LIVESPO® NAVAX PROBIOTICS CONTAINING *BACILLUS* SPORES EFFECTIVELY SUPPORT SYMPTOMATIC TREATMENT AND REDUCE THE CONCENTRATION OF RESPIRATORY SYNCYTIAL VIRUS (RSV) IN CHILDREN

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Background/Aims: Infection with the Respiratory Syncytial Virus (RSV) is one of the most common causes of respiratory tract diseases. This study was conducted to initially investigate the supportive therapeutic effects of probiotic product LiveSpo® Navax containing LS-III generation *Bacillus* spores in liquid-form and high concentration, in children having acute respiratory tract diseases caused by RSV at Vietnam National Children's Hospital. **Methods:** Totally 30 patients acquiring bronchiolitis participated in the blind, randomized and controlled clinical trial. The patients were divided randomly into 2 groups (n =15/group each): one using LiveSpo® Navax (Navax group) and the other using 0.9% NaCl physiological saline (Control group), and instructed to perform nasal spraying at frequency of 3 times a day in 6 days continuously, in combination with conventional drug therapy in the hospital. The patients were monitored their clinical indicators throughout the treatment period and underwent tests such as: (i) RSV rapid test at day 0, quantitative analysis of RSV, *B. subtilis* and *B. clausii* concentration in nasopharyngeal samples at day 0 and day 3 by real-time PCR. **Results:** The Navax group had about 1-day shorter recovery time of typical symptoms such as runny nose, difficulty breathing, dry rales, moist rales, chest depression than the Control group. After 3 days of treatment, the Navax group had about 300-fold reduction in RSV concentration in nasopharyngeal sample, while the Control group had only 15-fold reduction; in association with the presence of *B. subtilis* and *B. clausii* in the nasopharyngeal sample of Navax group but absence in Control group. 100% of patients using LiveSpo® Navax showed neither signs of abnormal breath, pulse, nasal mucosa irritation, nor digestion.

Conclusion: This study is the first clinical trial in children in the world to demonstrate safety and effects of nasal-spraying *Bacillus* spore probiotics. LiveSpo® Navax provided 1-day earlier treatment time of typical symptoms for respiratory diseases caused by RSV infection, as well as effectively reduced the concentration of RSV in nasopharyngeal sample of children by 20-fold higher than 0.9% NaCl physiological saline.

Keywords: LiveSpo® Navax, *Bacillus* probiotic spores, RSV, respiratory infection, children.

1. INTRODUCTION

Respiratory syncytial virus (RSV) is common virus cause acute respiratory tract infections (naso-pharyngitis, bronchiolitis, bronchitis, etc.), especially in children. Depending on the location of damage, the clinical symptoms vary from mild such as fever, cough, runny nose… to severe such as difficulty breathing, respiratory failure. The typical symptoms in children include runny nose, wheeze profusely and persisting for days after physical damage is gone. According to the record of the World Health Organization (WHO), there is about 3 billion people hospitalized due to RSV infection every year. The number of death cases can be up to

66.000 children. RSV often thrives throughout winter-spring and spring-summer season. RSV are easy to spread among community by entering eyes, nose or mouth and spread through the air by infected respiratory droplets. This virus can survive for hours in hard object surface such as table surface, crib rails and toys. The most at risk people includes premature babies, babies under 2 year old, immunocompromised adults and the elderly, especially people with latent heart and lung disease. According to recent report data (Nov, 2020), the number of hospitalized children due to respiratory infections at Vietnam National Children's Hospital (Hanoi) increase twice as many as normal, of these, one third was children infected with RSV [1-4].

Hitherto, there is no specific vaccine and treatment drug for RSV infected disease. Children only use antibiotics when superinfection appeared with other bacteria such as *Streptococcus pneumoniae* and *Haemophilus influenzae*... In recent years, the backup treatment methods have been strengthened, in which probiotics are considered as promising candidate for support treatment and reduce the drug and anti-biotic dependence [5-6].

