Proteomic plasma analysis of patients with ventilator-associated pneumonia

Análise proteômica do plasma de pacientes com pneumonia associada à ventilação mecânica

Lucianna Auxi Teixeira Josino da Costa[1], Marina Duarte Pinto Lobo[2], Semiramis Silva Santos[3], Renato de Azevedo Moreira[4], Tamara Soares de Oliveira Araripe[5], Ana Cristina de Oliveira Monteiro Moreira[6]

[1]Univerty of Fortaleza (UNIFOR), Fortaleza-CE, Brazil; [2]Federal University of Ceará(UFC), Biology Department, Fortaleza-CE, Brazil; [3]Ceará Cancer Institute (ICC), University of Fortaleza (UNIFOR), Fortaleza-CE, Brazil; [4]University of Fortaleza (UNIFOR) -CE, Labratory of Proteomic Analysys, Fortaleza-CE, Brazil; [5]Ceará Cancer Institute (ICC), University State of Ceará (UECE), Fortaleza-CE, Brazil; [6]University of Fortaleza (UNIFOR, Laboratory Proteomic Analysis, Fortaleza-CE, Brazil.

Abstract

Objective – The presence of biomarkers in ventilator-associated pneumonia (VAP) can help bedside diagnosis. Mass spectrometry can be used to detect both microorganisms and proteins associated with certain pathologies, called biological markers or biomarkers. **Methods** – In this study 50 patients with pulmonary infection associated with mechanical ventilators and 40 health subjects were investigated and 11 proteins were differentially expressed by ESI-TOF, five in the patients and six in both groups of subjects. **Results** – Kappa light chain immunoglobulin, serum amyloid A1/A2, C-reactive protein (CRP) and heparin cofactor 2 (HC2) were expressed in the plasma of patients hospitalized for lung infection. From the remaining six proteins, two were down-regulated in patients comparing to controls, the apolipoprotein AII and the alpha 2HS glycoprotein. Four proteins were up-regulated in patients: complement C9, alpha 1 acid glycoprotein, alpha 1 antichymotrypsin and leucine-rich alpha 2 glycoprotein. The group where the tracheal aspirate showed fungal growth did not express the proteins alpha 2HS glycoprotein and heparin cofactor 2. **Conclusions** – Leucine-rich alpha 2 glycoprotein was higher in the group with fungi comparing to the others, with a ratio of 5.75. This is the first Brazilian study, so far as we know, analyzing the plasma of patients with ventilator-associated pneumonia applying proteomics techniques (ESI-TOF).

Descriptors: Pneumonia; Proteomics; Lung diseases; Mechanical ventilator

Resumo

Objetivo – A presença de biomarcadores na pneumonia associada à ventilação mecânica (PAV) pode auxiliar no diagnóstico à beira do leito. A espectrometria de massa pode ser usada para detectar tanto microrganismos quanto proteínas associadas a determinadas patologias, chamadas de marcadores biológicos ou biomarcadores. **Métodos** – Neste estudo foram investigados 50 pacientes com infecção pulmonar associada a ventiladores mecânicos e 40 indivíduos saudáveis e 11 proteínas expressas diferencialmente por ESI-TOF, cinco nos pacientes e seis em ambos os grupos de indivíduos. **Resultados** – Imunoglobulina de cadeia leve kappa, amilóide sérica A1/A2, proteína C reativa (PCR) e cofator 2 de heparina (HC2) foram expressos no plasma de pacientes internados por infecção pulmonar. Das seis proteínas restantes, duas foram reguladas negativamente em pacientes em comparação aos controles, a apolipoproteína AII e a glicoproteína alfa 2HS. Quatro proteínas foram reguladas positivamente nos pacientes: complemento C9, alfa 1 glicoproteína ácida, alfa 1 antiquimotripsina e alfa 2 glicoproteína rica em leucina. O grupo onde o aspirado traqueal apresentou crescimento fúngico não expressou as proteínas alfa 2HS glicoproteína e heparina cofator 2. **Conclusão** – A alfa 2 glicoproteína rica em leucina foi maior no grupo com fungos em relação aos demais, com razão de 5,75. Este é o primeiro estudo brasileiro, até onde sabemos, analisando o plasma de pacientes com pneumonia associada à ventilação mecânica aplicando técnicas de proteômica (ESI-TOF).

