America. *R. prowazekii* was also detected in the remains of soldiers of Napoleon's Grand Army in Vilnius, Lithuania, indicating that Napoleon's soldiers had epidemic typhus [8]. It is estimated that, of the 25,000 soldiers who reached Vilnius, only 3000 survived (Figure 2). The majority of them were infected with louse-transmitted diseases and recently, DNA from soldier's dental pulp gave evidence of infection with either *R. prowazekii* or *B. Quintana* [9, 10].


**Figure 2** - A, Map showing the location of Vilnius. The "Grande Armée" retreated from Moscow to Paris. B, General view of the grave in Vilnius (from Raoult D. et al., 2006. Photo by P. Adalian, Centre National de la Recherche Scientifique, Unité Mixte de Recherche 6578) [10].

**Remedies**

*Mercurials*

Mercurials have been used in traditional medicine. Mercury and most of its compounds are extremely toxic. They have been used in antiseptics, laxatives and antisyphilitics. Topical mercury treatment was used to treat pediculosis.

In the Lemery's pharmacopoeia (1720) *Unguentum Neapolitanum simplex* made of quicksilver, Venice turpentine and lard is reported. It can be employed for treatment of scabies, phthiriasis, bedbugs, crabs. It is called *Neapolitanum*, and was also used against syphilis [11].

Campana in the *Farmacopea ferrarese* (several editions) recommends mercurial ointment of white or red precipitate of quicksilver [12].

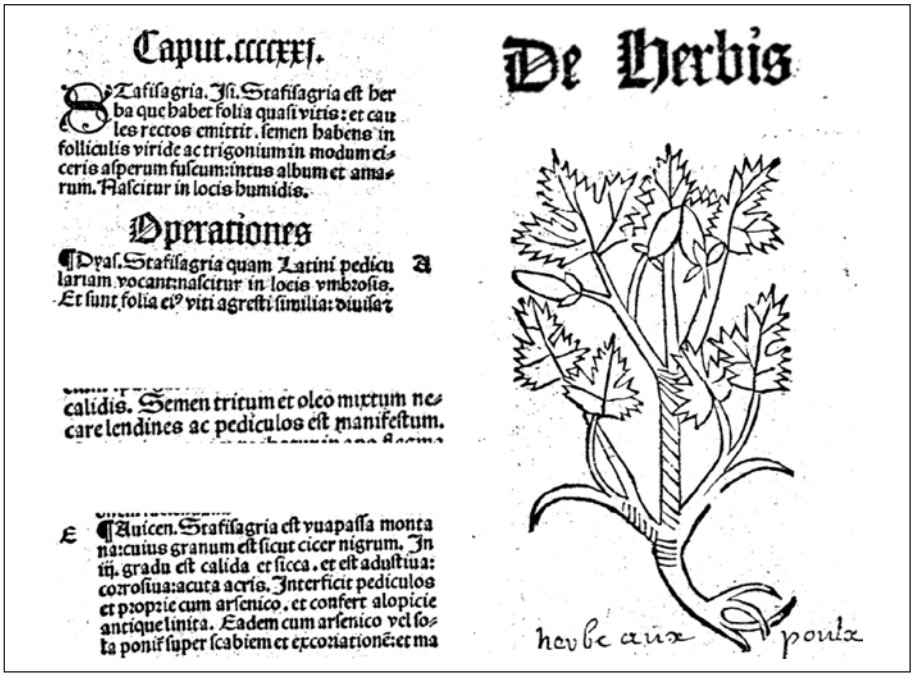
The same remedy is reported in Porati's *Manuale Farmaceutico* (1820): *Unguentum ad pediculos coeruleum* and *Unguentum ad pediculos rubrum* and in Orosi's pharmacopoeia (1856-57): *Pomata Antipedicolare: prec. Rosso o bianco* and lard [13, 14].

In the De Bruc's *Formulario* (1863), including many international pharmacopoeial preparations, simple mercurial ointment, mercurial rose-water and lotion of the bichloride of mercury (*Pomata mercuriale semplice, Acqua contro i pidocchi, Lozione mondificante)* are reported [15].

Brugnatelli in *Pharmacopeia Generale* (1817) cited red precipitate of mercury, also named red nitrated quicksilver, and mercurial creme, *Precipitato rosso e manteca mercuriale* [16].

