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European Social Fund Investing in PEOPLE!

SECTORAL OPERATIONAL PROGRAMME HUMAN RESOURCES DEVELOPMENT 2007- 2013
Project: “Professional training system for medical personnel in new technology for health care system (molecular diagnostic)” - POSDRU/81/3.2/S/58819
Project cofinanced from the European Social Fund by Sectoral Operational Programme Human Resources Development 2007- 2013

European training for the Romanian Health Care System

As announced early this year, we have started a European Structural Funded teaching project: “Professional training system for medical personnel in new technology for health care system (molecular diagnostic)” TDM. Project cofinanced from the European Social Fund by Sectoral Operational Programme Human Resources Development 2007- 2013, and we have chosen to inform you about the development of this complex project in a journal dear to us. There are several reasons for publishing this brief report. The first reason is the appropriate use of European funds engaged in the medical personal teaching for implementing state-of-the-art technologies in order to improve Romanian Health Care. The second reason is that, our trainees, medical personnel involved in diagnosis, are more open to learning in Romania the international recognized technologies for implementing up-to-date techniques. Last but not least, the anonymous evaluation of this course, brought great satisfaction to the management team, thus bypassing the inherent difficulties of implementing a project of such endeavour.

In an ever growing unsympathetic world, a few researchers with an internationally recognized scientific background have sacrificed their time and strength to put together the First National Proteomics Course intended to improve the diagnostic of the Romanian patients in their own country.

In the framework of project POSDRU/81/3.2/S/58819 „Victor Babes” National Institute of Pathology, Bucharest, organized in 2011 the course entitled Proteomics from research to clinic - basic course and hands-on applications.

For 6 months, 40 trainees, physicians, biologists, biochemists and chemists involved in Health Care System have gone through a round of theoretical lectures, followed by a series of applications of the latest proteomic technologies valid for implementation in the clinical laboratories.

The Proteomic course aimed the introduction of the trainees in the current proteomic technologies for laboratory investigations, hands-on applications for these methodologies that can improve diagnosis, prognosis and therapy monitoring on oncology, immunology, cardiology, rheumatology and so on.

80% of our trainees were women and were distributed between the development regions of Romania as follows: N-E was represented by 12.5% of the trainees, South - 17.5%, Centre - 10%, N-V - 5% and 55 % from Bucharest - Ilfov. We had 42.5% physicians, 22.5% biochemists, 17.5% biologists and 17.5% chemists.

Experts from „Victor Babes” National Institute Laboratories and highly renowned speakers lectured during the courses. The trainees had the opportunity to attend the lectures of Prof. Gary Coulton, Head of Omics Centre, St, George’s University of London (United Kingdom) and Prof. Ştefan Constantinescu, Head of Intracellular Signalling Department, Ludwig Institute for Cancer Research, Christian de Duve Institute, Bruxelles (Belgium). All the lectures were highly appreciated as seen in the anonymous evaluation of the course. All the theoretical courses had a homogenous structure: principles of proteomic technology, methodology description and application possibilities in clinical laboratories appropriate for Health Care improvement. After completion of the theoretical course, and in relation with their option, the trainees attended the practical modules in five series:

- Advanced electrophoretic techniques
- Post-electrophoresis analysis - Western blot;
- Immune-based detection - from ELISA to Protein microarray;
- Liquid phase multiplexing methods
- SELDI-TOF - Mass spectrometry for biomarker discovery.

The proteomic technology presented at the theoretical courses is available in the laboratories that hosted the applications modules. The hands-on courses lasted for one week. In the first day all the proteomic technologies were presented to the trainees, followed by 6 days when they actually used their chosen technology. Each of the trainees has been applying the technology, individually performing the technique on pathological/normal samples and ending with data interpretation and results conveying to a specific diagnostic.

After the final evaluation, all our trainees had good and very good grades and were entitled to receive a graduation diploma with credits from the Bucharest College of Physicians and the Order of Biochemists, Biologists and Chemists in the Health System in Romania.

The highly professional milieu in which the course has been developing for 6 months was created both by the lecturers and by the trainees that could accumulate international level scientific information in a short period of time. The human and professional bonds that were created during this time, the positive feed-back gave us confidence in the importance of this project. Sometimes the trainees' overall positive response was the only incentive that motivated us to bypass the inherent difficulties encountered during the project development. We do really hope that our trainees will be our future research collaborators.

What lies in our future? 2012 will open with two new series of proteomic courses, basic and applications. Related to the internationally evolving domain and connected to our trainees demands coming from the Health Care System, the course will be upgraded towards the fast implementation of these technologies in clinic, having always in mind the wellbeing of the patient and the improvement of their diagnostic.

Sometimes too optimistic, but always professional, we are eagerly awaiting our new trainees in 2012.

Monica Neagu - Proteomics Area Coordinator
Cristiana Tănase - Project Assistant Manager
Mihail Hinescu - Project Coordinator
IDENTIFICATION OF TREPONEMA DENTICOLA IN SUBGINGIVAL SAMPLES BY PCR TECHNOLOGY AND ITS CORRELATION WITH CLINICAL DIAGNOSIS

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ABSTRACT

Treponeema denticola has been associated with gingivitis and chronic periodontitis. The aim of this study was to identify Treponeema denticola in subgingival samples using PCR technology and to correlate it with clinical diagnosis of subjects. The study was carried out on seventy patients (20-84 years of age; mean age, 45.06 ± 12.58) of which 22 individuals with no detectable gingivitis or periodontitis, 4 subjects with chronic gingivitis and 44 subjects with chronic periodontitis. Subgingival plaque samples were collected from five sites in each patient. DNA was extracted from the samples using QIAamp® DNA Mini Kit (QIAGEN®). Treponeema denticola and other four periodontopathogens were found using multiplex polymerase chain reaction followed by a reverse hybridization. The relationship between clinical diagnoses and detection of Treponeema denticola was determined with Fisher exact test. The results showed significant differences between diagnostic groups regarding subject proportion. Treponeema denticola was detected in 2 out of 22 subjects with no detectable gingivitis or periodontitis, 2 out of 4 subjects with chronic gingivitis, and 40 out of 44 subjects with chronic periodontitis. Our findings suggest that Treponeema denticola is closely connected to the initiation and progression of periodontal disease.

Keywords: Treponeema denticola, periodontitis, polymerase chain reaction

INTRODUCTION

Recently there has been a remarkable expansion in the knowledge of the relationship between the large number of bacterial species in both the healthy sulcus and the inflamed periodontal pocket and of their potential role in the progression of the healthy sulcus to gingivitis and ultimately to periodontal disease with bone loss and tissue destruction [1]. The complexity of subgingival physiological and pathological inhabitants has hindered the identification of the precise microbial etiology of periodontitis, although very strong correlations between the amount and composition of the dental plaque biofilm and disease have been described [2].

Initial observations indicate a positive relationship between the presence and number of spirochetes in the oral environment and between oral health and disease [3-5].

Subgingival profile has been identified by several methods: anaerobic culture, microscopy, enzyme reaction, immunohistology, DNA-probes and polymerase chain reaction. Culture techniques require complex media and microaerophilic or strictly anaerobic growth conditions. The rapid development of molecular biology methods has facilitated the use of PCR for bacterial detection [9].

Treponeema denticola is present at significantly elevated levels in deep-pocket sites of patient with severe periodontitis [6, 7]. Treponeema denticola is often found in close proximity to Porphyromonas gingivalis and Tannerella forsythia forming together the “red complex”. These three bacterial species are generally found in the subgingival plaque of active periodontal lesions [1, 8].

The aim of this study was to identify Treponeema denticola in subgingival samples using PCR and correlate it with clinical diagnosis of subjects.

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MATERIALS AND METHODS

Subjects Distribution
Seventy patients (20-84 years of age; mean age, 45.06±12.58) of which 22 with no detectable gingivitis or periodontal disease, 4 with chronic gingivitis and 44 with chronic periodontitis, visiting two private dental clinics were followed up in this study between 2010 and 2011. Experimental protocol was approved by local ethics committee and informed consent was obtained from each patient.

Inclusion criteria: at least two sites with characteristic clinical findings for chronic gingivitis, extensive and generalized types of chronic periodontitis, periodontal pockets >4 mm, abnormal tooth mobility and characteristic radiographic findings.

Exclusion criteria: periodontal therapy and antimicrobial agents in the previous 3 months, any systemic condition which could influence the progression or treatment of periodontitis, localized or aggressive periodontitis.

Clinical Diagnostic Criteria
The subjects were divided into three groups: periodontal health status (lot 1), chronic gingivitis (lot 2) and chronic periodontitis (lot 3).

Periodontal health status means no detectable clinical findings of gingivitis or periodontal disease and no characteristic radiographic aspect. Chronic gingivitis is considered in the presence of spontaneous bleeding on probing, gum discoloration (bright red or purple red), gingival enlargement, no true periodontal pockets on probing and no alveolar bone loss on radiographs. Diagnostic criteria for chronic periodontitis include gum discoloration (from pale pink to red and purple red), abnormal gingival volume (retraction or enlargement), the presence of true or false periodontal pockets, abnormal dental mobility grade I, II or III, alveolar bone changes on radiographs from various halisteresis types to both horizontal and vertical bone loss [10].

Collection of Subgingival Plaque Samples
Prior to sampling, the supra-gingival plaque was carefully removed; the sample sites were isolated with sterile cotton rolls and gently air-dried. Each sterile paper point was inserted into pre-defined sites down to the base of periodontal pocket for 10 seconds. The probes were similarly gathered from gingival sulci of periodontally healthy and chronic gingivitis subjects. Five paper points were collected from each patient. Samples were stored in a refrigerator until assayed as HAIN Lifescience’s protocol indicates.

Detection of periodontal pathogens
After DNA extraction from the samples by using QIAamp® DNA Mini Kit (Qiagen®) according to manufacturer’s recommendations, Treponema denticola and several other periodontopathogenes were determined by microDent® test (Hain Lifescience, Germany). This kit identified five periodontopathogenic bacterial species (Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia and Treponema denticola).

PCR amplification was performed in a reaction volume of 50μL consisting of 5μL of template DNA and 45μL of master mix containing 35μL of primer-nucleotide mix (provided in microDent kit), 5μL of 10 x polymerase incubation buffer, 3μL of 25 mmol/L MgCl2 and 1U (0,2μL) Hot Start Taq DNA polymerase (Qiagen). PCR cycling was performed on GeneAmp PCR system 9700 (Applied Biosystem). The cycling conditions comprised an initial denaturation step at 95°C for 15 min; 10 cycles at 95°C for 30 s and at 58°C for 2 min; 20 cycles at 95°C for 25 s, at 53°C for 40s and at 70°C for 40 s; and a final extension step 1 cycle at 70°C for 8 min.

The subsequent reverse hybridization was performed according to manufacturer’s instructions on Beeblot (BeeRobotics). Briefly, the biotinylated amplicons were denatured and incubated at 45°C with hybridization buffer and strips coated with 2 control lines and 5 species-specific probes. After PCR products had bound to their respective complementary probe, a highly specific washing step removed any nonspecific bound DNA. Streptavidin-conjugated alkaline phosphatase was added, the samples were washed and hybridization products were visualized by adding a substrate for alkaline phosphatase. Interpretation of the banding pattern was ensured by a standard template. In order to validate the correct performance of the test and the proper functioning of reagents, two controls, one for amplification and the second for hybridization were included.

Data Analysis
The relationship between clinical diagnostic groups and detection of Treponema denticola was determined with Fisher exact test.

RESULTS
In our study the methods we used enabled the detection of Treponema denticola in 44 out of 70 tested patients. Treponema denticola was detected in 2 out of 22 subjects with no detectable gingivitis or
periodontitis, in 2 out of 4 patients diagnosed with chronic gingivitis, and 40 out of 44 patients diagnosed with chronic periodontitis. (Table 1)

In 42 out of 48 patients with marginal periodontium disease, Treponema denticola was detected. The results showed significant differences between groups as regards the proportion of subjects with Treponema denticola. The microIDent assay also detected another four species of bacteria (Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia). The patients found with any or all these 4 bacteria, not forming the subject of the present study, were referred to the specialist.

Our results showed a positive association between diagnosis and Treponema denticola (statistically significant p < 0.001 by Fisher exact test).

DISCUSSION

The periodontal microbiota is heterogeneous; over 700 species have been described in the oral habitat. A large number of reports have unequivocally established that the progress from health to gingivitis and hence to periodontitis is marked by a shift in the resident microbiota from a “healthy” biofilm to a “diseased” microbial ecology [1]. Examination of potential virulence characteristics shared by red complex bacteria demonstrated their ability to disrupt periodontal innate defense functions facilitating untoward host interactions with the entire dental plaque community which has been found to be potent activators of Toll-like receptor-2 (TLR-2) and Toll-like receptor-4 (TLR-4) [2].

Technological advances in microbial identification and classification based on the introduction of non-culture methods such as: DNA hybridization, PCR, Sanger sequencing, and the more recent developments of in high-throughput pyrosequencing-based analyses and metagenomics have allowed detection of microorganisms that are difficult or even impossible to culture, especially in case of spirochetes [2]. PCR methods have been increasingly used in the investigation of periodontal flora and are able to detect the presence of genomic DNA bacteria from subgingival sulcus [11, 12].

Subjects with severe periodontitis displayed significantly higher levels of Treponema denticola in their deep pockets compared to healthy periodontal subjects or those diagnosed with moderate periodontitis [13].

Literature suggests that a high salivary level of matrix metalloproteinase-8 (MMP-8) detected concomitantly with Treponema denticola in subgingival plaque displays a robust characteristic in predicting periodontal disease severity. Thus, monitoring these bacteria may prove to be an important tool in the assessment of periodontitis patients [14].

The results of this study indicate that the relationship between the presence of Treponema denticola and periodontitis patients was statistically significant. Detection rate of Treponema denticola was lower in chronic gingivitis group than in chronic periodontitis group. Some researchers (Ito et al. 2010, Mineoka et al. 2008) using molecular methods found Treponema denticola the most prevalent pathogen in both shallow and deep sites with increased numbers in sites with higher probing depths and bleeding on probing, which supports our result [15, 16].

Early identification of bacteria associated with the development of periodontitis using PCR technology may be helpful for screening periodontitis and healthy patients providing important information for diagnosis and treatment. As it also allows bacterial detection following antibiotic treatment, this method is able to detect residual bacterial load, helpful for patient monitoring. On the other hand, detection of residual bacteria can be a sign of inadequate treatment prescriptions or poor patient compliance.

In our study the low incidence of Treponema denticola in periodontally healthy subjects indicated

<table>
<thead>
<tr>
<th>Diagnostic groups</th>
<th>Absence of T. denticola</th>
<th>Presence of T. denticola</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No gingivitis or periodontitis (lot 1)</td>
<td>20 subjects</td>
<td>2 subjects</td>
<td>22 subjects</td>
</tr>
<tr>
<td>Chronic gingivitis (lot 2)</td>
<td>2 subjects</td>
<td>2 subjects</td>
<td>4 subjects</td>
</tr>
<tr>
<td>Chronic periodontitis (lot 3)</td>
<td>4 subjects</td>
<td>40 subjects</td>
<td>44 subjects</td>
</tr>
<tr>
<td>Total</td>
<td>26 subjects</td>
<td>44 subjects</td>
<td>70 subjects</td>
</tr>
</tbody>
</table>
that its presence alone was not responsible for the disease.