Descritores: Pneumonia; Proteômica; Doenças pulmonares; Ventiladores mecânicos

Introduction

Ventilator-associated pneumonia (VAP) is an infection that occurs after 72 hours in patients with ventilator-associated orotracheal intubation[1]. Cultures are important for microbiological rescue determination to direct treatment, but do not always show positive results. Growth takes from 24 hours to 7 days in the case of some fungi. This can delay treatment, even leading to death in extreme cases[2,3].

Because of this situation, some biomarkers have been found to be related to VAP, but still without achieving the desired specificity to corroborate the diagnosis. One of the reasons may be the lack of unanimity of definitions in the scientific community, leading to confounding factors in the use of biomarkers[4].

Some biomarkers have been used by researchers in clinical trials to make decisions on the treatment of VAP or nosocomial sepsis. The main biomarkers studied have been C-reactive protein, procalcitonin and sTREM-1, with controversial results for all three[5]. Proteomic analysis with mass spectrometry is also used to verify biological markers in the presence of a specific nosological entity, especially in infectious ones where the microbiological finding is slow[6].

Methods

This article reports a prospective, descriptive and analytical study conducted between January and May 2016.

The patients with pulmonary infection associated with mechanical ventilators were hospitalized in the Adult Intensive Care Unit of General Hospital of Fortaleza, where clinical data and plasma samples were collected. The proteomic analyses were performed in the Proteomic Analysis Laboratory of the University of Fortaleza (UNIFOR).

Sixty-seven patients with pulmonary infection were selected for this study, but 20 did not meet specific criteria for clinical diagnosis and 6 had other complications such as pneumothorax and acute respiratory distress syndrome (ARDS) or died in less than 48 hours, leaving 50 patients, who were followed until hospital discharge or death. Forty healthy people in the community made up the control group, with age and sex similar to the patients. Plasma was collected from patients diagnosed with ventilator-associated pneumonia within 48 hours after clinical worsening. The diagnosis of infection was given by worsening of the clinical aspects of hemodynamic instability and use of vasoactive drugs (noradrenaline and/or vasopressin), worsening of the respiratory condition with the increase of the inspired oxygen fraction (FiO2) and/or increased PEEP, worsening of the appearance of tracheal secretion and change of antibiotics by the attending physician. Patients with odds of death in the next 24-48 hours, under 18 years of age, without a family member, tracheostomized, with acute respiratory distress syndrome (ARDS) or new infection with more than 48 hours were excluded from the study. Results of cultures of the tracheal aspirate were recorded and their results were used to divide the patients into four groups, the fifth being the control group.

Group 1: Pulmonary infection and aspirated isolated gram-negative *bacilli*

Group 2: Pulmonary infection and aspirated isolated *fungi*

Group 3: Pulmonary infection and aspirated without bacterial/fungal growth

Group 4: Pulmonary infection and aspiration not tested

Group 5: Controls

Each patient was followed until discharge or death (ICU or hospital). The protocol was approved by the hospital's ethics committee.

Laboratory Techniques

Blood was collected by peripheral venipuncture in tubes containing sodium citrate. The samples were centrifuged at 5,000 xg for 5 min and the plasma obtained was stored at -80 ° C for later use.

Protein quantification

Plasma samples were individually quantified by absorbance at 280 nm using a NanoVue Plus (GE Healthcare) spectrometer.

Depletion chromatography

A 100 µL aliquot of the plasma with 200 µL of buffer was filtered through a 0.22 µM membrane and a volume of 150 µL of filtered plasma was applied to a HiTrap Albumin & IgG Depletion 1mL column (GE Healthcare, USA) Coupled to an ÄKTA 10 FPLC purifier system (GE Healthcare, USA). The column was equilibrated with 20 mM of Tris-HCl equilibration buffer, pH 7.4, containing 0.15 M NaCl, to elute the protein fraction that interacted with the matrix, i.e., the buffer (glycine-HCl, 0.1 M pH 2.7) was added to the albumin-rich fraction and IgG.

Chromatography of the pools was carried out at a constant flow rate of 1 ml/min, with 200 µl collections and monitoring of the protein elution by absorbance at 280 nm. Upon completion of the chromatographs, pool fractions were dialyzed against ultrapure water and concentrated by ultrafiltration in Vivaspin concentrators (GE Healthcare, USA) with a cutoff of 3 kDa.