Probiotics are useful bacteria for host, typically safe for human health. The recent studies show that probiotics are not only beneficial bacteria for digestive tract, but also able to protect and prevent respiratory infections [4]. There are many hypotheses about antiviral mechanism of probiotics have been discussed. However, this role is illustrated throughout three popular mechanisms: (i) Virus is captured through the direct interaction between probiotics and virus, (ii) Probiotics have ability to produce secondary substances, which inhibit the development of virus and (iii) Probiotics stimulate the immune system to capture the invading virus [7].

In the current market, only one probiotic product is recommended to spray directly into the nose, which is LiveSpo® Navax, this product was prepared in the form of suspension containing live spores of two strains *Bacillus subtilis* ANA4 and *Bacillus clausii* ANA39. These two probiotic strains were isolated from shrimp and chicken gut respectively with outstanding efficiency in spore and biofilm forming, thereby, enhancing their ability to adhere to the nasal mucosa and compete with bacteria-caused respiratory disease. Furthermore, this probiotic strains also stimulate the interferon-gamma (IFN-γ) synthesis and have antibacterial ability. On the basis of superior abilities and novelty of these two bacteria strains in LiveSpo® Navax, as well as the convenience of this product in spray model, this research was performed for initial assessment on safety and the supportive therapeutic effect in symptomatic treatment for children with RSV infection at National Children's Hospital of LiveSpo® Navax product. Besides, we also evaluate the decrease level of RSV concentration and appearance of *B. subtilis* ANA4 and *B. clausii* ANA39 in nasopharyngeal sample of patients.

2. OBJECT AND METHOD

2.1. Object

This study was performed on 30 pediatric patients aged from 4 to 24 months, volunteered for participate in study, including male (70%) and female (30%). All patients are diagnosed with bronchiolitis with RSV-positive and being treated as an inpatient at International Department S, National Children's Hospital from Jan 2nd, 2021 to Feb 8th, 2021.

LiveSpo® Navax (No XNCB: 190001347/PCBA-HN) is product of LiveSpo Pharma Co. Ltd., which is prepared as suspension form containing 0.9% NaCl physiological saline and LS-III generation of *B. subtilis* ANA4 and *B. clausii* ANA39 probiotic spores with extremely high purity and concentration ($\geq 5 \times 10^9$ CFU/5 mL), use it by directly spray into the nose and throat.

Diagnosis of RSV infection from nasopharyngeal swab sample by rapid test method using BD Veritor System for Rapid Detection of RSV (Becton Dickison), blood tests, C-reactive protein (CRP) and total white blood cells help determine the infection level. The cardiopulmonary X-ray image at day 0 was performed at International Department S, National Children's Hospital, RSV quantitative assay by real-time PCR Taqman

Probe from nasopharyngeal sample has been standardized and routinely used in the Department of Molecular Biology for Infectious Diseases.

2.2. Method

***Study design*:** The blind, randomized and controlled clinical trial.

***Sample size and sample selection*:** Randomly select 15 patients for each group. The samples were selected according to convenience sampling principle for all eligible patients during study period. Patients in Navax group were treated with LiveSpo® Navax and those in Control group were treated 0.9% NaCl physiological saline.

Study procedure: Study was conducted in parallel on 2 patient groups. Patients were provided with blind coding spray product to ensure the objectivity of study. Nurses were instructed to spray about 50µl/time 3 times a day directly into the nose for 6 days continuously, which simultaneously with conventional treatment drugs in hospital. So, every 50 µl LiveSpo® Navax contain ≥ 2.5 x 10^8 CFU of *B. subtilis* ANA4 and *B. clausii* ANA39, totally. During treatment period, patients were monitored the typical clinical symptoms of respiratory disease due to RSV, include: runny nose, dry rales, moist rales, chest depression and difficulty breathing, fast pulse, fast breathing, body temperature in 6 days; RSV quantitative in nasopharyngeal sample at day 3 in comparison with day 0 by real-time PCR Taqman probe; quantitative assay for *B. subtilis* ANA4 and *B. clausii* ANA39 in nasopharyngeal sample at day 3 by real-time PCR SYBR Green using specific primer for *B. subtilis* and *B. clausii*, which was optimized according to standard ISO 17025 at Key Laboratory of Enzyme and Protein Technology (KLEPT), Hanoi University of Science [8-9]. Efficacy of LiveSpo® Navax was evaluated based on RSV test results after 3 days of treatment and reduction level of clinical and sub-clinical symptoms. In there, the degree of RSV load reduction was calculated according to the $2^{\Delta Ct}$, with $\Delta C_t = C_{t\ day\ 3} - C_{t\ day\ 0}$; C_t is threshold cycle value of RSV-specified real-time PCR reaction. The concentration of *B. subtilis* and *B. clausii* in nasopharyngeal sample was calculated based on standard curve of relationship between concentration (CFU/mL) and C_t value of SYBR Green signal.