Based on our test results and data analysis, we may conclude that the presence of Treponema denticola is associated with chronic periodontitis diagnosis.

The identification method used in this study proved to be a reliable and sensitive assay.

ACKNOWLEDGEMENTS

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REFERENCES:


IN VITRO AND IN VIVO ANTIBACTERIAL ACTIVITY OF ACORN HERBAL EXTRACT AGAINST SOME GRAM-NEGATIVE AND GRAM-POSITIVE BACTERIA

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ABSTRACT

Background: The search for safe and effective antimicrobial agents, which treat, therapeutically and prophylactically, a wide variety of bacterial infections still represents a top priority for the biomedical field. This study was undertaken to investigate the antimicrobial properties of herbal extract (acorn) against bacterial pathogens in intestinal tract infections in in vitro and in vivo conditions and to study the effect of herbal extracts against bacteria in comparison with current antibiotics.

Findings: Ethanol extraction of acorn herb (Jaft) were evaluated against Klebsiella pneumoniae, Escherichia coli, Staphylococcus aureus, Salmonella typhi and Pseudomonas aeroginosa in in vitro and in vivo conditions. Minimal Inhibitory Concentration (MIC) was 10 μg/ml, 10 μg/ml, 5 μg/ml, 15 μg/ml and 15 μg/ml for K. pneumoniae, E. coli, S. typhi, S. aureus and P. aeroginosa, respectively. The in vivo results showed that the experimental infection produced by K. pneumoniae, E. coli, S. typhi and P. aeroginosa was totally inhibited in rats treated by the acorn extraction, while positive control rats died after five days.

Conclusion: The finding revealed that acorn extract has great potential as antimicrobial compounds against pathogenic microorganisms. Thus, acorn extract can be used in the treatment of infectious diseases caused by resistant bacteria.

Keywords: acorn extract, antimicrobial activity, pathogenic bacterial strains

INTRODUCTION

The search continues for safe and effective antimicrobial agents for treating, therapeutically and prophylactically, a wide variety of bacterial infections. This need has been urged recently by the emergence of many antimicrobial-resistant organisms like methicillin resistant staphylococci, multidrug, exendeded drug and pandrug resistant microorganisms [1]. The best therapeutic antimicrobial agents cause virtually no adverse reactions, have a wide spectrum of activity, and are not likely to select resistance.

Medicinal herbs are being increasingly studied by pharmacological researches, and many such herbs have a long history of medicinal use in Asia [2]. Herbs have many potential clinical and therapeutic applications in the modern medical setting, as numerous studies have revealed that they contain bioactive components, and have resulted in a better understanding of their physiological, therapeutic and clinical actions [3]. Antimicrobial agents can also be derived from herbs, and over 1000 plants exhibit antimicrobial effects [4]. Traditionally, these herbs are said to provide safe and effective treatments against many diseases.

Surface of acorn is traditionally used in Iran to treat gastrointestinal infections.

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This study was undertaken to investigate the antimicrobial properties of herbal extract (acorn) (that locally was called Jaft and obtained in Ilam Mountains) against bacterial pathogens isolated from intestinal tract infections, using in vitro and in vivo conditions and to compare their activity with that of current antibiotics.

**MATERIAL AND METHODS:**

**Bacterial strains:**

*K. pneumoniae, E. coli, S. aureus, S. typhi, P. aeruginosa* were analyzed against the acorn extraction (kindly provided by Department of Microbiology, Reference Laboratory of Ilam, Iran). The bacteria were resistant to the antibiotics, as follows: i) *K. pneumoniae* to aztreonam, cefazidime, ampicillin, ceftriaxone, cefotaxim, ciprofloxacin, amikacin; ii) *E. coli* to aztreonam, ampicillin, cefteraxone, ceftriaxone, amikacin; iii) *S. aureus* to oxacillin, tetracycline, amikacin, ceftriaxone, cefotaxim, vancomycin; iv) *S. typhi* to amikacin, ciprofloxacin, ceftriaxone, imipenem, cefotaxim; v) *P. aeruginosa* to oxacillin, amikacin, ciprofloxacin, ceftriaxone, imipenem, cefotaxim, tetracycline.

Plant extracts: The acorn, or oak nut, is the nut of the oaks and their close relatives (genera *Quercus* and *Lithocarpus*, in the family *Fagaceae*). Acorns vary from 1–6 cm long and 0.8–4 cm broad. Acorns take between about 6 and 24 months to mature the surface of acorn were powdered and soaked in the ethanol for about 10-15 days then this cold extract is subjected to distillation at low temperature under reduced pressure in rotary flash evaporator and concentrated on water bath to get the crude extract. The powder was subjected to soxhlation with ethanol for 48 hours. The solvent was distilled off at lower temperature under reduced pressure in rotary flash evaporator and concentrated on water bath to get the crude extract.

Screening for the antimicrobial potential of the plant extracts:

The bacterial cultures were grown in Brain Heart Infusion liquid medium at 37°C. After 6 h of growth, each microorganism, at a density of 10⁸ cells/mL, was inoculated on the surface of Mueller-Hinton agar plates. Subsequently, filter paper discs (6 mm in diameter) saturated with herbal extract (50 µL) were placed on the surface of each inoculated plate. To evaluate the efficiency of the methodology, each extract was distributed simultaneously in agar wells (50 µL). The plates were incubated at 37°C for 24 h. After this period, it was possible to observe the growth inhibition zone. Overall, cultured bacteria with growth inhibition area diameters equal to or greater than 7 mm were considered susceptible. DMSO and Tween 80 to 2% were used to dissolve the extracts in the culture media when necessary [5].

Confirmation for the antimicrobial potential of the plant extracts:

Minimal Inhibitory Concentration (MIC) for each bacterial sample has been performed. For this purpose, 100 µL of microbial suspension of 10⁸ cells/mL density were inoculated in tubes with nutrient broth supplemented with different concentrations (1-100 µg/mL) of the tested extracts. After 24 h of incubation at 37 °C, the MIC of each sample was determined by measuring the optical density using a spectrophotometer (620 nm), comparing the sample turbidity with that of non inoculated nutrient broth [6].

**Cell culture:** The extract was tested for its cytotoxicity effect on Vero Cell Line. The percentage viability of the cell line was carried out by using Trypan blue dye exclusion method and the MTT assay.

**Toxicity assay:** The cells were plated in 96-well flat bottom plates at a density of 5000-1000 cells/well and were allowed to adhere to the wells overnight. Then the cells were treated with different concentrations of the tannin ethanol herbal extracts (1-100 µg/mL) with a maximal final ethanol concentration of 1%. After 24 h, MTT assay was used to monitor cell growth. The absorbance of converted dye was measured at a wavelength of 570 nm with a background subtraction at 630 nm [7].

**Data interpretation:** Absorbance values lower than the control cells indicated a reduction in the rate of cell proliferation. Conversely, a higher absorbance rate indicated an increase in cell proliferation. Rarely, an increase in proliferation or morphological changes may be offset by cell death.

\[
\% \text{ inhibition} = \frac{1 - (\text{OD observed} / \text{OD control}) \times 100}{100}
\]

**In vivo assay:** Eighteen female rats aged between 4 and 5 weeks were used for all in vivo experiments. Rats were divided into five study groups, the positive control (infected rat without extract) and negative control. Throughout each experiment rats were given water containing streptomycin (5 mg/ml) to reduce the level of facultative anaerobic bacteria that normally colonize the intestine [8].
The bacteria were grown overnight in nutrient broth, centrifuged, washed in phosphate-buffered saline (PBS), and diluted to achieve the final suspension of $1 \times 10^8$ CFU density, further diluted in 20% sucrose and used to feed both control and study groups. One hour after infection, study group animals were given orally 5mg of the herbal extract daily whereas control animals were not.

Fecal samples were collected at 0, 1, 3, 4 days after administering the bacterial suspensions and the numbers of the bacteria per gram in feces were determined. For this purpose, aliquots (100 µl) of fecal suspensions were serially diluted in PBS and then were plated on special medium for each bacteria, which were then incubated overnight at 37 °C, and typical colonies were counted in plates containing between 30 and 300 colonies [9].

RESULTS AND DISCUSSION

The findings in screening stage revealed all that the tested bacteria were susceptible to the herbal extract of 10 µg/ml concentration for the Gram-negative bacterial and of 5 µg/ml for S. aureus. In the disk diffusion assay, the inhibition zone diameters around disks charged with the tested extract are presented in Table 1.

The cytotoxicity study was carried out for plant extract of surface of acorn (tannin). This extract was screened for its cytotoxicity against Vero cells line at different concentrations to determine the IC50 (50% growth inhibition) by MTT assay. It was found that the percentage of growth inhibition increasing with increasing concentration steadily up to 55 µg/ml and IC50 value of this assay was 0.245.

In the in vivo assay, the results showed the extract could remove the K. pneumoniae, E. coli, S. typhi and P. aeroginosa in rat faeces, thus that was effective against Gram-negative bacteria, while control rat infected by these bacteria died after 5 days. The control rat infected with S. aureus died after 8 days.

The results showed that the tannin extract is more effective against Gram-positive bacteria in in vitro conditions but the in vivo experiments revealed an increased tannin efficiency against the Gram-negative bacteria.

The antimicrobial properties of plants have been investigated by a number of researchers worldwide, especially in Latin America. In Argentina, a research tested 122 known plants species used for therapeutic treatments [10]. It was documented that among the compounds extracted from these plants, twelve inhibited the growth of S. aureus, ten inhibited E. coli, and four inhibited Aspergillus niger and also reported that the most potent compound was one extracted from Tabebuia impetiginosa. The antimicrobial properties of compounds obtained from Parthenium argentatum against Candida albicans, Torulopsis, Hansemula, K. pneumoniae and P. aeruginosa was detected [11]. Another work showed that the substances extracted from nine known plants in Uruguay did not show any activity against C. albicans and Saccharomycyes cerevisiae, but inhibited the growth of Bacillus subtilis, E. coli and P. aeruginosa [12]. Many studies have been conducted in Brazil. The inhibitory activity of Vatairea macrocarpa on Klebsiella spp. and S. aureus was observed [13]. Our findings revealed that jaft extract was effective against wide range of pathogenic Gram-negative bacteria while this herb in in vitro conditions could inhibit the growth of S. aureus but this result was not occurred in vivo. Our study performed in in vitro and in vivo conditions, but the majority of the research in herbal as mentioned above were only performed in in vitro condition, thus our results can strongly suggest the good effect of this extract against Gram negative bacteria.

The use of plants to heal diseases, including infectious ones, has been extensively applied by people. Our results reveal the great potential of plants for therapeutic treatment, in spite of the fact that they have not been completely investigated. Therefore, more studies need to be conducted to search for new compounds. Once extracted, and before being used in new therapeutic treatments, they should be tested for their toxicity in vivo. Bioassays have demonstrated the toxicity of extracts from different plants. Therefore, our results revealed the importance of plant extracts when associated with antibiotics, to

| Table 1. Antimicrobial activity of tannin by disk diffusion method |
|-----------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                        | 5 µg/ml          | 10 µg/ml        | 15 µg/ml        | 20 µg/ml        | 30 µg/ml        |
| K. pneumoniae          | 0                | 20 mm           | 31 mm           | 33 mm           | 38 mm           |
| E. coli                | 0                | 16 mm           | 30 mm           | 32 mm           | 33 mm           |
| P. aeruginosa          | 0                | 15 mm           | 24 mm           | 26 mm           | 29 mm           |
| S. typhi               | 0                | 17 mm           | 26 mm           | 26 mm           | 29 mm           |
| S. aureus              | 15 mm            | 25 mm           | 27 mm           | 30 mm           | 31 mm           |
control resistant bacteria, which are becoming a threat to human health.

The results showed that acorn extract has great potential as antimicrobial compounds against microorganisms. Thus, they can be used in the treatment of infectious diseases caused by resistant bacteria. This extract was more effective against the Gram-negative bacteria in the in vivo experimental model.

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REFERENCES:

INTRODUCTION

Acinetobacter baumannii (A. baumannii) is recognized as an important opportunistic gram-negative pathogen that is frequently associated with a variety of nosocomial infections worldwide [1].

Carbapenem is the first line drug against A. baumannii infections; however, the emergence of carbapenem resistance to A. baumannii strains has been reported worldwide [2,3].

THE ROLE OF BLAOXA-LIKE CARBAPENEMASE AND THEIR INSERTION SEQUENCES (ISS) IN THE INDUCTION OF RESISTANCE AGAINST CARBAPENEM ANTIBIOTICS AMONG ACINETOBACTER BAUMANNII ISOLATES IN TEHRAN HOSPITALS

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ABSTRACT

This study aimed to evaluate the occurrence and dissemination of blaoxa-like Carbapenemase genes and their insertion sequences among Acinetobacter baumannii isolates, taken from different hospitals in Tehran city and also their roles in the induction of resistance to carbapenem drugs. A total number of 100 non duplicate Acinetobacter baumannii with different origins, were isolated from patients with proved nosocomial infections at eight university hospital in Tehran city. Antimicrobial susceptibility of these strains was done by E-test against 7 antimicrobial agents according to CLSI guideline. PCR of blaoxa-51-like, blaoxa-23-like, blaoxa-24-like, blaoxa-58-like, ISAba-A1, ISAba-1133 was carried out by specialized primers and then these strains were typed by REP-fingerprinting. Colistin, imipenem and meropenem were the most sensitive antibiotics against Acinetobacter baumannii isolates with 96%, 51% and 51% sensitivity respectively. All the isolates had a blaoxa-51-like intrinsic to these species. The rates of blaoxa-23, 23 and 58-like were 38%, 32% and 1% respectively.

Coexistence of blaoxa-51/23/24-like was observed among 16% of these isolates. All blaoxa-23-like carbapenemase genes had only one ISAba-A1. REP fingerprinting showed 5 genotypes among carbapenem resistant isolates, 16 of them being genotype A.

This study emphasized on the major role of blaoxa-like carbapenemase, particularly blaoxa-23-like carbapenemase and their ISAba-A1, in the dissemination of carbapenem resistant Acinetobacter baumannii. This study confirmed a presumptive role of IS element neighboring the carbapenemase gene in the elevation of resistance to carbapenem drug among Acinetobacter baumannii isolates for the first time in Iran.

Keywords: A. baumannii, blaoxa genes, IS element, carbapenem drugs

INTRODUCTION

Acinetobacter baumannii (A. baumannii) is recognized as an important opportunistic gram-negative pathogen that is frequently associated with a variety of nosocomial infections worldwide [1].

Carbapenem is the first line drug against A. baumannii infections; however, the emergence of carbapenem resistance to A. baumannii strains has been reported worldwide [2,3].

The main mechanisms of resistance to carbapenem drugs are mainly due to production of metallo-beta-lactamase (molecular classes B) and OXA-type carbapenemases (molecular classes D) [4].

