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Research Article

Salt and Microbiota: Respiratory Tract Microbiota of Cystic Fibrosis Patients

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Abstract

Cystic fibrosis (CF) is an autosomal recessive disease most common in the Caucasian population. It is caused by mutation in Cystic Fibrosis Transmembrane Conductance Regulator (CFTCR). This protein is involved in the transport of anions in the cells but also the regulation of sodium absorption in the respiratory tract. The study of salinity in the airways of CF patients through sputum allowed us to show that sputum of CF patients are more saline than patients without the disease. Moreover, this difference in salinity has an impact on the microbiota of the respiratory tract, salt-tolerant fungi were isolated only in the sputum of CF patients.

Keywords: *Cystic fibrosis (CF); Cystic Fibrosis Transmembrane Conductance Regulator (CFTCR); Salt; Microbiota*

Introduction

Cystic fibrosis or mucoviscidosis is recessive autosomal disease that affects nearly 80,000 persons worldwide. It is widespread in the Caucasian population. In France this disease affects one out of 4,500 newborns. It is due to a gene mutation, CFTR on the long arm of chromosome 7 [1-3].

Clinical manifestations of the disease, including bronchopulmonary infections, intestinal malabsorption, male sterility, are caused by abnormal expression and function of a defective protein, CFTR [4].

The CFTR gene encodes the CFTR protein, a multifunctional glycoprotein of 1,480 amino acids. It is a cAMP anion channel expressed in a wide variety of tissues such as the lungs, pancreas, liver, reproductive tract and intestine. The CFTR protein forms a chlorine channel in the apical membrane of exocrine epithelial cells regulating and participating in the transport of electrolytes across cell membranes. It carries the anions inside and outside the cell. The transport of chlorine ions helps to control the movement of water in the tissues. This hydration is necessary for the production of fluid mucus. Mucus is a substance that lubricates and protects the walls of the respiratory tract, the digestive system and other tissues. CFTR is also involved in regulating the absorption of Na+ ion in the airways.

In the case of cystic fibrosis, the absence or dysfunction of the CFTR protein theoretically leads to a hyper-absorption of sodium from the airway fluid. This involves osmosis water absorption causing dehydration of the airway fluid preventing the flutter of the cilia which retains mucus.

There are more than 2000 types of CFTR gene mutations, the most common in populations in Europe and North America being a deletion of phenylalanine at position 508 (ΔF508) CFTR. There are currently 6 types of mutations in the CFTR gene. Class I muta-

tions that are most common mutations whose result is little or no expression of the protein. Class II mutations that result in the synthesis of a CFTR protein that is not adequately expressed within the apical membrane of cells. The CFTR mutation belongs to this second class.

There are several diagnostic methods for cystic fibrosis including the Nasal Potential Difference (NPD) and sweat test which is the standard diagnostic test. It is a minimally invasive method by which sweating is stimulated and the sodium chloride concentration of sweat is measured. The salinity threshold depends on the collection method used. There is also a test for measuring osmolarity in respiratory tract.

On the one hand, there are animal models that show that there is a difference in salinity in the airways of animals with the cystic fibrosis gene and a control group [5]. Also studies have been done *in vitro* [6]. However, the difference in osmolarity has never been studied in the respiratory tract in humans.

Patients with cystic fibrosis are prone to respiratory tract infections; however there are very few cases of *Mycobacterium tuberculosis* infections. A recent study in our laboratory shows the sensitivity of certain mycobacteria including *Mycobacterium tuberculosis* to salt showing an inverse correlation between salt tolerance and salt adaptation [7].

Objective for the Study

Our objective for this study is to determine the salinity in CF patients sputum and to compare them with a control group's. Also we will determine the impact of this salinity on the microbiota of the respiratory tract in patients with cystic fibrosis and the control group.

Materials and Methods

Ethical conditions

This study has been approved by the local ethics committee of IFR48, Faculty of Medicine of Marseille under the reference N° 07-008. No written consent was necessary for this study in accordance with the law N° 2004-800 relating to bioethics published in the official journal of the French Republic on August 6, 2004 because no additional sample was collected for this work.