***Data collection and analysis*:** Monitor and observe of patients' condition during study period and fill in record profile. The data was analyzed and processed by medical statistical method.

***Ethical issues*:** This study was conducted in accordance with ethical principle, which consistent to Declaration of Helsinki and ICH GCP guideline, appropriate to regulations and standards of Ministry of Health about study on human. This study was approved by Ethics Council in Medical Study, Vietnam National Children's Hospital under the Decision No. VNCH-RICH-2020-46.

3. RESULTS

3.1. Characteristics of patients before treatment

The collected data on 30 RSV-positive patients before treatment showed that there were 15/15 patients in both groups recorded symptoms of runny nose and dry rales, moist rales; some of patients showed chest depression and difficulty breathing (20-33%) and fever (6.7%). There were over 50% of patients showed high value of CRP (> 6mg/L) and about 20% of patients show high level of white blood cells (> 10.0g/L). The X-ray image showed that almost patients (93-100%) have lung lesions (Osler's nodes, hyperinflation…). The clinical symptoms of patients in both groups were collected and presented in Table 1 showed that the study's objects were bronchiolitis disease.

Table 1: Clinical and nonclinical properties of RSV-infected children before treatment

Indexes	Navax group (N = 15)	Control group (N = 15)
Age (months)		
≥ 4-12.n (%)	9 (60)	7 (46.67)
≥ 12-24.n (%)	6 (40)	8 (53.33)

Gender		
Male n (%)	11 (73.33)	10 (66.67)
Female n (%)	4 (26.67)	5 (33.33)
Clinical properties		
Runny nose. n (%)	15 (100)	15 (100)
Chest depression and difficulty breathing n (%)	5 (33.33)	3 (20)
Dry rales. n (%)	15 (100)	15 (100)
Moist rales. n (%)	9 (60.0)	11 (73.33)
Fever (>37.5^{0}C). n (%)	1 (6.67)	1 (6.67)
Nonclinical properties		
Cardiopulmonary X-ray (Osler's nodes, hyperinflation…)	15 (100)	14 (93.33)
Total white blood cell (>10.0 g/L)	8 (53.33)	10 (66.67)
CRP index (> 6.0 mg/L)	4 (26.67)	3 (20)

3.2. Safety and supporting effect in clinical symptomatic treatment in RSV-infected children of nasal-spraying probiotic LiveSpo® Navax.

Among the test results of LiveSpo® Navax on 15 patients, there were no recorded cases related to abnormal changes of breathing rate, pulse, body temperature and pulse oxymetry (SpO_2) before and after treatment. 100% of participated patients do not have any signs of nasal mucosa irritation or digestive disorder (vomiting, diarrhea).

The results of study showed that patients in Navax group had a faster decrease in clinical symptoms than those in Control group. Especially, the result in Figure 1 shows that the recovery time of patients is as follows: runny nose (in patients used Navax/NaCl 0.9% is 4 days/5 days), chest depression and difficulty breathing (in patients used Navax/NaCl 0.9% is 1 day/2 days), dry rales (in patients used Navax/NaCl 0.9% is 4 days /5 days) and moist rales (in patients used Navax/NaCl 0.9% is 3 days /4 days), which mean the recovery time in Navax Group is 1 day earlier than that of Control group. The difference is statistically significant (*p<0.05, **p<0.01; ***p<0.0001).