Most studies have shown that blaoxa-like carbapenemase has frequently induced resistance to carbapenem drugs [5, 6].

Four types of blaoxa-like carbapenemases including blaoxa-23, blaoxa-24, blaoxa-51 and blaoxa-58 have been identified in A. baumannii isolates so far [7].

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Apart from the intrinsic \( \text{bla}_{\text{OXA-51-like}} \) [8, 9] resistance to carbapenem drugs due \( \text{bla}_{\text{OXA-23}} \), \( \text{bla}_{\text{OXA-24}} \) and \( \text{bla}_{\text{OXA-58}} \) enzymes was reported worldwide [10]. The genes encoding OXA-like enzymes have been found to be linked to some insertion sequences (IS) elements. Insertion sequence (IS) including \( \text{IS}_{\text{Aba1}} \) and other IS may modulate the expression and transfer of the OXA-type carbapenemases genes. Insertion sequences are mobile genetic elements known to affect the evolutionary pattern of bacterial genomes. Upon integration, IS elements may cause DNA insertions/deletions, chromosomal rearrangement, modulate the expression of adjacent genes and thereby, influence the phenotype of a bacterium [11, 12].

Because of the rise in the multiresistant strains of \( \text{A. baumannii} \) in Iran [2, 13-14], nosocomial infections caused by this organism are difficult to be treated. Therefore, evaluation of antimicrobial resistance of \( \text{A. baumannii} \), especially carbapenem resistant strains, and detection of genes conferring resistance to carbapenems are necessary among different settings and hospitals of various countries.

This study was carried to evaluate the occurrence and dissemination of \( \text{bla}_{\text{OXA-like}} \) carbapenemase gene among \( \text{A. baumannii} \) isolates, detected from different hospitals in Tehran and their roles in the induction of resistance to carbapenem drugs.

**MATERIAL AND METHODS**

**Bacterial identification**

During a 6-month period, 100 non duplicate \( \text{Acinetobacter} \) spp. were isolated from patients with proved nosocomial infections at eight university hospitals (showed as H1 to H8 respectively) in Tehran. All positive samples were acquired from patients with 72h of hospitalization. Patients had no bacterial infections at the time of hospitalization. All the samples were then confirmed as \( \text{A. baumannii} \) by biochemical and API 20NE galleries and were enrolled in this study.

**Antimicrobial susceptibility tests**

Using E-test, the susceptibility of all the isolates was tested against the following antibiotics: imipenem, meropenem, ampicillin-sulbactam, cefepime, cefazidime, piperacillin-tazobactam and colistin.

The isolates were then interpreted according to the manufacturer’s instructions and CLSI guidelines [15]. \( \text{E. coli} \) ATCC 25922 and \( \text{Pseudomonas aeruginosa} \) ATCC 27852 were used as qualitative standard per reactions.

**DNA Extraction and PCR Assay**

DNA extraction was carried out by commercial DNA extraction kit (BioMerux, Republic of Korea). The presence of different \( \text{bla}_{\text{OXA-like}} \) carbapenemases (\( \text{bla}_{\text{OXA-51-like}}, \text{bla}_{\text{OXA-23-like}}, \text{bla}_{\text{OXA-24-like}} \) and \( \text{bla}_{\text{OXA-58-like}} \)), \( \text{IS}_{\text{Aba1}} \) and \( \text{IS}_{1133} \) was detected by PCR.

The PCR system (25 µl) was composed of 1X PCR buffer, 2 mM \( \text{MgCl}_2 \), 0.2 mM dNTPs, 10 pmol of primers, 1U Taq DNA polymerase (Ferments, UK) and 10 ng DNA template. PCR amplification was then carried out by thermal cycler (Bio-Rad, USA). The thermocycling parameters included an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing according to Table 1, extension at 72°C for 1 min, followed by a final extension at 72°C for 8 min.

**REP-PCR Fingerprinting**

DNA extraction was carried out by DNA extraction kit (BioMerux, Republic of Korea) and 4 µl of its extract was used as the DNA template. The primer pair REP1, 5'-11GCCCIGICATCGCIGGC-3' and REP2, 5'-ACGTCTTATCAGGCTAC-3' was used to amplify putative REP-like elements in the genomic bacterial chromosomes [16].

Amplification reaction was performed in a final volume of 25 µl. Each reaction mixture contained 2.5 µl of 10X PCR buffer, 1.25 U Taq DNA polymerase (Fermentase, UK), 0.8 µl of 2 mM mixed dNTPs (Fermentase, UK), 1.5 µl of 25 mM \( \text{MgCl}_2 \), 1 µl of 10 pmol primers and 50 ng of bacterial DNA. Amplification reaction was carried out by thermal cycler (Épendorf, Germany) with an initial denaturation at 94°C for 10 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 1 min and extension at 72°C for 1 min, followed by final extension at 72°C for 16 min.

Each sample aliquot was subjected to electrophoresis in 1.2% agarose gel. Amplified products were detected by Geldoc apparatus after staining with ethidium bromide (50 mg/L) and then photographed for analysis. Photograph results, were then analyzed visually.

**RESULTS**

Colistin, against which 96% of all the isolates were susceptible, was the most effective antimicrobial agent. Among beta-lactam antibiotics, imipenem and meropenem (46%) were the most effective agents followed by ampicillin-sulbactam (40%). The strains showed the lowest sensitivity rate against pi-
Distribution of OXA-carbapenemase genes among Iranian \textit{A. baumannii} strains

Table 1. Primer used for amplification of \textit{bla}_{\textit{OXA-like}} carbapenemase and ISs genes among Iranian \textit{Acinetobacter baumannii} isolates

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequences</th>
<th>Annealing Temperature (°C)</th>
<th>Amplicon size (bp)</th>
<th>References</th>
</tr>
</thead>
</table>
| \textit{bla}_{\textit{OXA-51-like}} | F: 5'- TAA TGC TTT GAT CGC CCT TG - 3'  
R: 5'- TGG ATT GCA CTT CAT CTT GC -3' | 53                         | 353                | [29]       |
| \textit{bla}_{\textit{OXA-23-like}} | F: 5'- GAT CGG ATT GGA AAA CCA GA-3'  
R: 5'- ATT TCT GAC CGC ATT TCC AT-3' | 53                         | 501                | [29]       |
| \textit{bla}_{\textit{OXA-24-like}} | F: 5'- GGT TAG TTG GCC CCC TTA AA-3'  
R: 5'- AGT TGA GCG AAA AGG GGA TT-3' | 53                         | 246                | [29]       |
| \textit{bla}_{\textit{OXA-58-like}} | F: 5'- AAG TAT TGG GGC TTG TGC TG-3'  
R: 5'- CCC TTC TGC GCT CTA CAT AC-3' | 53                         | 599                | [29]       |
| \textit{IS}_{\text{ABA1}}     | F: 5'- ATG CAG TTC TTT GCA GG-3'  
R: 5'- AAT GAT TGG TGA CAA TGA AG-3' | 55                         | 351                | [30]       |
| \textit{IS}_{1133}              | F: 5'- AGT ACA AAA AGC TGT GAG ATT TCA AG-3'  
R: 5'- GAT ATT CAT GAG CGC AAT ATT GGC T-3' | 58                         | 574                | [30]       |

Table 2. Susceptibility testings of 100 \textit{A. baumannii} isolates from Tehran Hospitals according to E-test results

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Range of MIC (µg/ml)</th>
<th>MIC50 (µg/ml)</th>
<th>MIC90 (µg/ml)</th>
<th>Sensitive</th>
<th>Intermediate</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin-sulbactam</td>
<td>0.125-256</td>
<td>16</td>
<td>32</td>
<td>40</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>Cefepime</td>
<td>0.125-512</td>
<td>32</td>
<td>64</td>
<td>8</td>
<td>-</td>
<td>92</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>0.125-512</td>
<td>32</td>
<td>64</td>
<td>4</td>
<td>-</td>
<td>96</td>
</tr>
<tr>
<td>Colistin</td>
<td>0.004-8</td>
<td>1</td>
<td>2</td>
<td>96</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>Imipenem</td>
<td>0.004-32</td>
<td>4</td>
<td>8</td>
<td>46</td>
<td>15</td>
<td>39</td>
</tr>
<tr>
<td>Meropenem</td>
<td>0.004-32</td>
<td>4</td>
<td>8</td>
<td>46</td>
<td>23</td>
<td>31</td>
</tr>
<tr>
<td>Piperacillin-tazobactam</td>
<td>0.125-256</td>
<td>16</td>
<td>32</td>
<td>11</td>
<td>-</td>
<td>89</td>
</tr>
</tbody>
</table>

Peracillin-tazobactam (11%), cefepime (8%) and ceftazidime (4%) respectively. The range of MIC to imipenem and meropenem was 0.004 to 32 µg/ml; while this range was 0.004 to 8 for colistin (Table 2). Totally, 54 isolates (54%) were resistant to at least five antimicrobial agents and were entitled as multidrug resistant \textit{A. baumannii}. All \textit{A. baumannii} strains harbor \textit{bla}_{\textit{OXA-like}} carbapenemase. The rate of \textit{bla}_{\textit{OXA-23,24}} and \textit{bla}_{\textit{OXA-51-like}} was 38%, 32% and 1% respectively. There was coexistence between two and three carbapenemase genes. A coexistence of \textit{bla}_{\textit{OXA-51/OXA-24}}, \textit{bla}_{\textit{OXA-51/OXA-23/OXA-24}} and \textit{bla}_{\textit{OXA-51/OXA-24}} carbapenemase was seen among 20%, 16% and 15% of the isolates, respectively.

Coexistence of \textit{bla}_{\textit{OXA-51/OXA-23/OXA-58}} was observed only in one strain. Totally, 45% of the strains harbored only \textit{bla}_{\textit{OXA-like}} carbapenemase. \textit{IS}_{\text{ABA1}} was detected among 40% of the isolates; 38 isolates harb-
boring \( \text{bla}_{\text{OXA-51/23}} \)-like carbapenemase and 2 isolates harboring only \( \text{bla}_{\text{OXA-51}} \)-like carbapenemase. There was no \( \text{IS}_{1133} \) among the isolates.

REP fingerprinting showed 15 dominant genotypes among \( A. \) baumannii isolates (Fig. 1).

REP fingerprinting could not differentiate between 15 strains of the \( A. \) baumannii isolates.

The correlation between existence of different \( \text{bla}_{\text{OXA-like}} \) genes and multidrug resistant \( A. \) baumannii isolates was shown in Table 3.

### DISCUSSION

Nowadays, treatment of hospital infections is one of the major problems in clinical settings. Many hospital infections are caused by gram-negative bacteria among which Acinetobacter spp., especially \( A. \) baumannii, are very important [17, 18]. \( A. \) baumannii is an opportunistic pathogen and plays an important role in colonization and infection in hospitalized patients. \( A. \) baumannii is also one of the most common bacterial agents causing ventilator associated pneumonia (VAP) in hospitalized patients particularly among those admitted to ICU [19]. Specific risk factors for acquisition of nosocomial infections caused by \( A. \) baumannii include a prolonged hospitalization especially in ICU and burn wards, surgery, trauma, previous infections without antibiotic therapy, the use of intravenous and urinary catheter and prolonged treatment with broad spectrum antibiotics [20, 21].

### Table 3. Distribution of \( \text{bla}_{\text{OXA-like}} \) genes among multidrug resistant \( A. \) baumannii isolates according to different hospitals

<table>
<thead>
<tr>
<th></th>
<th>H1</th>
<th>H2</th>
<th>H3</th>
<th>H4</th>
<th>H5</th>
<th>H6</th>
<th>H7</th>
<th>H8</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{bla}_{\text{OXA-51-like}} )</td>
<td>6</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>1</td>
<td>7</td>
<td>7</td>
<td>3</td>
<td>37</td>
</tr>
<tr>
<td>( \text{bla}_{\text{OXA-23-like}} )</td>
<td>7</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>2</td>
<td>3</td>
<td>30</td>
</tr>
<tr>
<td>( \text{bla}_{\text{OXA-24-like}} )</td>
<td>11</td>
<td>8</td>
<td>6</td>
<td>4</td>
<td>3</td>
<td>10</td>
<td>9</td>
<td>3</td>
<td>54</td>
</tr>
<tr>
<td>( \text{bla}_{\text{OXA-58-like}} )</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>ISABA1</td>
<td>11</td>
<td>8</td>
<td>6</td>
<td>4</td>
<td>3</td>
<td>10</td>
<td>9</td>
<td>3</td>
<td>54</td>
</tr>
<tr>
<td>IS1133</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Currently carbapenem is used as the first line drug against *A. baumannii* infections. *A. baumannii* strains, in this study, showed a high resistance rate against carbapenem agents. After colistin, as a non beta-lactam agent that was very effective against resistant isolates, a high degree of resistance to carbapenem and other beta-lactam drugs was observed. This fact indicated the risk of drug resistance spread among *A. baumannii* strains isolated from different infections in Tehran hospitals.

According to the results of this study, *A. baumannii* isolates showed a high degree of resistance against ampicillin-sulbactam as well as other beta-lactam antibiotics such as cefepime and ceftazidime. This finding was similar to the previous studies which reported an increased degree of resistance against carbapenem and other beta-lactam antibiotics among *A. baumannii* isolates in Tehran hospitals [2, 7-8, 13]. Unfortunately, apart from Iran, resistance to these drugs has also been reported in other parts of the world which has created a complexity in the treatment of the infections caused by these bacteria [22].

There are currently several mechanisms to induce resistance against carbapenem drugs, among which the most important and studied one is the expression of *bla*<sub>OXA-like</sub> carbapenemase among resistant *A. baumannii* strains [23-24]. In accordance with the previous reports, the PCR results showed that, during the recent years, *bla*<sub>OXA-23-like</sub> and *bla*<sub>OXA-24-like</sub> have become common genes among carbapenem resistant *A. baumannii* isolates in Tehran hospitals [2, 7, 13]. Compared with the previous studies, existence of *bla*<sub>OXA-58-like</sub> carbapenemase among *A. baumannii* in Tehran is dramatically decreased [7]. The reasons for these differences are unclear, but the variability of the hospitals from which samples have been collected, may play an important role in this reduction. Similar to other reports, there was a coexistence of *bla*<sub>OXA-51/bla*<sub>OXA-23</sub> and *bla*<sub>OXA-51/bla*<sub>OXA-24</sub> among these strains [7, 25-26]. In addition to *bla*<sub>OXA-51-like</sub>, all the strains that were resistant to imipenem and meropenem harbored *bla*<sub>OXA-23-like</sub> carbapenemase and IS<sub>ABA1</sub>. In agreement with the previous studies, the findings of current study showed that the existence of *bla*<sub>OXA-23-like</sub> and IS<sub>ABA1</sub> adjacent to this gene induces resistance against carbapenem drugs [11, 24]. These findings show that resistant strains which only harbor *bla*<sub>OXA-51-like</sub> gene have a low resistance against carbapenem drugs. Several factors that may be involved in the resistance of these strains are as follows: an increase in the gene expression and production of high concentrations of *bla*<sub>OXA-51-like</sub> carbapenemase, the existence of insertion sequences (IS) at the beginning of the gene sequences involved in the increase of gene expression and existence of other factors such as unknown carbapenemase genes, efflux pumps and defect permeability of outer membrane proteins [27-28].