In *Manuale di Pharmacopeia generale e speciale su Farmacopea Prussiana e lavori Alemanni e stranieri* (1871) Posner recommended the powder of bichloride of mercury mixed with starch and sugar [17].

*Stafisagria - Delphinium Staphisagria*
The *Ortus Sanitatis*, a treatise published since the end of 15th century, attributed the spread of pediculosis to … *balnei paucitatem* … and indicates hygiene as the main remedy … *precipuum remedium esse dicit frequens ablutio corporis* (Figure 3). As remedies it includes, in addition to mercury, staphisagria with arsenic, as Avicenna said [18].
Antonio Musa Brasavola, in *Examen omnium simplicium medicamentorum, quorum in officinis usus est* (1539), states that it is used by women *ad necandos pediculos*. The name comes from them in *strafusarium*. The name Staphisagria derived from the Greek and means wild raisin. It is the *herba pedicularis* of the Romans [19]. In *Librum quartum Dioscoridis Cap. CL* (*Commentarii*, Latin Edition of *I Discorsi*, 1554) it is illustrated according to Dioscorides *Staphys Agria*: *Trita, & ex oleo illita phthiriasi prodest.* Mattioli points out that *Latini ab effectu, quod pediculos necet, herba pedicularis dicitur.* Oil is effective agaist phthiriasis, Mattioli remembers that it is called by Latini *herba pedicularis* for its effect on lice. It is frequent in Istria, Dalmatia, Apulia and Calabria. It also has harmful effects on humans (Figure 4) [20].
Lemery in *Farmacopea Universale* reports lotion pediculicide *Lotio ad pediculos capitis enecandos (Staphysagria, Seminis contra, assenzio, tanaceti, betonica, centauria minor).* Hot decoction is used to wash the head. It kills lice and crabs [11].
As reported in Jourdan's *Parmacopea Universalis* (1837) stafisagria is a component in *Pulvis Capucinorum*, made of sabadilla, parsley and tobacco [21].
Orosi proposed an ointment: stafisagria powder mixed with lard in 1:8 ratio [14].
Ferrarini in the *Farmacopea* (1825) for the *Erba de Pidocchi* indicates the habitat in *Linguadocca*, Provence, and in the mountains of Spouthern Europe. He also suggests its use as powder, infusion, ointment [22].
Brugnatelli recommended its use as powder, decoction and ointment. An infusion of staphisagria may be made with vinegar [16].
The *Dizionario delle Droghe semplici e composte* (1831) and *Dizionario classico di Medicina interna ed esterna* (1838) report an ointment (*Pomata*) made of powder of stafisagra (fr. Staphisaigre) and lard [23,24]. Seeds can be macerated in vinegar. The isolation from the seeds of the active component is also cited: Lassaigne and Feneulle report that the seeds contain a basic substance, named *Delfinia*, with an excess of malic acid. The water-soluble fraction is more toxic to animals. Powdery seeds


Caput.cccxxxj.

Stafisagria. Jſi. Stafiſagria eſt herba que habet folia quaſi viris: et caules rectos emittit. ſemen habens in folliculis viride ac trigonium in modum ceris aſperum fuſcum: intus album et amarum. Naſcitur in locis humidis.

Operationes

Dyaſ. Stafiſagria quam Latini pediculariam vocant: naſcitur in locis vmbroſis. Et ſunt folia ei? viti agreſti ſimilia: diuſar

calidis. Semen tritum et oleo mixtum necare lendines ac pediculos eſt manifeſtum.