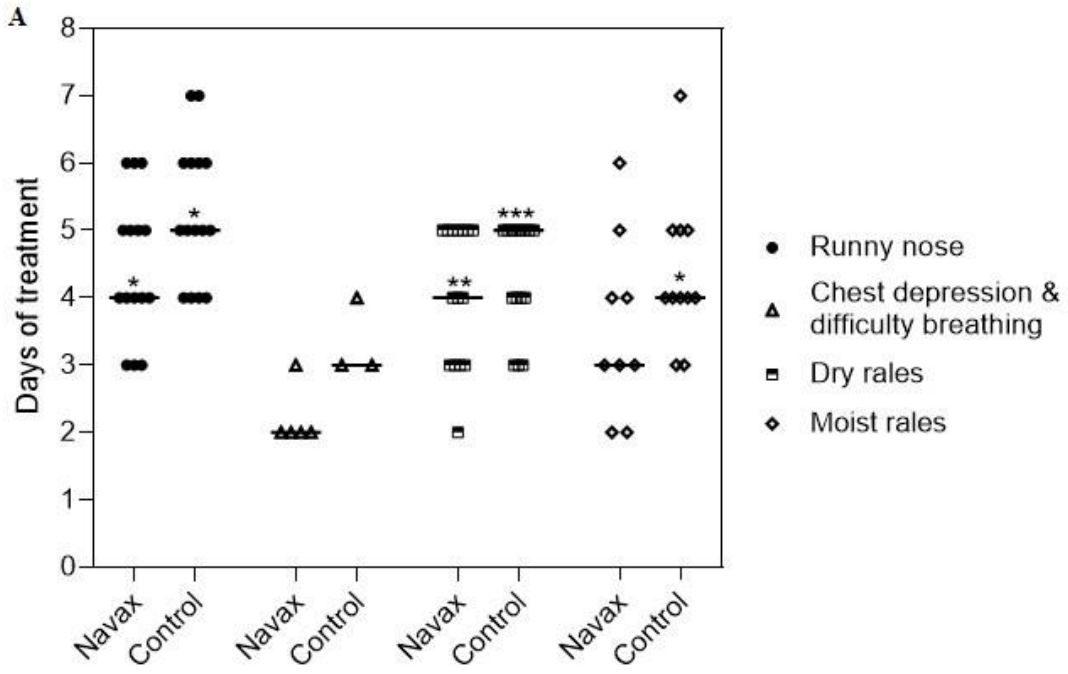

Figure 1: The time (days) to recovery from respiratory clinical symptoms of Navax group and Control group (*p<0.05, **p<0.01; ***p<0.0001).

3.3. Lowering-effect on RSV concentration in nasopharyngeal sample of patients

The statistical analysis results in comparison between $2^{\Delta Ct}$ value reflecting reduction degree of RSV load in nasopharyngeal sample of patients after 3 days using Navax in combination with other treatment methods and $2^{\Delta Ct}$ value prior to treatment on 30 patients in both groups showed that the RSV concentration of patients in Navax group has decreased significantly by about 300 folds. Meanwhile, the Control group has a decrease in RSV concentration at about 15 folds (Figure 2). Therefore, LiveSpo® Navax resulted in 20 folds more effectiveness in reduction of RSV concentration in comparison with physiological saline (NaCl 0.9%), statistically (****p<0.0001).

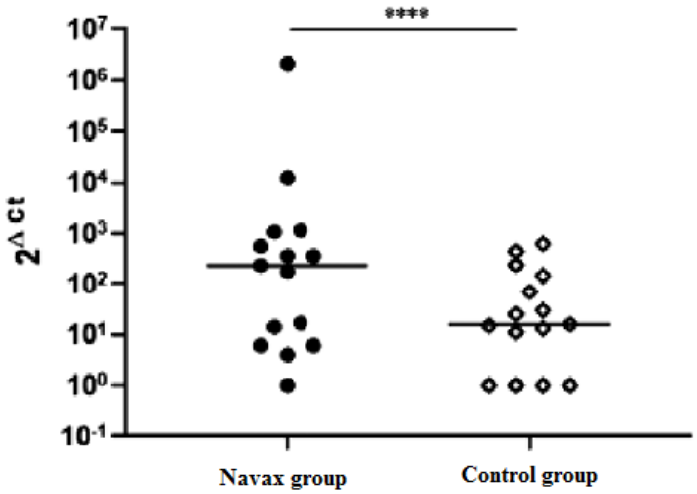

Figure 2: Reducing folds of RSV load after 3 days of treatment in Navax and Control group (**p<0.0001)**

3.4. Assessment of adhesion of *B. subtilis* ANA4 and *B. clausii* ANA39 on nasal mucosa of patients using LiveSpo® Navax.