Mass spectrometry

Initially, the dialyzed and concentrated pool fractions containing serum proteins were submitted to tryptic digestion. The samples were treated with a 0.2% surfactant solution (RapiGest SF, Waters, Milford, USA) added to a 50 mM NH_4HCO_3 solution containing a 100 µg protein fraction. The mixture was incubated for 15 min at 80°C. Then 5 µL 100 mM dithiothreitol was added to each sample for protein reduction, facilitating digestion. The tube was immersed in a dry bath at 60°C. After incubation for 30 min, 5 µL 300 mM iodoacetamide was added to alkylate the cysteins. The

Table 1. Single proteins found in groups according to tracheal aspiration

Gram-negative	Fungi	No growth	Not performed	Controls
Group I	Group II	Group III	Group I	X
Kappa light imunoglobulim chain VIII	Kappa light imunoglobulim chain VIII	Kappa light imunoglobulim chain VIII	Kappa light imunoglobulim chain VIII	X
Serum amyloid A1/A2	Serum amyloid A1/A2	Serum amyloid A1/A2	Serum amyloid A1/A2	X
C-Reactive Protein	C-Reactive Protein	Not found	C-Reactive Protein	X
Heparin Cofator 2	Not found	Heparin Cofator 2	Heparin Cofator 2	X

samples were stored for 30 min in a dark place at room temperature to allow for alkylation, followed by the addition of 1 µg trypsin and digestion in an incubator for ~16 hours at 37°C. Finally, the sample was centrifuged at 12000 g for 30 min and the supernatant transferred to appropriate vials. Tryptic peptides of yeast alcohol dehydrogenase (ADH, UniProt P00330) were added to the digested proteins to serve as standard in the absolute quantification of each sample. Chromatography was performed on a nanoACQUITY C18 UPLC BEH (1.7 µm, 100 µm×10 cm) reversed-phase column with 130 Å pores using a 0-40% gradient (v/v) of acetonitrile containing 0.1% formic acid at 500 nL/min. A 200 ng aliquot of sample containing the peptides obtained by protein digestion was used. All samples were applied in triplicate and protein identification and quantitative analyses relied on dedicated algorithms and searches in species-specific databases. The data were managed with the software MassLynx v4.1 and ProteinLynx v2.4, while database searches were performed with the package ProteinLynx Global Server (PLGs) v.2.4. containing the software ExpressionE v.2.4. Searches in the databases UniProtKB/Swiss-Prot 57.1 and UniProtKB/TrEMBL 40.1 were conducted specifying the taxonomy of Homo sapiens. The identified proteins were listed according to condition, i.e., proteins identified in the triplicates of the pools and found in 100% of the samples of a given group. Only proteins above the 95% confidence limit were considered acceptable results in database searches. The before/after immunonutrition ratios of the 3x3 filter were <0.66 for 'down' and >1.5 for 'up'.

Results

Fifty patients met the inclusion criteria and were divided according to the tracheal aspiration results into four groups (Gram-negative, Fungi, No growth and Not performed), the fifth being the control group. From hundreds of proteins expressed by the patients, 11 were chosen for being present in all the groups.

Five proteins appeared only in the patients and six appeared in both patients and healthy subjects, but in different patterns of expression (up-expressed or down-expressed) in the control group.

The proteins expressed only in patients with pulmonary infection were: kappa light chain immunoglobulin, serum amyloid A1/A2, heparin cofactor 2 and C-reactive protein. These proteins, however, were not found in all groups. Heparin cofactor 2 was not seen in the group with fungal growth and C-reactive protein was not seen in the patients without growth in the aspirate, as seen in Table 1.

Table 2. Common proteins in the groups according to tracheal aspirated results: Gram-negative, No growth and Not found

Proteins	Up	Down	Ratio		
			Gram-negative	No growth	Not Performed
Apolipoprotein A II		X	0,28	0,26	0,34
Alpha 2HS Glycoprotein		X	0,48	0,38	0,47
Complement C9	X		2,44	2,14	2,20
Alpha 1 Acid Glycoprotein	X		3,03	3,06	3,06
Alpha 1 Antichymotrypsin	X		4,06	3,82	3,97
Leucin Rich Alpha 2 Glycoprotein	X		4,18	3,60	3,25

Table 3. Common proteins in the groups according to tracheal aspirated results: Gram-negative, No growth and Not found