Auicen. Stafiſagria eſt vuapaſſa montana: cuius granum eſt ſicut cicer nigrum. In iij. gradu eſt calida et ſicca. et eſt adustiua: corroſiua: acuta acris. Interficit pediculos et proprie cum arſenico. et confert alopicie antique linita. Eadem cum arſenico vel ſota ponit ſuper ſcabiem et excoriationē: et ma

De Herbis



**Figure 3** - *De Herbis: Stafisagria*, Ortus sanitatis, 1517.

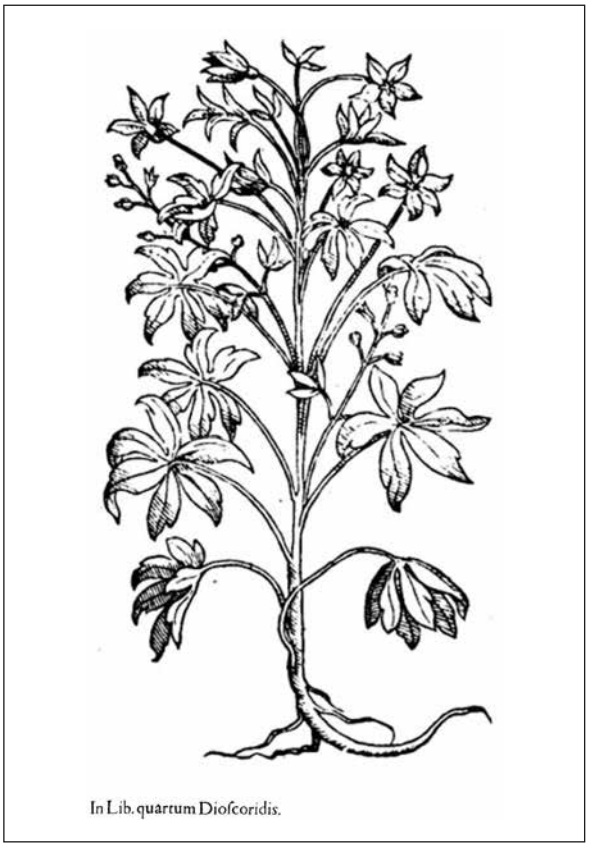



**Figure 4** - *Lib. quartum Dioscoridis Cap. CL: Staphys Agria*, Mattioli P.A., Commentarii, 1554.

are poisonous for ingestion in animals [23].
Campana itself mentions the isolation by Feneulle and Lassaigne of *Delfina*, its characterization and toxicity [12].
Posner suggests its use as powder, decotion and ointment, even in association with mercurials, and report that the seeds contain *Delfinia*, an acre alkaloid similar to *veratrina* [17].
*Delfina*, whose properties are attributed as a remedy, is described for its tossicity in treatise of Legal Medicin (1840) [25].

*Sabadilla - Veratrum sabadilla L.*
Sabadiglia *Veratrum sabadilla L.* is recommended in the treatment of pityriasis, as reported in Brugnatelli's pharmacopoeia [16].
The Jourdan's *Farmacopoea Universalis* contains many preparations of Sabadilla. Sabadilla is derived from the seeds of *V. sabadilla*, which grows in Mexico. Jourdan reported that the sabadilla's components isolated by Meissner, Pelletier Caventou are *acido cevadico* and *veratrina o sabadiglina*. The Galenical preparation are: *Pulvis capucinorum, Polvere dei Cappuccin*i, made of *Seminum sabadillae, Staphydis agriae, Petrosellini, Foliorum nicotianae* (seeds of sabadilla, staphisagria, parsley, tobacco); *Unguentum ad s. contra pediculos* (Ointment against lices) made of *Pulveris sabadillae, Synapis, Pyretri, Axungiae preparatae* (sabadilla in powder, mustard, pyrethrum, lard). The *Infusum ad cimices* is also reported, against the bedbugs, *per la distruzione delle cimici, lavando con questo le lettiere* [21].
*Pulvis* Capucinorum is well-known, reported also in Orosis's *Farmacopea* [14].
Antonio Campana in the *Farmacopea ferrarese* says that *veratrina* is an alkaline substance contained in sabadiglia and also in autumn crocus [12].
Meyer in *Manuale di Farmacologia* (1841) recommends that, if any excoriations exists, care must taken not to use the powder too freel [26].
As reported by Posner, Sabadilla in powder or infusion is a very good preparation. As ointment made mixing powder, lard and an essential oil of *lavandola*, can improve the odour (*Pharm austriaca* is cited). Instead, sabadilla's vinegar, obtained moisten the powder with diluted acetic acid, is not recommended as it causes skin irritation [17].
*Galla del Levante, Menispermum cocculus Lin. o Cocculo o Coccola del Levante* (fruit, powder) has been a substitute of sabadilla, as claimed by Ferrarini and Campana [12, 22]. Orosi reports that picrotoxin was first isolated by Couerbe and Pelletier and it is extremely toxic. [12]. As pediculicide, it may be applied in the form of ointment, according to *Farmacopea prussiana* [17].