The results of this study showed that resistance to carbapenem drugs among *Acinetobacter baumannii* in Tehran hospitals are gradually increasing. The incidence of carbapenem resistant genes can play a main role in the occurrence and circulation of resistant clones of *A. baumannii* among clinical setting in Tehran.

The infections caused by resistant *A. baumannii* are increasing throughout the world and this problem causes a serious challenge in the treatment of these strains in the public health. Therefore, annual antimicrobial susceptibility surveillance may be very helpful in the treatment of infected patients.

On the other hand, identification of resistant clones of pathogenic bacteria and their circulations among different parts of hospitals could result in identification of the main source of infection and its control.

**ACKNOWLEDGMENT**

This work was supported by a grant from the Ilam University of Medical Sciences. We gratefully acknowledge all the staff of clinical laboratory of microbiology in the Ilam University of Medical Sciences.

**REFERENCES**


ABSTRACT

Ten *Haemophilus influenzae* strains were isolated from patients aged between 1.6 - 24 years, with various diagnoses (acute meningitis, acute upper respiratory infection, otitis media and acute sinusitis). Identification was based on phenotypic and molecular characteristics; antibiotic susceptibility testing was performed by diffusion method according to CLSI standards 2011 for seven antibiotics. The results of molecular testing showed that all the studied strains produced an amplicon of 1000 bp with *omp*P2 primers indicating that all strains were *H. influenzae*. For six strains, the PCR amplicon obtained with *bex*A specific primers, proving that the strains were capsulated. The results of phenotypic testing showed that four strains were ampicillin nonsusceptible and β-lactamase-positive. The virulence potential of *H. influenzae* clinical strains was investigated by phenotypic methods, including the assessment of the soluble virulence factors on specific media containing the biochemical substratum for the investigated enzymatic factor, as well as the adherence and invasion capacity to HeLa cells monolayer using Cravioto modified method. The studied strains exhibited mainly a diffuse adherence pattern and different adherence indexes. Interestingly, two strains isolated from the same patient (blood and CSF) showed a different degree of invasiveness, the strain isolated from blood being 20 times more invasive than the one isolated from CSF.

Keywords: *Haemophilus influenzae*, serotyping, antibiotic resistance, β-lactamase, PCR capsular type, adhesion, invasion.

INTRODUCTION

*Haemophilus influenzae* belongs to the family *Pasteurellaceae* and is an important pathogen for humans, having the ability to colonize the upper respiratory tract mucosa in children and adults and to produce meningeal and respiratory tract infections, especially in children.

*H. influenzae* strains may be of different types of capsule (a, b, c, d, e, f) or noncapsulate (NC). Colonization with capsulated strains increases the risk of pathological manifestations such as: meningitis (the most common in unvaccinated children under 5 years), epiglottitis, septicemia, septic arthritis, pericarditis, cellulitis. However, NC strains have also been found responsible for different severe infections. In addition, NC *H. influenzae* strains are frequently implicated in the exacerbation of underlying lung disease, including chronic bronchitis, bronchiectasis and cystic fibrosis, representing an important aetiology of community-acquired pneumonia, especially among children in developing countries and elderly adults [1, 2]. Also, it can colonize the vaginal mucosa causing genital infections and neonatal sepsis [3].

Alteration and modification of respiratory mucosa in chronic bronchitis may also favor the growth and multiplication of bacteria to this level. Localized infections appear as a result of the reducing of local defense mechanisms, particularly impaired mucocilliary system after or during a viral infection or in smoking people. *H. influenzae* has adhesins and IgA proteases that promote colonization and infection.
Major virulence factors of *H. influenzae* are lipooligosaccharides and capsular polysaccharides. Pathogenic bacteria cell surface expose the carbohydrates and lipid molecules to the immune system recognition. During invasive manifestations, the capsule plays a major role, being an important virulence factor by its antiphagocytic action. A better ability to cause infection (to colonize host tissues) is associated with capsular polysaccharide synthesis. The fact is explained by the properties of the capsular polysaccharide negative chemotaxis for phagocytes. Capsular polysaccharide inhibits the action of phagocytes and protects cellular lysis mediated by complement. Due to encapsulation, the complement is activated at remote distance from the external membrane. The cells that are not synthesizing the capsular polysaccharide are capable of great variation in virulence and are found in the airway either as commensals or pathogens with the capacity to invade the airway epithelium; they have the advantage of not being recognized by specific antibodies.

The polysaccharide capsule of typeable *H. influenzae* strains is encoded by the *cap* locus, which is composed of three distinct regions I, II and III [4-7]. Regions I and III contain genes highly conserved across all capsular types, necessary for transport and process of the capsular material (including *bexA* gene). Region II is serotype-specific, being located between regions I and III. Enzymes encoded by region II synthesise the type-specific disaccharide [7].

In laboratory, differentiating capsulated from NC *H. influenzae* strains by slide agglutination can be challenging because this method many times has been shown to be unreliable. Therefore, there is a need for complemented slide agglutination by serotype-specific PCR, an unequivocal method.

The aim of this study was to optimize the identification and typing methods by molecular approach as well as the methodology for the *in vitro* investigation of *H. influenzae* strains virulence.

**MATERIALS AND METHODS**

**Bacterial isolates.** A total of 10 *H. influenzae* strains were collected from 9 patients aged between 1.6-24 years. The strains were isolated from cerebrospinal fluid (CSF) (*n*=4), blood (*n*=1) and upper respiratory tract (*n*=5) (Table 1). Patients from whom the strains were isolated had meningitis or community-acquired respiratory tract infections (otitis media or acute upper respiratory tract infection). All strains were identified according to the standard microbiological methods: X- and V- factor requirement (OXOID factor discs) and alternative method with API NH (bioMerieux) [8].

Beta-lactamase production was determined by the chromogenic cephalosporin test using nitrocephin as a specific substrate (Nitrocefin, OXOID).

Antibiotic susceptibility testing was performed by disk diffusion method according to CLSI 2011 recommendations [9] using the following antibiotics: ampicillin (10 μg), amoxicillin-clavulanic acid (10/20 μg), ceftriaxone (30 μg), tetracycline (30 μg), ciprofloxacin (5 μg), trimetoprim-sulphametoxazole (1.25 μg/23.75 μg) and chloramphenicol (30 μg) (OXOID discs). For confirmed ampicillin (AMP) susceptibility, we detected minimum inhibitory concentration (MIC) with E-test (bioMerieux).

**Serotyping.** The strains were serotyped by slide agglutination test using monovalent antisera according to the manufacturer’s instructions (DENKA sera).

DNA isolation. DNA isolation was performed from a 24-hour culture on chocolat Haemophilus Test Medium (HTM), by obtaining 0.5 McFarland density suspension of each bacterial culture in 1 mL of sterile distilled water and then boiled for 10 min.

**PCR for confirming H. influenzae and for molecular capsule typing.** In the single PCR amplifications was detected the presence of three genes: outer membrane protein *P*₂ (*ompP₂*) which confirms the isolate as *H. influenzae*, *bexA* gene (Van Ketel gene) which detects the strain’s ability to export capsule to the cell surface [8] and a capsular type specific gene (a-f) [4].

The *ompP₂*, *bexA* and capsule type specific genes were amplified with primer sets listed in Table 2. PCR was performed by using 2 μl of template DNA, 1.5 μM of each primer, 0.2 mM deoxyribonucleotides, 1 x reaction buffer, 1.5 mM MgCl₂ and 1,25 U of Taq polymerase (PRIMEGA) in a total volume of 50 μl. Reaction mixtures were subjected to an initial denaturation step for 5 min at 94°C, followed by 36 amplification cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. After the final cycle there was annealing 55°C for 1 min and a single final extension of 10 min at 72°C. Five μl of PCR products were electrophoresed through 1.5 % agarose in Tris-Acetate-EDTA buffer and visualized by ethidium bromide staining. The size of the amplified products (Table 2) was compared with a positive control, obtained from reference strains. The following strains provide internal quality assurance for the PCR: *H. influenzae* serotype b ATCC 10211 and *H. influenzae* serotype f ATCC 9833.
Investigation of virulence potential in *Haemophilus influenzae* clinical strains

Table 1. Phenotypic characterization and resistance mechanisms test results

<table>
<thead>
<tr>
<th>Isolate ID</th>
<th>Patient identification (sex, age)</th>
<th>Diagnostic specimen</th>
<th>Clinical specimen</th>
<th>Serotype</th>
<th>Nitrocefin test</th>
<th>Ampicillin MIC (µg/mL)</th>
<th>Resistance pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>F, 2</td>
<td>meningitis</td>
<td>CSF</td>
<td>cross reaction (b, f)</td>
<td>+</td>
<td>4</td>
<td>AMP&lt;sup&gt;R&lt;/sup&gt;, C&lt;sup&gt;R&lt;/sup&gt;, TE&lt;sup&gt;R&lt;/sup&gt;, SXT&lt;sup&gt;R&lt;/sup&gt;</td>
</tr>
<tr>
<td>17</td>
<td>M, 1.6</td>
<td>meningitis</td>
<td>CSF</td>
<td>NT&lt;sup&gt;2&lt;/sup&gt;</td>
<td>+</td>
<td>2</td>
<td>AMP&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>18</td>
<td>M, 2.8</td>
<td>meningitis</td>
<td>CSF</td>
<td>b</td>
<td>+</td>
<td>4</td>
<td>AMP&lt;sup&gt;R&lt;/sup&gt;</td>
</tr>
<tr>
<td>38 CSF</td>
<td>F, 24</td>
<td>meningitis</td>
<td>CSF</td>
<td>f</td>
<td>-</td>
<td>0.19</td>
<td>-</td>
</tr>
<tr>
<td>38 blood</td>
<td>F, 24</td>
<td>meningitis</td>
<td>blood</td>
<td>f</td>
<td>-</td>
<td>0.19</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>F, 11</td>
<td>acute upper respiratory tract infection</td>
<td>nasal exudate</td>
<td>NT</td>
<td>+</td>
<td>&gt;256</td>
<td>AMP&lt;sup&gt;R&lt;/sup&gt;, SXT&lt;sup&gt;R&lt;/sup&gt;</td>
</tr>
<tr>
<td>37</td>
<td>F, 4</td>
<td>otitis media</td>
<td>nasal exudate</td>
<td>NT</td>
<td>-</td>
<td>0.19</td>
<td>SXT&lt;sup&gt;R&lt;/sup&gt;</td>
</tr>
<tr>
<td>39</td>
<td>M, 4</td>
<td>acute upper respiratory tract infection</td>
<td>nasal exudate</td>
<td>cross reaction (b, f)</td>
<td>-</td>
<td>0.19</td>
<td>-</td>
</tr>
<tr>
<td>42</td>
<td>F, 23</td>
<td>acute sinusitis</td>
<td>nasal exudate</td>
<td>NT</td>
<td>-</td>
<td>0.125</td>
<td>SXT&lt;sup&gt;R&lt;/sup&gt;</td>
</tr>
<tr>
<td>43</td>
<td>M, 3</td>
<td>acute upper respiratory tract infection</td>
<td>nasal exudate</td>
<td>NT</td>
<td>-</td>
<td>0.125</td>
<td>-</td>
</tr>
</tbody>
</table>

Legend: * MIC interpretive standard, CLSI: ≤1 = susceptible; 2 = intermediate; ≥4 = resistant; F, female; M, male; ¹cerebrospinal fluid; ²nontypeable strain; Resistant, AMP, ampicillin; C, chloramphenicol; TE, tetracycline; SXT, trimethoprim-sulfamethoxazole.

The presence of soluble virulence factors has been performed by inoculating the strains on special culture media prepared in house [12]. The culture media were used to reveal the presence of the: caseinase, gelatinase, lipase, lecitinase, amilase, mucinase, aesculinase, DNA-ase.

Table 2. Oligonucleotides used to identify and typing *H. influenzae* strains

<table>
<thead>
<tr>
<th>Primer designation</th>
<th>Primer sequence (5’ to 3’)</th>
<th>Target gene</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>VK1</td>
<td>CGTTTGATATGATGTTGATCCAGAC</td>
<td>bex A</td>
<td>345</td>
<td>[10]</td>
</tr>
<tr>
<td>VK2</td>
<td>TGTCATCTTCTAAATGATG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O1</td>
<td>ATACAACGAAGGACTAAGGC</td>
<td>omp P&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1000</td>
<td>[11]</td>
</tr>
<tr>
<td>O3</td>
<td>ACCTACACCCACTGATTTT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b1</td>
<td>GCCTTTGTTGGACTCTTTATCTC</td>
<td>capsular type b</td>
<td>480</td>
<td>[4]</td>
</tr>
<tr>
<td>b2</td>
<td>GCTTTGTTGGACTCTTTATCTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>f1</td>
<td>GCTACTATCACTCCTCTCGGATAG</td>
<td>capsular type f</td>
<td>450</td>
<td>[4]</td>
</tr>
<tr>
<td>f2</td>
<td>CGCAATTATTGGGAAAGGCT</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
A bacterial suspension of 1 McFarland turbidity was prepared from each strain in 1 mL sterile PBS and used for infecting the HeLa cellular monolayer. The plates were incubated at 37°C for 2 hours allowing the microorganisms to adhere and invade the eukaryotic cells. Each bacterial strain was inoculated in four replica, two for highlighting the adhesion pattern, the third to quantify the adhesion and invasion capacity (adherence + invasion index) and the last one to quantify the capacity of invasion (invasion index).

The adherence pattern was revealed by optic/fluorescence microscopy performed on Giemsa/fluorescent stained preparations. After incubation, the infected monolayers were washed 3 times with PBS to remove nonadherent bacteria. The cells were fixed with methanol and stained with Giemsa solution (1:20) (Merck) for 20 min and respectively with a mix of ethidium bromide (EB) and acridine orange (AO) prepared in sterile PBS (15 mL PBS, 100 μg/mL EB, 100 μg/mL AO) (the plates were kept in dark, for 5 min). After staining the plates were examined in optic/UV light microscope. Microscopic examination was performed for semi-quantitative determination of the rate of adhesion and the adherence pattern on HeLa monolayer cells of studied strains. To determine H. influenzae association to eukaryotic cells was quantified the mean number of bacteria associated with one cell, determined by counting the number of bacteria associated with each of 100 cells.

Assessment of bacterial adhesion and invasion was performed by seeding HTM chocolate with 10 μl from every even serial ten-fold dilutions (10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}) obtained from cellular suspensions resulted after monolayer lyses and determination of colony forming unit per mL (CFU/mL). The adhesion and invasion indexes were established by viable cell counts expressed in CFU/mL.

To assess the bacterial invasion we treated monolayer cells with 1 mL gentamicin solution 100 μg/ml and incubated 1h at 37°C. Adding gentamicin was to kill all the bacteria found in the extra cellular environment and surviving only bacteria that invaded cells monolayer. Finally, we seeded serial ten-fold dilutions on HTM chocolate as in paragraph above.