Proteins	Up	Down	Ratio			
			Gram-negative	No growth	Not Performed	Fungi
Apolipoprotein A II		X	0,28	0,26	0,34	0,28
Complement C9	X		2,44	2,14	2,20	2,32
Alpha 1 Acid Glycoprotein	X		3,03	3,06	3,06	2,72
Alpha 1 Antichymotrypsin	X		4,06	3,82	3,97	3,56
Leucin Rich Alpha 2 Glycoprotein	X		4,18	3,60	3,25	5,75

The protein pool found in both groups (patients and healthy controls) consisted of six proteins, for this reason, they were classified as common proteins. This expression varied between both groups, namely: apolipoprotein AII and alpha 2HS glycoprotein had low expression in the infected group while alpha 1 acid glycoprotein, alpha 1 antichymotrypsin, complement C9 and leucine rich alpha 2 glycoprotein were highly expressed in the same group.

As occurred in the group of single proteins, this group also had some details that stood out. In patients with isolated fungi from the tracheal aspirate, the expression of alpha 2HS glycoprotein was not observed. Unlike the expression ratio of the leucine-rich glycoprotein, which was higher in these patients (5.75) than those with isolated gram-negative bacteria or those with no growth in the culture as show in Table 2 and 3.

Discussion

Following the analysis of the five research subject groups, the presence of a protein response pattern was observed in patients called Single proteins: kappa light chain immunoglobulin, Serum amyloid A1/A2 (SAA), heparin cofactor 2 and CRP. Exceptions occurred in the fungal group, where heparin cofactor 2 was not isolated, and in the non-growth group, where C-reactive protein was not expressed.

The other proteins, called Common – apolipoprotein AII (APO AII) and alpha 2HS glycoprotein –, were present in the patients and controls, but in the patients they were in low expression, and complement C9 (CC9), alpha-1 acid glycoprotein, alpha-1 antichymotrypsin and leucine-rich alpha 2 glycoprotein were found to more highly expressed in patients than healthy subjects (controls). An exception was the fungal group, where alpha 2HS was not found and leucine was more prominent.

Proteomic analyses have brought important observations in the field of sepsis and its different disease evolution stages. A Brazilian study found 14 proteins expressed in different stages: serum amyloid a, apolipoprotein A1 (2 isoforms), zinc finger protein 222, human albumin, PRO 2619, kappa light chain immunoglobulin, cold agglutination monoclonal immunoglobulin M 7 and protease inhibitors [7].

Nguyen et al. [8] found 76 different proteins among patients with acute lung injury, which they called the "pulmonary lesion proteomic signature" proteins: S100A8, lactotransferrin and actinin 8. The authors were unable to discern protein differences between patients with and without VAP. Due to the target population with acute lung injury, tissue remodeling proteins (sequelae) were also observed along with the inflammation proteins, a fact not seen in our study because of the absence of patients with acute lung injury.

Alpha-1 acid glycoprotein, also known as orosomucoid, is an acute phase protein produced by the liver, salivary glands, spleen, tongue, lymph nodes, uterus, ovary and lungs in response to infection and inflammation [9]. This protein was found in our work in infected patients. The presence of alpha 1 acid glycoprotein in all groups, even the group with the "no growth" of tracheal aspirate, suggests that even with the reduced number of bacterial colonies, there is expression of the immune response. Perhaps because of the reduction of the bacterial load due to the antimicrobial drug, the number of colonies was not enough for positivity, but the host responses are triggered. In a study conducted with cows suffering from mastitis, the alpha 1 acid glycoprotein was up-regulated in the initial hours of infection, a situation never shown before in animals [10].

Despite the increased detection of A1GPA in all groups (gram-negative, fungal, non-growth and untreated tracheal aspirates) of our study, it was unfortunately not possible calculate correlations with mortality or perform logistic regression because the proteomic analysis was group-wise. The vast majority of patients were in sepsis and all were being treated with antimicrobials. In sepsis, A1GPA gene regulation factors are activated by the cytokine network, involving tumoral necrosis factor-alpha (TNF-alpha), interleukin 1-beta (IL1-beta) and interleukin 6 (IL-6) [11].

We may suggest some explanations for the difference between their results and ours, such as: (i) time difference of sepsis; (ii) the presence of antibiotic treatment in our cohort; and (iii) immune system already activated in our sample by the new infection (hospital). A comparison between community and hospital sepsis with the same measurement technique would be interesting.