*Petrosellino nostrale - Apium petrosellinum L.*
A treatise comprehensive of the *Codice Farmaceutico Francese* and the *Farmacopea austriaca* (1838) praises the virtues of the parsley. The *Petrosellino nostrale, Apium petrosellinum*, which grows in Greece and Sardinia, contains *olio etereo* and *canfora*. The seeds were employed to prepare a ointment [27].
The powder is suggested by Brugnatelli and is a component in *Pulvis Capucinorum* previously described [16, 21].

*Rosmarino silvestre - Ledum palustre L.*
In *Farmacopea ferrarese,* it is reported that decotion of leaves of wild rosemary, Rosmarino silvestre

*Ledum palustre* Lin., which grows in swamps in North Europe, is also effective [12].

*Other compounds*
For treatment of phthiriasis, Brugnatelli makes a list of *Vegetabili e loro preparazioni*: Stafisagria (powder), Sabadilla (seeds) and also Colchico autunnale *Colchicum autumnale* L. (*sugo*), Lauro riccio *Laurus nobilis* L. (oil from berries and leave), Pepe nero *Piper nigrum* L. (powder), Tabacco *Nicotiana latifoglia* L. (powder or infusion) [16].
Campana suggested as insecticidal *Colchic Colchicum autumnale* Lin. and oil of *Carapa, Persoonia Guareoides Vild. Carapa oleifera Aubl.* [12].
As reported by Posner, decotion of *Evonimo Europeo*, european spindletree, and powder of seeds of *Datura stramonium* containing *daturina* are effective in the treatment of *phthiriasis* [17].
Throughout history, the treatment of phthiriasis is abundantly simple. Many are the medicines and varied the forms which are used for this purpose. The preparations of mercury, staphisagria and sabadilla are predominantly employed.
A number of these has been considered as homeopathic remedies: *S. staphisagria*, *V. sabadiglia*, *Petrosellinum*, *L. palustre*, *C. autumnale*, *Tabacum*, mercury [28, 15].

**The lesson of past remedies for future remedies**
The virtues boasted of raw materials conceived in past remedies in popular use or pharmacopoeia, can be certified with today's advanced techniques and tools. The insecticide action on the banal louse can now be exploited against vectors of dangerous infectious diseases, now rapidly spreading from one continent to another and often in a very short time. Some raw materials may cause high toxicity not compatible with their use, but others may reveal unexpected properties.

**Mosquito-borne diseases**
Mosquitoes are responsible for the transmission of several pathogenic microorganisms to humans, causing mosquito-borne diseases, such as malaria, dengue, yellow fever, West Nile virus (WNV) disease, Chikungunya fever, and Rift Valley fever (RVF). Although there are numerous native mosquito species present and thus able to transmit pathogens in Europe, other mosquito species (*e.g.* Aedes spp.) have recently been introduced and become established in the continent. Among them, the tiger mosquito, *Aedes albopictus*, is probably the major threat to public health in Europe.
The presence of these tropical species means that there is a risk of the appearance of autochthonous mosquito-borne diseases that have previously never or rarely been seen in Europe, acquired after importation from endemic countries [29].
Mosquito-borne diseases, remain a major source of illness and death worldwide, particularly in tropical and subtropical climates [30].
Although surveillance and diagnostic methods are available, control and preventive measures are still limited.
Protection against insect bites is best achieved by avoiding infested habitats, wearing protective clothing and applying insect repellent [31, 32].
Repellents that can be used anywhere at any time are the only feasible measure for preventing arthropod attacks in some situations. Economical and practical insect repellents have, therefore, become a viable and attractive alternative that is widely sold on the market. Commercially available insect repellents can be divided into three categories, synthetic chemicals, botanicals and alternatives such as the combination of synthetic and natural compounds [33].
Therefore, there is an urgent need to develop more efficient surveillance and control tools, and to support coordinated monitoring programmes, particularly in the Mediterranean region, to enable prompt recognition of the threat of potential introduction of some arbovirus into Europe.