RESULTS

Phenotypic characterisation

A total of 5 invasive H. influenzae and 5 noninvasive H. influenzae were identified. Patients with invasive H. influenzae disease ranged in age from 1.6 years to 24 years old (Table 1).

Serotyping identified 2 capsular types: b and f. Two strains cross-reacted with b and f antisera (Table 1).

The results of phenotypic testing showed that 4 strains were β-lactamase-producing and AMP nonsusceptible (Table 1). AMP nonsusceptible strains was confirmed by determining the minimum inhibitory concentration by E-test.

All strains were susceptible to amoxicillin-clavulanic acid (AMC), ceftriaxone (CRO), ciprofloxacin (CIP) and 9 were susceptible to chloramphenicol (C) and tetracycline (TE). Five strains were resistant to trimetoprim-sulphametoxazole (SXT), 4 of them being isolated from nasal exudates (Table 1). Two invasive strains were resistant to AMP and 1 was intermediate while just one strain isolated from nasal exudat was resistant (Table 1).

Molecular characterization

PCR results are presented in the Table 3. The tested strains, produced one amplicon with ompP2 primers indicating that the PCR reaction is functioning and all the isolates are H. influenzae. The PCR amplicon produced with VK primers was highlighted in the 16, 17, 18, 38 CSF, 38 blood and 39 strains, indicating that the strains are capsulated / typeable (Fig. 1, 2). The isolates 16 and 39, that have cross-reacted with b and f antisera, produced one amplicon with f1/f2 primers (Fig. 2) and the strain 17, that did not react with any specific antiserum, produced one amplicon with b1/b2 primers (Fig. 1). The strain 18 produced one amplicon with b1/b2 primers; the strains 38 CsF and 38 blood, isolated from the same patient, as expected, produced amplicons with f1/f2 primers (Table 3). The strains 14, 37, 42 and 43, were checked that they are NC / nontypeable both by phenotypic and genotypic methods. By the genotypic method they did not highlight any amplicon with b1/b2 primers (Fig. 1).

Virulence factors testing

The studied strains were streaked on special media for testing the presence of soluble virulence factors. The results, after 24h, 48h and 72h incubation in 5-10% CO₂ enriched atmosphere, were all negative.

Adherence and invasion of H. influenzae strains to the eukaryotic cell substrate

The study strains exhibited mainly a diffuse adherence pattern and different adherence indexes (Table 4). The adherence indexes were poor (20-40%) for the strains isolated from normally sterile sites and high
(50-95%) for the strains isolated from upper respiratory tract.

The majority of the tested strains were invasive (7 of 10 strains). The 38 blood and 38 CSF strains, isolated from the same patient, showed a different degree of invasiveness; the one isolated from blood being 20 times more invasive than the strain isolated from CSF (Table 4).

When comparing the invasion capacity of b type, f type and nontypeable strains, we can observe that f type H. influenzae strains have the highest invasivity, followed by nontypeable and b type strains.

The quantitative assessment of invasion and adherence was expressed in CFU/mL (Table 4).

The optic/fluorescence microscopy confirmed the strains capacity to adhere and invade the cellular substrate (Figs. 3-6, Figs. 7-10).

**DISCUSSION**

The phenotypic and molecular characterization shown that all strains isolated from the invasive infections and 2 from 5 isolated from noninvasive infections were capsulated, that confirms the fact that the capsule is an important virulence factor that can penetrate the nasopharyngeal mucosa and the capillary blood vessels endothelium, and can cause invasive infections. One strain isolated from CSF and another

---

**Fig. 1.** Agarose gel electrophoresis of PCR products from nontypeable H. influenzae (14, 37 strains) and capsulated, type b (17 strain) with ompP2, bexA and capsule type b specific primers (Hib)

**Fig. 2.** Agarose gel electrophoresis of PCR products from capsulated type f H. influenzae strain (39 strain) with ompP2, bexA and capsule type f specific primers
one isolated from nasal exudate cross-reacted with b and f antisera (Table 1) and required type confirmation by molecular methods. PCR capsular typing overcomes the problems of cross-reaction associated with the serotyping of studied strains.

The results of phenotypic testing showed that four strains were AMP nonsusceptible and β-lactamase-positive. No β-lactamase-producing and AMP resistant isolates were found. The first description of β-lactamase-mediated AMP resistance in H. influenzae in the United States was in 1974 [15, 16]. Since then, its prevalence has increased [17-20]. A U.S. national surveillance study conducted in 1993 revealed a prevalence of 33% β–lactamase production among non type b H. influenzae isolates [21].

Antibiotic susceptibility testing reveals that 1 strain is resistant to C, 3 strains are resistant to AMP and 1 intermediate, 5 to SXT and 1 to TE. The SXT, C and TE resistance also has been described in other studies [17, 18, 20, 22].

Molecular typing showed that the isolates 16 and 39, that have cross-reacted with b and f antisera, produced one amplicon with f1/f2 primers (Fig. 2) and the strain 17, that did not react with any specific antiserum, produced one amplicon with b1/b2 primers (Fig. 1). This fact is underlying the importance of

Table 3. Molecular characterization of study strains

<table>
<thead>
<tr>
<th>ID isolate</th>
<th>ompP2 gene</th>
<th>bexA gene</th>
<th>type b specific gene</th>
<th>type f specific gene</th>
<th>PCR capsular type</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>f</td>
</tr>
<tr>
<td>17</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>b</td>
</tr>
<tr>
<td>18</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>b</td>
</tr>
<tr>
<td>38 CSF</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>f</td>
</tr>
<tr>
<td>38 blood</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>f</td>
</tr>
<tr>
<td>14</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>NC</td>
</tr>
<tr>
<td>37</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>NC</td>
</tr>
<tr>
<td>39</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>f</td>
</tr>
<tr>
<td>42</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>NC</td>
</tr>
<tr>
<td>43</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>NC</td>
</tr>
</tbody>
</table>

Legend: +, amplicon present; -, no amplicon; ND, not determined, NC, noncapsulated strain.

Table 4. Adherence patterns, indexes and the quantitative assessment of invasion + adherence and invasion expressed in CFU/mL

<table>
<thead>
<tr>
<th>ID isolate</th>
<th>Adherence pattern</th>
<th>Adherence index (%)</th>
<th>Adhesion + Invasion CFU/mL</th>
<th>Invasion CFU/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>diffuse</td>
<td>30</td>
<td>1.3x10^7</td>
<td>1x10^4</td>
</tr>
<tr>
<td>17</td>
<td>diffuse</td>
<td>40</td>
<td>5.6x10^4</td>
<td>6.6x10^3</td>
</tr>
<tr>
<td>18</td>
<td>diffuse</td>
<td>30</td>
<td>1.4x10^5</td>
<td>&lt;10^4</td>
</tr>
<tr>
<td>38 CSF</td>
<td>diffuse</td>
<td>20</td>
<td>2.1x10^5</td>
<td>3.3x10^3</td>
</tr>
<tr>
<td>38 blood</td>
<td>diffuse</td>
<td>25</td>
<td>4.6x10^6</td>
<td>6.6x10^4</td>
</tr>
<tr>
<td>14</td>
<td>diffuse</td>
<td>75</td>
<td>2.3x10^8</td>
<td>3x10^4</td>
</tr>
<tr>
<td>37</td>
<td>localized</td>
<td>50</td>
<td>2.3x10^8</td>
<td>&lt;10^4</td>
</tr>
<tr>
<td>39</td>
<td>diffuse</td>
<td>50</td>
<td>1.3x10^7</td>
<td>6.6x10^3</td>
</tr>
<tr>
<td>42</td>
<td>diffuse-aggregative</td>
<td>95</td>
<td>4x10^8</td>
<td>&lt;10^4</td>
</tr>
</tbody>
</table>
Investigation of virulence potential in *Haemophilus influenzae* clinical strains

Fig. 3. Diffuse adherence pattern of strain number 14 on HeLa cells (IO, 1000x, Giemsa staining)

Fig. 4. Localized aggregative adherence pattern of strain number 37 on HeLa cells (IO, 1000x, Giemsa staining)

Fig. 5. Diffuse-aggregative adherence pattern of strain number 43 on HeLa cells (IO, 1000x, Giemsa staining)

Fig. 6. Diffuse-aggregative adherence pattern of strain number 43 on HeLa cells (IO, 1000x, Giemsa staining)

Fig. 7. Adherence to HeLa monolayer observed by fluorescence method for strain number 14
molecular confirmation of identification and typing; PCR detection representing an important tool for both typeable and nontypeable strains [23]. For phenotypic nontypeable strains, we have selected automatically b1 and b2 primers, because according to the literature, *H. influenzae* type b capsule is genetically unstable, and so it can be lost with a frequency of 0.1-0.3% [24].

The strains isolated from nasal exudates exhibited higher adherence ability than CSF and blood isolates, probably associated with the presence of pili on their surface. As we know, the adherence capacity of pathogenic microorganisms to host cells and tissues represents one precondition of the microbial coloni-
REFERENCES

17. Doern GV, Jorgensen JH, Thornsberry C, Preston DA, Tubert T, Redding JS, and Maher LA. National collabo-
**INTRODUCTION**

*Streptococcus pneumoniae* (S. pneumoniae) is an extracellular bacterium that colonizes the nasopharynx and remains pathogenic to humans despite antibiotic therapy. The incidence of pathologic entities linked to *S. pneumoniae* such as pneumonia, septicemia, meningitis and otitis media is very high in children below 2 years of age and in the elderly [1-7]. The increasing rate of antibiotic resistance makes the development of efficient and cost-effective vaccines, that provide broad serotype coverage, a high need and priority [8–10]. While protective adaptive immunity against *S. pneumoniae* mostly involves antibodies directed against serotype-specific capsular polysaccharides (PS), antibodies directed against some surface protein antigens have also been involved in protection [11, 12]. Systemic immunization with intact *S. pneumoniae* elicits an IgG, IgM response specific for a number of pneumococcal proteins, including surface protein A, as well as the capsular polysaccharide and the phosphorylcholine determinant of the cell wall C-polysaccharide [13, 10]. The development of protective vaccines against bacterial pathogens carrying polysaccharide (PS) capsules such as *S. pneumoniae* has encountered many difficulties including low immunogenicity, poor induction of memory responses and only temporary protection, some of them have been solved but others remain as immunological paradoxes. The studies performed in murine models can help to understand those paradoxical effects observed in humans and the mechanisms underlying such processes [14-17].

In this report, we described results of immunogenicity and efficacy tests on C57BL/6 mice immunized with heat inactivated *S. pneumoniae* serotypes (sT) 1, 3 and 6B in C57BL/6 mice by IgM and IgG detection, and by splenocyte in vitro 5-ethynyl-2'-deoxyuridine (EdU) incorporation after antigen specific stimulation, as a proposed method of cellular immune response evaluation. Antibody titer persistence after immunization was not lengthy while antigen specific proliferation response detected by EdU assay was remnant. Intraperitoneal (i.p.) challenge with serotype 6B *S. pneumoniae* proved that antibody titer and the detected specific cellular immune response do not cover seroprotective necessity and do not confer improved immunologic memory in comparison to non-immunized mice, which show natural resistance.
Antibody and splenocyte proliferation response to whole inactivated *Streptococcus pneumoniae* serotype 1, 3 and 6B in mice

Protection was evaluated by i.p. challenge with *S. pneumoniae* serotype 6B, the immune response being detected before and after infection.

**MATERIALS AND METHODS**

**Mice**

Female C57BL/6 mice (6-8 weeks old) from the Cantacuzino NIRDMI animal facility were housed according to the European recommendations, with free access to standard chow and tap water. All protocols were approved by Internal Ethical Committee.

**Antigens and immunization**

Mice were immunized i.p. with 1.5x10^8 CFU/ml heat inactivated whole antigen *S. pneumoniae* serotypes 1, 3 and 6B (Statens Serum Institute, Copenhagen, Denmark). Each strain was cultured on Columbia medium with 5% sheep blood for three passages and then in simple Columbia agar. Subsequently, 24 hours bacterial suspensions in 0.9% NaCl solution were set at McFarland 0.5 (1.5x10^8 CFU/ml), heated for one hour at 65°C and mixed together. The immunization protocol comprised two inoculations per week with the following doses: 0.1 ml of 1/10 and 1/5 dilution in the first week, 0.1 ml of vaccine the second week and 0.2 ml of vaccine for the remaining 4 weeks. The sera were collected by retroorbital bleeding before the study and after immunization at day 21, 42 and day 60.

**Challenge**

Immunized mice (4/group) were infected with 2x10^8 CFU/0.2ml pneumococcal serotype 6B by i.p. route at day 60 post-immunization. The control consisted in 2 groups of mice (4/group) which were infected with 2x10^8 CFU/0.2ml and 2x10^9 CFU/0.2ml, respectively of the same bacterium. Body weight and animal survival was recorded for 7 days after i.p. challenge. For surviving animals, IgG and IgM against pneumococcal whole antigen and PS serotype 6B detection was performed on day 10.

**Culture media and reagents**

Complete medium was RPMI 1640 medium (Sigma), supplemented with Glutamine 2mM (Sigma), 2-mercaptoethanol 50 microM (BioRad), 10% heat-inactivated fetal bovine serum (FBS) (Biochrom AG.) and 100 units/mL Penicillin–Streptomycin (Sigma), Click-iT EdU Flow cytometry assay kit (Invitrogen, Molecular Probes).

**Measurement of serum antigen-specific Ig titers by ELISA**

Sera from mice were individually analyzed by ELISA for corpuscular ST1, ST3, ST 6B *S.pneumoniae* antigens specific IgG and IgM levels (96-well flat-bottom microtiter plates were coated with 1x10^8/ml heat killed bacteria diluted in sodium saline buffer overnight at 4°C and blocked with phosphate-buffer saline containing 0.05% bovine serum albumin (Sigma) for 2h at room temperature). For the titration of anti-PS antibodies IgG and IgM, the plates were coated with pneumococcal polysaccharides (PS) ST1, ST3 and ST 6B (Statens Serum Institute, Copenhagen, Denmark) according to WHO ELISA-rev.2006 [18] for the quantification of *S. pneumoniae* serotype specific Ig. As positive control, we used immunized human serum with the same *S. pneumoniae* strains and peroxidase goat anti-human conjugates. Following the incubation of the sera, the plates were washed four times with PBS containing 0.05% Tween 20 and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG or IgM (Southern Biotech) for 2h at room temperature. The plates were then washed as above and incubated with 3,3',5,5'-tetramethylbenzidine (Sigma) for 20 min at room temperature. The reaction was stopped with the addition of 1M sulphuric acid (Merck) to each well. Plates were read at 450nm and 620nm (used to account for absorption by the plastic) using Tekan Sunrise reader. The titers of each sera diluted 1/400 in PBS with 0.05% BSA are expressed as optical density (OD).

**Measurement of EdU splenic antigen-specific incorporation by flow-cytometry**

Mice were sacrificed and spleen was harvested at various time points after immunization, respectively day 21, 42 and 60 after immunization (3 mice/group). As control were used non-inoculated mice. Spleen cells were isolated and resuspended in complete medium at 1x10^6 cells /ml. Cell viability was always greater than 98%, as determined by the tripan blue exclusion test.