Another up-regulated protein in the patients was the alpha 1 antichymotrypsin (A1ACT). It is involved in immunological control by inhibiting chymotrypsin through its cytotoxic effect, leading to elimination of the pathogen [12]. Leucine-rich alpha 2 glycoprotein or simply LEU is another acute phase protein detected in this study more prominently in the group of patients having tracheal aspirate with fungal growth (infection or colonization), in the ratio 5.75.

Leucine is expressed in granulocyte differentiation (versus infection) and is related to angiogenesis. It binds strongly to cytochrome C, similar to the binding of Apaf-1 protein (involved in the caspase pathway, which promotes apoptosis). Unlike Apaf-1, LEU acts as a "survival" factor, protecting against external toxic effects against lymphocytes, which can lead to apoptosis [13]. In this study, there was a strong correlation of LEU with the presence of fungi, with a higher ratio than the other aspirate results.

In addition to the reports of leucine binding with fungi, two studies by the same group regarding diagnosis of appendicitis found LEU in addition to the alpha 1 acid glycoprotein, plasminogen, LPS and alpha 1 antichymotrypsin binding proteins to have favorable diagnostic performance. However, LEU performed the best [14]. Alpha 2 HS glycoprotein (A2HS), also known as fetuin, is produced by hepatocytes and is involved in metabolic signaling pathways. It modulates

inflammation through cellular reuptake of pro-inflammatory inhibitors. Because of this, A2HS prevents strong sequelae of the inflammatory response that could result in the super-production of proinflammatory cytokines [15].

Alpha HS glycoprotein is temporarily depressed during the acute phase of systemic inflammation, classifying this protein as a "negative acute phase protein" [16].

Unlike our findings, in a study of cow mastitis, the authors found it to be up-regulated, along with alfa-1 acid glycoprotein (A1GPA). It is important to note that the difference between the healthy cows that were infected was only 18 hours, which might have influenced the results [10]. Our patients had new lung infection detected at most 48 hours previously, which could explain the down-regulation in our case.

The presence of alfa 2HS may act in counterregulatory signaling of infectious conditions and its administration can influence survival. Mice deficient in this protein were more susceptible to toxic shock [17].

In our study, we did not find A2HS in the group of patients with tracheal aspirate with fungi, perhaps because the presence of this substance inhibits the growth of other fungi such as *Aspergillus* and Histoplasma. A2HS was strongly correlated with this form of fungal protection. In turn, Al-Hakeim et al. [18] compared biomarkers by evaluating A2HS, procalcitonin (PTC) and C-reactive protein (CRP) in febrile seizures in children to determine the microbiological origin. A2HS was reduced when PCT levels increased, and a similar situation was found for CRP levels ($p < 0.0001$), meaning A2HS can be added to the procalcitonin markers for bacterial fever in children.

The second down-regulated protein in our study was apolipoprotein II (APO AII). APO AII is the main component of HDL (high-density lipoprotein) and is involved in innate immunity through the anti-endotoxin function, conferring resistance of the host through phagocytic function and organization of the coagulation function [8]. Apolipoprotein AII declined to a nadir on the third day and then returned to basal levels after four weeks. In contrast to the reduction of apolipoprotein AII, there was an increase in serum amyloid A [19]. These findings are similar to ours: we found low levels of APOAII in patients in the early collection (maximum 48 h) and found serum amyloid A1/A2 only in patients with pulmonary infection.

The complement C9 protein belongs to the membrane attack complex (C5b/9) and is part of the adaptive immune system, dilating pores on the target cells' surfaces, undergoing conformational modification and penetrating the bacteria.

Complement-deficient knockout mice had an inability to eliminate Pseudomonas aeruginosa from the respiratory tract even with the other two pathways intact, and also showed worsening elimination of pulmonary surfactant [20]. Heparin cofactor 2 (HCF2) is an inhibitor of a proteinase called extravascular thrombin and is activated by glycosaminoglycans, heparin or derman sulfate. In the presence of the last substance, HCF2 becomes predominantly a thrombin inhibitor in place of antithrombin III. It also inhibits chymotrypsin. N-terminal peptides of HCF2 have chemotactic activity against both monocytes and neutrophils.