**Remedies**
***Ledum palustre* L.**
***Rhododendron tomentosum* Harmaja**
*Insect repellent activity*
*Rhododendron tomentosum* Harmaja (previously: *L. palustre*) is a fragrant evergreen shrub found in peaty soils in northern Europe, Asia and North America, commonly referred to as wild rosemary, marsh tea, marsh rosemary or northern Labrador tea.
At least, since in the eighteenth century it has been used in ethnomedicine for the treatment of various sicknesses, such as rheumatism, cough, cold and insect bites, as well as a repellent. The essential oil of wild rosemary with the rich polyphenolic fraction possesses analgesic,

anti-inflammatory, antimicrobial, antiviral, antifungal, promising antidiabetic, antioxidant and anticancer properties. Its insecticidal potential has been demonstrated by *in vivo* and *in vitro* studies.
The ethyl acetate extract of *R. tomentosum* from southern Sweden, significantly reduced biting by *Aedes aegypti* L. mosquito in laboratory tests. The main volatile compounds, collected by solid phase microextraction (SPME) from leaves of wild rosemary, *i.e.* p-cymene, sabinene, and terpinyl acetate, were suggested to be responsible of this effect.
*R. tomentosum* may be a useful source of chemical compounds of medical, veterinary or agricultural importance for the control of insects [34].

***Apium petroselinum* L.**
***Petroselinum crispum* (Mill.) Fuss**
*Insecticidal activity*
The increasing and widespread resistance to conventional synthetic insecticides in vector populations, has underscored the urgent need to establish alternatives in the mosquito management system. The study was carried out with the aim to investigate the antimosquito property, larvicidal and adulticidal potential, of plant products against both the pyrethroid-susceptible and resistant strains of *A. aegypti*.
Seventeen plant products, including essential oils and ethanolic extracts, were obtained by steam distillation and extraction with 95% ethanol, respectively. Potential toxicity of the plant candidate was compared with that of synthetic temephos, permethrin, and deltamethrin. The highest efficacy was established from *P. crispum* fruit oil.
The profound larvicidal and adulticidal potential of *P. crispum* oil promises to form a new larvicide and adulticide against either the pyrethroid-susceptible or resistant strains of *A. aegypti*. Consequently, *P. crispum* oil and its constituents can be used or incorporated with other chemicals/measures in integrated mosquito management for controlling *A. aegypti*, particularly in localities with high levels of pyrethroid and organophosphate resistance.
GC-MS analysis of *P. crispum* oil demonstrated that 19 compounds, accounting for 98.25% of the whole oil, were identified as the main constituents such as thymol (42.41%), p-cymene (27.71%), and γ-terpinene (20.98%) [35].

***Veratrum sabadilla - Schoenocaulon officinale***
*Insecticidal activity*
Sabadilla is derived from the seeds of plant *Schoenocaulon officinale*, which grows in Venezuela. Sabadilla is one of the least toxic registered botanical insecticides, with mammalian LD50 of 5,000 mg/kg bw. Similar to other botanical insecticides, it has minimal residual activity and degrades rapidly in sunlight and moisture (rainfall). Purified veratrine alkaloids, however, are considered on par with the most toxic synthetic insecticides.
Data from systemic poisoning by sabadilla preparations used as insecticide are rare or nonesixtent [36]. This compound is effective against caterpillars, leafhoppers, thrips, sting bugs and squash bugs. The major insecticidal components of sabadilla are the alkaloids cevadine and veratridine which are inside the seeds. The extracted alkaloids are highly poisonous.

***Nicotiana tabacum* L**
*Insecticidal activity*
Beside traditional use of botanicals, their commercial use began in the nineteenth century with the introduction of pyrethrum from *C. cinerariaefolium* and also nicotine from *Nicotiana tabacum.* This last is another well-established botanical insecticide. Nicotine analogues also possess insecticidal properties. Nicotine is active against piercing-sucking insects such as *aphids, leafhoppers, whiteflies, thrips,* and *mite*s. However, because of the high mammalian toxicity and detrimental effect on human health, its use as an insecticide has decreased considerably [36].