Samples

The samples used are sputum collected at adult and child hospital of Timone (Marseille) from January to June, 2016. The samples were collected spontaneously (without the use of medication). Bronchial aspirations and bronchopulmonary lavage were excluded from the collection.

Salinity measurement

For each sample 500 µL of sample were used. In a 1.5 mL Eppendorf tube we put 500 µL of sterile distilled water and 500 µL of sample. The diluted samples were vortexed and incubated for 1 hour at 95°C. Then they were cooled for 5 minutes at room temperature. Samples are then centrifuged at 10,000g for 10 minutes. The supernatant was used for Gram stain to ensure that no cellular or bacterial debris interfered with the salinity measurement. This same supernatant was used for the measurement of salinity by spectrophotometry using the Digital Refractometer (PR-100SA, Atago). With a Pasteur pipette, about 200 µL/300 µL of the supernatant were deposited on the surface of the prism. After each sample the prism was decontaminated with alcohol and the zero was carried out by sterile distilled water.

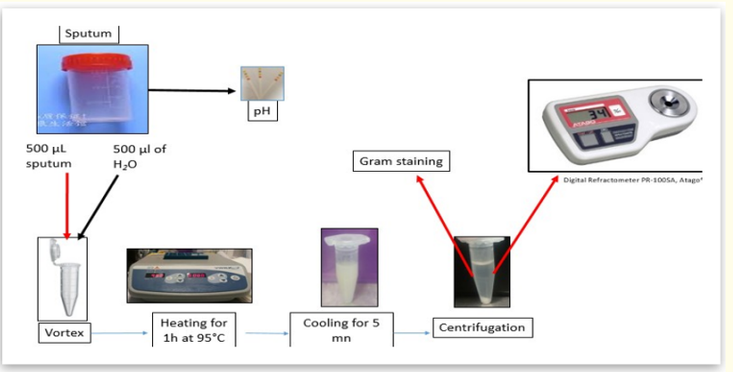

Figure 1: *Sputum salinity and pH measurement*

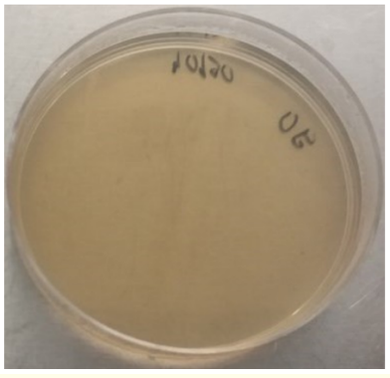

Figure 2: *Seawater medium 2%.*

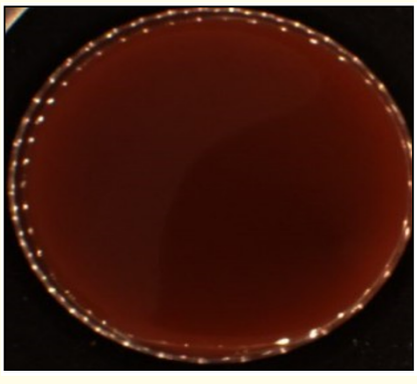

Figure 3: *Seawater medium+sheep blood 2%.*

pH measurement

For each sample the pH was measured with pH paper according to the supplier's instructions (Sigma-Aldrich, Saint-Quentin Fallavier, France).

Culture media

Two types of culture media were used:

For 1l of medium

- We put in 500 ml of distilled water with magnetic stirring: Casein hydrolyzate: 12g, Proteose peptone: 5g, Yeast Extract: 3g, Beef Extract: 3g, Potato Starch: 1g Sodium Chloride: 5g, Agar: 13.5g. The medium is autoclaved. After 1 hour at 56°C, 500 ml of sea water filtered at 0.4 µm and then 0.2 µm was added and 50 ml of defibrinated sheep blood and the media arepoured.
- We put in 500 ml of distilled water with magnetic stirring: Casein hydrolyzate: 12g, Proteose peptone: 5g, Yeast Extract: 3g, Beef Extract: 3g, Potato Starch: 1g Sodium Chloride: 5g, Agar: 13.5g. The medium is autoclaved. After 1h of time at 56°C, 500 ml of sea water filtered at 0.4 µm and then 0.2 µm was added and the media were cast. 1 µL of each sample is inoculated on each medium.