As the results showed in Figure 3, the detected average concentration of spores in 1 mL of nasopharyngeal sample of patients in Navax group is $\geq 1 \times 10^5$ CFU/mL for *B. subtilis* and $\geq 1 \times 10^2$ CFU/mL for *B. clausii* with 96.48% reliability. Thus, the average concentration of *Bacillus* spore on the cotton swab of each sample collection is 0.04% compared to amount of spores at the initial spray each time ($\geq 2.5 \times 10^8$ CFU of both *B. subtilis* ANA4 and *B. clausii* ANA39 in total). However, in case of recovered patients (RSV negative), the concentration of *Bacillus* spores in nasopharyngeal sample is up to $\geq 10^7$ CFU/mL. In other word, the amount of *Bacillus* spores on the sampled cotton swab is equivalent to 4% of the initial spray each time. Meanwhile, *B. subtilis* and *B. clausii* spores do not found in any nasopharyngeal sample of patients in Control group.

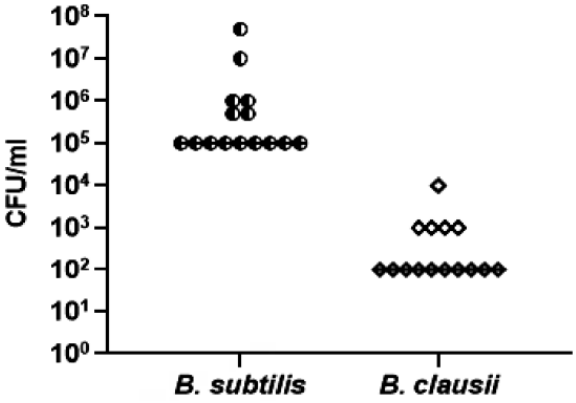

Figure 3: Concentration of probiotics *B. subtilis* and *B. clausii* in nasopharyngeal sample (1 mL) of patients in Navax group.

4. DISCUSSION

This is the first blind, randomized and controlled clinical trial on safety and efficacy of nasal-spray probiotic LiveSpo® Navax, which was initial performed on 30 pediatric inpatients with RSV-positive at International Department S, Vietnam National Children's Hospital. The screening results of participated patients showed that they had abnormal signs of clinical symptoms and sub-clinical indexes related to bronchiolitis disease. 100% of children showed runny nose and dry rales, 66.67% showed moist rales, 26.67% showed chest depression and a few patients showed difficulty breathing. The cardiopulmonary X-ray results showed that 29/30 patients have lung lesions, mostly osler's nodes or both nodes and hyperinflation. The test of total white blood cells and CRP index also showed certain infection in patients. Thus, the selected patients were all eligible to participate in the study.

RSV is common respiratory disease-caused virus. However, currently, there have not been had any vaccine or specific drug for clinically treatment to patients. Recent researches suggest that probiotics are one of the promising therapies to prevent RSV infection due to anti-virus mechanisms, which were discussed in previous researches. The study of Chiba et al. (2013) on mice model showed that the oral administered probiotics containing *Lactobacillus rhamnosus* CRL1505 at 10^8 CFU/micro/day can effectively reduce RSV concentration in lung at day 4 by 32 times and protect lung cells through ability to stimulate the immune system to produce interferon IFN-γ and IL10 [10]. Study of Eguchi et al. (2019) on assessment of probiotic efficacy of acid lactic bacteria *Lactobacillus gasseri* LG2055 on mice model, used orally, had effect on prevention of viral infection. The results showed that RSV load in lung of mice had slightly reduction (8 folds) after 4 days of treatment [11]. However, these two studies were limited to animal (mice) model. Although *Lactobacillus* and *Bifidobacterium* have been proved to have many advantages in prevention of viral infection, but probiotic products containing these strains remain some disadvantages such as heat instability through transportation