***Delphinium staphisagria* L**
*Trypanocidal properties in Chagas disease*
Chagas disease or american trypanosomiasis, is a devastating disease caused by the kinetoplastid protozoan *Trypanosoma cruzi* commonly transmitted to humans and other mammals by an insect vector, the blood-sucking bug of the subfamily Triatominae (family Reduviidae) most commonly species belonging to the *Triatoma infestans* (vinchuca bug), Rhodnius, and Panstrongylus genera. The disease may also spread through blood transfusion and organ transplantation, ingestion of food contaminated with parasites, and from a mother to her fetus. Chagas disease may lead to chronic and systemic stages, which can affect severely heart, esophagus, and colon.

Chagas disease is endemic throughout Latin America and it is the third most widely spread tropical disease after malaria and schistosomiasis according the World Health Organization (WHO). It is estimated that about 100 million people are at risk of infection and from 15 to 20 million are infected, with some 50 000 persons dying yearly from this disease [37].

*D. staphisagria* is an endemic annual or biennial herb from the Mediterranean Basin. Due to its historical medicinal uses, this plant has probably become widespread in the Mediterranean area. Human-mediated distribution could have promoted few migrant genotypes. The limited genetic variability, the high genetic similarity among populations and the dysploidy of this species, make it worthy of conservation [38].

The molecular structure of major alkaloid isolated from seeds by Lassaigne and Feneulle in 1819, delphinine (Figure 5), was determined and synthesized in 1970 [39].

Diterpenoid alkaloids isolated from the aerial parts of *D. staphisagria* has also been studied extensively [40, 41].

Tissue cell cultures of *D. staphisagria* L. produced dianthramide glucosides. Their formation in cellus tissue of a Delphinium species appears to be unprecedented and may be a response to unknown pathogens. Dianthramides are generally considered to be phytoalexins [42].

Finally, the *in vitro* and *in vivo* trypanocidal activities of nine flavonoids (Figure 6) isolated from the aerial parts of *D. staphisagria,* have been studied in both the acute and chronic phases of Chagas disease [43, 37].

The antiproliferative activity of these substances against *T. cruzi* (epimastigote, amastigote, and trypomastigote forms) exhibited in some cases more potent antitrypanosomatid activity and lower toxicity than the reference drug, benznidazole.

*In vitro* studies using ultrastructural analysis together with metabolism-excretion studies were also performed in order to identify the possible action mechanism of the compounds tested. Alterations mainly at mitochondria level may explain metabolic changes in succinate and acetate production, perhaps due to the disturbance of the enzymes involved in sugar metabolism within the mitochondrion. *In vivo* studies provided results consistent with those observed *in vitro*. No signs of toxicity were detected in mice treated with the flavonoids tested (Figure 6), and the parasitic charge was significantly lower than in the control assay with benzimidazole. The effects of these compounds were also demonstrated with the change in the anti-*T. cruzi* antibody levels during the chronic stage [37].

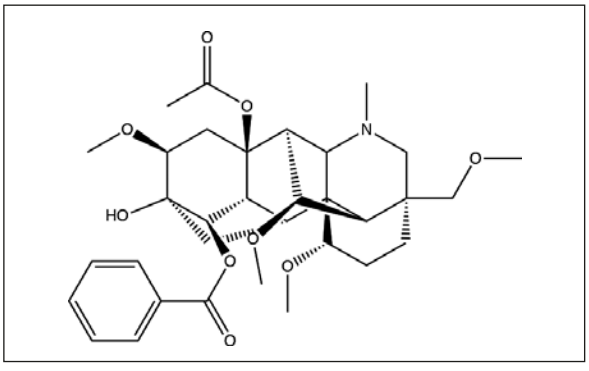



**Figure 5** - Delphinine.

*Leishmanicidal properties in Leishmaniasis*

Leishmaniasis is an infection caused by different species of the protozoan genus *Leishmania*, which is transmitted by dipterans of the genera *Phlebotomus* in the Old World and *Lutzomyia* in the New World. Leishmaniasis represents one of the most significant neglected tropical diseases which according to WHO latest report (2013) affects 350 million people in 88 countries. Approximately 12 million individuals are currently infected with *Leishmania spp.* with an estimated 2 million new cases occurring every year (44). Major endemic areas are in the tropics and subtropics; however, leishmaniasis exclusively due to *Leishmania infantum* is also endemic in large parts of southern Europe.