MALDI-TOF MS Mass Spectrometry

Identification by MALDI-tof mass spectrometry was done in triplicate using a Vitek-MS spectrometer (bioMérieux, La Balme les Grottes, France). The results have been validated when the identification score is greater than or equal to 1.7 and the first spot must be in agreement with the other 2 spots. In each plate, Escherichia coli was used as a positive control and the colony-free (a-cyano-4-hydroxycinnamic acid) matrix solution was used as a negative control.

Statistical analysis

The Student t test parametric test is used to compare the cystic fibrosis group and the non-cystic fibrosis group. A value of $p < 0.05$ is used to evaluate the significant difference in comparisons.

Results

30 samples have been analysed: 15 of cystic fibrosis patients and 15 of non-cystic fibrosis patient. The average salinity in patients with cystic fibrosis is 9.8 ± 3 g/L while it is 6.76 ± 2.84 g/L in the control group. This difference in salinity is significant ($p < 0.05$, t Student test).

	Cystic fibrosis		Non-cystic fibrosis	
	Men	**Women**	**Men**	**Women**
Age	22.3 ± 18.5	18.6 ± 13.4	42.4 ± 21	25.6 ± 21.9
Gender	9	6	9	6
Salinity	10 ± 2.6	9.5 ± 4.8	6.9 ± 3.1	6.5 ± 2.6
pH	5.8 ± 0.7	5.9 ± 1.1	5.8 ± 1.2	5.5 ± 1.6

Bacteria in cystic fibrosis patients sputum

With our seawater media, 15 bacterial species have been isolated among which the most represented species are *Staphylococcus aureus* (43%), *Escherichia coli* (13%), *Rothia dentocariosa* (11%) and *Pseudomonas aeruginosa* (10%).