In turn, Kalle et al. [21] detected with a flow cytometer (FACScalibur) the release by HCF2a of a 50 kDa structure (KEY28) with high hydrophobicity that led to the disintegration and expulsion of gram-negative intracytoplasmic components by blocking the action of the generated intracellular NF-kB/AP-1 by LPS. We did not find this protein in the group of patients with fungi in the tracheal aspirate. As seen in the second work of Kalle and colleagues, protein transcription only occurred in the presence of gram-negative bacteria.

Free light chains are produced by B lymphocytes during the process of antibody synthesis, as a reflex of activation of plasma cells. They function as a signal that adaptive immunity was triggered [22]. In addition to antigens, they also bind directly to neutrophils, reducing their apoptosis and increasing in plasma during inflammation/infection [23].

The presence of the kappa free light chain in our work indicates the activation of acquired immunity in hospital infection cases directed to certain specific agents, such as *Acinetobacter* and *Pseudomonas* (mainly resistant strains), making it possible to propose vaccine prophylaxis, either against gram-negative bacteria or reverse, protective for the patient [24].

C-reactive protein (CRP) in our study was present in patients but not in healthy subjects. It is involved directly in the elimination of microorganisms, degranulation of platelets and increased NK cell activity. In addition to the liver, CRP can be produced and released into the lungs and can be measured [25].

The suspicion of infection is related to its serial increase (2-3 days), so its quantification associated with clinical scores can help diagnosis and monitoring of responses [26]. The release of CRPs such as serum amyloid can impair the restoration of lung cells [27]. This also applies to partially resistant strains, where antimicrobial treatment is not effective in eliminating the pathogen [28]. The change of the CRP decline over the course of days, the high concentration ratio and the amplitude variation were significantly associated with the appearance of VAP, showing that their kinetics are more important than the relative number [29].

CRP was not found in our study in patients in the group with no growth in tracheal aspirate cultures, perhaps because of the low bacterial load.

Serum amyloid A (SAA) was found in all our groups, even in patients who did not have microbiological growth. This is also an acute phase protein, involved in the chemotaxis of leukocytes and macrophages, IL1 secretion, platelet activation, cell adhesion and regulation of other proteins. The production of SAA can be associated with VAP or not [30].

SAA was found in the first 48 h of respiratory worsening in our group, contrasting with the results of Paiva, who found it in the second stage of sepsis [7].

The use of biomarkers to diagnose community or hospital infection is still very controversial. The innate immune system still needs to be better understood. In hospital environments, the relationship with resistant bacteria is increasingly reported. For a while the analysis of this relationship involved mainly bacterial genetics, which brought many advances. Despite this knowledge, mortality is still very high and independent of the biomarker discovered. Therefore, before clinical use a biomarker should be submitted to laboratory tests and statistical analysis.

Conclusions

In the ESI-TOF proteomic analysis, the profile of 11 infection-related proteins was found. Five proteins were seen only in patients with ventilation-associated infection: kappa light chain immunoglobulin, C-reactive protein, serum amyloid A1/A2 and heparin cofactor 2. Six proteins were found differently in healthy controls and patients, two being down-regulated in the patients (apolipoprotein A-II and alpha 2 HS glycoprotein) and four up-regulated in the same group (alpha 1 acid glycoprotein, alpha 1 antichymotrypsin, complement c9 and leucine-rich alpha 2 glycoprotein).

In the six patients with fungal growth in the tracheal aspirate, the findings regarding three proteins were noteworthy: heparin cofactor 2 and alpha 2HS glycoprotein were not found and leucine-rich alpha 2 HS glycoprotein was higher in the group of patients with bacteria isolated in a ratio of 5.75, suggesting a differential between microorganisms.

In summary, we found a plasma protein profile of infected patients, detected microbiologically or not, with some peculiarities between bacteria and fungi. The presence of proteins of the immune system and interconnected systems such as lipid metabolic, coagulation, complementary, adaptive, protease inhibitor and acute phase systems, demonstrates an inflammatory reaction and anti-inflammatory situation, and can be interpreted as indicating acute infection with a counterregulatory response. The joint profile was approached and related to similar studies for better understanding.

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Corresponding author:

Tamara Soares de Oliveira Araripe
Street Papi Júnior, 122
Fortaleza-Ce, CEP 60430-230
Brazil

Email: tamara.araripe@icc.org.br

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