Drug treatment for leishmaniasis has been available since the beginning of the 20th Century, but only a few drugs have been developed for use and there are numerous drawbacks to each of the treatments: antimonials (meglumine antimoniate or glucantime and sodium stibogluconate or pentostan), amphotericin B, paromomycin (aminosidine) and pentamidine isethionate, miltefosine, now used in combination with different classes of azole oral antifungal agents including ketoconazole, juconazole, and itraconazole. In addition to the adverse effects of the drugs, resistance to these treatments is appearing in the parasites.

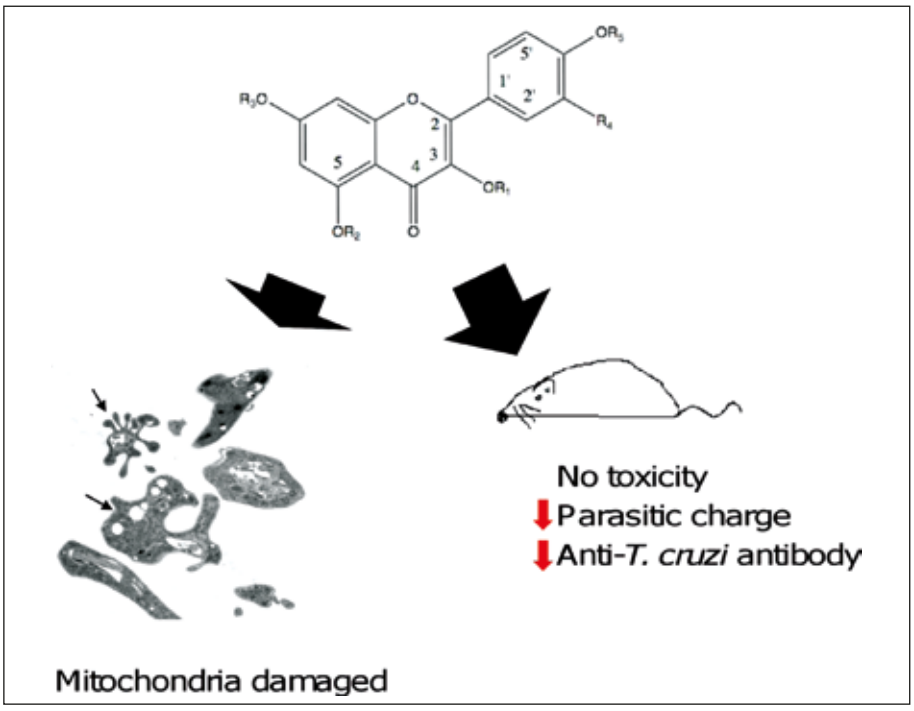



**Figure 6** - In vitro and *in vivo* trypanocidal activity of flavonoids from *D. staphisagria* against Chagas Disease [37].

For all these reasons, further novel drug development is necessary to treat these infections. Considerable attention is currently being paid to phytotherapy in the search for new drugs.
Leishmanicidal properties may reside in phytochemicals such as javonoids, which are hence strong candidates for use in combination therapy against these infections.
Flavonoids (Figure 6) from aerial parts of *D. staphisagria* L. (Ranunculaceae) showed leishmanicidal activity against promastigote as well as amastigote forms of *L. infantum* and *Leishmania braziliensis*. These compounds were nontoxic to mammalian cells and were effective at similar concentrations up to or lower than that of the reference drug (Glucantime) [45].

***Anthemis pyrethrum* L.**
***Anacyclus pyrethrum* (L.) Lag**
*Antiprotozoan activity*
*Piretro, Anthemis pyrethrum*, is *Anacyclus pyrethrum*. Pyretrum does not contain pyrethrins, which are presents in several plants of the genus Chrysanthemum. The ethnomedicinal use of *Anacyclus pyrethrum* as an antimalarial might be explained by the activity of lipophilic constituents present in roots. Alkamides were isolated and were tested *in vitro* for antiprotozoal activity against *Plasmodium falciparum*, *Trypanosoma brucei rhodiense, T. cruzi* and *Leishmania donovani*. Their overall antiparasitic activity is low [46].