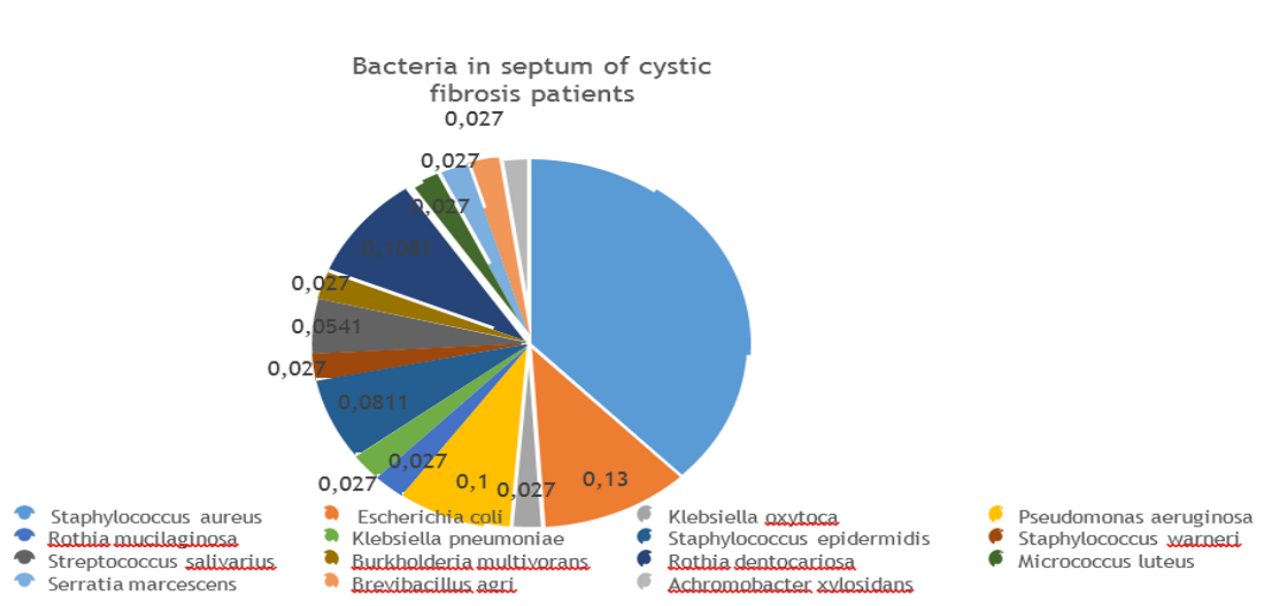

Figure 4: *Bacteria in cystic fibrosis patients sputum.*

Fungi in cystic fibrosis patients sputum

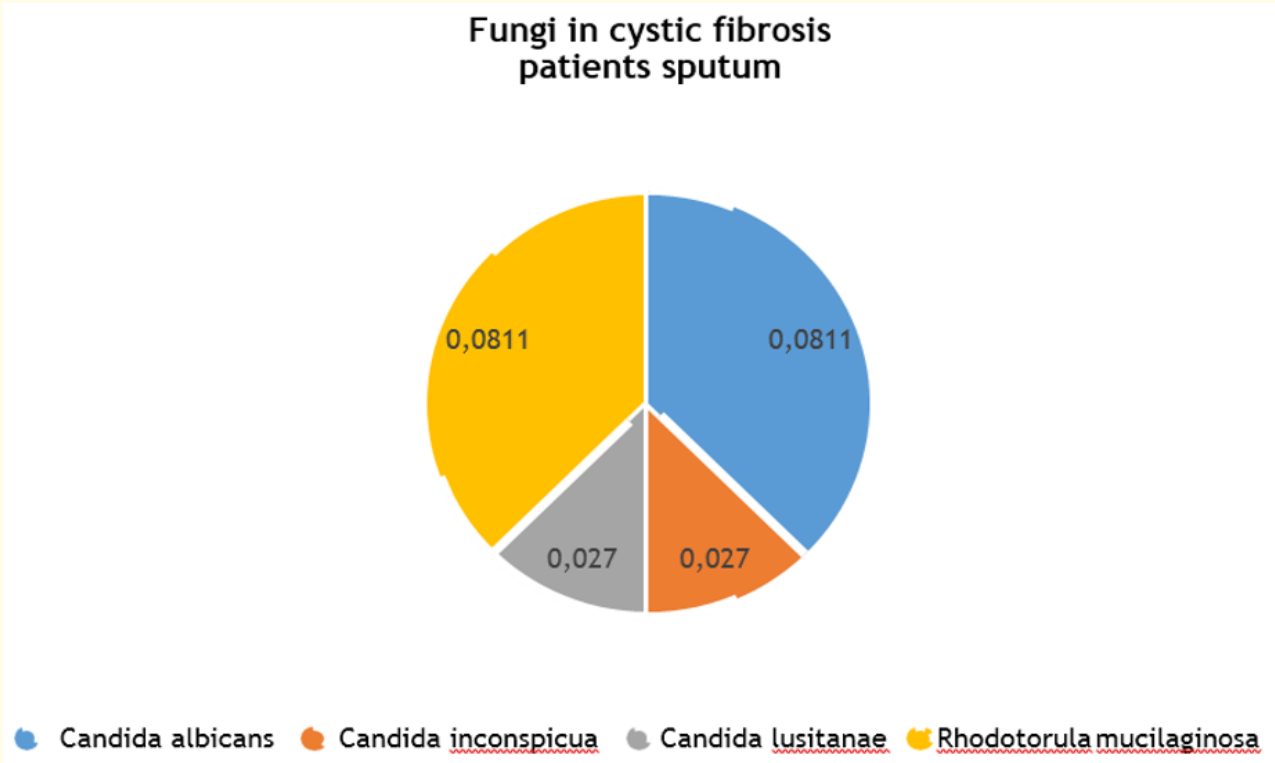

Figure 5: *Fungi in cystic fibrosis patients sputum.*

Fungi species have also been isolated in patients with cystic fibrosis, namely 8% *Candida albicans* and *Rhodotorula mucilaginosa* and 3% *Candida inconspicua* and *Candida lusitaniae*.