The cells were cultured for 7 days at 1x10^6 cells/ml in the absence or presence of 1x10^5/ml pneumococcal antigen mixture which was also used in the immunization.

EdU (from the Click-iT™ EdU Flow Cytometry Assay Kit, Invitrogen™) was added at a 10 μM final concentration 24 h before harvesting the cells. For the Click reaction, cells were collected and washed in 3 ml of PBS, centrifuged and fixed with 2% paraformaldehyde for 15 min. Cells were washed again and incubated with 100 μl of saponin-based perme-
abilization buffer for 15 min. After additional washing, cells were incubated with 500 μl Click-iT reaction buffer for 1 h and washed again with 3 ml permeabilization buffer. All procedures were performed according to the manufacturer’s instructions.

Flow cytometric data (20,000 ungated events) were acquired on a FACS Calibur (Becton-Dickinson, San Jose, USA) using CellQuest software. Cells were first gated according to their scatter characteristics and then analyzed for the presence of EdU incorporated cells on fluorescence channel 1 (FL1). The background proliferation was calculated in the absence of antigen. The proliferating fraction (PF) for each moment of detection (day 20, 42 and 60) was calculated by subtracting the mean background proliferation from the mean proliferating fraction in response to specific antigen and designate a positive response when PF ≥ 1.0 %. The stimulation index (SI) was calculated by dividing antigen-induced PF of immunized mice to that of non-inoculated mice and it was considered as positive response for a value ≥ 2.0 [19].

Statistical analysis

The results were expressed as mean ± standard deviations (SD) of the individual serum Ig type titers (no less than 4 mice/group). Significance was determined by Student t test, p-values of ≤0.05 were considered statistically significant. Splenic cells antigen specific proliferation response was calculated as previously described, through proliferating fraction (PF) and stimulation index (SI).

RESULTS

Persistence of anti-pneumococcal ST 1, ST 3 and ST 6B antibodies in immunized mice

The IgG and IgM serum antibody level detection were performed before (day 0) and after immunization at day 21, 42 and 60 against corpuscular ST1, ST3, ST 6B pneumococcal antigens and also against characteristic capsular polysaccharides. Fig. 1 shows the increase levels of antigen specific antibodies from day 21 to day 42 at significant titers (p≤0.05) after immunization and the decrease of both IgM and IgG against pneumococcal antigens detected at day 60. The IgG and IgM titers against pneumococcal capsular polysaccharides were lower compared with that detected against whole bacteria wall. From all the 3 strains of S. pneumoniae used for immunization serotype 6B seems to be the most immunogenic and ST 1 the lowest.

Fig. 1. The titers of antibodies against pneumococcal antigens (IgM and IgG) in immunized C57BL/6 mice. The serum samples were collected before immunization (day 0) on day 21 (middle of immunization protocol), 42 (after immunization) and on day 60 from the start. The mice were i.p. inoculated with 10⁹ CFU/ml heat inactivated whole S. pneumoniae ST 1, ST 3 and ST 6 B, and the results are presented as mean (4 mice/group) ± SD. The results showed significantly increased anti-pneumococcal IgG, IgM titers on day 21 (p≤0.05) with the peak values attended on day 42 (p≤0.02) compared to the concentration detected before immunization and the decreased of specific antibody titers on day 60.
Kinetics of antigen specific cellular immune response by EdU incorporation

Click-iT EdU flow cytometry assay was used to detect antigen specific proliferation response of splenocytes isolated from immunized mice versus control. EdU, 5-ethyl-2'-deoxyuridine is a nucleoside analog to thymidine and is incorporated into DNA during active DNA synthesis. Detection is based on the alkyne group of EdU, which reacts with fluorescent azides in a copper catalyzed reaction, enabling detection of EdU incorporation into cells by flow cytometry.

The antigen specific proliferating immune response was performed on immunized mice (3/group) at day 21, 42 and day 60 and in non-inoculated mice considered as control. The results expressed as fraction of proliferation (PF) and stimulation index (SI) showed the antigen specific proliferation response of splenocytes isolated from the immunized mice which increase from day 21 to day 42 (Fig. 2).

Seroprotection of immunized mice

In order to evaluate the seroprotection conferred by immunization, the challenge was performed by i.p. inoculation of 2x10^9 CFU/0.2ml pneumococcal type 6 group B of immunized mice and by i.p. inoculation with 2x10^9 CFU/0.2ml respectively 2x10^{10} CFU/0.2ml of same bacteria in the mice from control groups. The challenge was performed on day 60 after immunization and the results were as follow: one mouse from immunized group died on day 5 after challenge and none from the control group infected with the same dose, and 2 of 4 mice died from the control group infected with 2x10^{10} CFU/0.2ml on the day 3. The sera from the surviving mice were collected at 10 days after challenge and assayed for IgG and IgM against pneumococcal antigens serotype 6B used in the experiment. The results showed in Table 1 revealed the development of the secondary immune response in immunized mice and increase of Ig M against whole pneumococcal ST 6B antigen in the naïve mice as consequence of primary immune response development.

DISCUSSION

Animal models of pneumococcal infection are an essential tool for study of infection and protection, for clarifying mechanisms of disease pathogenesis, testing vaccines and novel drugs but are hampered by low virulence of pneumococcal serotypes most frequently encountered in human diseases and by differences of virulence between serotypes and strains [20-22]. The vaccine prevention is based on the induction of a rapid and efficient immune response against pathogens encountered in the field. In the present work, using a mouse model, we investigated the immunogenicity induced by three S. pneumoniae strains ST 1, ST 3 and ST 6B causing frequent human infections and the level of protection conferred by the vaccination against ST 6B infection. We first observed the generation of antigen specific antibodies IgG and IgM against corpuscular antigen but also PS at significant titers against all the strains used in immunization. The data showed the ascending anti-pneumococcal antibodies titer (IgM, IgG) as were detected in day 21 (p≤0.05) and 42 (p≤0.02) compared to the values before immunization but with low persistence revealed by the decrease of antibody titers on day 60 for all 3 strains used in immunization. It seems that the most immunogenic was the ST 6B pneumococcal antigen used in the experiment and the lowest was ST1. In parallel, we monitored antigen-specific immune cell response by EdU incorporation, which to our knowledge is the first

![Fig. 2. Kinetics of antigen specific proliferation immune response in spleen upon immunization of C57BL/6 mice (3 mice/group) with heat inactivated S. pneumoniae ST 1, ST 3 ST 6B. Click-iT EdU flow cytometry assay was performed and analyzed on day 21, 42 and 60. Results are expressed as proliferation fraction which is the difference between specific proliferation and the background, whereas the stimulation index (SI) is the ratio of specific proliferation of immunized mice to control. A response with a PF of at least 1.00% and an SI of at least 2.0 was considered positive.](image-url)
application of this method to evaluate the antigen-specific proliferation response. Spleen antigen-specific response showed a tendency to increase from day 21 to day 60. The method may be useful in the exploration of specific cellular immune response in vaccination studies. The results are expressed as proliferation fraction (PF) and stimulation index (SI). The background and unspecific response obtained in non-immunized mice of both values have been excluded. The values were considered positive for PF of 1.0 % or more and for an SI of 2.0 or more.

The challenge performed with 6B on immunized mice revealed that the detected antibody titers do not cover the seroprotective necessities. The data showed a variability of virulence of capsular S. pneumoniae serotype 6B tested on C57BL/6 mice as previously described [23-25], one death was registered in vaccinated and none in control mice group when they were infected i.p. with $10^5$ CFU/0.2 ml and 2 of 4 mice died in $10^{10}$ CFU/0.2 ml infected control group. Our experiments, trying to establish MLD50 for S. pneumoniae serotype 6B after i.p. administration by the Reed-Muench method, did not conduct to clear results. The mortality rate being extremely irregular within the administered concentration ($10^{10}$-$10^4$ CFU/inoculum) or volume (0.2ml-0.5ml) range. In those experiments, signs of neurologic involvement (hind limb paralysis, head shaking and moving around in circles) were noticed in some mice starting at 5 days after infection (data not shown). According to previous data, the longer survival time caused by most capsular 6B strains suggests rather a difference in the nature of the disease than in the ability of these strains to kill and perhaps that, these bacteria could elicit significant growth in protected sites [26-28]. The presence of preexisting, naturally occurring antibodies against serotype 6B antigen was not detected in the control mice group. Ten days after challenge, pneumococcal antigen specific antibody detected by ELISA showed the presence of secondary immune response in immunized mice and the development of primary specific humoral immune response in control mice.

These observations, like previous publications [8, 17, 26, 27, 29], indicate that there are differences in some of the properties that make pneumococci virulent in humans and mice and also regarding the evaluation of immunization. Our data, revealed a low persistence of antigen specific antibodies and uncertain seroprotection after immunization as a consequence of the low immunogenic capacity of the strains used in mice vaccination or due to the pathogen-host interaction C57BL/6 mouse model [14]. The presence of splenocyte antigen-specific proliferation response could not be correlated with a protective memory response. These facts suggest that more emphasis should be given to the mechanisms involved in the host pathogen interactions in order to improve the efficiency of immunization against pneumococcal diseases.

ACKNOWLEDGEMENTS

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Antibody and splenocyte proliferation response to whole inactivated Streptococcus pneumoniae serotype 1, 3 and 6B in mice

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GLUTEN SCREENING OF SEVERAL DIETARY SUPPLEMENTS
BY IMMUNOCHROMATOGRAPHIC ASSAY

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ABSTRACT
Celiac disease (CD) is a chronic intestinal disorder of public health concern caused by gluten ingestion in sensitive individuals. Gluten is a protein found not only in gluten-containing food but also as normal component of drugs and dietary supplements. Detection of gluten in dietary supplements is a very important task required for establishing their gluten status, which is highly important for the safety of products consumed by CD and gluten-sensitive patients.
In this paper, we investigated the presence of gluten in twenty one common dietary supplements from the national market using the immunochromatographic assay. This visual assay proved to be an efficient rapid tool for gluten screening as an alternative to the ELISA techniques. The results have shown the presence of gluten in 23.8% of the investigated samples (vitamins, minerals, plant extracts, probiotics supplements, lactoferrin, propolis supplements). The results provide information which may contribute to the completion of the existing lists of gluten-free pharmaceuticals. It is known that for CD patients obtaining accurate information about the gluten content of a particular item is a difficult and time-consuming process.

Keywords: Celiac disease, gluten, immunochromatography, dietary supplements

INTRODUCTION
Celiac disease (CD) is a gluten-sensitive enteropathy developed under an immunological mechanism driven by human leukocyte antigens (HLA-DQ2 or DQ8)-restricted T cells [1]. Gluten ingestion in genetically susceptible individuals causes severe injury of intestinal mucosa and atrophy of the intestinal villi. Clinically, CD may manifest in very different ways from being asymptomatic to serious problems of malnutrition. The main clinical manifestations consist of abdominal pain, increased gastrointestinal peristalsis, diarrhea, weight loss, and anemia. There is an atypical form of CD with no gastrointestinal disorders, but with signs of anemia, bone and endocrine disorders, infertility and neurological dysfunction [2-5].

Literature data from several National Institutes of Health describe that 1% of the total Europe and USA population is suffering from CD. Recent studies have estimated that CD occurs more frequently worldwide than previously shown, with a prevalence of up to 3% in Western populations [6-8].

Untreated CD patients are at increased risk of osteoporosis, type I diabetes, reproductive disorders, lymphoma and other autoimmune disorders [9]. The only efficient treatment for CD patients is leading a gluten-free diet during their lifetime. Studies investigating the potential nutritional risks of gluten-free diet have shown that children that follow a gluten-free diet generally have the same developmental trends as healthy children which are on a normal gluten-containing diet [10]. According to that study, CD children may show high intakes of saturated fat and sucrose and low intakes of dietary fiber, vitamin D and magnesium, compared to general diet recommendations.

Gluten is a widely spread component of our daily diet not only in grains (wheat, rye, barley and triticale) but also in food ingredients, pharmaceutical products, dietary supplements. Some research suggested that some grains (oats) considered as gluten-free might be contaminated with wheat, barley, and/or rye during harvest, transport, and/or processing [11-12].

Therefore, efficient, rapid and reliable assays of gluten screening are essential to detect traces of gluten in food, medicinal or pharmaceutical products. The analysis of gluten in foodstuffs is sometimes a difficult approach because of the heterogeneity of

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gluten, the diversity of the products and the interference with some food processing (heat, enzymatic digestion). Several bio-analytical methods for qualitative and quantitative determination of gluten have been established. Classical techniques are based on the measurement of nitrogen content of starch preparations, but modern available detection methods are based on immunological assays, such as ELISA [13], immunochromatography biochemical techniques [14-15], PCR-based methods [16] and electrochemical magneto-immunosensor for the quantification of gliadin or small gliadin fragments [17].

In the present study, we investigated the presence of potentially hidden gluten ingredients in some dietary supplements using the immunochromatographic visual test. This assay is a qualitative and semi-quantitative sensitive method for the detection of gluten acting in good correlation with the ELISA assay [18]. It has been shown that immunochemistry becomes an increasingly important field as it finds good application in many food- and health-related area.

MATERIALS AND METHODS

Sample extraction and preparation

Twenty one dietary supplements samples purchased from the national pharmacy market were used in the present investigation, as follows: multivitamins (Brands A, B), multiminerals & vitamins (Brands C, D, E), syrup vitamins for children (Brand F), vitamins & eukalyptus (Brand G), calcium supplements (Brands H, I, J, K), vitamin C (Brands B, L, M), plant extracts (Brands N, O, P, R), probiotics supplement (Brand S), lactoferlin (Brand T), propolis & added essential oils (Brand U).

The samples were grinded and extracted in 60% (v/v) aqueous ethanol at room temperature. After 30 minutes of incubation at room temperature under stirring, the mixtures were filtered and aliquots of samples were diluted 1:10 in test tubes with PBS buffer pH 7.4.

Gluten detection

The immunochromatographic visual assay was used for qualitative determination of gluten in the selected samples. Immunochromatographic sticks (purchased from OPERON S.A., Spain) which contain monoclonal antibodies specific for gluten of wheat, barley and rye, were dipped into diluted solution samples. Results were read after 5 min. of chromatographic running. Development of a red band in addition to the control blue band on the reaction strips is considered a positive result. Each sample was determined in duplicate.

RESULTS AND DISCUSSION

CD patients can manage their condition only by following a strict gluten-free diet. As there are less obvious sources of gluten, in particular in ingredients used in food or pharmaceuticals production, CD patients must check carefully the information label of food, pharmaceutical or medicinal products. Codex Alimentarius Commission (2008) and U.S. Food and Drug Administration FDA proposal (2007) consider any food product as gluten-free if the gluten content is below 20 ppm. This standard has been adopted in regulations by the 27 EU countries, but some national organizations may have other restrictions regarding this issue. The 20 ppm standard is based on current methods for gluten detection, but there are not validated methods to detect smaller amounts.

It is known that small amounts of gluten cause intestinal damages in susceptible individuals. As today, CD and gluten-sensitive patients may find on the market an increased number of food products labeled as gluten-free, the situation regarding pharmaceuticals, herbs or food supplements is not the same. Despite few common gluten-free medications are known, most dietary supplements might contain gluten because of the excipients used.