## CONCLUSIONS

Natural remedies have served as a source of inspiration in the development of new drugs. This pathway is "experience-driven" and mainly based on traditional uses. Thus, ancient studies may serve as a valuable starting point to develop plant driven discovery of new drugs. This has been proven possible for some important remedies (*i.e. Artemisia annua*) [47]. Starting from existing hystorical proofs, one can envisage a stage of discovery from herbal ingredients, which includes the preparation of extracts, structure/composition elucidation, and *in vitro* bioactivity evaluation.
In the present work, the cross-survey of basic raw materials in the preparation of pastile products in the past and the most used medical database, has led to interesting findings in the fight against pe-

**Table 1** - Past and present medical remedies active against lice and other current Infectious Diseases and vectors.

| | *Activity* | *Actual denomination* | *Ongoing studies* |
|---|---|---|---|
| Mercurials | Lice, scabies, bedbugs, crab louse | | |
| Rosmarino silvestre *Ledum palustre* L. | Lice | *Rhododendron tomentosum* Harmaja | Insect repellent *Aedes aegypti* |
| Petrosellino nostrale *Apium petroselinum* L. | Lice | *Petroselinum crispum* (Mill.) Fuss | Insecticidal against pyrethroid-susceptible and resistant strains of *Aedes aegypti* |
| Sabadilla *Veratrum sabadilla* L. | Lice, bedbugs | *Schoenocaulon officinale* | Insecticidal caterpillars, leafhoppers, thrips, sting bugs and squash bugs |
| *Nicotiana tabacum* | | | Insecticidal piercing-sucking insects such as aphids, leafhoppers, whiteflies, thrips, and mites |
| Stafisagria *Delphinium staphisagria* L. Active principle: Delphinine | Lice, crab louse | | Trypanocidal and leishmanicidal activities *Trypanosoma cruzi* *Leishmania infantum* *Leishmania braziliensis* |
| Piretro *Anthemis pyrethrum* L. | Lice | *Anacyclus pyrethrum* (L.) Lag. | Antiprotozoal activity (low) *Plasmodium falciparum* *Trypanosoma brucei rhodiense* *Trypanosoma cruzi* *Leishmania donovani* |

diculosis (Table 1). To this end, the search had to be matched with today's denomination.

Therefore, the *L. palustre*, now termed *R. tomentosum*, revealed repellent insect activity, particularly against *A. aegypti*, responsible for Dengue fever, Chikungunya, Zika fever, Mayaro, yellow fever and other infectious diseases. *A. petrosellinum*, today *P. crispum*, is insecticide for resistant strains of *A. aegypti*. In some cases, lately, interest in the insecticide activity of *V. sabadilla*, today *S. officinale*, and *N. tabacum*, has dropped dramatically.

In the case of *D. staphisagria*, the phytochemical profile was further investigated with the identification of additional molecules in addition to Delphinine. The latter have shown interesting activities against *T. cruzi* and *Leishmania*.

*Anthemisis pyrethrum*, today *Anacyclus pyrethrum*, although not containing pyrethrins present in several plants of the genus Chrysanthemum, had revealed pediculicidal activity but did not produce satisfactory results in antiprotozoan activity.

From the past and from the old louse treatment, seem to emerge therefore cues to combat some of today's diseases and vectors that support them, such as repellents or insecticides, even in cases of resistance.

The use of insecticide-treated nets has become a key malaria control strategy, although more efficacious than in the past. All pesticids have been shown to be toxic.

We need implementation of strategies to minimize potential risk through careful selection and discovery of new products, considering the parasite resistance to current antimalarian drugs and to insecticide by vector mosquitoes [48].

In summary, this context, an approach that takes into consideration past experience, such as those deriving from tradition, can be plentiful of suggestions and may shorten time to discovery, thus reducing overall investment.

Traditional uses still represent the largest clinical study ever conducted by human beings.

Principal drawbacks are related to the preparative methodologies in use in ancient times, which are not easily understandable and do not make clear what part of the activity is linked to original mole-

cules or their by-products that are formed during preparation and storage.
This is the most challenging task in the re-investigation of ancient remedies and ethnobotany preparations in the light of the most recent techniques and equipments [49].

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