Bacteria in non-cystic fibrosis patients sputum

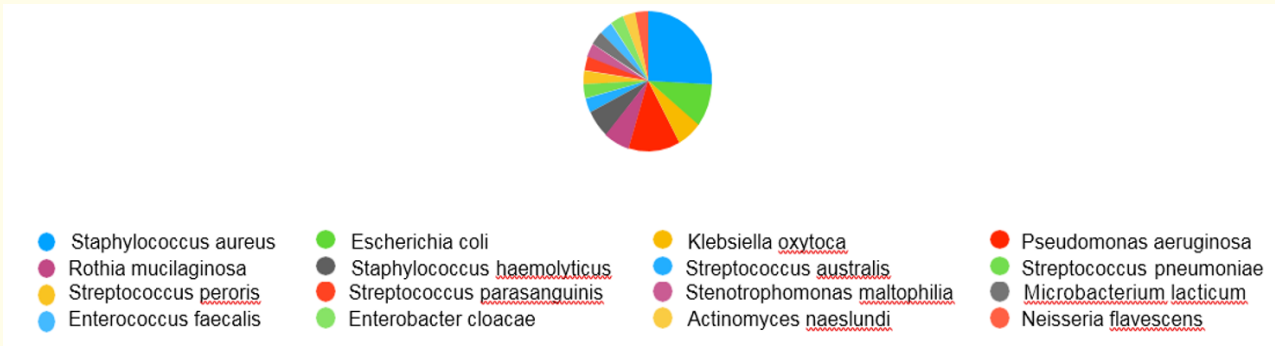

Figure 6: *Bacteria in non-cystic fibrosis patients sputum.*

In the sputum of patients of the control group, we were able to isolate 14 bacterial species, the most represented being *Staphylococcus aureus* (24%), *Pseudomonas aeruginosa* (12%) and *Escherichia coli* (9%).

Five bacteria: *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella oxytoca* and *Rothia mucilaginosa* were isolated both in the sputum of patients with cystic fibrosis than the control group. We did not isolate fungi in the sputum of the control group.

Discussion

This study shows a significant difference in sputum salinity in cystic fibrosis patients compared to patients in the control group. Sputum from patients with a salinity average 9.8 g/L higher ($p < 0.05$) than those from the control group. And we have a higher salinity trend in male patients with cystic fibrosis compared to male control group patients ($p < 0.05$). This difference is not correlated with the sex of the patients because the sex ratio of the patients used in this study to that of the control group do not differ significantly.

In this study, patients with cystic fibrosis are significantly younger than the control group, however one observation shows that in patients with cystic fibrosis, salinity increases with age. Thus this age difference would not influence the general conclusion of this work.

pH and salinity measurements were performed after protein extraction by heating to limit interference on the measurements. Protein extraction can release cell salts and cause bias in the measurement. We have experimented with the same measurement method without prior protein extraction and it does not affect the salinity of the sample. Also, exactly the same protocol was used to analyze the control group samples.

Unexpectedly, no comparable work has been published in the literature. The study of the osmolarity of the airways during cystic fibrosis has been carried out in animal models or *in vitro* [7].

There is no significant difference in pH between our two groups compared which is in line with the data from the literature [8].