In the present study, we have screened for gluten a number of twenty one samples of common dietary supplements (vitamins, minerals, plant extracts, probiotics supplements, lactoferlin, and propolis supplements) with unknown or not specifically mentioned gluten status. These samples are not recognized as gluten-free by CD patients.

After ethanol extraction and PBS dilution of the samples, qualitative determination of gluten was performed by the sensitive immunochromatographic assay. Gluten reaction strips immobilized with R5 monoclonal antibody were placed in diluted samples and results were read after 5 min. of chromatographic running. The development of a red band indicates gluten positive sample, while the blue control band appears in both positive and negative samples. The sensitivity of the test is of 10 ng/ml of gliadins equivalent to 2 ppm of gluten in the sample.

The obtained results are given in Table 1.

As shown in Table 1, among the investigated twenty ones samples, five samples produced by different manufacturers were found positive to gluten, giving a distinguishable pink-red band on the reac-
tion strips. Four of these samples are vitamins/calcium supplements including syrups for children use, while the other sample is a complex plant extract. The other great part of the investigated dietary supplements (vitamins, minerals, plant extracts, probiotics supplements, lactoferrin, propolis supplements) does not contain gluten in concentration above 2 ppm, the sensitive limit of the test.

The obtained results have shown that some of the dietary supplements might be of concern for CD patients as they may contain small amounts of gluten. Our results are important in terms of completing the existing databases regarding items safe for the consumption of CD patients. However, even when a pharmaceutical/supplement product has no gluten-containing ingredients added, manufacturers cannot guarantee the product as gluten-free since they do not test for its absence.

Therefore, detection methods for routine analysis should be considered for a rapid gluten screening of items in order to be safely consumed by CD patients. The method of immunochromatography used in our investigation proved to be a sensitive, simple, fast and reliable test for a rapid determination of gluten in foodstuffs.

**CONCLUSIONS**

CD is a chronic intestinal disorder of public health concern as untreated CD patients develop disease complications and suffer from the negative impact on their quality of life. The disease results from ingestion of gluten from wheat, barley and rye. Gluten can be found not only in food products but also as normal component of drugs and dietary supplements.

Detection of gluten in dietary supplements is a very important task required for displaying the gluten status of products as avoidance of gluten remains the only efficient treatment for celiac patients. In this paper, we investigated the presence of gluten in twenty one common dietary supplements using

<table>
<thead>
<tr>
<th>No.</th>
<th>Type of dietary supplement</th>
<th>Gluten</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>multivitamins (Brand A)</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>multivitamins (Brand B)</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>multiminerals and vitamins (Brand C)</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>multiminerals and vitamins (Brand D)</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>multiminerals and vitamins (Brand E)</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>syrup vitamins for children (Brand F)</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>B vitamins with eukalyptus oil (Brand G)</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>calcium D3 supplement (Brand H)</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>calcium + potassium, supplement (Brand I)</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>marine calcium, supplement (Brand J)</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>calcium, supplement for good bone structure (Brand K)</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>vitamin C (Brand B)</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>vitamin C (Brand L)</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>vitamin C (Brand M)</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>detoxifiant plant extracts (Brand N)</td>
<td>+</td>
</tr>
<tr>
<td>16</td>
<td><em>Nasturtium officinale</em> plant extract (Brand O)</td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td><em>Oenothera Biennis</em> plant extract (Brand P)</td>
<td>-</td>
</tr>
<tr>
<td>18</td>
<td><em>Lepidium meyenii</em> plant extract (Brand R)</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>lactic ferments (Brand S)</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>lactoferrin (Brand T)</td>
<td>-</td>
</tr>
<tr>
<td>21</td>
<td>propolis and essential oils (Brand U)</td>
<td>-</td>
</tr>
</tbody>
</table>

Note: "+" = positive gluten content
"-" = negative gluten content

*Table 1. Gluten content of selected samples of dietary supplements determined by immunocromatographic assay*
a sensitive procedure, the immunochromatographic assay. The results have shown the presence of gluten in five from the twenty one investigated samples.

The results provide information about the absence of gluten in the selected products contributing to the completion of the existing lists of gluten-free pharmaceuticals. It is known that for CD patients obtaining accurate information about the gluten content of a particular pharmaceutical is a difficult and time-consuming process.

This assay provides a convenient opportunity to detect gluten not only in foodstuffs but also in drugs and dietary supplements.

In conclusion, the recommendation for CD patients and also for medical practitioners and dietitians is to exclude these food components from the diet. Also, unless clearly “gluten-free” labeled food is consumed by CD patients, it is important for them to avoid any foodstuff that seems doubtful regarding gluten content.

REFERENCES

INTRODUCTION

Antiendomisium antibody (eMA) discovery meant a step forward in celiac disease screening. But then, the method has been replaced by determination of tissue transglutaminase antibodies (tTG), due to increased sensitivity. Today antiendomisium antibodies are used to confirm results of tissue transglutaminase antibodies due to the specificity raised. But because of their false positive results is recommended confirmation of positive results with intestinal biopsy [1]. Discovery of tissue transglutaminase marked a new era in the diagnosis of celiac disease. With all the new challenges, immunoenzymatic assay for determining tissue transglutaminase remained the most effective in celiac disease screening [2]. Discovery of tissue transglutaminase ability to catalyze deamidation reaction of gliadin, with formation of gliadin peptides, marked another new step in the development of serological tests in celiac disease. Use of deamidated gliadin peptides (DGP) in immunoenzymatic reactions increased performance of antigliadin antibody (AGA) [3]. Based on these findings, the aim of our study was to make a detailed analysis of celiac disease. The work presents a review of abstracts of our research in the context of clinical data. Research area was evaluation of a diagnostic procedure. So we made a point making of what is old and new in celiac disease diagnosis. Performance of serological tests were analyzed in celiac disease, in risk groups of celiac disease, type 1 diabetes mellitus, in particular manifestations of celiac disease, dermatitis herpetiformis but also in biochemistry, allergy, viral and immunology mani-

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Romanian experience in child celiac disease diagnosis

festations of celiac disease. The new tests, deamidated antigliadin antibodies and determination of HLA molecules in celiac disease in children are news for our country. Also, studies on the importance of gluten-free diet in the development of celiac disease in families of children with celiac disease were not performed in our country.

MATERIAL AND METHODS

- RESEARCH GROUP - children from Regional Center for Cluj celiac disease management, held in the structure of the Emergency Hospital for Children, Cluj-Napoca, Pediatric Clinic II, according to Government Decision no. 168/2005 on the organization and functioning of the Ministry of Health in the counties of Cluj, Satu Mare, Maramures, Bihor, Salaj, Bistrita, Mures, Harghita, Covasna, Brasov, Sibiu, Alba.

The inclusion criteria were: a) patients with old celiac disease, serologically monitored for their adherence to a gluten-free diet; b) patients with signs suggestive of celiac disease: chronic diarrhea, weight loss, statural growth retardation, malabsorption syndrome, frequent anemia, as well as patients with any disorders with primary or secondary intestinal involvement.

- Methods

The serological tests were provided by INOVA Diagnostics Inc., (San Diego, USA). IgA EMA were determined by indirect immunofluorescence, using type esophageal mucosal sections taken from the monkey as a substrate. Reading was performed using the Olympus CX31 fluorescence microscope. IgA tTG were evidenced by ELISA, with the Chem Well 2910 Awareness Technology Inc. analyzer, and the results obtained were validated only after internal quality control. The main interferences of the method were with free bilirubin (19.3 mg/dl), conjugated bilirubin (19.9 mg/dl), hemolyzed hemoglobin (485 mg/dl) and rheumatoid factor (45 IU/ml), such sera being avoided during testing. The linearity of the method showed a regression coefficient R² higher than 0.997. The minimum quantitative detection limit, i.e. the analytical sensitivity of the method was 1.23 U/ml. The method had a cutoff value of 25 U/ml, samples with a higher concentration being considered positive, and samples with a lower concentration being considered negative.

The genetic tests were performed using DNA extraction kits and HLA typing kits, by polymerase chain reaction (PCR), produced by the Inno-Train Diagnostik company, Germany. PROTRANS Domino System HLA kits were used, which determine the following haplotypes specific for celiac disease:

1. pattern 1 DR3-DQ2- and pattern 5 DR4-DQ8 as single haplotypes;
2. pattern 2 DR3-DQ2/DR3-DQ2 as two identical homozygous haplotypes;
3. pattern 3 DR3-DQ2/DR7-DQ2 and pattern 4 DR5-DQ7/DR7-DQ2 as combinations of two haplotypes.

- RESEARCH infrastructure

1. Medical Analysis Laboratory, Department of Virology and Immunology Clinic, Children's Hospital Cluj-Napoca - ELISA tests, indirect immunofluorescence tests, DNA extraction. 2. Research and Grants Laboratory, Dermatology Clinic Cluj-Napoca - direct fluorescent antibody testing. 3. Immunogenetics and Virology Center, Institute of Clinical Fundeni Bucharest - polymerase testing chain reaction (PCR) - HLA determinations.

PERSONAL RESEARCH AND OURS CONCLUSIONS

Chapter 1. SEROLOGICAL DIAGNOSIS

1.1. New Para-Clinical Investigations in the Celiac Disease

Deficiencies of native antigliadin antibodies tests lead to the decrease of interest but, at the same time, to more serious studies of molecular biology in connection with this test. The discovery of specific B lymphocytes epitopes on certain deamidated gliadin molecules has led to a new serological test, the deamidated antigliadin antibodies. We aimed to evaluate these antibodies and to determine the possible connections with immunoglobulin A deficit in the child's celiac disease. During 2008 we carried out an observational analytical study that determined both IgG and IgA immunoglobulin isotypes of DGP in a group of 102 children from Cluj area, of which 31 children had celiac disease, under gluten-free diet, and 71 children, without diagnosis but with clinical signs of celiac disease. After evaluating the qualities of the deamidated antigliadin antibodies, we obtained a sensitivity (Se) of 80 % (95 % CI 28-99) and a specificity (Sp) of 88.4 % (95 % CI 74-96) for the 0-3 years age group (p=0.007). In older children, the test's sensitivity decreased but the specificity remained at close values, as the children got older. At the same time, in 4 % of the children we observed a good correlation with the A immunoglobulin deficit. Assessment of the IgA + IgG DGP represents a useful test in
the celiac disease screening, mainly for the 0-3 years age group. This test also helps detecting the A immunoglobulin deficit [4].

The good results of our study given by DGP IgA+IgG in the immunoglobulin A deficit screening, confirm the existent studies: Korponay-Szabó [5] stated that DGP IgA+IgG detected all 57 patients diagnosed with celiac disease and with immunoglobulin A deficit.

1.2. IgA tTG, first line in the diagnosis of celiac disease

According to the 2008 celiac disease working group run by Dr. A. Fassano under the auspices of the Federation of International Societies of Pediatric Gastroenterology, Hepatology and Nutrition, celiac disease is a chronic immune-mediated enteropathy characterized by gluten sensitivity, which can affect any organ or system, having a wide range of clinical manifestations of variable severity. The serological diagnosis of celiac disease is based on high sensitivity and specificity tests. The measurement of IgA tTG by ELISA is universally accepted in the screening of celiac disease. Using the gold standard represented by IgA EMA in a group of 890 children investigated during 2008-2009, we aimed to evaluate IgA tTG, as well as to establish their prevalence in associated diseases. Following the measurement of IgA tTG in the entire group, we obtained: Se 77.3 %, positive predictive value 55.2 %, Sp 93.1 %, negative predictive value 97.3 %, p=0.000, and in IgA tTG associations we obtained the value 0.51 for the ROC curve area. We found associations of IgA tTG with type I diabetes mellitus (2.35 % prevalence), protein-calorie malnutrition (0.89 % prevalence), and intestinal malabsorption (0.56 % prevalence). Our results have a high specificity and sensitivity in the screening of celiac disease, while requiring a second method of confirmation [6].

Why is the screening of celiac disease with tTG IgA and the evaluation of celiac disease after EmA IgA necessary? Our work protocol is comparable to other European studies. In order to find the prevalence of celiac disease in children and adults, Mustalahti et al. [7] carried out a study in four European countries (Finland, Germany, Italy and Great Britain). The authors performed the measurement of tTG IgA in all the participants in the study 29212, while EmA IgA were only measured in positive patients or patients with borderline tTG IgA values. Intestinal biopsy was recommended for persons with positive tTG IgA and EmA IgA.

1.3. Difficulties in Celiac Disease Diagnosis in Children - A case report

Duodenal biopsy on the American continent remains the gold standard in diagnosing celiac disease, according to North American Society for Pediatric Gastroenterology, Hepatology and Nutrition, protocol, which also joined the European Society of Pediatric Gastroenterology, Hepatology and Nutrition. However, the need for rapid diagnosis of celiac disease by avoiding intestinal biopsy and the benefits of the introduction of gluten-free diet persists in the current studies, studies that are too numerous to go unnoticed. We were in favor of a diagnosis of celiac disease based on IgA EMA positivity and thus in favor of a faster gluten free diet but with the mention to repeat IgA EMA, intestinal biopsy and HLA DQ2/DQ8 typing to the next medical examination [8].

However, due to the complexity of this disease, Catassi et al. [9] recently proposed a new diagnostic algorithm, which could confirm diagnosis of celiac disease if at least 4 of the following 5 criteria are positive: 1) typical symptoms of celiac disease, 2) serological positivity of immunoglobulin classes, 3) a class of celiac disease-specific antibodies at a high titer, 4) human leukocyte antigens DQ2 or DQ8, 5) diagnosis of celiac disease by the biopsy of the small intestinal mucosa and the response to a gluten-free diet.

Chapter 2. HUMAN LEUKOCYTE ANTIGENS TYPING

2.1. The importance of determining human leukocyte antigens in preventing intestinal lymphoma in patients with celiac disease

Identification of celiac disease, by determining human leukocyte antigens DQ2/DQ8, is important since recent long-term studies have shown that the mortality of celiac disease is increased, if it is unrecognized and untreated. In this sense, we wanted to see the usefulness of genetic tests in celiac disease diagnosis and screening. During 2010 we determined by PCR, DQ2/DQ8 haplotype, in a group of 27 children with celiac disease and 9 of their brothers, serologically negative for celiac disease. 22 children and 7 of their brothers confirmed the diagnosis of celiac disease, DR3-DQ2 haplotype was predominant in children with celiac disease and DR4-DQ8 to their brothers. Genetic testing to determine human leukocyte antigens remain the most reliable test in the diagnosis of celiac disease but also in identifying family risk for people with celiac disease [10].

Srivastava et al. [11], by evaluating the prevalence and the role of HLA DQ2/DQ8 testing in first degree relatives of children with celiac disease,
found that 85% of the first degree relatives were HLA DQ2 positive and thus, presented the risk of developing celiac disease - same conclusion as in our study.