leading to rapidly reduction in live-count and product efficacy. Moreover, products are prepared only in powder or tablet form for oral administration but not in liquid form for spraying directly to the nose. That why, it is difficult to promote the highest efficacy in directly inhibiting/killing viruses or bacteria that cause upper respiratory disease. Now a day, there are a few researches to evaluate the role of *Bacillus* sp. spore forming bacteria in prevention of viral infection. Research of Wang et al. (2017) proved that *Bacillus subtilis* OKB105 strain can capture and resist to gastritis virus (*Transmissible gastroenteritis virus*-TGEV) belong to *Coronaviridae* family, attach the porcine intestinal epithelial cells [12]. However, this study was just conducted *in-vitro*, not in human model.

Our study is the first study on clinical trial of clinical effects of nasal-spray product containing *Bacillus* probiotic on children. Although it is initial trial in small scale (n = 30), the statistically significant results showed that the recovery time from symptoms such as runny nose, difficulty breathing, dry rales, moist rales, chest depression were shortened by 1 day due to LiveSpo® Navax. After 3 days of treatment, RSV load in nasopharyngeal sample in Navax group was 300-fold reduction, much more than that of Control group (15-fold reduction). In addition, the presence of *B. subtilis* and *B. clausii* in nasopharyngeal sample was detected only in Navax group but not in Control group. The analytical results of RSV load in nasopharyngeal sample showed that LiveSpo® Navax containing two types of probiotic spores (*B. subtilis* and *B. clausii*) provided 300-fold reduction of RSV load after 3 days of treatment, which is 20-fold higher than 0.9% NaCl physiological saline. Especially, there is one case has strong RSV load reduction to negative (considered as > 10^6-fold reduction) after 3 days using LiveSpo® Navax (Figue 2). In accordance to negative result in this case, the concentration of probiotic spore *B. subtilis* in nasopharyngeal sample of this patients is highest among the group (> 10^7 CFU/mL, equivalent to 4% of initial sprayed spores) (Figure 3). This result suggests that strict compliance to product instructions will help spores adhere to nasal mucosa better and bring into play the efficacy in preventing RSV replication in patients' nose, subsequently, support to reduce the symptoms of respiratory inflammation and shortening the recovery time for patients.

Analysis of real-time PCR using SYBR Green showed that the presence of *B. subtilis* ANA4 and *B. clausii* ANA39 were detected in nasal mucosa of all patients who used LiveSpo® Navax but not in Control group. The average concentration of spores in nasopharyngeal sample is ≥ 10^5 CFU/mL for *B. subtilis* and ≥ 10^2 CFU/mL for *B. clausii* (Figure 3). This result showed that the participated patients in both groups were provided with right trial product, properly instructed and followed the nasal spray procedure. In addition, these two spores have ability to adhere on nasal mucosa of patients.

These results provide the initial assessment of nasal-spraying probiotic product LiveSpo® Navax. The product is completely safe, even for children under 2 years old with recommended dose as spray to nose 3 times/day in 3 days continuously, combined with clinical and sub-clinical indexes monitoring. During the trial period, there were neither abnormal signs related to breathing rate, pulse, body temperature, SpO_2, nor abnormal change in CRP index and total white blood cells of participated patients. In 100% of patients using LiveSpo® Navax, no abnormalities in digestion (vomiting, diarrhea) or nasal mucosa irritation were found.

5. CONCLUSION

This is the first clinical trial studied on safety and efficacy of nasal-spraying probiotic spores *Bacillus* on children in the world. Probiotic product LiveSpo® Navax, in liquid-form, contains probiotic spores LS-III generation of two strains *B. subtilis* ANA4 and *B. clausii* ANA39 is completely safe for children. This product helps shorten the treatment period for the typical symptoms of upper respiratory tract disease such as runny nose, chest depression, difficulty breathing, dry rales and moist rales by 1 day and reduce the RSV concentration in nasopharyngeal sample by 20-fold more effectiveness than 0.9% NaCl physiological saline. The clinical trial needs to be expanded in larger scale, higher sample size for more comprehensive assessment on safety and efficacy of LiveSpo® Navax, as a scientific basis for product development with the effect of preventing and supporting the treatment of respiratory infectious diseases.

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