This study revealed the usually isolated bacteria in the sputum of patients with cystic fibrosis [9], mainly *Pseudomonas aeruginosa* (10%), *Staphylococcus aureus* (43%) and *Escherichia coli* (13%). The culture medium used for the culture of the micro-organisms has a salinity of 2%, four mushrooms were isolated only in the sputum of the patients with cystic fibrosis in particular *Candida albicans*, *Candida inconspicua*, *Candida lusitaniae*, and *Rhodotorula mucilaginosa*. Tests on four *Candida* species: *C. albicans, C. dubliniensis*, *C. glabrata*, and *C. parapsilosis* have a salt affinity. *Candida albicans* that we found in the sputum of patients with cystic fibrosis is a halophile mushroom [10]. This is in line with our findings and our initial hypothesis that sputum of patients with cystic fibrosis is more salty than that of patients free of the disease is confirmed. The growth of halophilic mushrooms such as *Candida albicans* is proof of this.

Rhodotorula mucilaginosa is also a fungus that we could isolate only within the sputum of patients with cystic fibrosis. It is also a halophilic mushroom [11].

According to our study, the difference between the sputum of patients with cystic fibrosis and non-cystic fibrosis patients resides in the level of salinity and by extension on the colonization of the microbiota by halophilic fungi. This salinity could be the factor protecting cystic fibrosis patients against certain pathogens such as *Mycobacterium tuberculosis*. As a prospect, it would be interesting to culture *Mycobacterium tuberculosis* in sputum from cystic fibrosis patients and to compare them with a control group.

Sputum salinity could be a non-invasive diagnostic test for cystic fibrosis just like the sweat test.

Conclusion

The study of the salinity and pH in the respiratory tract of cystic fibrosis patients through sputum allowed us to show that the sputum of patients with cystic fibrosis is significantly saltier than those of patients free of the disease, without significant difference in pH. This

difference in salinity is correlated with the composition of the respiratory tract microbiota, as we have isolated halophilic fungi from the sputum of cystic fibrosis patients.

Bibliography

1. Lubamba B., *et al*. "Cystic fibrosis: insight into CFTR pathophysiology and pharmacotherapy". *Clinical Biochemistry* 45.15 (2012): 1132-1144.

2. Dalcin Pde T., *et al*. "Cystic fibrosis in adults: diagnostic and therapeutic aspects". *Jornal Brasileiro de Pneumologia* 34.2 (2008): 107-117.

3. Hou Y., *et al*. "Emerging role of cystic fibrosis transmembrane conductance regulator - an epithelial chloride channel in gastrointestinal cancers". *World Journal of Gastrointestinal Oncology* 8.3 (2016): 282-288.

4. Asmar S., *et al*. "Inverse correlation between salt tolerance and host-adaptation in mycobacteria". *BMC Research Notes* 9 (2016): 249.

5. Chen JH., *et al*. "Loss of anion transport without increased sodium absorption characterizes newborn porcine cystic fibrosis airway epithelia". *Cell* 143.6 (2010): 911-923.

6. Zabner J., *et al*. "Loss of CFTR chloride channels alters salt absorption by cystic fibrosis airway epithelia in vitro". *Molecular Cell* 2.3 (1998): 397-403.

7. Cantin AM. "Cystic Fibrosis Transmembrane Conductance Regulator. Implications in Cystic Fibrosis and Chronic Obstructive Pulmonary Disease". *Annals of the American Thoracic Society* 13.2 (2016): S150-S155.

8. Bittar F., *et al*. "Molecular detection of multiple emerging pathogens in sputa from cystic fibrosis patients". *PLoS One* 3.8 (2008): e2908.

9. Krauke Y and Sychrova H. "Four Pathogenic Candida Species Differ in Salt Tolerance". *Current Microbiology* 61.4 (2010): 335-339.

10. Lahav R., *et al*. "Alterations in protein synthesis and levels of heat shock 70 proteins in response to salt stress of the halotolerant yeast Rhodotorula mucilaginosa". *Antonie Van Leeuwenhoek* 85.4 (2004): 259-269.

11. Turk M., *et al*. "Extremophilic yeasts: plasma-membrane fluidity as determinant of stress tolerance". *Fungal Biology* 115.10 (2011): 950-958.

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