2.2. Controversies in the laboratory diagnosis of celiac disease; New haplotypes discovered [12].

The latest consensus on celiac disease in 2008, under the auspices of the International Societies of Pediatric Gastroenterology, Hepatology and Nutrition [13], shows that HLA DQ2/DQ8 typing indicates the highest negative predictive value for celiac disease, which would exclude the diagnosis of celiac disease. In Romania, there are no studies on the implication of HLA-DQ2/DQ8 in celiac disease in children. The aim of our study was to analyze the significance of genetic tests, with a focus on negative HLA-DQ2/DQ8 cases, as well as to determine the main haplotypes involved in celiac disease in children. We tested in 37 children with old celiac disease, confirmed based on the presence of intestinal villi changes on duodenal biopsy, the IgA tTG by ELISA and the IgA EMA by indirect immunofluorescence, compared to HLA-DQ2/DQ8 typing by PCR. In 25 children, the determined HLA haplotypes predominantly belonged to DQ2, and in 3 children we report the presence of a new haplotype, DR3-DQ2/DR4-DQ8, formed by pattern 1, DR3-DQ2 - the DQA1*0501 and DQB1*0201 alleles, and pattern 5, DR4-DQ8 - the DQA1*0301 and DQB1*0302 alleles. In 9 children, genetic tests were negative for celiac disease. The identification of HLA-DQ2/DQ8 provides additional data in the diagnosis of celiac disease, but a rigid algorithm in the diagnosis of celiac disease has no practical applicability.

Chapter 3. ASSESSMENT OF RISK GROUPS: TYPE 1 DIABETES MELLITUS

3.1. Unfavorable prognosis markers in the association of type 1 sugar diabetes with celiac disease

The association of celiac disease with sugar diabetes has been demonstrated by the common genetic aspect (HLA-DR3) which correlates with the serious evolution of the type 1 diabetes. During 2008-2009 we followed the quantification of the associated autoimmune phenomena through immunoenzymatic reactions on a group of 40 children with sugar diabetes of type 1. The immunologic markers’ prevalence was: liver-kidney anti-microsomal antibodies 2.5 %, soluble liver antigen 0 %, glutamic acid decarboxylase antibodies (GAD) 35 %, anti-tyrosine phosphatase antibodies 55 %, anti-insulin antibodies 50 %, cytoplasmic anti-insulin antibodies 2.5 %, antithyroglobulin antibodies 11.1 %, antithyroidperoxidase antibodies 23.1 % and IgA tTG 25.7 %. The main associations of the found IgA tTG were with GAD \( r = 0.33 \) (\( p = 0.027 \)). Since the presence of GAD associated with the IgA tTG, these could be used as predictive markers in the unfavorable development of the type I sugar diabetes [16].

Among the most recent studies on autoimmune manifestations we mention the one of Karavanaki & collab. [17], who, in the case of 144 children with type 1 sugar diabetes obtained a prevalence of 53.2% of anti-GAD, 11.1% of anti-TT, 17.4% of anti-TPO and 7.6% of anti-tTG IgA. At the same time, they admitted the connection between anti-GAD and anti-TPO (\( p = 0.01 \)) and recommended the use of anti-GAD as marker in the development of autoimmune manifestations associated with type 1 sugar diabetes at children.

3.2. Prevalence of IgA tTG antibodies in children with type 1 diabetes mellitus

The association of celiac disease with type 1 diabetes mellitus is known, but the evolution of celiac disease is most frequently asymptomatic, without any
clinical signs. Thus, diagnosis is impossible to make in the absence of serological tests. Our study aimed to determine the prevalence and the efficiency of IgA tTG in the screening of celiac disease in children with type 1 diabetes mellitus. During the course of 2008-2009, we performed an analytical clinical study that included the determination of IgA tTG in a group of 119 children with type 1 diabetes mellitus. Fifty-seven percent of the subjects were male and 43% were female, with a mean age of 11.74 years. By evaluating IgA tTG, we obtained a prevalence of 9.2 % in children with type 1 diabetes mellitus, with a Se and Sp of 80 and 82.6 %, respectively. There is an increased prevalence of IgA tTG, which suggests the need to use this method as an effective first-line test in the screening of celiac disease in children with type 1 diabetes mellitus [18].

In Romania Cev et al. [19] in 2009 in the Banat region on a lot of 307 patients with type 1 diabetes mellitus achieve a prevalence of 5.5% tTG, comparable to that we found in Cluj area and surrounding areas [19].

Chapter 4. DERMATITIS HERPETIFORMIS

4.1. Rare disease: dermatitis herpetiformis - description, laboratory diagnostic, stage results

Dermatitis herpetiformis is a gluten-sensitive disease with a symmetrical distribution of papua erythematous in the skin. Association with celiac disease is supported by the high rate of immunoglobulin A autoantibody to endomysium in patients with dermatitis herpetiformis, which is a marker with high sensitivity and specificity for celiac disease. Detection IgA EMA by immunofluorescence, although it was considered “golden standard” depends on the experience of the laboratory. tTG was identified as a major antigen recognized by EMA. We selected 10 patients with dermatitis herpetiformis who were previously diagnosed by direct immunofluorescence on presence granular immunoglobulin IgA deposits in papillary dermis to assess Se and Sp of ELISA kits for IgA tTG and biological parameters IgA and IgG AGA. ELISA kits for IgA tTG have a high specificity, comparable to the IgA EMA higher than AGA and can be used as an alternative to EMA in the near future [20].

Elisa Detection methods of anti-IgA tTg tissue has been established for screening gluten enteropathy [21], but the data and correlations presented indicate that the dosage IgA transglutaminase antibodies lead to an accuracy diagnosis in association gluten enteropathy-dermatitis herpetiformis, alongside anti-endomisium antibody determination.

4.2. Dermatitis herpetiformis - a case report [22]

Dermatitis herpetiformis is diagnosed traditionally by direct immunofluorescence and histopathology by skin puncture. Dermatitis herpetiformis direct immunofluorescence shows deposits of IgA along the epidermal junction with dermal papilla concentration towards the ends. The existence of IgA deposits in dermal papillae was accepted as the main diagnostic criterion for dermatitis herpetiformis. But deposits of IgA found by direct immunofluorescence occur in other dermatitis: IgA dermatitis type bulbs, along the base membranous skin areas [23]. In conclusion, diagnosis by direct immunofluorescence depends on laboratory specialist experience.

Chapter 5. BIOCHEMICAL EVENTS IN CELIAC DISEASE

5.1 Biochemical nutritional status in children with celiac disease

Gluten-free diet is the only treatment of patients with celiac disease. This however changes to both the child and family life and its failure leads to nutritional deficiencies and malnutrition. The purpose of this study was to evaluate biochemical nutritional status of children with celiac disease. Our study group consisted of 28 patients with celiac disease and gluten-free under and a group of 11 patients with newly diagnosed celiac disease, that during 2009 we determined immunoenzymatic: tissular IgA tTG, IgA EMA and IgA+IgG DGP in serum and biochemical: aspartate aminotransferase (AST), alanine aminotransaminase (ALT), total calcium (CaT), magnesium (Mg), iron (Fe) in serum and hemoglobin (Hb) in whole blood. The first group of patients we obtained correlations between tissular IgA tTG and IgA EMA, AST and ALT, CaT and Mg, Fe and Hb, with 33.1 % share of factor specific antibody, followed by factors: liver 21.9 %, 18.6 % mineral nutritional status and anemic 18.4 %. The patients in the second we found correlations between tissular IgA tTG and IgA EMA, AST and ALT but no correlations occur between CaT and Mg, Fe and Hb, with 33.1 % share of the liver factor combined with IgA+IgG DGP, followed by the factors: specific antibodies 26.1 % and anemic 23.8 %. In case of celiac disease occur liver function disturbances and mineral nutrients absorptions and iron. If compliance with diet, correlations are normal as in healthy individuals [24].

Studies on nutritional deficiencies in children with celiac disease are rare. Kuloglu et al. [25] and on a lot of 109 children observed iron deficiency, prolonged prothrombin time, hypoalbuminemia, and elevated transaminases significantly more common.
in typical classical forms of celiac disease than in atypical forms of celiac disease ($p < 0.005$).

**Chapter 6. ALLERGY EVENTS IN CELIAC DISEASE**

6.1. IgE-Mediated immune response in celiac disease to the child

Celiac disease appears when there is an inadequate immune response to gluten, a protein that finds itself in wheat. Adverse reactions may also be of allergic nature, because of the production of IgE antibodies against soluble proteins from wheat. The liaison between the two of them, realized by T lymphocytes from the intestinal mucosa, with the possibility of passing from an immune Th1 to a Th2 type during the free-gluten regime in patients with celiac disease is over debated. We found an IgE-mediated immune response in patients with celiac disease and gluten-free diet, 28.5 %, respectively in 8 patients, mostly female, mean age of occurrence of atopic manifestations 5-9 years. But there is no relationship of association between celiac disease and atopic manifestations. Presented clinical observations reveal the existence of moderate IgE immune response in celiac disease after gluten-free diet, and family character of this. We suggest the need to impose a screening program of atopic manifestations during gluten-free diet for patients with celiac disease, by determining total IgE [26].

"Is celiac disease an allergy to wheat?" It’s a question that has opened many discussions. Today, celiac disease and food allergies are recognized as distinct entities. However, Silano et al. [27] observed in vitro, p10mer, a decapeptide from durum wheat, has shown the ability to switch a patient’s immune response disease with celiac disease by a Th1 type immune response to a Th2 type, and these molecules able to move the immune response from Th1-type one to a Th2-type immune response could be used as therapeutic agents in autoimmune diseases with Th1 immune response.

**Chapter 7. VIRAL ASSOCIATIONS IN CELIAC DISEASE**

7.1. Adenoviruses in celiac disease in children

Celiac disease or gluten intolerance is often triggered by viral infections, caused by hepatitis C virus and Adenovirus 12. There are authors who say that the disease becomes active after such infections. Viral agent acting through different pathogenic mechanisms, such as molecular mimicry, which results in modulation of immune tolerance of the host organism. Understanding the relationship between infectious agents and the immune response may help predict an early diagnosis and also to prevent celiac disease. The purpose of this study is to determine the presence of serum antibodies to other subtypes of IgA adenovirus (IgA Ad) in patients with celiac disease and see if it can establish a relationship of association between these two elements. Our study was conducted in a group of 80 children diagnosed with celiac disease, treated and untreated, which we highlighted in 2010, IgA Ad by ELISA. Of the total patients, 25 patients (31.2 %), mostly female, aged between 6 and 9 years, were found with IgA Ad. A statistical significant association between celiac disease and IgA Ad ($p = 0.002$) can be reported in patients with treated celiac disease, which had values of IgA tTG between 0-100 U/ml. There is an IgA Ad activity in celiac disease in children, being in close relationship and with occurrence in celiac disease regression [28].

Involvement of infectious agents in the pathogenesis of many autoimmune diseases through different pathogenic mechanisms such as molecular mimicry, resulting modulation of the host immune tolerance has long remained speculative level [29]. Our study brings some clarifications.

7.2. Screening for celiac disease in chronic viral hepatitis C.

Understanding the relation between the infectious agents and autoimmunity is important for early diagnosis and also for preventing celiac disease, and for its clinical implications during interferon treatment. As such, our proposal in celiac disease screening is immunoassay determination of IgA tTG in hepatitis C before and after interferon treatment [30].

Why we decided to make this bibliographic study? In 2009, Silano M. et al. [31] identified that of the 3725 patients enrolled in the Celiac Disease Italian Register, 34 persons (0.91%), which had hepatitis C on celiac diagnosis. For the diagnosis of Hepatitis C Virus, they have taken in consideration only serological antibody positivity anti-Hepatitis C Virus, because some patients have been diagnosed with hepatitis C at the beginning of ‘80s when molecular tests detection of viral antigens were not yet available.

**Chapter 8. IMMUNOLOGICAL ASSOCIATIONS IN CELIAC DISEASE**

8.1. IgG-F-actin antibodies in celiac disease and dermatitis herpetiformis

Anti-actin antibodies are found in 52-85% of patients with autoimmune hepatitis or chronic active hepatitis and in 22 % of patients with primary biliary
Cirrhosis. In patients with celiac disease, anti-actin antibodies correlate with the degree of villous atrophy. Studies on their involvement in celiac disease and dermatitis herpetiformis in Romania have not been done. The purpose of this study was to evaluate the quality of IgG anti-F-actin antibodies (IgG AAA) tests compared with IgA tTG having IgA EMA as gold standard in celiac disease and dermatitis herpetiformis and to see if there is any relationship between them. The study included 70 pediatric patients with celiac disease under gluten-free diets and 10 adult patients with dermatitis herpetiformis, during 2010. The IgG AAA antibodies levels were determined by ELISA. Assessing the qualities of IgG AAA compared to IgA tTG, we obtained the following values Se 27.8 %, Sp 79.4 %, respectively Se 88.9 %, Sp 79.4 % in celiac disease and Se 33.3 %, Sp 100 %, respectively Se 100 %, Sp 100 % in dermatitis herpetiformis. Also, there was a prevalence of 24.3 % and 30 % of IgG AAA in the two groups of patients, but no statistically significant associations were found. Therefore, we concluded that IgG AAA can not replace IgA tTG in children patients with celiac disease under gluten-free diets and in adult patients with dermatitis herpetiformis. IgG AAA serum activity in both diseases exist, but without a relationship of association with them [32].

Our results are comparable with other European studies. In 2007, Bonaci-Nikolic et al. [33] compared serological and clinical presentation of 38 adults (5 males, 33 females) and 37 children (15 boys, 22 girls) with anti-endomysial antibodies. They found no differences in frequency of antinuclear, anti-parietal, anti-thyroglobulin, anti-smooth muscle, anti-mitochondrial and anti-actin antibodies in children and adults.

8.2. Celiac disease and variable immunodeficiency (case report)

Chronic variable immune deficiency (CVID) syndrome is an entity that should be considered in patients with recurrent infections. When it is associated marked weight hypotrophy is a possible comorbidity. The aspect of Marsh 3C celiac disease found in our patient is not yet reported in Romania [34].

Duodenal villous atrophy is very frequent in symptomatic CVID patients, with relevant clinical and immunological implications [35], but the relationship between primary immunodeficiency syndromes and gastrointestinal manifestations is not yet well defined.

Chapter 9. HEALTH EDUCATION

9.1. Importance of the Educational Environment in the Evolution of Celiac Disease

We serologically monitored the evolution of patients with celiac disease under a gluten-free diet, in correlation with the family’s level of education. Our study was performed in a representative sample of 50 children with celiac disease, in whom we monitored the evolution of IgA tTG during 2008-2009. In the children of parents with a primary education (38 %), the evolution of IgA tTG was intermittent; in the children of parents with a secondary education (44 %), the evolution of IgA tTG was decreased but with many intermittences; and in the children of parents with a higher education (18 %), the evolution of IgA tTG was decreasing, without intermittences. The role of a gluten-free diet is not completely understood in the families of children with celiac disease, particularly in those with primary education [36].

Non-adherence to a gluten-free diet is frequently reported in adolescents with celiac disease. Olsson et al. [37], in a study performed in 47 adolescents with celiac disease, showed that these experienced various dilemmas related to the gluten free diet (ie, insufficient knowledge, reluctance to accept gluten-free food products, and insufficient social support).

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